Epidemiological investigation of Chlamydiaceae in poultry of Mexico and comparison of growth characteristics of Chlamydia gallinacea in different experimental trials

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Étude épidémiologique des *Chlamydiaceae* chez les volailles au Mexique et essais expérimentaux pour comparer des caractéristiques de croissance de *Chlamydia gallinacea*

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ABSTRACT

Epidemiological investigation of *Chlamydiaceae* in poultry of Mexico and comparison of growth characteristics of *Chlamydia gallinacea* in different experimental trials

Chlamydiaceae infections in poultry are mainly due to *Chlamydia psittaci* and *Chlamydia gallinacea*. While *C. psittaci* has long been known to affect birds and to have zoonotic potential, *C. gallinacea* is a newly described species that has been found to be widespread in chickens and whose pathogenicity and zoonotic potential have not yet been shown. Good husbandry and biosecurity practices, coupled with antimicrobial treatment in punctual cases, are the most effective means to control chlamydial infections among poultry. Few studies had been conducted on Mexican poultry farms assessing on-farm biosecurity practices implementation and no data were available regarding the presence of *Chlamydiaceae* in poultry of Mexico. The aims of this thesis were to detect the presence of *Chlamydiaceae* on commercial and backyard farms, to identify risk factors associated with their potential presence on poultry and to characterize the on-farm management and biosecurity practices. Results showed that apparent prevalence of *Chlamydiaceae* increased inversely to the level of poultry confinement. *C. gallinacea* was the only chlamydial species found. On backyard poultry, the lack of antimicrobial usage and an impaired health status were found to be associated with the presence of *Chlamydiaceae*, while on commercial farms laying hens had higher odds of being *Chlamydiaceae*-infected than chicken broilers. Five biosecurity practices were identified as the most significantly associated with farm clustering. Three of these practices concerned directly the staff or visitors while two were related to farm facilities. Farms within the cluster in which laying hen farms predominated were more prone to not implement some of these five biosecurity practices. Recommendations were provided to improve the on-farm biosecurity. Finally, *in vitro* studies carried out to optimize the growth protocol for *C. gallinacea* were not conclusive. However, an enhanced growth of one *C. gallinacea* positive field specimen was identified. The cause of this enhanced growth needs to be determined; differences at genetic or transcriptional level should be considered, as well as the influence of external parameters. If *in vitro* observations can be extrapolated to an *in vivo* phenomenon, it could shed light on the pathogenic potential of this newly described species.

Keywords: *Chlamydiaceae*, *Chlamydia gallinacea*, *Chlamydia psittaci*, poultry, risk factor, biosecurity, Mexico, *in vitro* growth.

RESUME

Étude épidémiologique des *Chlamydiaceae* chez les volailles du Mexique et essais expérimentaux pour comparer des caractéristiques de croissance de *Chlamydia gallinacea*

Les espèces *Chlamydia psittaci* et *Chlamydia gallinacea* sont les principales espèces de *Chlamydiaceae* détectées chez les volailles. Contrairement à *C. psittaci* qui est décrite depuis longtemps et pour laquelle le pouvoir zoonotique est avéré, *C. gallinacea* est une espèce nouvellement décrite qui est très répandue dans les élevages de poulets et dont la pathogénicité et le pouvoir zoonotique n'ont pas encore été démontrés. De bonnes pratiques d'élevage et de biosécurité, associées à un traitement antimicrobien dans les cas ponctuels, sont les moyens les plus efficaces pour contrôler les infections à chlamydia chez les volailles. Peu d'études avaient été menées dans les exploitations avicoles mexicaines pour évaluer la mise en œuvre des pratiques de biosécurité et aucune donnée n'était disponible concernant la présence de *Chlamydiaceae* dans ces élevages. Les objectifs de cette thèse visaient à détecter la présence de *Chlamydiaceae* dans des fermes commerciales et des basses-cours, à identifier les facteurs de risque associés à la présence de *Chlamydiaceae* chez ces volailles et à caractériser les pratiques de gestion et de biosécurité au sein de ces fermes. Les résultats ont montré que la prévalence apparente des *Chlamydiaceae* augmentait de manière inversement proportionnelle au niveau de confinement des volailles. *C. gallinacea* est la seule espèce de *Chlamydiaceae* détectée. Pour les volailles de basse-cour, l'absence d'utilisation d'antimicrobiens et un statut sanitaire dégradé ont été associés à la présence de *Chlamydiaceae*, tandis que dans les exploitations commerciales, les poules pondeuses avaient plus de risque d'être infectées par *Chlamydiaceae* que les poulets de chair. Cinq pratiques de biosécurité ont été identifiées comme étant les plus significativement associées au classement des fermes. Trois de ces pratiques concernaient directement le personnel ou les visiteurs, tandis que deux étaient liées aux équipements et installations de ces fermes. Les fermes du « cluster » (groupe) où prédominaient les élevages de poules pondeuses étaient plus susceptibles de ne pas appliquer certaines de ces cinq pratiques de biosécurité. Des recommandations ont été formulées pour améliorer la biosécurité dans les fermes. Enfin, les études *in vitro* réalisées pour optimiser le protocole de croissance de *C. gallinacea* n'ont pas été concluantes, mais des différences individuelles dans la croissance de *C. gallinacea* à partir de prélèvements positifs ont été identifiées. La différence de croissance soulève la nécessité de déterminer si elle est due à des aspects génétiques voire transcriptionnels, ou si elle implique d'autres paramètres externes. Si les observations *in vitro* peuvent être extrapolées à un phénomène *in vivo*, ceci pourrait avoir des implications sur la compréhension de la pathogénicité potentielle de cette nouvelle espèce.

Mots-clés : *Chlamydiaceae*, *Chlamydia gallinacea*, *Chlamydia psittaci*, volailles, facteur de risque, biosécurité dans les fermes, Mexique, croissance *in vitro*.

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INDEX OF ABBREVIATIONS

AIC	Akaike information criterion
AMU	Antimicrobial usage
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under the ROC curve (receiver operating characteristic)
BGM	Buffalo green monkey kidney cells
BLAST	Basic local alignment search tool
Caco-2	Human colon adenocarcinoma cell line
CFT	Complement fixation test
CPAF	Chlamydial protease activity factor
DEAE	Diethylaminoethyl dextran
DF-1	Chicken fibroblast spontaneously transformed cell line
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EB	Elementary bodies
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EMEM	Eagle's Minimum Essential Medium
FAO	Food and Agriculture Organization of the United Nations
FDA	United States Food and Drug Administration
FLI	Friedrich-Loeffler-Institut
FMVZ	Faculty of Vterinary Medicine and Zootechnics
GMPs	Good Manufacturing Practices
GXM	Glucuronoxylomannan
HACCP	Hazard Analysis and Critical Control Points
HCA	Hierarchical cluster analysis
HPAI	Highly pathogenic avian influenza
ICSP	International Committee on Systematics of Prokaryotes
IFN-ɣ	Interferon gamma
InDRE	Mexican Institute of Epidemiological Diagnosis and Reference
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
MACPF	Membrane attack complex/perforin protein
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MCA	Multiple correspondence analysis
MEM	Minimum Essential Medium
MLVA	Multi-locus VNTR analysis
MOMP	Major outer membrane protein
OIE	World Organization for Animal Health
OR	Odds ratio
PCR	Polymerase chain reaction
Pmp	Polymorphic membrane protein
PZ	Plasticity zone
RB	Reticulate bodies
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SAGARPA	Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food of Mexico
SEMARNAT	Secretariat of Environment and Natural Resources of Mexico
SENASICA	National Service for Agrifood Health, Safety and Quality of Mexico
SPF	Specific-Pathogen-Free
SPG	Sucrose phosphate glutamine medium
SSOPs	Sanitation Standard Operating Procedures
T3SS	Type III secretion system
TCA	Tricarboxylic acid
TIF	Federal inspection type processing plants
UNA	National Association of Poultry Farmers
UNAM	National Autonomous University of Mexico
VNTR	Variable number of tandem repeats
WAHIS	World Animal Health Information System
WHO	World Health Organization
WTO	World Trade Organization

RESUME DE LA THESE

Étude épidémiologique des *Chlamydiaceae* chez les volailles au Mexique et essais expérimentaux pour comparer des caractéristiques de croissance de *Chlamydia gallinacea*

INTRODUCTION

Le phylum *Chlamydiae* comprend actuellement une seule classe, *Chlamydiia*, qui ne contient qu'un seul ordre, les *Chlamydiales*, qui comprend la famille *Chlamydiaceae*, qui est de loin la famille la plus étudiée et qui abrite d'importants agents pathogènes humains et animaux (Bayramova et al., 2018; Borel et al., 2019; Borel and Greub, 2019; Horn, 2010; Ruggiero et al., 2015). Les *Chlamydiaceae* sont des bactéries intracellulaires obligatoires dites à Gram négatif, avec un cycle de vie biphasique distinctif impliquant un corps réticulé intracellulaire répliquant et un corps élémentaire infectieux non divisible (Bachmann et al., 2014; Elwell et al., 2016; Omsland et al., 2014). Leur capacité à persister chez leur hôte associée à un certain nombre de pathologies chroniques, est la caractéristique la plus notable chez certaines espèces de cette famille bactérienne (Borel et al., 2018; Elwell et al., 2016; Omsland et al., 2014). À ce jour, 18 espèces ont pu être identifiées et ont été regroupées en un seul genre *Chlamydia*, certaines de ces espèces étant encore candidates.

Les infections des volailles dues aux *Chlamydiaceae* sont cosmopolites (Donati et al., 2018; Gaede et al., 2008; Guo et al., 2016; Hulin et al., 2015; Li et al., 2017; Szymańska-Czerwińska et al., 2017; Zocevic et al., 2012). Parmi la diversité des espèces que l'on peut trouver chez les volailles, *C. psittaci* présente un intérêt particulier car son potentiel zoonotique est bien reconnu, avec des manifestations cliniques graves chez l'homme qui peuvent entraîner la mort si un traitement antibiotique approprié n'est pas administré à temps (Knittler and Sachse, 2015). Les volailles semblent être moins sensibles à l'infection par *C. psittaci*, même si de nombreux cas de psittacose zoonotique signalés dans le monde ont été attribués à des volailles infectées de façon subclinique par cette espèce de *Chlamydia* (Durfee et al., 1975; Hedberg et al., 1989; Hogerwerf et al., 2020; Hulin et al., 2015; Laroucau et al., 2015, 2009; Newman et al., 1992; Newman, 1989; Shaw et al., 2019; Yin et al., 2013). Les études menées sur la présence de *Chlamydiaceae* chez les volailles ont révélé des résultats différents selon le pays et les espèces de volailles concernées. Il est prouvé que *C. psittaci* tend à être plus présent dans les élevages de canards mulets que dans les élevages de dindes ou de poulets (Guo et al., 2016; Hulin et al., 2015). Quelques autres études réalisées dans des élevages commerciaux de poulets aux Pays-Bas (Heijne et al., 2018), et des élevages de dindes en Suisse (Vogler et al., 2019), ainsi que chez les poulets de basse-cour aux États-Unis (Li et al., 2017), ont même montré que *C. psittaci* pouvait être absent. Au contraire, la prévalence de *C. gallinacea* semble dépasser celle de *C. psittaci* dans les élevages de volailles, selon l'espèce aviaire échantillonnée, car *C. gallinacea* est plus souvent trouvée dans les élevages de poulets et de dindes (*Galliformes*) que dans les élevages de canards (*Anseriformes*) (Guo et al., 2016; Hulin et al., 2015; Szymańska-Czerwińska et al., 2017). La présence de *C. gallinacea* a également été décrite chez un passereau de compagnie en captivité dans un foyer en Argentine (Frutos et al., 2015), et dans un psittacidé sauvage australien (Stokes et al., 2019). Bien qu'il ait été signalé que l'infection persistante par *C. gallinacea* entraîne

une réduction de la prise de poids chez les poulets de chair, sa virulence doit encore être clarifiée car aucune manifestation clinique spécifique n'a été signalée à ce jour (Donati et al., 2018; Guo et al., 2016; Heijne et al., 2018).

Le Mexique est un grand pays producteur de volaille, avec une industrie avicole commerciale intensive à grande échelle et une production de basse-cour d'autoconsommation importante. La production d'œufs et de poulets place le Mexique au 4e et au 6e rang des producteurs mondiaux, respectivement, selon les données de production moyenne de 2010 à 2018 enregistrées par la base de données de l'Organisation des Nations Unies pour l'alimentation et l'agriculture (FAOSTAT) (FAO, 2020). Les volailles représentaient près de 64% du cheptel national en 2018 (35% de poulets de chair, 29% de poules pondeuses et 0,01% de dindes) (UNA, 2018). En 2018, la valeur de l'aviculture mexicaine s'élevait à près de 7 milliards de dollars (UNA, 2018).

Avant 2016, les infections à chlamydia chez les animaux étaient considérées comme exotiques au Mexique par les autorités nationales de santé animale, tant chez les oiseaux que chez les petits ruminants. Quelques études ponctuelles ont été menées pour détecter la présence de chlamydia chez les oiseaux, principalement dans les animaleries, les zoos et à l'hôpital aviaire du FMVZ-UNAM. Certaines de ces études ont révélé la présence de *Chlamydia* spp. (Morales Luna, 2006; Rojas Martinez, 1996)(Vázquez Machorro, 2015)(Vázquez Machorro, 2015)(35), alors que dans d'autres, il n'a pas été possible de la détecter (López Yelmi, 2011; Pérez Olmedo, 2018). Par ailleurs, de nombreux cas d'infection à chlamydia ont été suspectés ces dernières années suite à un examen post-mortem et des résultats d'histopathologie chez les oiseaux rélisés au Laboratoire de diagnostic et de recherche des maladies aviaires du FMVZ-UNAM), mais ils sont restés sans aucune confirmation du diagnostic (Ornelas-Eusebio and Ledesma-Martínez, 2017). Ce n'est qu'en 2015 qu'avec le soutien financier du Secrétariat de l'Environnement et des Ressources Naturelles (SEMARNAT) un test moléculaire permettant de réaliser le diagnostic de la chlamydiose aviaire a été mis en place et intégré au catalogue de diagnostic du laboratoire précité du FMVZ-UNAM. Ce test a permis de rassembler les éléments pour signaler pour la première fois *C. psittaci* en 2016 chez des individus appartenant à une espèce endémique de psittacidés en voie de disparition qui étaient hébergés dans une unité de gestion pour la conservation de la faune au Mexique (Ornelas-Eusebio et al., 2016). Cette constatation a été notifiée aux autorités nationales de santé animale. Les différents rapports sur la présence de *Chlamydia* au Mexique ont pu entrainer une révision de la réglementation zoosanitaire du pays qui a abouti à la reclassification définitive en 2016 de la chlamydiose aviaire en tant que maladie endémique.

Une étude menée en 2016 en coopération avec l'ANSES a montré la circulation de *C. psittaci* ainsi que d'espèces de chlamydia non identifiées chez les oiseaux sauvages et de compagnie au Mexique (Ornelas-Eusebio et al., 2017), mais aucune étude n'avait été menée concernant les infections à *Chlamydia* chez les volailles au Mexique. D'autre part, les bonnes pratiques d'élevage et de biosécurité, sont considérées comme les mécanismes les plus efficaces pour contrôler les infections à *Chlamydia* chez les volailles (Balsamo et al., 2017); peu d'études ont fourni des informations concernant les pratiques de biosécurité dans les élevages de

volailles au Mexique (Absalón et al., 2019; Afanador-Villamizar et al., 2017; Cardenas Garcia et al., 2013; Peña Aguilar et al., 2016).

Différentes études suggèrent que les conditions qui favorisent la croissance in vitro d'une espèce/un spécimen de *Chlamydia* semblent ne pas être applicables pour les autres espèces (Onorini et al., 2019; Schiller et al., 2004). Bien que plusieurs optimisations aient été apportées au protocole de croissance de *Chlamydia* - toutes visant à augmenter le taux d'infectivité et l'amélioration de la croissance *in vitro* -, la propagation de certaines souches/espèces reste difficile. De telles difficultés sont rencontrées pour *C. gallinacea* (communications personnelles de plusieurs chercheurs européens), même si peu d'études ont abouti à la culture *in vitro* de cette nouvelle espèce identifiée (Guo et al., 2016; Hölzer et al., 2016; Laroucau et al., 2009; You et al., 2019).

Dans ce contexte, les objectifs de cette thèse étaient : (i) de détecter la présence de *Chlamydiaceae* et d'identifier la diversité des espèces de *Chlamydia* trouvées dans les volailles dans les fermes commerciales et les basses-cours du Mexique, (ii) d'identifier les facteurs de risque potentiels associés, (iii) de caractériser les pratiques de gestion et de biosécurité dans les fermes de volailles et (iv) d'optimiser le protocole de croissance in vitro de *C. gallinacea* pour finalement caractériser les isolats mexicains de *C. gallinacea*.

CHAPITRE I. ÉTUDE EPIDEMIOLOGIQUE DES CHLAMYDIACEAE CHEZ LES VOLAILLES AU MEXIQUE

Matériels et méthodes

Plan d'échantillonnage pour l'étude transversal

Une étude transversale a été menée entre juin 2017 et juin 2018 sur des exploitations commerciales et des basses-cours dans huit États fédéraux du Mexique caractérisés par une forte densité de volailles. Nous avons cherché à inclure les fermes commerciales avec différents degrés de confinement : bâtiments à ventilation dynamique (milieu fermé) vs bâtiments à ventilation statique (milieu semi-ouverts), et aussi des fermes élevant des poulets de chair et des poules pondeuses. Les bâtiments à ventilation dynamique correspondent à un milieu fermé dont le flux d'air est assuré par un système de ventilation automatique en tunnel et un éclairage artificiel, tandis que les bâtiments à ventilation statique sont des bâtiments dont les murs sont ouverts permettant une ventilation naturelle, modulée par des rideaux actionnés manuellement. Les basses-cours sont caractérisées par un petit nombre de volailles (principalement de races indigènes) élevées en liberté sur le sol avec un abri fourni par un toit de base.

La taille des échantillons d'exploitations et de volailles a été calculée pour détecter une prévalence entre exploitations de 20 % dans chacun des trois groupes d'exploitations, et une prévalence au sein de l'exploitation de 30 % avec un niveau de confiance de 95 %, en tenant compte des valeurs de prévalence signalées précédemment (Donati et al., 2018; Heijne et al., 2018; Szymańska-Czerwińska et al., 2017) et en utilisant le calculateurs épidémiologique Epitools (Sergeant, 2017). Cela a permis d'obtenir un échantillon minimum de 14 fermes et de 9 oiseaux par troupeau. Comme il n'était pas disponible une base de données nationale sur les

exploitations avicoles commerciales, les vétérinaires qui apportaient un soutien technique aux exploitations situées dans les États où se trouvent la plupart des producteurs de volaille mexicains ont été contactés lors d'un congrès national sur la filière avicole. Les éleveurs de basse-cour inclus dans l'étude, ont été contactés grâce à des étudiants en médecine vétérinaire de la FMVZ-UNAM. Dans les fermes commerciales et les basses-cours, les volailles ont été sélectionnées au hasard et soumises à un prélèvement cloacal. Deux écouvillons ont été prélevés en parallèle sur chaque volaille ; le premier écouvillon cloacal a été conservé dans 1 ml de milieu saccharose-phosphate-glutamine (SPG), tandis que l'autre a été conservé à sec. Les deux spécimens ont été transportés à basse température. Les écouvillons secs ont été congelés à -20°C jusqu'au traitement pour l'extraction de l'ADN et les écouvillons en milieu SPG ont été conservés à -80°C jusqu'au traitement pour la croissance de la chlamydia en culture cellulaire.

Suivi hebdomadaire des poulets de chair pour étudier l'excrétion de *Chlamydiaceae*

Afin d'étudier la dynamique d'excrétion des Chlamydiacées dans les troupeaux de poulets de chair commerciaux, deux troupeaux de poulets de chair hébergés dans des poulaillers à environnement contrôlé ont été échantillonnés à l'aide de tampons cloacaux au cours de leur processus de production habituel. Quinze écouvillons cloacaux ont été prélevés au hasard sur des poulets de chair à cinq reprises par les travailleurs présents dans l'exploitation. Les échantillons ont été prélevés lors d'une manipulation dans le troupeau, par exemple lors de la réception des poussins, d'une vaccination ou d'une procédure de rappel de vaccin.

Collecte de données

Un questionnaire comprenant 48 questions à compléter et des questions fermées a été conçu pour recueillir des informations concernant (i) les caractéristiques des exploitations, (ii) le type de logement et la description des installations, (iii) les caractéristiques des élevages/volailles échantillonnés, (iv) le statut sanitaire et la gestion sanitaire des élevages/volailles, (v) les pratiques d'élevage, (vi) les procédures de nettoyage et de désinfection, et (vii) les pratiques de biosécurité. Le questionnaire a été conçu en tenant compte des manuels de bonnes pratiques d'élevage pour les poulets de chair et les poules pondeuses publiés par le gouvernement mexicain (SAGARPA-SENASICA, 2016a, 2016b). Les questionnaires ont été administrés par la même personne dans le cadre d'un entretien semi-structuré dans l'exploitation, mené soit avec le manager de l'exploitation, soit avec le vétérinaire chargé de la santé des volailles. Les dernières sections du questionnaire (c'est-à-dire les pratiques de gestion des exploitations et des volailles, les procédures de nettoyage et de désinfection, et les mesures de biosécurité) comportaient des questions ouvertes, permettant au répondant de donner une réponse détaillée. Les personnes interrogées ont donné leur consentement verbal avant le début de l'entretien.

Analyse de laboratoire

Tous les écouvillons cloacaux secs ont été soumis à une extraction d'ADN comprenant une extraction interne et un contrôle d'inhibition par PCR. Le dépistage préliminaire de tous les échantillons d'ADN a été effectué à l'aide d'une technique de PCR en temps réel spécifique à *Chlamydiaceae* (rt-PCR) ciblant le gène ARNr 23S (Ehricht et al., 2006). Tous les échantillons de *Chlamydiaceae* positifs au rt-PCR ont ensuite été analysés par

des systèmes de rt-PCR spécifiques aux espèces de *Chlamydia* ciblant le gène *omp*A pour *C. psittaci* et le gène *eno*A pour *C. gallinacea*, avec les conditions, les amorces et les sondes décrites précédemment (Laroucau et al., 2015; Pantchev et al., 2009).

Analyses statistiques

Prévalence des *Chlamydiaceae*

Une exploitation était considérée comme positive si au moins un animal était testé positif. Les valeurs de prévalence entre les exploitations et la prévalence animale ont été comparées en fonction du système d'élevage en utilisant un test exact de Fisher.

Facteurs de risque : régression logistique et modèle de régression logistique à effets mixtes

Étant donné que certaines variables ne s'appliquaient qu'aux exploitations commerciales et d'autres aux exploitations de basse-cour, deux analyses distinctes ont été menées pour identifier les facteurs de risque potentiels de la présence de *Chlamydiaceae*. Comme toutes les variables des fermes commerciales étaient des prédicteurs au niveau de la ferme, une régression logistique a été effectuée pour comparer les fermes commerciales avec des bâtiments à ventilation dynamique (milieu fermé) vs fermes avec des bâtiments à ventilation statique (milieu semi-ouverts), en utilisant la ferme comme unité épidémiologique.

Pour les basses-cours, un modèle de régression logistique à effets mixtes (modèle linéaire mixte généralisé avec un lien binomial) a été mis en œuvre pour prendre en compte les informations collectées soit au niveau de l'animal, soit au niveau de l'exploitation. Pour ce deuxième modèle, les facteurs de risque ont été considérés comme des effets fixes et l'exploitation comme un effet aléatoire.
Les variables quantitatives ont été catégorisées en tenant compte des événements physiologiques et du type d'exploitation. Le diagnostic de multicolinéarité des variables a été effectué en utilisant le facteur d'inflation de la variance (VIF) garantissant une valeur VIF < 2. Lorsqu'une paire de variables s'est avérée colinéaire, seule la variable la plus biologiquement plausible a été conservée pour une analyse plus approfondie.

Analyse multivariée des exploitations avicoles commerciales

Cette analyse a été menée sur des exploitations commerciales ; toutes les variables du questionnaire administré aux exploitations commerciales ont été incluses, à l'exception de l'âge et du sexe de la bande échantillonnée. Différentes catégories pour les variables quantitatives ont été établies (nombre de volailles par bâtiment, nombre de bâtiments par exploitation, nombre d'ouvriers par exploitation et durée de la période de vide sanitaire).

Une analyse des correspondances multiples (ACM) a été effectuée pour résumer et visualiser l'ensemble des données multidimensionnelles en tenant compte des individus (c'est-à-dire les exploitations agricoles) et les variables catégorielles les décrivant. Les objectifs de cette analyse sont d'abord d'étudier les similitudes entre les individus et ensuite d'étudier les relations entre les variables, tout en évaluant les associations entre chacune

des catégories de variables. Ceci permet de caractériser les individus d'après leur schéma de variables (Husson and Josse, 2018). En utilisant les dimensions avec la plus grande variance (inertie) générée par l'ACM, les exploitations ont été classées en clusters par une analyse de classification ascendante hiérarchique (CAH) basée sur la méthode de Ward, qui consiste à ajouter deux groupes (clusters) de telle sorte que la croissance de l'inertie intra-groupe soit minimale à chaque étape de l'algorithme. L'algorithme de regroupement hiérarchique peut être visualisé à l'aide d'un dendrogramme. L'inertie intra-groupe caractérise l'homogénéité d'un cluster (Husson et al., 2016; Kassambara, 2017).

Résultats

Au total, 59 élevages de volailles ont été visités, 43 élevages commerciaux et 16 élevages de basse-cour, et 59 questionnaires ont été remplis. Des échantillons biologiques provenant de six fermes (94 écouvillons cloacaux en double) ont dû être éliminés à cause d'une contamination.

Analyse descriptive de la population étudiée pour l'étude transversal

Au total, 37 exploitations commerciales et 586 échantillons de volailles (9 à 22 oiseaux par exploitation) ont été incluses dans l'étude transversale visant à étudier la prévalence de *Chlamydiaceae* chez les volailles et les facteurs de risque associés. Dans les fermes commerciales, nous avons décidé d'échantillonner les poules pondeuses et les poulets de chair, car ce sont le type de volailles le plus couramment élevées au niveau national. Sur les 37 fermes commerciales incluses dans l'étude, 14 étaient des fermes avec de bâtiments à ventilation dynamique (milieu fermé) et 23 des fermes avec de bâtiments à ventilation statique (milieu semi-ouvert). Les fermes échantillonnées étaient situées dans des zones tempérées, sèches ou tropicales, ayant une altitude allant de 0 à 2 250 m au-dessus du niveau de la mer.

En ce qui concerne les basses-cours, 16 fermes ont été choisies, en cherchant à en inclure différents types selon les espèces élevées : uniquement des élevages de poulets, uniquement des élevages de dindes, et des fermes avec différentes espèces d'oiseaux. Il n'y a eu aucun refus de participer à l'étude. Au total, 293 échantillons ont été collectés (10 à 20 oiseaux par ferme). Tous les éleveurs de basse-cour et les vétérinaires contactés ont accepté de participer à l'étude.

Dans les fermes commerciales, la seule espèce échantillonnée était le poulet (*Gallus gallus*) de races/souches commerciales. Les poules pondeuses étaient logées dans des cages en batterie et les poulets de chair étaient élevés au sol dans un système de litière profonde (la litière s'étendait uniformément sur le sol jusqu'à une profondeur de 2 à 5 cm). Les litières déclarées dans les 28 élevages de poulets de chair étaient la paille de riz (21,5%), la paille de café (42,8%) et la paille coupée (35,7%).

Dans les élevages de basse-cour, les poulets et les dindes (*Meleagris gallopavo*) ont été les principales espèces échantillonnées. Deux fermes de basse-cour abritaient des races spécialisées de coqs de combat et dans une ferme, la seule espèce élevée était la dinde. Des canards domestiques (*Anas platyrhynchos*), des cailles

(*Coturnix coturnix*) et des faisans (*Phasianidae*) ont également été échantillonnés dans trois élevages de basse-cour. La coexistence des volailles avec d'autres espèces domestiques telles que les bovins, les ovins, les caprins ou les porcins a été observée dans huit des seize élevages de basse-cour échantillonnés.

En ce qui concerne le statut sanitaire des volailles, des signes cliniques respiratoires ont été observés dans six des 16 élevages de basse-cour. Les signes observés comprenaient des plumes ébouriffées, une dyspnée, un écoulement larmoyant ou purulent des yeux et des narines, des éternuements et un gonflement léger à grave du visage et de la région périorbitaire. Tous les élevages de volailles commerciales étaient apparemment en bonne santé au moment de l'échantillonnage.

L'utilisation d'antibiotiques a été signalée dans 28 des 37 fermes commerciales (75,7 %). L'utilisation d'antibiotiques comme traitement thérapeutique a été signalée dans 8 des 16 fermes de basse-cour. Les antibiotiques n'étaient utilisés comme stimulateurs de croissance dans aucune ferme. Les pratiques de biosécurité n'étaient mises en œuvre que dans les exploitations agricoles commerciales. Les aliments pour animaux dans les basses-cours étaient principalement un mélange d'aliments industrialisés et d'aliments faits maison. Un plan de gestion de la mortalité a été signalé dans neuf des seize exploitations agricoles de basse-cour : l'enfouissement était la principale méthode d'élimination ; sinon, les volailles mortes étaient éliminées avec les ordures ménagères.

Estimation de la prévalence apparente

Sur les 879 oiseaux analysés, 104 ont été considérés comme positifs à la *Chlamydiaceae* par rt-PCR. La prévalence animale apparente était de 0,4 % dans les exploitations commerciales avec des bâtiments à ventilation dynamique, de 5,4 % dans les exploitations commerciales avec bâtiments à ventilation statique et de 28,7 % dans les élevages de basse-cour. Ces valeurs de prévalence apparente étaient significativement différentes ($p<0,0001$). Les valeurs de la prévalence apparente entre les exploitations étaient également significativement différentes ($p=0,03$) : 7,1 % pour les exploitations commerciales à environnement contrôlé, 26,1 % pour les exploitations commerciales à ciel ouvert et 75 % pour les exploitations de basse-cour.

Un seul poulet de chair a été testé positif dans la seule ferme commerciale avec des bâtiments à ventilation dynamique. La prévalence apparente au sein de l'exploitation variait de 5 à 55 % dans les exploitations commerciales et de 5 à 80 % dans les exploitations de basse-cour. Aucune volaille n'a été testée positivement dans l'élevage de basse-cour avec uniquement de dindes et dans l'élevage de basse-cour avec uniquement de mâles (coqs de combat). Toutes les espèces de volailles échantillonnées qui n'étaient pas des poulets ont été testées négatives (4 faisans, 4 cailles et 15 canards).

Résultats sur les *Chlamydiaceae* par rt-PCR

Étude transversale

En termes de charges d'excrétion de *Chlamydiaceae*, les valeurs de Cq par rt-PCR dans les exploitations commerciales avec des bâtiments à ventilation statique (milieu semi-ouverts) allaient de 27,3 à 39,9, et de 21,4 à 38,2 dans les exploitations de basse-cour. Des volailles positives avec des charges d'excrétion élevées (Cq<28) ont été détectées dans une ferme avec des bâtiments à ventilation statique accueillant des pondeuses âgées de 40 semaines. Des charges d'excrétion élevées ont également été détectées dans quatre basses-cours accueillant plusieurs espèces (Cq<29).

Suivi hebdomadaire de l'excrétion des *Chlamydiaceae*

En raison des ressources disponibles, seul un troupeau de poulets de chair des deux troupeaux surveillés hebdomadairement a été analysé. Un seul poulet de chair dans le troupeau analysé excrétait des Chlamydiacées à la deuxième semaine du processus de reproduction avec un faible niveau d'excrétion (Cq 39.6).

Identification des espèces de *Chlamydiaceae*

Aucun ADN de *C. psittaci* n'a été détecté. *C. gallinacea* était la seule espèce de *Chlamydia* trouvée. A noter que certaines volailles étaient *Chlamydiaceae*-positives mais *C. gallinacea* négative (avec des valeurs de Cq de *Chlamydiaceae* rt-PCR supérieures à 36).

Analyse des facteurs de risque

Dans l'analyse multivariable pour les volailles commerciales, seul le type de production était significativement associé à la présence de *Chlamydiaceae*. Les élevages de poules pondeuses avaient 6,7 fois plus de chances d'être infectés que les élevages de poulets de chair (OR=6,7 [95% CI : 1,1 - 44,3], p=0,04).

Deux facteurs de risque potentiels significatifs ont été associés à l'infection à *Chlamydia* : le manque d'utilisation d'antibiotiques (OR=8,4 [95% CI : 1,84 - 38,49, p=0,006]) et un état de santé dégradé (OR=8,8 [95% CI : 1,9 - 38,9, p=0,004]).

Caractérisation des pratiques de biosécurité dans les exploitations avicoles commerciales

Cette analyse a été réalisée à partir des informations recueillies dans les 43 exploitations avicoles commerciales visitées.

Analyse des correspondances multiples

Sur les 50 variables générées à partir du questionnaire, 19 ont été retenues pour l'analyse ACM, dont sept décrivaient les caractéristiques des exploitations, six les pratiques de gestion et cinq les mesures de biosécurité adoptées dans les exploitations. Les 31 variables restantes ont été rejetées pour les raisons suivantes : homogénéité de la réponse des personnes interrogées (13), variables permettant d'identifier l'exploitation et décrire son emplacement (4), variables binaires pour lesquelles 5 % ou moins des personnes interrogées ont donné la même réponse (3), variables qui ont été transformées en une nouvelle variable (4) et faible pertinence des informations obtenues (7). Le type de bâtiments et le type de production ont été introduits comme variables

supplémentaires (ou illustratives) dans l'analyse, ce qui signifie qu'elles n'ont eu aucune influence sur la construction des dimensions mais elles ont aidé à l'interprétation des résultats.

L'AMC a été réalisée en conservant les cinq premières dimensions couvrant 80,8% de la variance des données, aucune des autres dimensions n'expliquant plus de 5% de la variance des données. Les valeurs propres obtenues à partir du tableau de Burt ont montré que trois dimensions couvraient déjà 89,1% de la variance des données, tandis que les autres dimensions expliquaient <5%.

Les variables les plus significativement liées à la construction de la première dimension (p<0,001) étaient : (i) la stratégie d'élimination de la mortalité (R^2=0,67) ; (ii) l'utilisation de dérivés de l'acide phosphonique comme traitement antimicrobien (R^2=0,66), et (iii) l'utilisation de vêtements de travail exclusifs par le personnel et les visiteurs (R^2=0,52). Pour la deuxième dimension, les variables les plus significativement liées à sa construction (p<0,001) étaient : (i) l'utilisation d'équipements de protection individuelle par le personnel et les visiteurs (par exemple, masques faciaux, charlottes et protection des yeux) (R^2=0,82), (ii) l'exigence d'un protocole d'hygiène pour le personnel et les visiteurs avant et après leur entrée dans l'exploitation (R^2=0,53), (iii) et l'utilisation de quinolones comme traitement antimicrobien (R^2=0,51).

Classification ascendante hiérarchique

En tenant compte de la perte relative d'inertie la plus élevée au sein d'un groupe, la partition consolidée du dendrogramme hiérarchique a mis en évidence trois groupes. Les pratiques de biosécurité les plus significativement liées à la partition en clusters (p<0,001) étaient : (i) l'utilisation d'équipements de protection individuelle par le personnel et les visiteurs (par exemple, masques faciaux, charlottes et protection des yeux) ; (ii) protocole d'hygiène obligatoire pour le personnel et les visiteurs avant et après l'entrée dans l'exploitation ; (iii) utilisation par le personnel et les visiteurs de vêtements de travail exclusifs, (iv) présence d'un pédiluve à l'entrée de chaque bâtiment d'élevage et (v) méthode d'élimination de la mortalité. D'autres variables ont contribué à la caractérisation de chacun des trois groupes avec des valeurs de p < 0,05.

L'utilisation d'antimicrobiens comme stimulateurs de croissance n'a été signalée dans aucune exploitation agricole. L'utilisation d'antimicrobiens était plus répandue dans les exploitations appartenant aux groupes 1 et 3, avec respectivement 100 % (n=12/12) et 85 % (n=11/13), tandis que seulement 45 % (n=8/18) des exploitations du groupe 2 ont signalé leur utilisation. Quatre classes d'antimicrobiens ont été signalées comme étant utilisées dans les exploitations, par ordre décroissant : les dérivés de l'acide phosphonique (n=15/31), les tétracyclines (n=13/31), les macrolides (n=11/31) et les quinolones (n=9/31). Dans certaines exploitations, l'utilisation de plus d'une classe d'antimicrobiens a été signalée : trois dans le cluster 1, six dans le cluster 2 et un dans le cluster 3.

L'utilisation ou la non-utilisation de certaines classes d'antimicrobiens dans les exploitations agricoles a été associée de manière significative aux exploitations de chaque groupe. L'antimicrobien le plus utilisé dans

toutes les exploitations du cluster 1 (n=12/12) était un dérivé de l'acide phosphonique (fosfomycine). En outre, trois de ces exploitations ont également signalé l'utilisation de tylosine, un antibiotique macrolide. Ainsi, l'interaction potentielle de la fosfomycine et de la tylosine dans ces 3 fermes était possible. L'absence d'utilisation de tétracyclines et de quinolones a été associée de manière significative aux exploitations appartenant au cluster 1. Les quinolones étaient significativement associées aux exploitations utilisant des antimicrobiens (n=8) au sein du cluster 2. Dans six de ces exploitations, des tétracyclines et des macrolides ont également été administrés. Ainsi, l'interaction potentielle des tétracyclines, des quinolones et des macrolides dans les troupeaux de ces six exploitations était possible. À l'inverse, l'absence d'utilisation de dérivés d'acide phosphonique comme antimicrobiens a été associée de manière significative aux exploitations appartenant à ce cluster.

Discussion du premier chapitre

Chlamydia gallinacea, était la seule espèce de chlamydia identifiée dans cette étude. Ce résultat est cohérent avec les études précédentes montrant que cette espèce est endémique chez les poulets (Donati et al., 2018; Guo et al., 2016; Heijne et al., 2018; Hulin et al., 2015; Szymańska-Czerwińska et al., 2017; Taylor-Brown and Polkinghorne, 2017).

Les valeurs de prévalence obtenues dans les exploitations commerciales de notre étude (7,1 % dans les exploitations commerciales avec des bâtiments à ventilation dynamique et 26,1 % dans les exploitations commerciales avec des bâtiments à ventilation statique) étaient comparables aux valeurs de prévalence entre fermes trouvées dans les exploitations avicoles commerciales polonaises (15,9 %) et néerlandaises (47 %) (Heijne et al., 2018; Szymańska-Czerwińska et al., 2017). Néanmoins, la prévalence globale entre les exploitations de basse-cour (75 %) était plus élevée que celle rapportée dans les études menées aux États-Unis (12,4 %, n=66/531), en Italie (15 %, n=24/160) et en Chine (16,7 %, n=384/2 300) (Donati et al., 2018; Guo et al., 2016; Li et al., 2017).

Un statut sanitaire dégradé s'est avéré être un facteur de risque associé à la présence de Chlamydiaceae chez les volailles de basse-cour. Cependant, *C. gallinacea* a également été détectée dans des élevages de basse-cour où les volailles étaient cliniquement saines. Ceci est conforme aux résultats d'études précédentes dans lesquelles aucune maladie clinique n'était directement associée à l'infection par *C. gallinacea* (Donati et al., 2018; Guo et al., 2016; Heijne et al., 2018; Taylor-Brown and Polkinghorne, 2017). Néanmoins, Guo *et al.* (2016) ont pu montrer la présence de cette bactérie dans des prélèvements oropharyngés et cloacaux, ainsi que dans le sang, les poumons, le cœur, le foie, la trachée, les reins, le pancréas et la rate de poulets naturellement infectés, ce qui suggère que cette bactérie n'est pas seulement un commensal, mais aussi un agent pathogène de virulence modérée (Guo et al., 2016).

L'absence d'utilisation d'antibiotiques comme traitement thérapeutique a été associée de manière significative à la présence de *Chlamydiaceae* dans les basses-cours. Il a été signalé que quelques antibiotiques

(principalement les oxytetracyclines) peuvent éliminer les infections à *C. psittaci* et *C. gallinacea* (Gaede et al., 2008). L'utilisation d'antibiotiques dans la plupart des fermes commerciales échantillonnées dans le cadre de notre étude peut également expliquer la proportion plus faible de *Chlamydia* chez les volailles dans les fermes commerciales.

Le type de production a été le seul facteur de risque associé à la présence de Chlamydiaceae dans les fermes commerciales. Les élevages de poules pondeuses étaient 6,6 fois plus exposées au risque d'infection que les élevagesde poulets de chair (p=0,04). Ce facteur de risque pourrait s'expliquer par l'exposition plus longue des poules pondeuses ou par des pratiques particulières de gestion/biosécurité dans les élevages de poules pondeuses. Curieusement, même si l'âge de la bande n'a pas été considéré comme un facteur de risque, le seul troupeau commercial de volailles ayant une charge d'excrétion élevée de *Chlamydiaceae* était une bande de poules pondeuses de 40 semaines (avec des oiseaux atteignant une valeur Cq de 24,4). Cet âge se situe dans la catégorie d'âge pour laquelle l'étude menée dans les élevages de poules pondeuses aux Pays-Bas a révélé un risque plus élevé d'infection par *C. gallinaceae* (Heijne et al., 2018).

Le fait que seul le type de production a été identifié comme facteur de risque associé à la présence de *Chlamydiaceae* dans les élevages commerciaux et qu'aucune variable concernant la biosécurité n'ait pas pu être exploitée lors de l'analyse des facteurs de risque, nous a incité à effectuer une analyse multivariée des pratiques de gestion et de biosécurité des exploitations. Cette analyse nous a permis de mieux caractériser les pratiques de biosécurité qui pourraient exposer les poules pondeuses à la présence de *Chlamydia* et d'autres agents pathogènes aviaires, comme l'a montré l'épidémie de virus de l'influenza aviaire hautement pathogène (IAHP) de sous-type H7N3 qui s'est produite pour la première fois en 2012 dans des exploitations avicoles commerciales au Mexique (Kapczynski et al., 2013). Ce premier foyer a été détecté dans des élevages de poules pondeuses dans une région à forte densité de volailles, puis il s'est propagé en quelques mois aux poulets de chair, aux reproducteurs et aux élevages de basse-cour (Lu et al., 2014).

L' étude sur la biosécurité a donné un aperçu des pratiques critiques de biosécurité dans les exploitations avicoles qui risquent le plus de ne pas être mises en place ou d'être enfreintes (si elles ont été mises en place). Des variations significatives dans l'application des pratiques de biosécurité ont été observées dans les groupes d'exploitations avicoles identifiés dans notre étude. Cette conclusion est conforme aux résultats d'études précédentes qui montrent que l'application des mesures de biosécurité dans les exploitations avicoles tend à être variable et peut souvent être intermittente (Racicot et al., 2011; Tanquilut et al., 2020), que ce soit dans les élevages de poulets de chair (Gibbens et al., 2001; Gifford et al., 1987; Tablante et al., 2008) ou dans les élevages de poules pondeuses (Durr et al., 2016; Lestari et al., 2011; Ssematimba et al., 2013). Nous avons mené une analyse exploratoire multidimensionnelle en considérant que l'évaluation des pratiques de biosécurité est mesurée par un grand nombre de variables. Comme un grand nombre de ces variables peuvent être corrélées, cette méthodologie permet de découvrir les relations entre les variables catégorielles au sein des exploitations et entre elles, pour finalement trouver des profils (Husson et al., 2016; Sourial et al., 2010).

L'analyse de regroupement hiérarchique effectuée par la suite nous a permis de regrouper objectivement les exploitations en fonction de ces profils précédemment identifiés. Cette approche a été adoptée au lieu de décrire les pratiques de biosécurité mises en œuvre en fonction de caractéristiques spécifiques des exploitations, telles que le degré de confinement (bâtiments à ventilation dynamique vs bâtiments à ventilation statique), la taille de l'exploitation ou l'objectif de l'exploitation (poulets de chair ou pondeuses).

Cinq pratiques de biosécurité ont été identifiées comme étant les plus significativement associées à la classification des exploitations agricoles en trois clusters. Trois de ces pratiques étaient liées à des mesures concernant directement le personnel ou les visiteurs (utilisation appropriée d'équipements de protection individuelle, protocole d'hygiène avant et après l'entrée dans l'exploitation, utilisation de vêtements de travail exclusifs), tandis que les deux dernières étaient liées aux installations générales de l'exploitation (présence d'un pédiluve à l'entrée des bâtiments) et aux méthodes d'élimination de la mortalité des volailles. Des études antérieures ont établi que la mise en œuvre et le respect des mesures de biosécurité concernant le personnel sont essentiels pour prévenir la transmission d'agents pathogènes dans une bande (Gifford et al., 1987; Nespeca et al., 1997; Racicot et al., 2011; Ssematimba et al., 2013; Tablante et al., 2008; Volkova et al., 2012).

Aucune utilisation d'antimicrobiens pour stimuler la croissance n'a été signalée dans aucune exploitation agricole, ce qui est conforme aux mesures nationales et internationales mises en œuvre pour prévenir la résistance aux antimicrobiens (Maron et al., 2013). Quatre classes d'antimicrobiens ont été signalées comme étant utilisées pour le traitement dans 31 des 43 exploitations visitées : tétracyclines, quinolones, macrolides et dérivés de l'acide phosphonique. Selon la liste des agents antimicrobiens d'importance vétérinaire publiée par l'OIE, les classes d'antimicrobiens utilisées dans les exploitations incluses dans notre étude sont approuvées pour une utilisation chez les animaux destinés à la consommation (OIE, 2019). Au Mexique, il existe une liste d'antimicrobiens autorisés en médecine vétérinaire depuis 2012 (SAGARPA, 2012). Toutefois, cette liste ne classe pas les classes d'antimicrobiens en fonction du risque pour la santé publique que représente leur utilisation chez les animaux, laissant le choix thérapeutique à la discrétion du vétérinaire qui apporte un soutien technique à l'exploitation.

La proportion d'exploitations utilisant des antimicrobiens diffère selon les groupes ; plus les mesures de biosécurité sont strictes dans les exploitations d'un même groupe, moins il y a d'exploitations qui utilisent des antimicrobiens. Plus précisément, les antibiotiques n'ont été utilisés que dans 45 % des exploitations du groupe 2, contre 85 % dans le groupe 3, et même 100 % dans le groupe 1. En outre, nous avons constaté que plus il y avait d'infractions aux pratiques de biosécurité dans les exploitations, plus il était probable d'observer l'utilisation d'antibiotiques essentiels pour la santé humaine. À titre d'exemple, la fosfomycine était l'antibiotique le plus utilisé dans les exploitations avicoles ayant signalé l'utilisation d'antimicrobiens dans notre étude. Son utilisation était très répandue dans les exploitations des groupes 1 et 3, les deux groupes d'exploitations où des mesures de biosécurité moins strictes étaient appliquées, tandis que l'absence d'utilisation de la fosfomycine était significativement associée aux exploitations du groupe 2.

CHAPITRE II. ESSAIS EXPERIMENTAUX POUR COMPARER DES CARACTERISTIQUES DE CROISSANCE DE *CHLAMYDIA GALLINACEA*

Plusieurs études ont suggéré que les conditions qui favorisent la croissance in vitro d'une espèce/un spécimen de chlamydia semblent ne pas être applicables aux autres. Malgré les améliorations apportées au protocole de croissance de la chlamydia visant à augmenter le taux d'infectiosité et à favoriser la croissance in vitro, la propagation de certaines souches/espèces de chlamydia reste difficile : par exemple, les souches de *C. pecorum* provenant du tractus intestinal des ruminants (Sachse et al., 2009), souches de *C. suis* (Schiller et al., 2004), et souches de *C. pneumoniae* (Kuo and Grayston, 1988; Wong et al., 1992). Les difficultés sont décrites comme des restrictions soit dans la culture d'une souche à partir d'un spécimen, soit dans la propagation d'une souche lors des passages, après un premier isolement réussi.

De telles difficultés sont rencontrées pour *C. gallinacea*, même si peu d'études ont jusqu'à présent porté sur la culture in vitro de cette nouvelle espèce. Au cours des études menées pour caractériser en profondeur les isolats de *C. gallinacea*, il a été observé que ces isolats perdaient progressivement leur infectivité par les passages ultérieurs dans les embryons de poulet ou dans les cellules BGM ; plus les passages sont nombreux, moins les bactéries se développent (résultats non publiés). Des observations similaires ont été faites dans d'autres laboratoires de recherche (Dr Marloes Heijne de l'Université de Wageningen et Dr Daisy Vanrompay de l'Université de Ghent, communications personnelles).

Compte tenu du fait que la croissance in vitro de *C. gallinacea* a tendance à être fastidieuse, et afin d'effectuer une caractérisation génétique plus poussée permettant de répondre aux inconnues qui entourent encore cette espèce de *Chlamydia*, l'étude présentée dans ce deuxième chapitre visait (i) à comparer les caractéristiques de croissance de la souche de référence *C. gallinacea* 08-1274/3 (08DC63) selon trois protocoles expérimentaux d'infection différents, et (ii) à mettre en œuvre un protocole alternatif proposé pour cultiver des spécimens de *C. gallinacea* positifs sur le terrain.

Matériels et méthodes

Souches et spécimens de *Chlamydia*

Pour réaliser les protocoles d'infection expérimentaux, nous avons utilisé la souche 08-1274/3 de *C. gallinacea*, isolée à partir d'un écouvillon cloacal prélevé sur un poulet ne présentant aucun signe clinique de maladie dans un élevage de volailles en France (Laroucau et al., 2009).

Le protocole retenu a été mis en œuvre sur huit spécimens de terrain de *C. gallinacea* positifs. Des spécimens du Mexique ont été collectés au cours de l'étude présentée au chapitre I. Des spécimens d'Italie ont été envoyés par l'Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta dans le cadre d'un projet de collaboration visant à optimiser la croissance de *C. gallinacea* en culture cellulaire.

Systèmes et milieux de culture cellulaire

La lignée cellulaire utilisée dans cette étude comme référence pour la croissance de la chlamydia était la BGM (Buffalo Green Monkey epithelial kidney cells). Deux autres lignées cellulaires ont été testées pour comparer la croissance de C. gallinacea : DF-1 et Caco-2. Chaque système de culture cellulaire a nécessité un milieu de culture différent, complété par différents composants. Toutes les lignées cellulaires ont été testées pour confirmer l'absence de *Mycoplasma* spp.

Pour réaliser les infections à chlamydia, les cellules ont été ensemencées dans des lamelles de verre rondes de 12 mm contenues dans des récipients en polystyrène bijou (Sterilin, Thermo Scientific™ 129AX/1, France), et cultivées de manière synchrone jusqu'à confluence le jour de l'inoculation dans les conditions et les milieux de croissance indiqués pour chaque lignée cellulaire.

Infections expérimentales utilisant la souche de référence 08-1274/3 (08DC63)

Pour préparer les inocula, les cryotubes originaux contenant les cellules infectées par l'une des deux souches de référence, ou les tubes contenant des spécimens de terrain (tous conservés à -80°C), ont été rapidement décongelés à 37°C puis soniqués. Pour les souches de référence, des dilutions décuplées de chaque souche ont été effectuées dans un milieu SPG. Pour chaque souche de référence, il y avait au total quatre inocula, c'est-à-dire la souche non diluée, la dilution 10^{-3}, la dilution 10^{-4} et la dilution 10^{-5}.

Trois protocoles d'infection expérimentaux différents ont été mis en œuvre pour comparer la croissance de *C. gallinacea*, appelés protocoles d'infection expérimentaux 1 à 3 (E1 à E3). Dans chaque protocole expérimental d'infection, différents paramètres ont été comparés : système de culture cellulaire (E1), température d'incubation (E2) et infections simples contre infections quotidiennes assistées par centrifugation (E3).

Après inoculation, les monocouches cellulaires ont été soumises au protocole de croissance chlamydiale standard décrit par Sachse et al., (2003). Pour chaque protocole d'infection expérimental, le protocole standard de croissance de la chlamydia a été applique dans un groupe de cellules infectées tandis que dans l'autre groupe, un paramètre spécifique a été modifié (c'est-à-dire une température d'incubation différente ou des centrifugations quotidiennes).
Au bout de 48 h ou 72 h pi, une lamelle avec des cellules infectées a été fixée avec du méthanol, et colorée par immunofluorescence directe. Pour certains protocoles d'infection expérimentaux, jusqu'à deux passages des monocouches infectées ont été effectués pour analyser la croissance de *C. gallinacea* à travers le nombre de passages.

Infections avec des échantillons de *C. gallinacea* positifs à la PCR

Différents volumes de spécimens décongelés et soniqués, provenant du Mexique et de l'Italie, ont été inoculés sur des tapis cellulaires confluents.

Après inoculation, les monocouches cellulaires ont été soumises à l'un des deux protocoles de croissance de la chlamydia. Le protocole de croissance chlamydiale standard décrit par Sachse et al., (2003) a été suivi pour les spécimens du Mexique, tandis qu'un protocole alternatif de croissance de chlamydia proposé, modifié d'après Donati et al., (2010) a été mis en œuvre pour les spécimens provenants d'Italie.

Entre 48 et 96 h pi, une lamelle avec des cellules inoculées avec 100 µl de chaque échantillon a été fixée avec du méthanol et colorée par immunofluorescence directe. Pour certains isolats, jusqu'à quatre passages des monocouches infectées ont été effectués pour analyser la croissance de *C. gallinacea* à au cours des différents passages.

Résultats et discussion

Optimisation du protocole de croissance de *C. gallinacea*

Dans l'ensemble, les inclusions chlamydiales typiques étaient rarement visibles. Par conséquent, la croissance de *C. gallinacea* n'a pas pu être évalué quantitativement en utilisant les approches classiques, c'est-à-dire le nombre et la taille des inclusions telles que présentées couramment dans la littérature.

Comparaison de la croissance de *C. gallinacea* dans trois systèmes de culture cellulaire différents (E1)

Les immunocolorations effectuées à 48 h pi des trois lignées cellulaires (DF-1, Caco-2 et BGM) infectées par la souche de référence non diluée de *C. gallinacea* et les trois dilutions (10^{-3}, 10^{-4} et 10^{-5}), ont montré que toutes les lignées cellulaires permettaient la croissance de *C. gallinacea*. D'innombrables grandes inclusions chlamydiales typiques, bien délimitées, d'un vert vif, occupant presque tout le cytoplasme cellulaire, ont été observées dans des cellules infectées par la souche de référence non diluée de *C. gallinacea* (7,3 x 10^7 IFU/mL) à 48 h pi. Cependant, seules quelques structures chlamydiales ressemblant à des inclusions ont été observées dans les trois lignées cellulaires (DF-1, Caco-2 et BGM) infectées par la dilution 10^{-3} de la souche de référence à 48 h pi. Aucune inclusion de *Chlamydia* n'a été observée dans les cellules infectées par les dilutions 10^{-4} et 10^{-5} de la souche de référence de *C. gallinacea*.

En ce qui concerne la taille des inclusions de *Chlamydia* dans les trois lignées cellulaires, de légères différences ont été observées uniquement pour la première inoculation de la souche non diluée. Les inclusions de *C. gallinacea* dans Caco-2 semblent être plus importantes que celles observées dans les deux autres lignées cellulaires (les tailles réelles n'ont pas été mesurées), comme l'ont montré des études antérieures réalisées à l'aide de cette lignée cellulaire pour l'infection à *C. suis* (De Puysseleyr et al., 2017; Schiller et al., 2004), et l'infection à *C. trachomatis* (Lantos et al., 2018).

Dans nos conditions expérimentales, les cellules BGM semblent être plus résistantes que les DF-1 et les Caco- 2, car elles restent viables plus longtemps sans changer de milieu de culture une fois qu'elles ont été infectées. C'est pourquoi nous avons décidé de conserver la lignée cellulaire BGM pour les autres protocoles d'infection.

Comparaison de la croissance de *C. gallinacea* à deux températures d'incubation différentes (E2)

Comme il a été suggéré que certaines espèces de *Chlamydia* pourraient mieux se développer à des températures similaires à celles que l'on peut trouver chez leurs hôtes naturels, nous avons décidé d'étudier l'impact de la température d'incubation sur la croissance de *C. gallinacea*. En effet, la formation d'inclusions matures de *C. poikilothermis*, une espèce de chlamydia obtenue à partir de serpents (un animal poikilothermique), a été améliorée à 28°C plutôt qu'à 37°C, la température standard pour la croissance des *Chlamydia* (Onorini et al., 2019). Comme la température naturelle des oiseaux se situe entre 38,5° et 43°C (Prinzinger et al., 1991), nous avons décidé de réaliser un test avec une incubation à 39°C afin de ne pas dépasser la température limite fixée pour les cellules BGM (Barron et al., 1970). Aucune différence de croissance de *C. gallinacea* n'a été observée entre les deux températures différentes (37° contre 39°C) pendant la durée évaluée de l'infection (48 h contre 72 h contre 96 h). Cependant, comme nous n'avons pas mesuré quantitativement la croissance de la chlamydia, nous ne pouvons pas conclure avec certitude sur l'impact de la température sur la croissance de *C. gallinacea*.

Comparaison de la croissance de *C. gallinacea* en utilisant des infections simples ou quotidiennes assistées par centrifugation (E3)

Le mécanisme par lequel l'utilisation des infections assistées par centrifugation pourrait favoriser la croissance in vitro de la chlamydia n'est pas bien élucidé. On a émis l'hypothèse que la centrifugation pourrait produire des modifications structurelles de la surface cellulaire qui favoriseraiennt certaines voies d'entrée des cellules, mais aussi qu'elle favoriserait la fixation des corps élémentaires chlamydiens aux cellules hôtes, ce qui augmenterait les infections productives (Allan and Pearce, 1979; Prain and Pearce, 1985; Weiss and Dressler, 1960), Les deux théories ne s'excluent pas mutuellement et peuvent se produire simultanément.

D'innombrables structures chlamydiales vert clair, de taille irrégulière, ressemblant à des inclusions, ont été observées dans le cytoplasme des cellules infectées. Aucune différence de croissance de *C. gallinacea* n'a été observée entre les deux protocoles d'infection assistée par centrifugation au cours de l'infection (jusqu'à 168 h). Les infections assistées par centrifugation ont été incluses au fil des ans en tant que technique facile à mettre en œuvre en laboratoire pour renforcer les infections à chlamydia en culture cellulaire. En considérant qu'aucun impact négatif d'une centrifugation quotidienne n'a été observé, nous avons décidé de conserver cette étape supplémentaire.

Application de la norme et d'un protocole alternatif de croissance de la chlamydia aux échantillons de terrain positifs pour *C. gallinacea*

Des échantillons de *C. gallinacea* positifs provenant d'écouvillons cloacaux de poulets du Mexique ont été soumis à un isolement en culture cellulaire en utilisant le protocole standard de croissance chlamydiale. Les premières immunocolorations effectuées entre 48 et 72 h pi ont révélé d'innombrables structures chlamydiales vert clair, de taille irrégulière et ressemblant à des inclusions, dans le cytoplasme des cellules infectées de tous les spécimens mexicains. Lors du 1er passage de tous les isolats, l'immunocoloration à 48 h n'a montré aucune

amélioration des structures de type inclusion chlamydiale pour aucun d'entre eux. Au contraire, les structures chlamydiales à inclusion ont eu tendance à diminuer en taille et en nombre.

Mise en œuvre d'un protocole alternatif de croissance de la chlamydia pour les spécimens de terrain provenant d'Italie

Suite aux essais d'optimisation réalisés afin d'améliorer la croissance de *C. gallinacea*, nous avons décidé de conserver (i) les cellules BGM comme lignée cellulaire de référence, (ii) une température d'incubation de 37°C une fois les cellules infectées, et (iii) d'effectuer des centrifugations quotidiennes pour favoriser le taux d'infectiosité de l'inoculum. En outre, nous avons décidé de tester un nouveau milieu d'infection à *Chlamydia* qui avait été référencé dans la littérature (Donati et al., 2010). Cet ensemble de paramètres a été utilisé dans un protocole alternatif pour la croissance de chlamydia. Ce protocole alternatif proposé a été appliqué à quatre spécimens de *C. gallinacea* positifs provenant d'écouvillonnages cloacaux de poulets d'Italie.

De façon remarquable, dès le premier passage, un des quatre isolats d'Italie (60260-3) a montré une évolution distinctive de la croissance de la chlamydia ; peu d'inclusions chlamydiales de taille moyenne, bien délimitées et d'un vert brillant ont été observées à 48 h pi. Pour le deuxième passage de cet isolat, plusieurs grandes inclusions chlamydiales typiques, bien délimitées, vert vif, occupant presque tout le cytoplasme cellulaire, ont été observées à 48 h pi. Comme la croissance de l'isolat 60260-3 de *C. gallinacea* était exceptionnelle, nous avons confirmé par PCR spécifique à l'espèce (i) qu'il s'agissait bien d'un isolat de *C. gallinacea* et (ii) qu'aucun *C. psittaci* n'était présent dans le spécimen.

Il convient de mentionner que l'isolat 60260-3 provenant d'Italie a présenté une contamination initiale détectée dans les premières 24 h pi. La contamination était principalement fongique (structures ressemblant à des champignons, de couleur blanche comme les mycéliums) mais aussi par d'autres bactéries. Après avoir effectué les passages et renouvelé toutes les 24 h le milieu d'infection à *Chlamydia* (avec des antibiotiques et des antifongiques), la contamination bactérienne a disparu mais le champignon est resté jusqu'au deuxième passage. Une concentration quatre fois supérieure d'antifongiques ajoutés au milieu d'infection à *Chlamydia* (10 µg/ml d'amphotéricine B et 100 U/ml de nystatine) était insuffisante pour contrôler sa prolifération.

Le surnageant du deuxième passage de l'isolat 60260-3 d'Italie a été prélevé et cultivé sur une gélose au sang. La culture pure du champignon a été soumise à une identification par le service de désorption-ionisation laser assistée par matrice (MALDI-TOF) de l'École nationale vétérinaire d'Alfort. Le champignon a été identifié comme étant *Trichosporon asahii*. Le séquençage de la prochaine génération a révélé que jusqu'à 125 genres de champignons pouvaient être trouvés dans le tractus gastro-intestinal des poulets, cependant, *Microascus* sp., *Trichosporon* spp. et *Aspergillus* spp. représentent plus de 80% de la diversité totale de la population fongique, *Trichosporon asahii* étant l'espèce prédominante parmi les *Trichosporon* genus (Robinson et al., 2020). Dans notre étude, la présence de ce champignon dans la culture de cet isolat de *C. gallinacea* à

croissance atypique, soulève la question de l'impact que ce champignon pourrait avoir sur la croissance de cet isolat.

Notre étude visant à améliorer la croissance de *C. gallinacea* présente plusieurs limites. Premièrement, comme les inclusions typiques de chlamydia n'ont été que rarement observées dans nos infections expérimentales, la croissance de *C. gallinacea* n'a pas pu être mesurée quantitativement à l'aide des approches classiques (c'est-à-dire le nombre et la taille des inclusions). Enfin, étant donné la limitation de notre plan d'étude, consistant en l'impossibilité de lancer toutes les infections expérimentales avec le même inoculum (c'est-à-dire des aliquotes de la même dilution de la souche de référence), les résultats de E2 et E3 ne pouvaient pas être reproduits, comme nous l'avons fait pour les deux inoculums expérimentaux dérivés de E1.

Conclusions générales

Nous avons montré que *C. gallinacea* est présent à la fois chez les volailles commerciales et de basse-cour au Mexique ; par conséquent, le diagnostic de la *Chlamydiaceae* chez les volailles ne doit plus être négligé. Son association avec les poules pondeuses des exploitations commerciales souligne la nécessité d'approfondir les recherches concernant son impact sur la qualité des œufs, les performances de production, ainsi que sa pathogénicité potentielle. Fait remarquable, *C. psittaci* n'a été détecté dans aucune des fermes mexicaines échantillonnées.

Notre étude sur la biosécurité fournit une analyse exploratoire des modèles de pratiques de biosécurité à la ferme dans les différents groupes d'exploitations avicoles du Mexique identifiés par notre analyse. Cette analyse pourrait être utile aux vétérinaires de terrain ou aux éleveurs pour comprendre comment orienter les stratégies visant à renforcer la formation du personnel, ainsi que la mise en place et le respect des pratiques de biosécurité dans les exploitations, en donnant la priorité à celles qui ont été identifiées comme essentielles dans notre analyse. Cette étude offre également des informations caractérisant l'utilisation des antimicrobiens dans l'industrie avicole, et contribue ainsi au besoin national d'information sur ce sujet. Ces données peuvent aider à consolider une stratégie nationale visant à améliorer l'utilisation d’antimicrobiens et à contenir la résistance aux antimicrobiens. Nous espérons que nos résultats pourraient également être utiles à d'autres industries avicoles présentant des conditions similaires en dehors du Mexique. D'autres études sur l'efficacité des dispositions officielles publiées ces dernières années devraient être menées, afin de suivre l'évolution des pratiques de biosécurité dans les exploitations et l'utilisation des antimicrobiens dans le secteur avicole mexicain.

Les différents protocoles expérimentaux d'infection testés dans cette étude pour optimiser la croissance de *C. gallinacea* n'ont pas permis d'obtenir un protocole optimisé pour la culture de cette espèce. De même, les deux protocoles appliqués aux spécimens de terrain n'ont pas permis une propagation adéquate de ces isolats pour produire des quantités suffisantes pour les étudier plus en détail, à l'exception d'une souche. En effet, nous avons constaté qu'il existait des différences individuelles dans la croissance des spécimens de *C. gallinacea*

positifs. Ces différences ne sont pas liées à la charge de *Chlamydia* dans l'échantillon, ni à la durée et aux conditions de stockage (car les échantillons italiens ont été manipulés de manière similaire et avaient une charge bactérienne et durée de stockage similaires). Nous pensons que les différences pourraient être associées (i) à la mise en œuvre du protocole alternatif pour la croissance des *Chlamydiae*, qui aurait pu fournir les conditions optimales pour le développement d'un microenvironnement favorisant la croissance d'autres microorganismes (comme *T. asahii*), ou (ii) au fait que nous avons dû confronter une levure résistante aux antifongiques habituels, alors que pour d'autres échantillons, le cocktail antibiotique/antifongique était suffisant pour éliminer les microorganismes dès le début. *C. gallinacea*, une espèce récemment décrite, a encore plusieurs inconnues concernant sa pathogénicité, ses mécanismes de transmission, son potentiel zoonotique, etc. L'isolement des souches est encore nécessaire pour étudier la relation de la bactérie avec l'hôte, pour réaliser des études microbiologiques fondamentales, pour mettre au point des tests de diagnostic et pas seulement pour effectuer la caractérisation génétique.

Ce travail de thèse représente la base de la recherche sur les infections à *Chlamydia* chez les volailles au Mexique, tout en enrichissant la connaissance des pratiques de gestion et de biosécurité à la ferme mises en œuvre dans l'un des plus grands pays producteur de volailles.

INTRODUCTION

1. *Chlamydiae*: from environmental bacteria to animal pathogens

1.1. The phylum *Chlamydiae,* an overview

There is no way to talk about the history of *Chlamydiae* without talking about its taxonomy, or the other way around. Phylogenetic analyses based on the 16S rRNA gene have shown that *Chlamydiae* are a unique group among prokaryotes that diverged about two billion years ago (Horn et al., 2004). There is evidence that approximately 700 million years ago, the last common ancestor of the pathogenic and symbiotic *Chlamydiae* was already adapted to the intracellular lifestyle in early eukaryotes and, moreover, that they already contained many virulence factors found in modern pathogenic *Chlamydiae* (Horn et al., 2004).

Although the study of chlamydia-related diseases began in the late 18th century, it was from the 1980s onwards that ultrastructural and later molecular evidence started to emerge indicating that *Chlamydiae* were symbionts of free-living amoebae (Horn, 2008). All *Chlamydiae*, either pathogenic or environmental, share an obligate intracellular lifestyle within eukaryotic hosts, from protozoa to animals including humans. They are nonmotile, have small genomes (1–2.4 Mb) and a unique developmental cycle characterized by morphologically and physiologically distinct stages, even though with differences among the members of the phylum. These differences will be addressed in a specific section later on (Bayramova et al., 2018; Horn, 2010; Horn et al., 2004). However, the true diversity of *Chlamydiae*, their host range and their occurrence in the environment are just beginning to be discovered. Metagenomics has made a tremendous contribution leading to new species description without prior strain isolation, which still remains a restrictive step, especially for some of the newly described species (Onorini et al., 2019).

The most recent evidence of *Chlamydiae* diversity is shown in the study carried out by Dharamshi et al. (2020) in the Arctic Mid-Ocean Ridge. They found that in deep anoxic marine sediments, chlamydial lineage dominates with up to 43% of the microbial communities abundance, with a maximum diversity of 163 different species-level taxa. Thus, the current known genomic diversity of this phylum was expanded up to a third, without identifying a conceivable eukaryotic host. Undoubtedly, the most important contribution of this study was the demonstration of the high abundance and diversity of this phylum in such a setting, reinforcing the idea that maybe *Chlamydiae* could play an important ecological role and perhaps might have an alternate lifestyle strategy.

The phylum *Chlamydiae* currently comprises a single class, *Chlamydiia*, containing only one order, the *Chlamydiales* (Bayramova et al., 2018; Borel et al., 2019; Borel and Greub, 2019; Horn, 2010; Ruggiero et al., 2015). The current accepted taxonomy of the phylum *Chlamydiae* according with the Subcommittee on the Taxonomy of *Chlamydiae*, of the International Committee on Systematics of Prokaryotes (ICSP) is presented in **Figure 1**.

Super kingdom	**Prokaryota**
Kingdom	**Bacteria**
Phylum	***Chlamydiae***
Class	***Chlamydiia***
Order	***Chlamydiales***
Family	***Candidatus* Actinochlamydiaceae**
	***Candidatus* Clavichlamydiaceae**
	***Candidatus* Parilichlamydiaceae**
	***Candidatus* Piscichlamydiaceae**
	Chlamydiaceae
	Criblamydiaceae
	Parachlamydiaceae
	Rhabdochlamydiaceae
	Simkaniaceae
	Waddliaceae

Figure 1. Current accepted taxonomy of the phylum *Chlamydiae*, according with the Subcommittee on the taxonomy of *Chlamydiae*, of the International Committee on Systematics of Prokaryotes (ICSP). Adapted from (Bayramova et al., 2018; Borel et al., 2019; Borel and Greub, 2019; Horn, 2010; Ruggiero et al., 2015).

In parallel, another chlamydial genome-based phylogeny has been proposed by Parks et al., (2017), in which the taxonomy of *Chlamydiae* was remarkably expanded. This classification is available at the Genome Taxonomy Database (GTDB) (Chaumeil et al., 2019). Although the GTDB classification has several commonalities with the ICSP proposed taxonomy, there are some major differences highlighted by the Subcommittee on the Taxonomy of *Chlamydiae* (Borel et al., 2019). To solve conflicting nomenclatures and to avoid confusion in the literature, the proposal of intercommunication between both research groups is on the table. There is no doubt that the taxonomy of *Chlamydiae* will continue to evolve in the near future.

The classification of a microorganism within the order *Chlamydiales* was initially suggested by Storz and Page (1971) to group all bacteria sharing the obligate intracellular lifestyle, the absence of flagella and peptidoglycan, but above all the characteristic developmental cycle. This order includes the family *Chlamydiaceae*, that is by far the most studied family harboring important human and animal pathogens. It includes as well nine other family-level taxa, initially called *Chlamydiae*-related bacteria, which have an untold diversity (Bayramova et al., 2018). They are ubiquitous in the environment but in some cases they could be associated to human and animal pathologies (Bayramova et al., 2018; Wheelhouse and Longbottom, 2012). An outline of the diversity of the order *Chlamydiales* is presented in **Figure 2**.

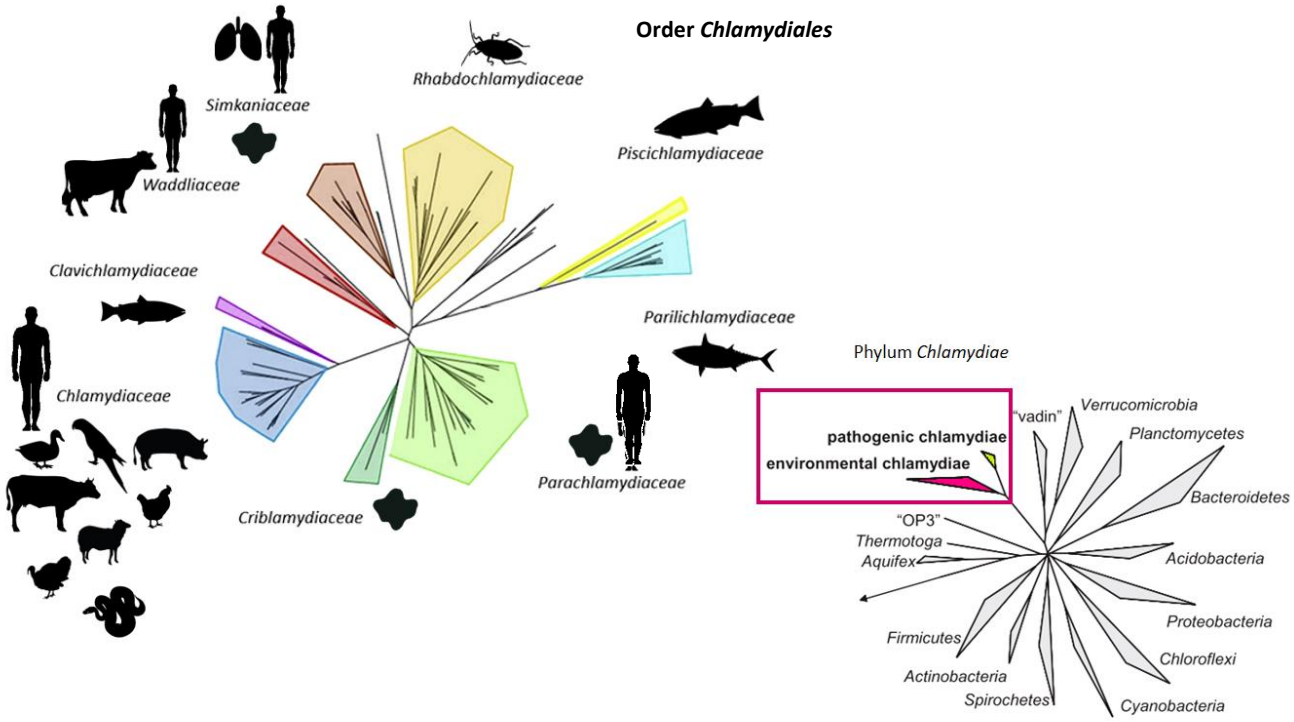

Figure 2. Reconstruction of the *Chlamydiae* phylogeny. Neighbor-joining trees were inferred based on the 16S rRNA gene analysis. On the top left is shown the unrooted tree showing the nine families considered as pathogenic *Chlamydiae*. Next to each family, the most representative eukaryotic host for each chlamydial family has been indicated. On the bottom right is shown the reconstructed phylogeny of *Chlamydiae* in relation with the major bacterial phyla and the intra-phylum partition of pathogenic and environmental *Chlamydiae* (red square). Adapted from (Bayramova et al., 2018; Horn et al., 2004).

It is assumed that pathogenic *Chlamydiae* have lost more genes than the environmental ones because pathogenic chlamydiae have colonized niches that still tend to homeostasis and therefore they have adapted by losing genes, even if intracellular survival is also a challenge requiring adaptations (Horn et al., 2004). It has been proposed two major genetic adaptations of pathogenic chlamydiae that need to happen in order to have a successful intracellular lifestyle: the reduced biosynthetic metabolic pathways leading to their inability to produce most of the amino acids and nucleotides they need, and the impaired tricarboxylic acid (TCA) cycle. Thus, *Chlamydiae* are forced to use the host cell's metabolites and adenosine triphosphate (ATP) (Horn, 2010, 2008; Omsland et al., 2014).

As the world of the so-called *Chlamydiae*-related bacteria is just beginning to be unveiled, as well as its environmental role and its potential as animal and human pathogens, hereafter are presented the most medically relevant members of this phylum.

Probably, the best known chlamydial-related family is *Waddliaceae*, composed by a single genus *Waddlia* and so far three species. The first one, *W. chondrophila*, was first described from an early aborted bovine fetus in 1990 and accepted as a member of a separate chlamydial family in 1999 (Rurangirwa et al., 1999). It was later associated with zoonotic cases involving miscarriages and male infertility (Baud et al., 2020, 2011; Wheelhouse and Longbottom, 2012), as well as pneumonia (Haider et al., 2008). A study conducted with 414

human serum samples from Switzerland, England and Israel revealed a seroprevalence ranging from 8.6% up to 32.5% (Vouga et al., 2018). The other two species were isolated from fruit bats in Malaysia and in Mexico, named *Waddlia malaysiensis* (Chua et al., 2005) and *W. cocoyoc* (Aguilar Pierlé et al., 2015), respectively. The species *W. cocoyoc* caused severe lesions in the lungs and spleen of bats from which it was isolated.

Inside the *Simkaniaceae* family, *Simkania negevensis* is the type species (Horn, 2010). It was first described as a cell culture contaminant (Kahane et al., 1993) and later it was associated to human respiratory infections such as bronchiolitis in the UK (Friedman et al., 2006), and community acquired pneumonia associated to a water source in Brazil (Nascimento-Carvalho et al., 2009). More recently, its persistent infection has been implicated with the pathogenesis of Crohn's disease (Scaioli et al., 2019). Similarly, within the *Parachlamydiaceae* family the species *Parachlamydia acanthamoebae* and *Protochlamydia naegleriophila* have been associated to respiratory infections (Bühl et al., 2018; Casson et al., 2008; Greub, 2009). More research is needed to investigate the involvement of these *Chlamydiae*-related bacteria as pathogens, which most of the time have an environmental origin.

1.2. History and taxonomy of *Chlamydiaceae*

The *Chlamydiaceae* family encompasses important pathogens of humans and animals. Descriptions of diseases in animals and humans now known to be caused by *Chlamydiaceae*, such as trachoma, psittacosis and spontaneous abortion, has been found as early as in Egyptian and Chinese documents (Pospischil, 2009). However, the study of this group of bacteria only started in the late 18th century. It was Jacob Ritter, a Swiss physician, who first described seven human pneumonia cases with three fatalities related to recently imported parrots and finches. He recognized the spontaneous transmission of the etiological microorganism, described the incubation period, the non-transmissibility from man to man and identified these birds as the source of infection. He published these results in 1879 as the diagnosis of typhoid (or typhus), hence naming the disease pneumotyphus (Pospischil, 2009).

Later on, Edmond Nocard in 1893 proposed the name *Bacillus psittacosis* for the Gram-negative bacterium presumably responsible for psittacosis disease. A disease whose name is derived from the Latin *psittacus* meaning parrot and the suffix *–osis* coming from ancient Greek meaning an abnormal condition. The first time the term “psittacosis” was applied to name this disease was in 1895 by the physician Antonin Morange. It was later confirmed that Nocard had isolated *Salmonella* sp. from the bone marrow of parrots dying of psittacosis. Microorganism identification was not consistently achieved from men and parrots concurrently presenting clinical signs of psittacosis, hence, these findings elicited the idea that the causative agent of psittacosis could be a virus (Page, 1966; Pospischil, 2009).

During expeditions to study the transmission and treatment of syphilis in Batavia, Java, Ludwig Halberstaedter and Stanislaus von Prowazek infected, independently, orangutans with conjunctival scrapings from trachoma patients. Microscopic investigation of Giemsa-stained smears of conjunctival scrapings revealed intracytoplasmic inclusions containing several small particles that were initially considered to be protozoa but

later were recognized as elementary and reticulate bodies. They called them *chlamydozoa*, from the Greek word chlamys "χλαμῦς", meaning mantle, because of the mantle appearance that seems to surround the particles in the Giemsa staining. This findings were published in 1907 (Pospischil, 2009).

It is assumed that psittacosis originated in South America, from the numerous endemic psittacine species that inhabit the forests, and that the causative agent spread to Europe and North America through bird trade (Harkinezhad et al., 2009; Pospischil, 2009). It was not until 1930 that five physicians and their colleagues, separately and concurrently, described the filterability, the inability to grow in common microbiological media, and the obligate intracellular nature of the microorganism responsible for the psittacosis epidemic that occurred in Europe and North America related to diseased psittacine birds (Bedson, 1930; Bedson et al., 1930; Page, 1966).

In 1932, Samuel P. Bedson and J.O.W. Bland recognized for the first time the biphasic intracellular developmental cycle of the microorganism responsible of psittacosis, describing it as having an "initial body" and an "elementary body", this very first schematic description figures in their manuscript (Bedson and Bland, 1932). The next stage, the *in vitro* isolation, was achieved several years later by at least five research teams that successfully grew the agent of psittacosis on different animal tissues as well as the chorioallantoic membrane and the yolk sac of embryonated chicken eggs (Bedson and Bland, 1932; Burnet and Rountree, 1935; Yanamura and Meyer, 1941). In 1942, a group of scientists proposed that the etiologic agents of some human diseases i.e. trachoma and lymphogranuloma, were related to the zoonotic microorganism that causes psittacosis (Rake et al., 1942).

Since 1945 the taxonomy of *Chlamydiae*, that share such unique biphasic developmental cycle among prokaryotes, has undergone several modifications (Page, 1966; Pospischil, 2009). Numerous genera names were proposed to name bacteria belonging to this group (*Chlamydia*, *Miyagawanella*, *Bedsonia*, *Rickettsia*, *Rakeia*, *Chlamydozoon*, *Ehrlichia*, *Rickettsiaformis*, *Colesiota*, *Ricolesia* and *Colettsia*) and were classified within different bacterial families such as *Rickettsiaceae*, *Chlamydozoaceae*, *Ehrlichiaceae* and *Chlamydiaceae*, among others. Time after, based on bacteria morphology, developmental cycle and antigenic similarities, Page (1966) proposed to unify the taxonomy in a single genus *Chlamydia*. Furthermore, Page (1966) provided details regarding the biochemistry, metabolism and host range of members of the genus *Chlamydia*. Two years later, Page (1968) proposed two species within the genus *Chlamydia*: *Chlamydia psittaci* and *Chlamydia trachomatis*, based on their morphology, biochemical characteristics and host range.

With the development of DNA-based classification methods during the 1980s, a new classification of the chlamydial group was proposed (Everett et al., 1999). First, two additional species were added: *C. pneumoniae* (Grayston et al., 1989) and *C. pecorum* (Fukushi and Hirai, 1992). Then, Kaltenboeck et al. (1993) demonstrated a large *C. trachomatis* intra-species heterogeneity, depending on the host from which the strain had been isolated; he noticed that porcine isolates consistently diverged from human isolates.

Phylogenetic analyses using the ribosomal 16S rRNA operon, revealed that the four species classification could be splitted into more genetic groups (Pudjiatmoko et al., 1997). In 1999, Everett et al.(1999) proposed a reclassification of the *Chlamydiaceae* family based almost exclusively on the phylogenetic analysis of the full-length 16S and 23S rRNA operons, without further biological markers for genus differentiation. Using solely this rRNA operon-based criterion, the analysis supported the division of *Chlamydiaceae* into two genera: *Chlamydia* and *Chlamydophila*, as well as the inclusion of five new species: the first two were *Chlamydia muridarum* and *Chlamydia suis* that, together with *C. trachomatis*, together composed the *Chlamydia* genera. The three others species were the result of the split of *C. psittaci* into *Chlamydophila abortus*, *Chlamydophila caviae* and *Chlamydophila felis* that, together with *Chlamydophila pecorum*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci*, composed the *Chlamydophila* genera. However, this classification aroused several controversies among the research community working on human and animal chlamydiosis, especially the genus partition, quickly leading to a first letter stating that the new genus designation overlooks the unique and highly conserved biology shared by these bacteria (Schachter et al., 2001).

By the comparative phylogenetic analysis of 110 concatenated conserved genes of *Chlamydiaceae*, Stephens et al. (2009) demonstrated, nine years later, the close evolutionary relationship within this family. Considering the lack of use of the two-genus nomenclature by the scientific community, in the same study Stephens et al. (2009) therefore suggested the reunification of the family into a single genus: *Chlamydia*, as all members within the *Chlamydiaceae* are sufficiently genetically, biologically and ecologically closely related.

After over 15 years of reflections and compilation of research community opinion, the current classification of the *Chlamydiaceae* family was finally proposed by Sachse et al. (2015a), in order to unify this controversial taxon. Based on genomic data, but considering the unique developmental cycle as well as biological properties, Sachse et al. (2015a) concluded that the phylogeny constructed with the 16S rRNA operon and other genetic markers was not enough to support the division of the *Chlamydiaceae* family. Therefore, and since 2015, the 11 species recognized so far were grouped into a single genus *Chlamydia*.

Since then, the development of broad-spectrum molecular identification methods has enabled the discovery of several new species of chlamydia, notably in birds or reptiles, most often without clear association with a clinical condition. In birds, Sachse et al. (2014) proposed the existence of two other chlamydial species of avian origin: *Chlamydia avium* and *Chlamydia gallinacea*, based on phylogenetic analysis of ribosomal RNA operons, *omp*A genes and the multi-locus sequence analysis of pigeon and psittacine-origin strains (Gasparini et al., 2011; Sachse et al., 2012; Zocevic et al., 2013), and poultry strains (Gaede et al., 2008; Laroucau et al., 2009b; Zocevic et al., 2012), respectively. The increasingly use of broad range DNA-based diagnosis assays allowed their detection. Similarly, Vorimore et al. (2013) demonstrated the presence of an atypical chlamydial strain related to *Chlamydiaceae* family in feral African Sacred Ibises (*Threskiornis aethiopicus*) and suggested a new candidate species named *Candidatus* Chlamydia ibidis. The latest and, certainly not the last, bird-origin

new species came from a sample taken from a red-shouldered hawk who had conjunctivitis before it died, as well as mild hepatitis and splenitis at the histopathological post mortem analysis (Laroucau et al., 2019). Interestingly, this new taxon named *Chlamydia buteonis* is phylogenetically more closely related to *C. psittaci* and *C. abortus*.

Regarding new taxa coming from reptiles, unidentifiable chlamydial species were initially detected from *Chlamydiaceae*-positive samples taken from snakes. Sequences of the 16S rRNA placed these new taxa phylogenetically nearby *C. pneumoniae*, but as the isolation couldn't be performed, no further research on these microorganisms was conducted (Taylor-Brown et al., 2015a). In 2016, the sequencing of the complete genome of a new chlamydial strain coming from a choanal sample of captive snake (Taylor-Brown et al., 2016), opened the way to the description of new species within *Chlamydiaceae* through their molecular characterization, without the need to isolate the strain and consequently revolutionizing the paradigm of first cultivating the strain in order to further characterize it within the chlamydial research community. Few months later, using the same approach, three new species/candidate species were proposed, all isolated from snakes: *Candidatus* Chlamydia corallus (Taylor-Brown et al., 2017), *Chlamydia serpentis* and *Candidatus* Chlamydia poikilothermis (Staub et al., 2018). The later reptile-origin species described is *Chlamydia testudinis* (Laroucau et al., 2020b), isolated from turtles presenting severe conjunctivitis in a recovery center in Spain. This new taxon, closely related to *C. pecorum*, was previously detected in Polish and probably in German turtle samples, originally reported as *Chlamydia*-related bacteria. However, no clinical disease could be clearly attributed to this new species as it has been detected in both healthy and sick animals (Hotzel et al., 2005; Mitura et al., 2017).

The accelerated discovery over the last 10 years of new taxa within the *Chlamydiaceae* family as well as within the phylum *Chlamydiae* illustrates the incredible diversity inside this group of bacteria. The phylogeny of the *Chlamydiaceae* family, including all the species identified so far, is presented in **Figure 3**.

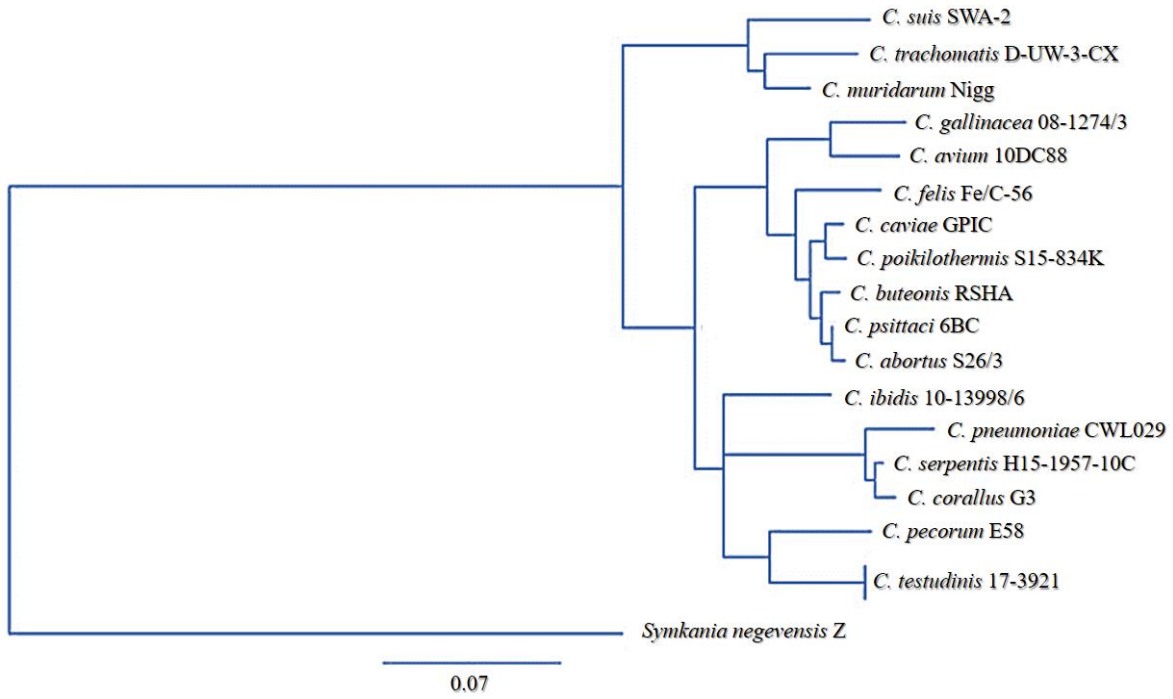

Figure 3. Phylogenetic reconstruction of current identified taxa within the *Chlamydiaceae* family. The topology was inferred based on the 16S rRNA gene analysis using the Neighbor-Joining method. The scale bar indicates the number of substitutions per site. Adapted from (Laroucau et al., 2020b).

1.3. *Chlamydiaceae* as animal pathogens

A wide spectrum of diseases has been associated with *Chlamydiaceae* infections in animals, including humans (Cheong et al., 2019; Longbottom and Coulter, 2003; Nunes and Gomes, 2014). There is evidence that some of the chlamydial species are potentially zoonotic, for some others the range of susceptible hosts could be extensive, and for some more, the infection of a host is not always associated with disease presentation (Borel et al., 2018; Cheong et al., 2019; Longbottom and Coulter, 2003; Ramakers et al., 2017). The range of hosts, as well as an associated zoonotic potential, have not been yet described for the newly described chlamydial species. The non-exhaustive compendium of the *Chlamydiaceae*-associated infections and diseases in animals is presented in **Table 1**. As the subject of this thesis are the avian associated chlamydial species, they will be reviewed in a specific section (1.5. Chlamydial infections in birds).

Table 1. Chlamydial species and non-exhaustive compendium of associated diseases in animals or humans. Modified and adapted from (Borel et al., 2018; Cheong et al., 2019; Sachse et al., 2015a).

Chlamydial species	Main host	Diseases in main non-human host	Other hosts	Zoonotic potential and associated disease in humans
C. abortus	Sheep, goat	Abortion in late gestation or weak/dead fetus delivery, vaginitis, endometritis, seminal vesiculitis, (latent) mastitis, respiratory disease, subclinical carriers	Cattle, swine, birds	Yes: abortion, stillbirth, reproductive pathologies, atypical pneumonia
C. avium	Birds	Enteritis, hepatosplenomegaly, respiratory disease	Unknown	Unknown
C. buteonis[1]	Birds	Conjunctivitis, hepatomegaly, splenomegaly	Unknown	Unknown
C. caviae	Guinea pigs	Follicular conjunctivitis, keratitis, respiratory disease	Cat, dogs, rabbits, horses	Yes: conjunctivitis, severe community-acquired pneumonia
C. corallus	Snakes	No associated pathology	Unknown	Unknown
C. felis	Cats	Conjunctivitis, rhinitis, mild respiratory disease, upper reproductive tract infection	Other felids, dogs	Yes[2]: conjunctivitis
C. gallinacea	Birds	Decrease in body weight gain	Cattle	Possible[2]: atypical pneumonia
C. ibidis	Birds	No associated pathology	Unknown	Unknown
C. muridarum	Rodents	Pneumonia, ileitis, cervicovaginal infection, oviduct occlusion	Birds[3]	No
C. pecorum	Cattle, sheep, goats,	Encephalitis, polyarthritis, pneumonia, enteritis, cystitis, nephritis, vaginitis, endometritis	Birds[3], water buffalo	No
	Swine	Polyarthritis, serositis, enteritis, pneumonia		
	Koala, other marsupials	Keratoconjunctivitis, blindness, vaginitis, ovarian cyst, infertility		
C. poikilothermis[4]	Snakes	No associated pathology	Unknown	Unknown
C. pneumoniae	Koala, other marsupials, horses	Rhinitis, pneumonia, conjunctivitis	Birds[3]	Unknown[5]
	Amphibians, reptiles Human*	Conjunctivitis, enteritis, granulomatous inflammation of internal organs		
C. psittaci	Birds	From subclinical carriers to psittacosis, conjunctivitis, rhinitis, and blepharitis	Ruminants, horses	Yes: From subclinical to mild respiratory infection or atypical pneumonia
C. sanzinia	Snakes	No associated pathology	Unknown	Unknown
C. serpentis[4]	Snakes	Hepatitis, splenitis, stomatitis, esophagitis, enteritis, myocarditis, oophoritis, pneumonia and encephalitis[6]	Unknown	Unknown
C. suis	Swine	Respiratory disease, conjunctivitis, enteritis, reproductive failure, polyarthritis	Ruminants, birds[3]	Yes[2,7]: Subclinical infection
C. trachomatis	Human*	No associated pathology	Birds[3,8]	Unknown
C. testudinis[9]	Reptiles	Conjunctivitis, respiratory disease	Unknown	Unknown

[1]Described by (Laroucau et al., 2019). [2]Associated to punctual reports. [3]Reported by (Guo et al., 2016). [4]Described by (Staub et al., 2018). [5]The detection of animal genotypes of *C. pneumoniae* in humans suggests a possible zoonotic transmission. [6]Reported by (Laroucau et al., 2020a). [7]Reported by (De Puysseleyr et al., 2014). [8]Reported by (Sachse et al., 2012). [9]Described by (Laroucau et al., 2020b).*Humans are considered the main host.

The most investigated chlamydial species is undoubtedly *C. trachomatis*, a primary human pathogen. It is the leading infectious cause of blindness, causing an ocular infection called "trachoma" and, depending on the chlamydial serovar, it could cause lymphogranuloma venereum (LGV) in both genders (Mohseni et al., 2019).

In women, the cervix is the primary site of infection, leading to mucopurulent cervicitis, urethritis, and salpingitis. Among the more commonly observed complicated pathologies if the infection remains untreated are salpingitis and tubal factor infertility, thus increasing the risk of infertility and ectopic pregnancy, and, more importantly, cervical and/or ovarian cancer (Zhu et al., 2016). In pregnant women, it can cause preterm delivery, premature rupture of membranes, and spontaneous abortion. Exposure of newborns to the bacterium can cause conjunctivitis and/or pneumonia. In men, the most common pathologies observed are urethritis, prostatitis, epididymitis, orchitis, seminal vesiculitis, and similarly, the chronic untreated infection may lead to infertility due to a reduced semen volume, apoptosis of spermatozoa, and sperm DNA fragmentation (Cheong et al., 2019; Mohseni et al., 2019). Additionally, the chronic disseminated infection could derivate in reactive arthritis in both men and women (Cheok et al., 2020).

The other human-related chlamydial species is *C. pneumoniae*, whose infection in the respiratory tract is mostly asymptomatic, but associated diseases include from pharyngitis, to community-acquired pneumonia, or even chronic obstructive pulmonary disease (Cheong et al., 2019). Remarkably, its chronicity has been implicated with the onset and progression of asthma, as well as with several pathologies, e.g. primary biliary cirrhosis, atherosclerosis, reactive arthritis, multiple sclerosis, Alzheimer's disease and lung cancer (Cheok et al., 2020; Cheong et al., 2019).

1.4. *Chlamydiaceae* biology and infection

1.4.1 The developmental cycle: the chlamydial hallmark

The chlamydial structural forms, composition and biology are strongly related to the *Chlamydia* developmental cycle that can be divided into three phases. The first one is the attachment and entry of elementary bodies (EB) into the susceptible host cells with their consequent reorganization into reticulate bodies (RB). The second one is the multiplication of RB by binary fission, and the third is the conversion of the majority of the RB population into a new generation of EB, that are released from the infected host cell and are already infectious. The cycle is not synchronized within the infected cell, and each of the first two phases overlaps with the next (Elwell et al., 2016; Kuo and Stephens, 2010). The schematic representation of the development cycle is shown in **Figure 4**. Particular characteristics of chlamydial structural forms are presented in **Table 2**.

EB have a spore-like morphology, they have long been considered as metabolically inactive, although nowadays it has been shown that they have metabolic and biosynthetic activities depending on D-glucose-6-phosphate as a source of energy (Kuo and Stephens, 2010; Omsland et al., 2014). The initial interaction of EB with the susceptible host cell is mediated by electrostatic interactions, is enhanced by polycations and inhibited by polyanions (Kuo and Stephens, 2010). *In vitro*, this interaction can be facilitated by centrifugation to improve the rate of infection by producing cell surface changes and contacts (Allan and Pearce, 1979).

Table 2. Characteristics of chlamydial elementary and reticulate bodies. Adapted and modified from (Kuo and Stephens, 2010).

Characteristic	Elementary body (EB)	Reticulate body (RB)
Diameter (µm)	0.2 – 0.4	0.5 – 1.5
Infectivity	+	–
Cell wall rigidity	Rigid	Flexible
Trilaminar structure	+	+
Projections on surface	+	+
Generation of ATP	–	–
DNA nature	Compact	Disperse
RNA/DNA ratio	1	3 – 4
Ribosomes	Scarce	Abundant
Synthesis inhibited by penicillin	–	+
Resistance to mechanical stress	+	–
Resistance to osmotic stress	+	–

EB binding to the host cell involves several bacterial ligands and host receptors which. On contact, pre-synthesized effectors are injected into the host cell through a type III secretion system (T3SS), a mechanism used by Gram-negative bacteria to translocate proteins. T3SS facilitates the host–pathogen interaction, thus allowing the EB internalization in phagosomes and the remodeling of the inclusion membrane derived from the cytoplasm membranes of the host cell (Kuo and Stephens, 2010). The inclusion is non-acidified and remains dissociated from the endosomal/lysosomal pathways throughout its existence (Omsland et al., 2014). For some chlamydial species, the infection of a single cell by several EB generates individual inclusions which are subsequently merged with each other (Elwell et al., 2016). The chlamydial inclusions are rapidly transported to the perinuclear region inside the host cell (Kuo and Stephens, 2010). In a chlamydial strain-dependent time (6–8 hours post-infection), the transition from EB to RB takes place and early genes start to be transcribed (Elwell et al., 2016).

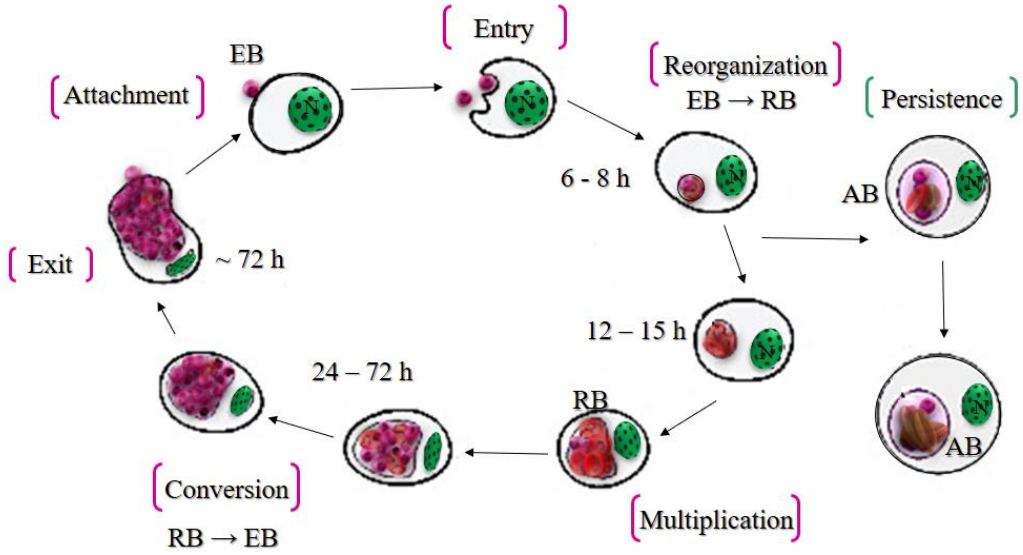

Figure 4. Developmental cycle of *Chlamydia*. EB are elementary bodies, RB are reticulate bodies, and AB are aberrant bodies. Main steps are in brackets: attachment and entry of EB into the susceptible host cells with their consequent

reorganization into RB, multiplication of RB by binary fission, and, conversion of the majority of the RB population into a new generation of EB which are released from the infected host cell by extrusion or host cell lysis. Several stimuli can drive *Chlamydia* into a persistent state (on the right) in which RB undergo a transition into aberrant bodies (AB), once infection is reactivated from this state, RB will ultimately reorganize back into EB. Modified from *(Stephens et al., 2011)*.

Starting from eight to 16 hours post-infection, the mid-cycle bacterial genes begin to be expressed, including effectors that mediate the acquisition of nutrients, while maintaining the viability of the host cell. In particular, macromolecules produced by the Golgi apparatus are intercepted, thus ensuring the distribution of lipids towards the inclusion (Elwell et al., 2016; Kuo and Stephens, 2010). Mitochondria appear to be closely associated with the inclusion, probably for the acquisition of energy metabolites by *Chlamydia* (Omsland et al., 2014). The chlamydial DNA starts to decondense within each RB, ribosomes increase in number, and the bacterial cell wall begins to thin and become more flexible (Kuo and Stephens, 2010). Then, RB begin to divide by binary fission and the inclusion is substantially expanded (Elwell et al., 2016). Considering a mean doubling time of two to four hours, a progeny of approximately 200 to 500 new RB per cell, after seven to eight generations, are expected (Nunes and Gomes, 2014). Depending on the chlamydial strain, 12–15 hours after infection, intracellular inclusions are large enough to be seen by ordinary light microscopy using histological stains or immunostainings (Kuo and Stephens, 2010).

After 24 to 72 hours post-infection, RB transition to EB begins asynchronously inside the membrane-bound inclusion (Elwell et al., 2016). Late-cycle bacterial genes (e.g. histones or transcriptional repression of sigma factor) condense the DNA and switch-off the transcription of the majority of the chlamydial genes, late-cycle effectors are packaged in the newly formed EB, those who no longer divide (Elwell et al., 2016). RB continue to divide and reorganize into EB until the host cell can no longer withstand the inclusion. At the end of the cycle, the chlamydial inclusion can occupy almost the entire cytoplasm of the host cell (Kuo and Stephens, 2010). Depending on the chlamydial strain, a developmental cycle could last from 48 to 72 hours, however, as the RB to EB transition is asynchronously, there is no clear end of it (Elwell et al., 2016).

The release of *Chlamydia* from the host cell takes place from 30 to 72 hours post-infection and involves two mechanisms which have been observed to happened *in vitro* equally often: the host cell lysis and the extrusion of the inclusion (Kuo and Stephens, 2010; Nunes and Gomes, 2014). The numerous attempts to purify *Chlamydia* from the eukaryotic host, have revealed the extreme fragility of the inclusion membrane, nowadays these protocols are successful. (Elwell et al., 2016). Lytic exit leads to cell death, while extrusion is a process similar to exocytosis, with the associated energetic and metabolic costs. Extrusion prevents the activation of an immediate inflammatory process, thus protecting the new generation of EB from the host's immune response. In addition, extrusion can also contribute to the persistence of *Chlamydia*, as some bacteria may remain within the already infected host cell (Elwell et al., 2016; Kuo and Stephens, 2010). A typical chlamydial inclusion is illustrated in **Figure 5**.

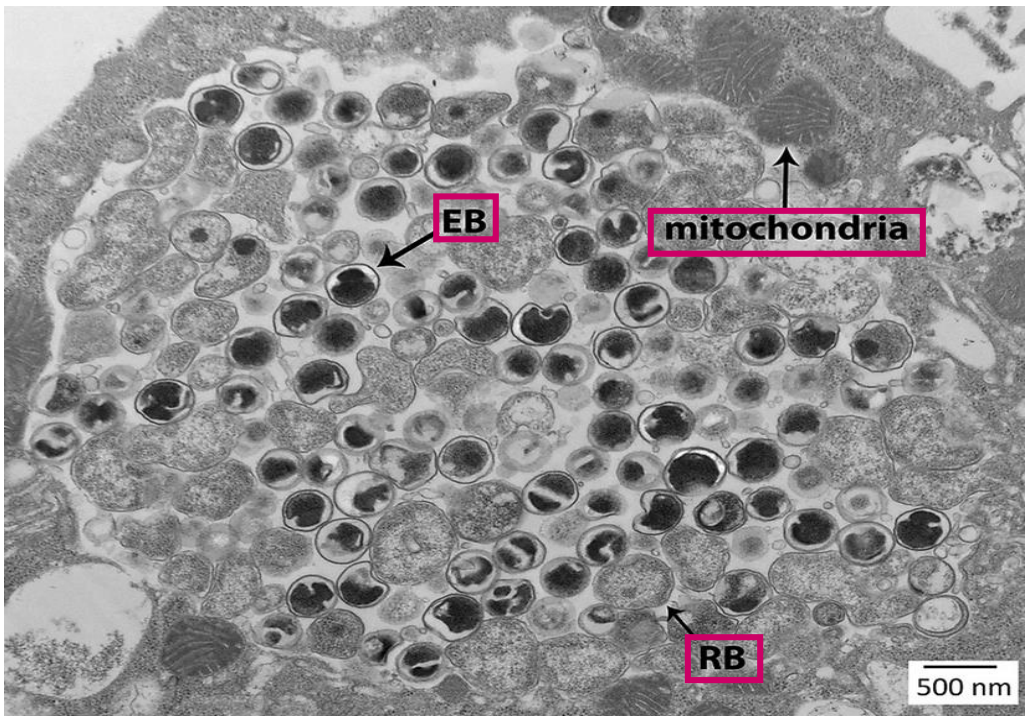

Figure 5. Ultramicrophotography of a Vero cell infected with *C. ibidis* at 48 hours post-infection. A typical inclusion is observed, with a mixture of EB and RB at different stages of reorganization. The closely association of mitochondria with the inclusion membrane is noted. Adapted from (Vorimore et al., 2013).

1.4.2. Persistence, a unique feature for intracellular survival

The persistence of *Chlamydiae* was first described by Meyer et al., (1935), in parrots infected with *C. psittaci*. While, a minority of birds showed clinical signs, for some others the clinical signs were intermittent. Years later, these observations where correlated to reports following the *in vitro* cultivation of the human strains of *C. trachomatis*. Indeed, Lee and Moulder, (1981) observed that after 100 days of cultivation of persistently infected Mc Coy cells, the bacterium managed to find a balance between periods of host cell death and chlamydial proliferation. Therefore, chlamydial persistence has been described as a condition in the developmental cycle where the bacterium has a viable but non cultivable growth that results in a long-term relationship with the infected host cell (Hogan et al., 2004).

The developmental cycle can be reversibly stopped in a bacterial subpopulation by numerous environmental factors (Elwell et al., 2016). The so far well identified *in vitro* conditions that trigger the chlamydial persistence are: stress derived from nutrient deprivation, e.g. amino acid or iron deficiency, the exposure to host cell cytokines produced as part of the immune response (IFN-ɣ), as well as the antibiotic exposure i.e. penicillin (Hogan et al., 2004; Panzetta et al., 2018). Under these conditions, RB undergo a transition to aberrantly enlarged, non-dividing persistent forms, which are called aberrant bodies (AB) (Bavoil, 2014; Elwell et al., 2016). Clinical importance of this arrestment of the developmental cycle implies the maintenance of the infection, which goes unnoticed by the host and sometimes escapes diagnostic tests. Persistent infections may contribute to chronic inflammation and all diseases that are associated with it (Bavoil, 2014). As an example

of this phenomenon, there have been *in vivo* observations where the same strain of *C. trachomatis* has been isolated several times over a large-period (4 years) from the same patients, even after facing an aggressive diagnosis and antimicrobial-based treatment protocols, strengthening the idea that some *C. trachomatis* infected patients can remain persistently infected (Rockey et al., 2019).

1.4.3. Chlamydial genome

It was in 1998 when, for the first time, a full-genome of a chlamydial species (*C. trachomatis*) was published (Stephens et al., 1998), unveiling its small size and the distinguishing features that would later be found to be shared across the *Chlamydiaceae* family. Stephens et al., (1998) proposed that the chlamydial intracellular lifestyle and all that it entails, has resulted in the lack of many biosynthetic pathways but with sufficient gene retention to perform key steps and use host cell metabolites. A clear example of that is the absence in all *Chlamydiaceae* of at least three enzymes required for the tricarboxylic acid (TCA) cycle operation: citrate synthase (GltA), aconitase (Acn) and isocitrate dehydrogenase (Icd), forcing *Chlamydia* into a constant metabolite exchange with the host cell (Omsland et al., 2014). So far, all the known chlamydial genomes comprise a single chromosome ranging from 1 Mbp for *C. testudinis*, 1.04 Mbp for *C. trachomatis* and *C. gallinacea* as the smallest, to 1.23 Mbp for *C. pneumoniae* as the largest genomes (Laroucau et al., 2020b; Sachse et al., 2015a).

A distinctive feature of the *Chlamydiaceae* genomes is that they are highly conserved in terms of gene content and order, what is called genome synteny, with very few pseudogenes and mobile genetic elements (Bachmann et al., 2014; Kuo and Stephens, 2010). They share approximately two-thirds of the predicted proteins in their genome (Elwell et al., 2016), which range from 907 for *C. gallinacea* to 1113 for *C. pneumoniae* (Sachse et al., 2015a). One remarkable exception of the highly conserved chlamydial genome, is a specific region of high inter-species diversity called “plasticity zone”, which fluctuates between 18 to 81 kb, in which many virulence factors are encoded (Bachmann et al., 2014; Elwell et al., 2016). Furthermore, the DNA repair and recombination systems are well represented all over the chlamydial genome, indicating that recombination are common events, especially but not exclusively, within and around genes with known roles in virulence, e.g. the polymorphic membrane protein gene (*pmp*), the *tarp* gene and the *omp*A gene (Bachmann et al., 2014). The latter is of particular interest because it encodes for the major outer membrane protein (MOMP), which is in constant changing in an attempt to elude the different host immune system response present in different tissues, resulting in several genotypes for some chlamydial species. This is particularly true for *C. trachomatis* (Bachmann et al., 2014), *C. psittaci* (Sachse et al., 2009a), *C. suis*, *C. pecorum* (Kuo and Stephens, 2010), and has been recently described for *C. gallinacea* (Guo et al., 2017; Sachse et al., 2014).

As an obligate intracellular bacterium, *Chlamydia* is thought to have low rates of horizontal gene transfer events, however recent studies have demonstrated that both *C. trachomatis* and *C. psittaci* can actively exchange DNA (Kim et al., 2018; Read et al., 2013). Indeed, Read et al., (2013) found that *C. psittaci* horizontal gene transfer is not restricted to few hotspots, but its genome contains complete sets of genes that necessarily

come from horizontal transfer. Interestingly, *C. suis* is so far the only species found to have naturally acquired genes encoding for tetracycline resistance, a tetracycline class C gene cassette. It has been hypothesized that recombination could be the leading mechanism for its transmission from a Gram-negative donor after the introduction of tetracycline into animal feed in the 1950s decade (Marti et al., 2017). Although the cassette transfer frequency observed during *in vitro* assays is low (~5%), it is estimated that about 90% of *C. suis* field strains are tetracycline resistant (Marti et al., 2017). The architecture of the genomic island is variable and yet, the *tet*(C) gene is always intact, with at least two insertion sites having been detected in the chlamydial genome (Marti et al., 2017; Seth-Smith et al., 2017). In an unpublished study, Laroucau et al. analyzed different strains of *C. psittaci*, *C. avium* and *C. gallinacea* from poultry farms, both with the molecular test developed to corroborate the presence of the *tet*(C) cassette as well as evaluating the *in vitro* tetracycline susceptibility in cell culture assays. So far, no resistant chlamydial strains of avian origin have been found.

The majority of chlamydial species have a conserved 7500 bp plasmid that is not required for *in vitro* growth but is crucial during *in vivo* infection, as it contains eight predictable proteins, so-called plasmid glycoproteins, that contribute to the virulence of the strain and possibly to promote chlamydial adaptation to different animal tissues (Kuo and Stephens, 2010; Zhong, 2016).

1.4.4. Virulence factors and antigen structure

Chlamydiaceae encode several virulence factors, which comprise approximately 10% of their genome depending on the species (Elwell et al., 2016). *Chlamydiaceae* have a well-developed and highly conserved T3SS, which is expressed throughout the chlamydial cycle as a mechanism to interact with the host cell (Knittler et al., 2014; Nunes and Gomes, 2014). There is a great variety of pre-packaged effectors which are injected into the cytoplasm of the host cell since early stages of the infection, inducing cytoskeletal rearrangements that promote invasion, inhibit phagocytosis, and modulate intracellular trafficking, among others (Elwell et al., 2016).

The plasticity zone (PZ) is a highly variable genome region among *Chlamydiaceae* species encoding several virulence factors and including the chlamydial cytotoxin, the membrane attack complex/perforin protein (MACPF), the phospholipase D and the tryptophan biosynthesis operon (*trp*) (Elwell et al., 2016). It has been described that encoded proteins coming from this PZ may have a role not only as virulence factors but as a key influence for adaptation to different environmental niches and tissue tropism (Knittler et al., 2014). For example, although tryptophan is an essential amino acid, the complete functional operon to synthesize it is a key inter- and intra-species difference. A functional *trp* operon indicates the strain capability to produce its own tryptophan, eluding the amino acid depletion as part of the host immune response to the intracellular infection. Otherwise, to obtain tryptophan, *Chlamydia* has to rely on the metabolites produced by other microorganisms which are present in specific tissues (e.g. microflora in the genitourinary tract), resulting in a strain-associated capability to invade different tissues (Bachmann et al., 2014; Nunes and Gomes, 2014).

The cytotoxin gene, which is found also within the PZ, has not been found in all chlamydial species, and even in some strains of the same species, it could be interrupted by mutations and deletions (Bachmann et al., 2014). The encoded chlamydial cytotoxin is analogous to the *Clostridium* cytotoxins, and it is directly involved in the cytoskeletal actin-filaments disassembly, being a major responsible of the cytopathic effects (Nunes and Gomes, 2014). Other major proteins encoded in the PZ are the phospholipase D enzymes and the membrane attack complex/perforin protein (MACPF), both are more extensively represented among *Chlamydiaceae* but with some species particularities (Nunes and Gomes, 2014). It is thought that their function for the acquisition and processing of lipids by the inclusion is interconnected and may have a role in host tropism (Elwell et al., 2016).

The Pmp proteins are probably unique to *Chlamydiae*. They are present in all *Chlamydiaceae* and are encoded by the 3 to 5% of the chlamydial genome (Nunes and Gomes, 2014).Their number depends on the species: there are nine to 21 clusters of the Pmp proteins in *C. trachomatis* and *C. pneumoniae*/*C. psittaci*, respectively (Bachmann et al., 2014). The proteins of the Pmp family participate in the adhesion of EB to the host cell, in molecular transport and provide antigenic diversity for immune evasion (Knittler et al., 2014).

CPAF (chlamydial protease activity factor) is one of the major chlamydial virulence factors. It is a protease responsible for the degradation of various host factors required for chlamydial intracellular survival and plays an essential role in maintaining the structural integrity of chlamydial inclusions (Knittler et al., 2014). Additionally, the Inc (from inclusion) proteins are a group of chlamydial effector proteins that, through direct interaction with the host cell, may directly affect the cytoplasmic signaling and trafficking pathways to promote the development of the inclusion, e.g. lipid acquisition and the mobilization of the inclusion to a perinuclear location (Elwell et al., 2016; Nunes and Gomes, 2014).

Regarding the antigenic structure of *Chlamydiaceae*, the most outstanding is the highly variable major outer-membrane protein (MOMP), which is the quantitatively predominant surface antigen. It is a porin with immunogenic determinants that confer species- or serotype- specificity. Additionally, the chlamydial LPS (lipopolysaccharide), a genus-specific endotoxin, has become the target for several commercially available monoclonal antibodies for many diagnosis tests. Other important surface antigens present in all *Chlamydiaceae* are the aforementioned Pmp proteins (Bachmann et al., 2014; Elwell et al., 2016; Kuo and Stephens, 2010).

1.5. Chlamydial infection in birds

1.5.1. Diversity and prevalence

As mentioned in 1.2 history and taxonomy of *Chlamydiaceae*, the first identified chlamydial infection in birds was described, back in the 1930's, as a respiratory disease that occasionally was fatal (Harkinezhad et al., 2009; Pospischil, 2009). It was only with the advent of broad-range molecular detection tools that new species

began to be described, especially in birds and reptiles. Several species of *Chlamydia*, whose main hosts are not birds, have been occasionally detected in birds. Therefore, transmission of these species to birds may occur when birds have close contact with natural hosts of those chlamydial species, e.g. cattle, pigs (Guo et al., 2016). Thus, it has been hypothesized that host barrier of the different chlamydial species is probably wider than previously thought (Taylor-Brown and Polkinghorne, 2017).

Presence of *Chlamydia* has been confirmed by isolation followed by antigenic detection, serological tests, or molecular identification in over 400 bird species belonging to at least 30 different orders (Kaleta and Taday, 2003; Sachse et al., 2015b). **Figure 6** is showing host range and diversity of chlamydial infections in the class Aves.

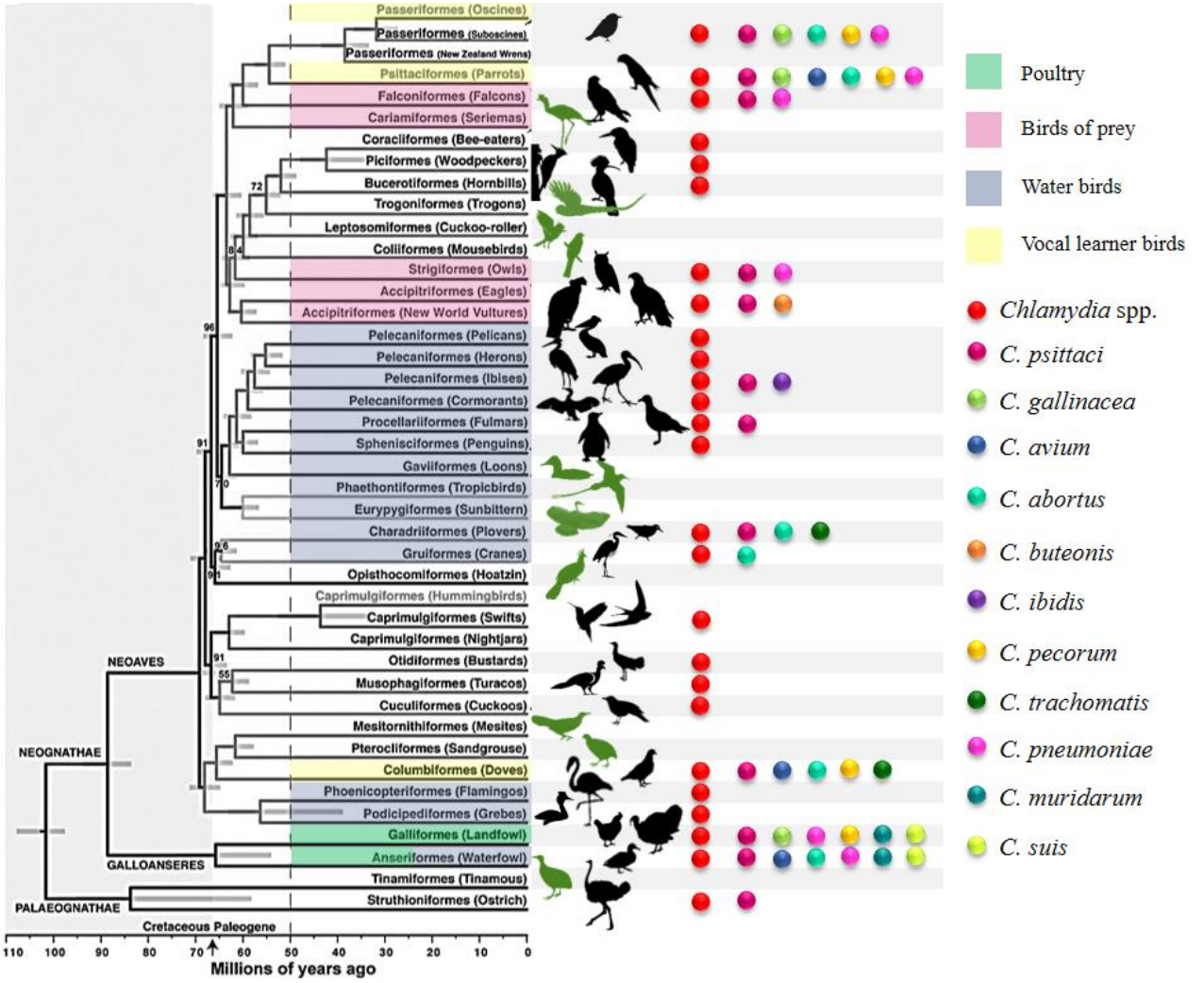

Figure 6. Diversity and host range of chlamydial infections in the class Aves. First *Chlamydiae* are thought to have emerged 2,000 million years ago, while pathogenic *Chlamydiae* 700 million years ago. Avian silhouettes in green indicate the absence of chlamydial identification within this order. The cladogram of the class Aves was adapted from (Jarvis et al., 2014).

Historically, the order within the class Aves in which the presence of *Chlamydia* has been mostly detected is the order *Psittaciformes* (cockatoos, parrots, parakeets and lories), followed by *Columbiformes* (doves,

pigeons), *Anseriformes* (ducks, geese, and swans) and, to a lesser extent, *Galliformes* (turkeys, chickens, quails, partridges, pheasants). The high rate of investigation of diseases in domestic or companion birds, coupled with the zoonotic cases linked with these bird species, are the main reasons hypothesized by Kaleta and Taday, (2003) explaining the high frequency of chlamydial infections in birds belonging to this orders.

Chlamydia psittaci is the most important chlamydial species from a public health point of view, due to its well-recognized zoonotic potential. Infection in birds by this species is also called psittacosis or ornithosis (Sachse et al., 2015b). Numerous studies have been performed assessing the prevalence of *C. psittaci* and other chlamydial species in several countries and type of birds all over the world, showing variable prevalence values. One of the most exhaustive efforts to compile these studies is the review performed by Kaleta and Taday, (2003). However, this review was conducted including studies using isolations with antigenic detection, as well as serological analysis. Therefore, information compiled was not specific for *C. psittaci*, as nowadays it is known that such antigenic determinants are shared across the genus *Chlamydia*.

In the review carried out by Sachse et al., (2015b), it is mentioned that in psittacines kept as pet birds, in breeding facilities, or in exhibition at zoos, *C. psittaci* prevalence could vary from 16% to 81%. However, this range values are continuously expanding as more and more studies are conducted. For example, in a recent study conducted in psittacines of 64 species belonging to 31 different genera in Taiwan, *C. psittaci* prevalence, detected through PCR analysis, was as low as 3.1% (n=16/514) and most of the positive parrots were kept at breeding facilities (Liu et al., 2019). In Brazil, the prevalence of *C. psittaci* was as high as 72% (n=152/212) among dead parrots rescued from illegal wildlife trafficking (Vilela et al., 2019).

C. psittaci prevalence in pigeon flocks differs if birds are domesticated, feral or wild and goes from 12% to 95 % (Sachse et al., 2015b). In recent studies carried out in feral pigeons, prevalence can be as low as 2.5% (n=4/156) as reported by Perez-Sancho et al., (2020) in a study conducted in Madrid, or 4.9% (n=4/81) from samples taken during 2017 in two Dutch cities (Burt et al., 2018).

Studies conducted on *Chlamydiaceae* occurrence in poultry have revealed different results depending on the country and the poultry species involved. If we consider only the studies performed using molecular tools for the specific detection of *C. psittaci* infection, there is evidence that *C. psittaci* tends to be more present in mule duck flocks than in turkey or chicken flocks (Guo et al., 2016; Hulin et al., 2015). In fact, mule ducks are thought to be the main poultry species associated with human cases of psittacosis in France, especially those related to the production of *foie gras* (Laroucau et al., 2009a). Some other studies carried out in commercial chicken flocks in Netherlands (Heijne et al., 2018), and turkey flocks in Switzerland (Vogler et al., 2019), as well as in backyard chickens in the United States (Li et al., 2017), even showed that *C. psittaci* could be absent.

Four years after its admixture as new taxon within *Chlamydiaceae* in 2014, *C. gallinacea* has been found in four continents: America, Europe, Asia and Oceania. Its presence has been mainly described in chickens,

turkeys but also in a captive pet passerine bird from a household in Argentina and in a wild Australian psittacine bird (Frutos et al., 2015; Guo et al., 2016; Heijne et al., 2018; Hulin et al., 2015; Li et al., 2017; Stokes et al., 2019; Vogler et al., 2019). Prevalence of *C. gallinacea* seems to exceed that of *C. psittaci* in poultry flocks, depending on the avian species sampled, as *C. gallinacea* is more likely to be found in chicken and turkey flocks (*Galliformes*) than in duck flocks (*Anseriformes*) (Guo et al., 2016; Hulin et al., 2015; Szymańska-Czerwińska et al., 2017a). To date, few studies have assessed the specific prevalence of this microorganism and results are variable. Prevalence values can be as low as 0.7% (n=7/1008) in Swiss fattening turkeys (Vogler et al., 2019), 12.4% (n=66/531) in backyard chickens in the United States (Li et al., 2017), or 15% (n=24/160) in backyard chickens in Italy (Donati et al., 2018), or as high as 47% (n=71/151) in Dutch commercial egg layer farms (Heijne et al., 2018), or 65.5% in the *Chlamydiaceae*-positive poultry flocks in Poland (Szymańska-Czerwińska et al., 2017a). It is worth mentioning that the host range of *C. gallinacea* seems not to be restricted to birds, as its presence has been shown in most vaginal swabs taken from cattle in China (M. Li et al., 2016).

Chlamydia avium was proposed as a new species by Sachse et al. (2014), simultaneously with *C. gallinacea*, to identify those atypical *Chlamydia* detected in pigeons and psittacines in previous studies. Earlier occurrence of atypical chlamydial species has been reported at least since 1996 in isolates from urban pigeons in Italy that were later identified as *C. avium* (Sachse et al., 2014). Since 2014, *C. avium* has been identified in pigeons, psittacines and mallards (Gasparini et al., 2011; Sachse et al., 2012; Szymańska-Czerwińska et al., 2017b; Zocevic et al., 2013). Regardless of the host, *C. avium* prevalence within *Chlamydiaceae*-positive samples are comparatively low with the rest of chlamydial species, ranging from 0.8% (n=1/132) in samples from wildfowl in Poland (Szymańska-Czerwińska et al., 2017b), 2.3% (n=1/44) in samples from feral pigeons in Thailand (Sariya et al., 2015), 3.1% (n=4/128) in samples from urban pigeons in Germany (Sachse et al., 2012), 6.8% (n=6/73) in samples from urban and feral pigeons in Switzerland (Mattmann et al., 2019), and 8% (n=10/125) in samples from Parisian pigeons (Gasparini et al., 2011; Zocevic et al., 2013). However, a recent study identified a prevalence of 28.4% for *C. avium* in samples of feral pigeons from two cities in Netherlands, this prevalence even exceeded the one for *C. psittaci* in the same study (4.9%) (Burt et al., 2018). Mixed infections of *C. avium* and *C. psittaci* have already been reported (Burt et al., 2018; Mattmann et al., 2019).

Chlamydia ibidis was first detected by Vorimore et al. (2013) in feral African sacred ibises (*Threskiornis aethiopicus*). As a part of a program to eliminate invasive species, many apparently healthy feral sacred ibises were culled, and the presence of an atypical chlamydial strain was evidenced and later confirmed as a new candidate species. Recently, *C. ibidis* was detected in all (n=20/20) the *Chlamydiaceae*-positive (n=20/99) samples coming from the endangered crested ibis (*Nipponia nippon*) taken in a Wildlife Rescue and Breeding Research Center in China (Z. Li et al., 2019). The authors of this study suggested that*C. ibidis* could be a species of high genetic diversity probably related to the host and/or geographic origin. A mixed infection of *C. ibidis* and *C. psittaci* was also reported in this study.

It was proposed to include the avian isolates misidentified as atypical *C. psittaci* within the *C. abortus* species (Szymańska-Czerwińska et al., 2017b). These atypical strains found in wild birds in Poland, mainly from *Anatidae*, *Rallidae* and *Corvidae* bird families, were firstly identified as *C. psittaci*, but it was until their deep genetic characterization that they could be formally classified as *C. abortus*. Previous studies have shown the presence of *C. abortus* in seabirds (*Charadriiformes*) (Herrmann et al., 2000), pigeons (Sachse et al., 2012) and psittacines (Madani and Peighambari, 2013; Pantchev et al., 2009). *Chlamydia buteonis*, in particular, is the latest bird-origin chlamydial species described (Laroucau et al., 2019). It was isolated from a red-shouldered hawk (*Buteo lineatus*) and to date no other study has reported its presence. However, with hindsight as related by Laroucau et al., (2019), an unidentifiable *Chlamydia* had been isolated in 1990 from a red-tailed hawk (*Buteo jamaicensis*) showing respiratory signs of disease and diarrhea (Mirandé et al., 1992). The strain was sequenced and, after the *in silico* analysis of its genome, a high similarity with *C. buteonis* was revealed (Joseph et al., 2015).

Different studies have evidenced the presence in birds of chlamydial species normally associated with mammals or even humans, raising the question about the role of birds as potential carriers/transmitters or their susceptibility to the infection, e.g. *C. pecorum* and *C. trachomatis* found in urban pigeons from Germany (Sachse et al., 2012). *C. pecorum* has also been found in feral pigeons in Japan (Tanaka et al., 2005), and *C. trachomatis* in an Eurasian coot (*Fulica atra*, *Rallidae*) in Poland (Krawiec et al., 2015). *C. pneumoniae* was the main species detected in captive birds from eight different avian orders in Argentina. In the same study, *C. pecorum* or mixed infections of *C. pneumoniae*/ *C. pecorum* were found in three different avian orders (Frutos et al., 2015). Similarly, *C. suis*, *C. muridarum* and *C. pecorum* were found in backyard poultry in China (Guo et al., 2016). In fact, in the large nationwide study performed by Guo et al., (2016) in China, which involved 2,300 poultry (chickens, ducks, geese and pigeons), pigeons followed by chickens were the most frequently *Chlamydiaceae*-infected, while the most common chlamydial species found were *C. gallinacea* and *C. psittaci*. Naturally infected chickens with *C. gallinacea*, *C. suis* and *C. psittaci* from this study were confined for seven months under laboratory conditions, and during this time, *C. gallinacea* was the only chlamydial species persistently found in cloacal swabs of these chickens.

Interestingly, many studies have reported the presence of atypical *Chlamydiaceae* or new *C. psittaci* genotypes in wild birds, mostly seabirds (Aaziz et al., 2015; Blomqvist et al., 2012a), birds of prey (Blomqvist et al., 2012b), and pigeons (Krawiec et al., 2015). Their full characterization was truncated at the time mainly due to their unsuccessful *in vitro* cultivation attempts. With the development of increasingly powerful molecular tools, it is now possible to unveil the great diversity of the chlamydial infections in birds without this constraint.

1.5.2. Transmission routes and pathogenesis

The most studied chlamydial species infecting birds is *C. psittaci*. Several genotypes of *C. psittaci* have been identified, each genotype exhibiting more or less restricted host specificity. Most *C. psittaci* genotypes are thought to predominantly occur in a particular order of the class Aves while others in non-avian hosts. Most

of the avian genotypes have been identified in cases of zoonotic transmission, and some of them are thought to be more virulent than the others. *C. psittaci* genotypes are going to be reviewed with more detail in a later section (see 1.5.3.6 chlamydial characterization methods).

1.5.2.1. Transmission routes

Depending on various factors attributed to both bacteria and host, infection of birds by *C. psittaci* can take significantly different courses: the infection can be severe in the acute phase in some individuals and cause even death, while in others it can be subclinical. In some other cases individuals can remain chronically infected (see 1.4.2 chlamydial persistence) (Sachse et al., 2015b). In any case, birds either symptomatic or subclinically infected, shed intermittently massive amounts of the bacterium through respiratory secretions, ocular discharges and feces. Therefore, direct contact with infected birds, as well as with the contaminated environment by them, are the main source of infection for other birds and humans, e.g. poultry workers, pet bird owners, breeders and veterinarians (Harkinezhad et al., 2009; Hulin et al., 2016; Vorimore et al., 2015).

Depending on factors such as the bird species (e.g. ducks and chickens *vs* psittacines), the chlamydial species (e.g. *C. psittaci vs C. gallinacea)*; and the stage of the infection (i.e. acute *vs* chronic infections), two main route of transmission have to be considered: fecal-oral and respiratory routes (Balsamo et al., 2017). Hence, for *C. psittaci* in ducks, it has been shown that fecal-oral route of transmission could be the most important. In fact, Thierry et al., (2016) experimentally infected ducks through the oral route with different doses of *C. psittaci* genotype E/B. Over ten days of infection, mock-inoculated ducks that were put in the same facility with inoculated ones, get contaminated and showed systemic chlamydial dissemination with bacteria loads equivalent to the orally *C. psittaci* inoculated ducks. Moreover, cecum was the organ with the highest chlamydial load and fecal shedding was evidenced throughout the evaluation period while ducks remained subclinically infected. Similarly, for *C. gallinacea*, an experimental infection was conducted with the strain JX-1 in chickens. The study carried out by You et al., (2019) showed that fecal-oral transmission is more efficient than the respiratory route in experimentally infected chickens with *C. gallinacea*. Mock-inoculated chickens got infected by *C. gallinacea* only when they were co-housed with experimentally infected ones and not when the only connection between them was through a ventilation system, which has proved to be effective to transmit well-known respiratory pathogen such as infectious bronchitis virus. Infection was corroborated in both groups through *C. gallinacea* positive oropharyngeal and cloacal swabs.

Other routes of transmission have been suggested. There is an experimental study in which the transmission of *C. psittaci* through eggshell penetration with the eventual infection of the growing embryo was evidenced (Ahmed et al., 2017). While other studies showed, through the isolation of *C. psittaci* of the growing embryo, that naturally infected chickens and turkeys can transmit the bacterium to their progenies (Dickx and Vanrompay, 2011). Vertical transmission has been also demonstrated for ducks, geese, gulls and psittacines, although the frequency appears to be low (Harkinezhad et al., 2009). Vertical transmission has been evidenced for *C. gallinacea* in experimentally infected chickens via penetration of the bacterium from eggshell to

albumen, yolk, and the growing embryo (You et al., 2019). More research is needed to investigate the importance and frequency of this transmission route.

Transmission of *C. psittaci* in the nest is also possible. In several birds such as pigeons, and some aquatic birds, e.g. cormorants, egrets and herons, transmission from parent to chicks may occur through feeding by regurgitation, while a contaminated nesting site might be the most important route of transmission for other species, such as snow geese, gulls, shorebirds and even for backyard poultry (Sachse et al., 2015b). Less efficient transmission routes might be blood-sucking ectoparasites such as lice, mites and flies or, less commonly, through bites or wounds (Longbottom and Coulter, 2003).

1.5.2.2. Pathogenesis

Incubation period varies widely, it could range from as short as 3 days and as long as several weeks according to diverse studies carried out with *C. psittaci* infections (Balsamo et al., 2017). Studies conducted in poultry have shown that most of the time *C. psittaci* cause subclinical to mild infections that are revealed only when human outbreaks occur, e.g. in duck (Hulin et al., 2015; Laroucau et al., 2009a) and chicken flocks (Laroucau et al., 2015, 2009b; Shaw et al., 2019). Conversely, it has been observed a significant association between the detection of *C. psittaci* in psittacine birds and the manifestation of clinical signs of psittacosis. In a study carried out in 90 psittacines from Buenos Aires, Argentina, kept as pet birds, half of them showed clinical signs of psittacosis while the others were apparently healthy, Origlia et al., (2019). *Chlamydiaceae* was detected only in 30% of the birds showing clinical signs. However, this study showed that 7.7% of the asymptomatic birds also tested positive for *Chlamydiaceae*. Clinical signs in psittacine birds as well as in pigeons ranges in severity from subtle upper respiratory disease, conjunctivitis, to even acute death in infected individuals (Balsamo et al., 2017). This mostly depends on the virulence of the chlamydial strain and the immune system of the host. Fecal shedding of *Chlamydia* can occur intermittently and can be activated by stress factors, including shipping, crowding, breeding, treatment/handling, poor nutrition and changes in the environment. Moreover, the development of an overt disease can be induced by these stress factors too, as well as coinfections with other pathogens (Sachse et al., 2015b).

Numerous studies have reported natural or experimental coinfections of *Chlamydia* with other pathogens in birds. In some of these studies, disease caused by *C. psittaci* would be even exacerbated. In experimentally infected chickens with different combinations of an avian influenza virus, *Ornithobacterium rhinotracheale* and *Aspergillus fumigatus*, severe pneumonia and high mortality were observed only in the groups of chickens simultaneously co-infected with *C. psittaci*, otherwise, a transient respiratory diseases without mortality was observed (Chu et al., 2017). Likewise, in experimentally infected turkeys, *C. psittaci* pathogenicity was exacerbated with *E. coli* co-infection (Van Loock et al., 2006). Similar results were obtained when an experimental infection with *Bacillus cereus* (food-borne pathogen) was conducted in chickens, in which the presence of both bacteria aggravated the gizzard erosion and ulceration syndrome and triggered severe respiratory lesions (Zuo et al., 2020). Natural co-infections have also been reported, e.g. in a commercial egg laying flock with avian poxvirus (Karpińska et al., 2014) or turkey flocks with avian pneumovirus and

Ornithobacterium rhinotracheale (Van Loock et al., 2005). Coinfections have been also reported in psittacines, e.g. parrots coinfected with a psittacine adenovirus in which the viral load was positively correlated with the bacterial load (To et al., 2014), and a case of reovirus in budgerigars (Perpiñán et al., 2010).

To investigate the pathogeny in birds due to the infection by *C. psittaci*, experimental infections have been conducted to improve the understanding of the processes occurring in the natural host (Sachse et al., 2015b). Few studies compile the most important data generated on this matter. Two studies concern *C. psittaci* and *C. abortus* infection in chickens (Kalmar et al., 2015), and chicken embryos (Braukmann et al., 2012), another one describes the infection of two different genotypes of *C. psittaci* considered of low and high virulence in chickens (Yin et al., 2013b), and the last two correspond to *C. psittaci* infection in turkeys (Vanrompay et al., 1994), and ducks (Thierry et al., 2016).

After the experimental infection of seven day–old chickens through aerosolized *C. psittaci* strain DC15 (isolated from an abortion episode in cattle) (Kalmar et al., 2015), chickens showed lethargy, rhinorrhea and dyspnea at six days post infection (dpi). At 14 dpi, all *C. psittaci*–infected chickens remained prostrated and showed severe dyspnea (breathing with open beaks). No deaths occurred. At the post mortem examination, birds showed severe congestion of the lungs, pneumonia, fibrinous airsacculitis, fibrinous pericarditis and severe enlargement of the liver. When low and highly virulent avian strains (Yin et al., 2013b), genotypes B and D, respectively, were inoculated into chickens through aerosolization, pharyngeal and cloacal excretion, indicative for systemic dissemination, as well as clinical signs such as conjunctivitis, rhinitis, and dyspnea, were observed in both experimentally infected groups. However, genotype D infected group showed higher mortality rate and more severe clinical signs and lesions as compared to genotype B infected group.

When comparing the experimental infection caused by *C. psittaci* strain DC15 *vs C. abortus* strain S26/3 in both chicken embryos and chickens at immunological level (Braukmann et al., 2012; Kalmar et al., 2015), it can be noticed that the expression of relevant factors, (including IFN-ɣ) was significantly stronger up-regulated in *C. psittaci*– than in *C. abortus*–infected birds, but equivalent in chicken embryos. These results reveal a more intense immune response following infection with *C. psittaci*. Clinical signs and lesions at the postmortem examination, only measured in chickens, appeared sooner and were more severe in the *C. psittaci*–infected group. A more intense systemic dissemination of *C. psittaci* in chickens, was positively correlated with higher and faster infiltration of immune cells. Likewise, *C. psittaci* displayed a significantly better capability of dissemination in the chorioallantoic membrane (CAM) and internal organs of the chicken embryo (liver, spleen, bursa of Fabricius, stomach, intestine, heart, and brain) than *C. abortus*.

An experimental infection was carried out in ducks with the genotype E/B of *C. psittaci* (Thierry et al., 2016). Oral inoculation with various infectious doses caused mild diarrhea only in the group with the highest challenge dose; no further clinical signs were observed. Oral intake of *C. psittaci* resulted in systemic dissemination of the bacterium, leading to its detection in all analyzed organs particularly in cecum, liver, and

spleen but without histopathological lesions. Bacterial colonization of the organs reached a plateau after a few days, depending on the infectious dose, and then decreased. Interestingly, control birds left in contact with the infected ones showed an equivalent level of infection when euthanized at 10 dpi.

Turkeys experimentally infected by aerosolization with genotypes A, B and two strains of genotype D (Vanrompay et al., 1994), showed lethargy, depression, sneezes, sinusitis, respiratory distress, conjunctivitis with unilateral corneal opacity, head shaking and diarrhea. The clinical condition of birds improved from day 17 to 21 pi and then, they became lethargic with respiratory distress again, by day 29 to 31 it seemed to have improved but finally it deteriorated again by day 34 pi. Some birds of the group inoculated with genotype A and a strain of genotype D died. At the postmortem examination, mild to moderate lesions were present in the conjunctiva, cornea, sinus, kidney and gut, the most affected organs were lungs, airsac, pericardium, spleen, liver, especially in the groups inoculated with genotype A and D. An unusual clinical presentation of a natural infection caused by *C. psittaci* genotype B in turkey flocks occurred during an outbreak in California, in which 3-5% of the flock manifested mild to severe inflammation of the nasal glands, without further respiratory signs or mortality, except for a slight drop in water and feed consumption (Shivaprasad et al., 2015).

1.5.3. Other chlamydial species infecting birds

Pathogenesis and clinical manifestations of the infection in birds by the newly described chlamydial species have not been fully elucidated. So far, *C. gallinacea* could not be linked to any clinical disease in naturally infected birds (Donati et al., 2018; Heijne et al., 2018; Szymańska-Czerwińska et al., 2017a). However, in the study of Guo et al., (2016), experimentally inoculated broiler chickens with *C. gallinacea* showed a significantly lower body weight gain than the control group, especially from week three pi to week five. Remarkably, *C. gallinacea* seems not to be restricted to birds, as it was the main chlamydial species detected in vaginal swabs, whole blood and milk from cattle in China (J. Li et al., 2016).

In contrast, it has been suggested that *C. avium* might be associated with clinical signs of disease. For example, a psittacine from Germany showed digestive signs of disease before dying and the post-mortem examination revealed catarrhal enteritis and hepatosplenomegaly, while several other birds of the same flock had also died with similar signs. Other *Chlamydiaceae* species were not detected (Sachse et al., 2014). However, in pigeons *C. avium* role as a primary pathogen seems to be more controversial. Indeed, it has been linked with respiratory disease and diarrhea in German *C. avium*-positive pigeon flocks, but other microorganisms were simultaneously identified, such as *Mycoplasma* spp., *Candida albicans*, *Trichomonas gallinae*, and *Spironucleus columbae* (Sachse et al., 2014). Conversely, *C. avium* strain PV 4360/2 was isolated from a dead pigeon without pathological findings at the post-mortem examination (Floriano et al., 2020). Probably, the role of *C. avium* as a pathogen could comprise a wide range of presentations, from asymptomatic to overt clinical diseases.

So far, the presence of *C. ibidis* has not been found to cause disease in any of the birds in which it has been detected (Z. Li et al., 2019; Vorimore et al., 2013). *Chlamydia buteonis* has been associated with signs of disease in the red-shouldered hawk from which it was first isolated, the clinical signs include conjunctivitis before the bird died, as well as mild hepatitis and splenitis at the histopathological post mortem analysis (Laroucau et al., 2019).

1.5.4. Diagnosis and characterization

According with the last version of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019, issued by the World Organization for Animal Health, in the chapter 2.3.1 for avian chlamydiosis (OIE, 2019), the available methods for chlamydial infection diagnosis in birds can be divided in those detecting directly the microorganism (either by isolating the bacterium or by detecting its antigens or its nucleic acids), and those detecting the immunological response of the host. A selection of these diagnosis assays and their epidemiological purpose is presented in **Table 3**. considering that there are no policies to eradicate avian infections caused by *Chlamydia*. Each method is going to be reviewed in the following sections.

Table 3. Selected methods for the diagnosis of chlamydial infections in birds and their epidemiological purpose. Adapted from the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 (OIE, 2019).

Method	Epidemiological purpose			
	Population freedom from infection	Individual freedom from infection prior to movement	Confirmation of a clinical case	Prevalence of infection – surveillance
Direct bacterial identification				
Conventional PCR[1]	–	–	++	+
Real–time PCR	–	–	+++	++
In vitro isolation	–	–	++	+
Antigen detection (IHC[2]/IF[3])	–	–	++	–
Detection of host immune response				
CFT[4]	+	+	+	+
ELISA[5]	++	+	+	++

[1]PCR: polymerase chain reaction, [2]IHC: immunohistochemistry, [3]IF: immunofluorescence, [4]CFT: complement fixation test, [5]ELISA: enzyme–linked immunosorbent assay. –: not appropriate for this purpose, + = could be used in some situations, but cost, reliability, etc. severely limits its application, ++: suitable method but may need further validation, and +++: recommended method.

Chlamydia diagnosis in birds can be conducted in different situations (Balsamo et al., 2017). If psittacosis human cases have been confirmed, it will be relevant to identify the source of contamination and, therefore, direct detection in birds should be carried out, preferably through molecular tests. If *C. psittaci* infection has been diagnosed in birds within a flock (wild birds in captivity or pet birds), it will be necessary to identify the infected animals, to isolate them and, if a treatment is applied, to ensure the absence of chlamydial excretion at the end of the treatment. Chlamydial detection could also be performed on each bird before its incorporation

into a flock (at the breeding or selling facilities), or before its transport. Chlamydial screening of birds at frequent contact with public should also be performed, e.g. bird encounters, long-term care facilities, exhibition, schools, etc. Special cases for chlamydial detection include specific-pathogen-free birds (SPF), or before reintroduction of a rehabilitated wild birds.

1.5.4.1. Samples

There are two approaches for the diagnosis of chlamydial infection in birds. The first one is the direct detection of the bacterium in tissue, fecal or swab samples (choanal/oropharyngeal or cloacal) and the second one is the demonstration of anti-chlamydial antibodies through a serological screening of blood samples.

Some studies have pointed out that the positivity rate of choanal/oropharyngeal swabs tend to be higher than that of cloacal swabs. Moreover, in birds from which both oral and cloacal swabs were collected, only few tested positive in both samples. Birds having a mixed infection with two chlamydial species, could even shed a different species in each sample (Andersen, 1996; Guo et al., 2016). Additionally, it has been found that choanal/ oropharyngeal swabs are more consistent for the isolation of the bacterium than feces or cloacal swabs, especially during early stages of infection (Harkinezhad et al., 2009). However, most studies privilege the use of cloacal swabs because of the ease of taking this sample, particularly at the slaughterhouse to avoid unnecessary handling of birds. Besides, most of the chlamydial environmental or facility contamination comes from feces (Hulin et al., 2016; Vogler et al., 2019). In the study performed by Guo et al., (2016), it was shown that shedding via intestinal tract is especially important for *C. gallinacea*, as the bacterial load was higher in cloacal swabs, followed by oropharyngeal swabs, and samples of lung, heart, kidney and liver. Conjunctival swabs could also be taken with successful odds for bacterial isolation when there is conjunctivitis (Laroucau et al., 2019).

If the study is conducted in birds whose health status must be corroborated to prevent zoonotic cases or the introduction of a chlamydial agent into a flock, e.g. companion birds, birds in rehabilitation centers, etc., it is recommended to take individual specimens from multiple anatomical sites. This is due to the fact that some infected birds can be detected only from one sample that can be either choanal/oropharyngeal or cloacal (Andersen, 1996). From dead animals it is possible to take multiple organs to perform molecular assays and even to isolate the strain.

Preservation of samples is crucial to achieve bacterial isolation. For strain isolation, organs and swabs are recommended to be stored in a specific medium based on sucrose/phosphate/glutamate (SPG medium), first developed for *Rickettsiales*, but suitable for *Chlamydiales* as well (Andersen, 2008). Depending on the type of sample submitted, if the specimen is for isolation, the use of antimicrobials with specific antibacterial and antifungal activity is recommended. Once taken, samples intended for the isolation of the strain should preferably be stored in refrigeration, or stored at -80°C, for the shortest possible time until its processing in the

laboratory (Sachse et al., 2009b). The more time a sample is stored since it was taken, or the more freeze and thaws cycles a sample undergoes, the less likely it will be to isolate the strain.

For molecular assays, a simple dry swab could be used. However, bacterial recover and adequate test performance depends on a correct sampling technique using the right materials. In a study carried out by Panpradist et al., (2014), it was shown that rayon swabs performed well for excess-volume samples (beyond swab saturation), but showed poor recovery for low-volume samples (tending to dry surfaces). Nylon and polyester swabs showed intermediate bacterial recovery for low-volume and excess-volume samples. While polyurethane swabs showed excellent recovery for all sample types.

1.5.4.2. In vitro isolation and propagation

Due to the obligate intracellular lifestyle of *Chlamydia*, it requires an eukaryotic cell to be isolated and propagated. So far, attempts to achieve an efficient axenic culture, although promising, still need to be further developed (Omsland et al., 2008). Thus, chlamydial *in vitro* growth relies either on primary or permanent cell lines, chicken embryos or animal models. Isolation has been considered as the gold standard test in chlamydial diagnosis (Sachse et al., 2009b). Chlamydial *in vitro* growth has been considered to be of paramount importance for bacterial characterization by molecular methods. Nowadays, isolation is still considered ideal to demonstrate bacterial viability of a field strain. In order to achieve the isolation, all considerations must be taken to preserve chlamydial viability in the sample during transportation and storage. The presence of contaminating bacteria and fungi should be limited adding antimicrobials such as streptomycin, gentamicin, vancomycin and nystatin (Andersen, 2008).

The first method to cultivate *Chlamydia* from bird specimens, other than their natural host, was developed in early 1930's. At this time, *Chlamydia* was successfully grown into the chorioallantoic membrane of chicken embryos. Soon after, other chlamydial agents were successfully isolated using this route of inoculation, thus the yolk-sac became the most extensively used method to isolate chlamydiae in diagnosis laboratories (Sachse et al., 2009b). Chicken embryos must be between six to seven days old. Once inoculated, embryos should be checked daily through ovoscopy, as dead is expected between 72 hr to 14 days post infection, depending on the chlamydial strain, then the yolk sac is collected. If the embryo does not die, up to two passages could be performed before declaring a sample as negative. At the present time, chicken embryos are still being used for chlamydial isolation, particularly for massive antigen production to use in experimental infections and, in to a lesser extent, to grow difficult clinical/field samples (Sachse et al., 2009b).

Table 4. Cell lines commonly used for *in vitro* chlamydial growth.

Name	Origin	Comments	References
McCoy	Human synovial fluid with degenerative arthritis, subsequent sub-lines shown to be fibroblast of mouse origin	*C. psittaci*, *C. trachomatis*, *C. abortus*	(Croy et al., 1975) (Wills et al., 1984) (Barnes, 1989) (Johnston and Siegel, 1992)
Buffalo Green Monkey Kidney (BGM or BGMK)	African green monkey (*Cercopithecus aethiops*) epithelial-like cells from kidney	*C. psittaci*, *C. trachomatis*, *C. abortus*	(Barron et al., 1970) (Wills et al., 1984) (Barnes, 1989) (Johnston and Siegel, 1992)
HeLa 229	Human epithelial cells from cervical cancer	*C. trachomatis*	(Croy et al., 1975) (Barnes, 1989)
HL	Human myeloblastic from acute promyelocytic leukemia	Used for some strains of *C. pneumoniae* difficult to grow in cell culture	(Roblin et al., 1992)
HEp-2	Human epithelial carcinoma, *prob*. larynx carcinoma	Used for some strains of *C. pneumoniae* difficult to grow in cell culture *C. gallinacea*	(Käding et al., 2017) (Guo et al., 2016) (Wong et al., 1992) (Roblin et al., 1992)
H 292	Human epithelial mucoepidermoid pulmonary carcinoma	Used for some strains of *C. pneumoniae* difficult to grow in cell culture	(Wong et al., 1992)
African green monkey kidney (Vero)	African green monkey (*Cercopithecus aethiops*) fibroblast-like cells from kidney	*C. psittaci*, *C. abortus*	(Croy et al., 1975) (Sachse et al., 2009b)
Baby hamster kidney (BHK)	Newborn hamster fibroblast kidney cells	*C. abortus*	(Croy et al., 1975)
Fibroblast L cells	Mouse fibroblast from skin	*C. psittaci*, *C. abortus*	(Croy et al., 1975)
Caco-2	Human epithelial from colon adenocarcinoma	Used for *C. suis*, which is difficult to isolate and propagate in cell culture. Also tested for *C. pecorum*	(Schiller et al., 2004)
HD11	Chicken hematopoietic cells (monocytes/ macrophages) transformed by avian leukemia viruses	*C. psittaci*	(Beug et al., 1979) (Beeckman et al., 2010)

As revised by Sachse et al., (2009b), from 1940's decade, several monolayer tissue cultures have been tested to isolate chlamydial strains. Due to the advantages provided by cell culture over chicken embryo to achieve chlamydial isolation, e.g. easier handling, less laborious, no mortality issues due traumatism and ethical concerns; the isolation of *Chlamydiae* in confluent monolayers gradually took advantage. *Chlamydia* has the ability to infect different cell types, thus, several cell lines have been used for chlamydial isolation. **Table 4** shows the main cell lines that have been used for *in vitro* chlamydial growth, among the most used are Buffalo Green Monkey (BGM), McCoy, HeLa and HEp–2. Successful propagation of the strain depends on factors relying on the specimen (e.g. storage and transportation conditions, the time the specimen has been stored) but

also on cell line, chlamydial species, cultivation medium, additives used, incubation temperature and other technical conditions such as the use of centrifugation assisted infections. Chlamydial growth is going to be reviewed in chapter II. After the inoculation either in chicken embryo or in cell monolayers, specimens must be further analyzed with an antigenic detection method and/or tested through molecular assays to corroborate the presence of bacteria.

1.5.4.3. Antigenic detection: immunohistochemistry and immunofluorescence

Specimens for antigen detection can be impression smears from fresh tissues taken during necropsy, formalin-fixed and paraffin-embedded tissues (e.g. lungs, liver, gut, spleen, airsacs, sinus, etc.) after bacterial isolation on cell culture or chicken embryo, impression smears of infected yolk sac or chorioallantoic membrane, as well as coverslips with inoculated cell monolayers.

Depending on the specimen and the objective of the study, different techniques can be used, i.e. immunohistochemistry is more commonly performed in formalin-fixed paraffin-embedded tissue, while immunofluorescence is performed from impression smears of tissues, methanol-fixed cell cultures, or formalin-fixed paraffin-embedded tissues. Both methods, immunohistochemistry and immunofluorescence, are based on the use of labeled antibodies directed against chlamydial epitopes allowing the detection of *Chlamydiaceae*. Many of the commercially-available tests are based on antibodies against the chlamydial lipopolysaccharide (LPS), or monoclonal antibodies targeting MOMP (Sachse et al., 2009b).

1.5.4.4. Antibodies detection: serological tests

Antibody detection tests to identify a chlamydial infection in birds are more suited for epidemiological studies than for diagnosis purposes, especially in individual birds, and still there are some constraints to be considered. As long-term persistence of anti-chlamydial antibodies has been reported when a birds has been infected by *Chlamydia*, examination of paired sera is needed, coupled with further antigenic or molecular detection of the bacterium from swabs (OIE, 2019).

The complement fixation test (CFT) was a routinely used assay that nowadays is falling into obsolescence. This test is based on the detection of anti-chlamydial antibodies in sera from birds. The complement, a set of proteins in the sera, making part of the innate mechanisms of immunity, is added to the pre heat–inactivated serum of the bird to be tested in the presence of the antigen. If specific antibodies are present in the serum, they will bind to the antigen and take up the added complement. Then, a hemolytic system composed of antibodies and red blood cells is added that will interact with the complement if it has remained free. The test is negative when the lysis of the red blood cells occurs (pink coloration), and positive when red blood cells remain intact (sedimentation) (Sachse et al., 2009b). The CFT test has the major disadvantage that sera from different species may react differently in the test, being sera from pigeons the most reliable. Furthermore, CFT antigen for *Chlamydia* is no longer commercialized.

No commercial enzyme-linked immunosorbent assays (ELISA) to be used in birds are available, and few methods have been developed by research laboratories. Existing ELISA assays have variable levels of sensitivity, specificity and reproducibility (Verminnen et al., 2006).

1.5.4.5. Bacterial nucleic acids detection methods

Molecular methods have revolutionized the knowledge on *Chlamydiae*. For illustration, they have allowed the discovery and description of new species based on the sequencing of their genome from biological samples. That is the case for *C. corallus* (Taylor-Brown et al., 2017) and *C. testudinis* (Laroucau et al., 2020b). In addition, the high stability of the DNA makes it possible to analyze poorly preserved samples.

According with the last version of the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, the preferable diagnosis test to detect chlamydial infections in birds, is no longer isolation of the bacterium but the molecular tests searching to amplify and characterize the nucleic acids, e.g. PCR as the most evenly used method, DNA microarray–based detection systems and DNA sequencing (OIE, 2019).

The PCR consists on amplifying a targeted sequence using specific primers, then visualizing this amplification after migration on agarose gel. Real–time PCR, on the other hand, share the same basis but the amplification is measured throughout the reaction using a probe coupled to a fluorochrome or an intercalating agent. This technique makes it possible to rapidly obtain an amplification kinetics curve and, more importantly, to quantify the amount of DNA present in the sample (Sachse et al., 2009b). It has become the diagnostic method of choice in many veterinary laboratories because of its speed and superior sensitivity compared to conventional PCR. Several different real-time PCR protocols targeting different genes have been suggested in the literature, although the most extensively used is the broad range real time PCR targeting a well conserved region in the 23S rRNA operon shared among *Chlamydiaceae* (Ehricht et al., 2006). This technique allows the identification of all *Chlamydiaceae* rather than targeting specific species. Some other systems have been developed and they have been reviewed by Sachse et al., (2009b).

As it was mentioned in section 1.5.1 diversity of chlamydial infections found in birds, several chlamydial species can be present in birds and even mixed infections can occur. Since some chlamydial species have a well–recognized zoonotic potential, e.g. *C. psittaci* and *C. abortus*, thus it is important to identify the chlamydial species when a bird is *Chlmydiaceae*-positive. For this purpose, it is recommended to follow a hierarchical approach in which the first step would be a *Chlamydiaceae* screening by PCR, followed by species specific PCR systems (OIE, 2019). Several PCR systems and DNA microarrays have been developed by research groups over the years. In **Table 5** are shown some PCR detection systems described for the main chlamydial species of veterinary interest.

Table 5. PCR detection systems described for the detection of main chlamydia species of veterinary interest.

Chlamydial species	Targeted gene	PCR system	Nucleotide sequence 5'- 3'	Reference
Chlamydiales	16S rRNA	panCh16F2 panCh16R2 panCh16S	CCGCCAACACTGGGACT GGAGTTAGCCGGTGCTTCTTTAC FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1	(Lienard et al., 2011)
Chlamydiaceae	23S rRNA	Ch23S-F Ch23S-R Ch23S-p	CTGAAACCAGTAGCTTATAAGCGGT ACCTCGCCGTTTAACTTAACTCC FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA	(Ehricht et al., 2006)
C. abortus	*omp*A	CpaOMP1-F CpaOMP1-R Cpa-OMP1-P	GCAACTGACACTAAGTCGGCTACA ACAAGCATGTTCAATCGATAAGAGA FAM-TAAATACCACGAATGGCAAGTTGGTTTAGCG-TAMRA	(Pantchev et al., 2009)
C. avium	*eno*A	APC-Fw APC-Rv APC-Pr	CATGCAAGCTATTGAGAAAAGTGGT CCTTGATATGTACGTGTTTTCTCG FAM-CACCCCTGGTGAAGATATTTCCTTAGCAT-TAMRA	(Zocevic et al., 2013)
C. buteonis	*oxa*A	RSHA-F RSHA-R RSHA-P	ATTTCCAACACGCACTGCAT TGGGACTAGGTGTTTCTCCCT FAM-GGACAACATGCCTAGATGAAGA-TAMRA	(Laroucau et al., 2019)
C. caviae	*omp*A	CpcavOMP1-F CpcavOMP1-R CpcavOMP1-S	GAATAACATAGCCTACGGCAAACATA CGATCCCAAATGTTTAATGCTAAGA FAM-CAAGATGCAGAATGGTCCACAAACGC-TAMRA	(Pantchev et al., 2010)
C. felis	*omp*A	CpfOMP1-F CpfOMP1-R CpfOMP1-S	TCGGATTGATTGGTCTTGCA GCTCTACAATGCCTTGAGAAATTTC FAM-ACTGATTTCGCCAATCAGCGTCCAA-TAMRA	(Pantchev et al., 2010)
C. gallinacea	*eno*A	enoA_F enoA_R enoA_P	CAATGGCCTACAATTCCAAGAGT CATGCGTACAGCTTCCGTAAAC FAM-ATTCGCCCTACGGGAGCCCCTT-TAMRA	(Laroucau et al., 2015)
C. ibidis	*omp*A	Forward Reverse Probe	TCTTTGGGAATGTGGTTGG GGTATCCTTCTC CGTCCCAG FAM-CCGCAGCGCAATTCAAGTGCAC-TAMRA	(Z. Li et al., 2019)
C. pecorum	*omp*A	Cppec-F Cppec-R Cppec-P	CCATGTGATCCTTGCGCTACT TGTCGAAAACATAATCTCCGTAAAAT FAM-TGCGACGCGATTAGCTTACGCGTAG-TAMRA	(Pantchev et al., 2010)
C. psittaci	*omp*A	CppsOMP1-F CppsOMP1-R CppsOMP1-S	CACTATGTGGGAAGGTGCTTCA CTGCGCGGATGCTAATGG FAM-CGCTACTTGGTGTGAC-TAMRA	(Pantchev et al., 2010)
C. serpentis	*gat*C	Serpentis_192 F Serpentis_291R Serpentis_251 P	TGAAGACTTAAGAGAAGATGCGGT TGCGGGGACTTTTACTAGCC FAM- ACGTTCCAG AGT CTT TAG GGG-TAMRA	(Laroucau et al., 2020a)
C. suis	23S rRNA	Csuis23S-F Csuis23S-F Csuis23S-F	CCTGCCGAACTGAAACATCTTA CCCTACAACCCCTCGCTTCT FAM-CGAGCGAAAGGGGAAGAGCCTAAACC-TAMRA	(Pantchev et al., 2010)
C. testudinis	*isp*E	Forward Reverse Probe	TTTCGGCTTCGTCCAGATCTC AGGTTGCTCCAGATCCTGAC FAM-TGGAATCCCTTTCAAGGTAAAGTCT-TAMRA	(Laroucau et al., 2020b)

1.5.4.6. Chlamydial characterization methods

First, if *C. psittaci* infection was confirmed, the specimen could be further subtyped. Initially, typing of *C. psittaci* strains was performed by microimmunofluorescence using monoclonal antibodies targeting epitopes of the major outer membrane protein (MOMP). Six avian serovars (A to F) and two non-avian serovars (M56 and WC) were identified using this technique (Andersen, 1991; Vanrompay et al., 1993). Later on, a conventional PCR method amplifying the *omp*A gene followed by RFLP (Restriction Fragment Length Polymorphism) was used. This technique is based on restriction enzymes (e.g. *Alu*I and *Mbo*II) in the PCR product generating different restriction profiles patterns revealing high correlation with serotyping results (Sayada et al., 1995; Vanrompay et al., 1997).

Then, DNA–based microarrays were developed; this molecular technique is based on hybridization between a labelled probe and a specific gene sequence. This technique was initially designed for identifying all species in the *Chlamydiaceae* family (Ehricht et al., 2006; Sachse et al., 2005) and was later developed for *C. psittaci* typing (Sachse et al., 2008). Microarrays allowed the simultaneous testing of a large number of samples, as

well as the identification of six potentially new genotypes and subgenotypes for the A, D and E/B genotypes (Sachse et al., 2009a). Genotypes of *C. psittaci* are based on sequences of the *omp*A gene, which encodes the major outer membrane protein MOMP (see 1.4.4 antigenic structure of *Chlamydia*). So far, at least 15 genotypes have been identified, for some of them subgroups have been identified (i.e. genotype A, subgroup VS1, 6BC and 84-55; genotype D, subgroup NJ1 and 9N; genotype E/B, subgroup WS/RT/E30, 06-859/1 and KKCP-1) **(Table 6)**. Each genotype has been predominantly found infecting one host species, nevertheless they have also been found in other host species.

MLVA (Multi-Locus VNTR Analysis) was the first technique applied to typing chlamydial strains targeting other genetic regions than the *omp*A gene. The MLVA technique is based on the analysis of VNTRs (Variable Number of Tandem Repeats). VNTRs are short sequence of nucleotides (less than 100 bp) repeated in tandem, a variable number of times in the genome. This technique was developed for *C. psittaci* (Laroucau et al., 2008), and then for *C. abortus* (Laroucau et al., 2009c), allowing the direct and fast typing of clinical samples.

Sequencing era was just starting and MLST (Multi-Locus Sequence Typing) technique was developed. Pannekoek et al., (2008) proposed a scheme using sequences of seven housekeeping genes (*eno*A, *fum*C, *gat*A, *gid*A, *hem*N, *hlf*X, *opp*A) to elucidate chlamydial strain diversity and possible associations between sequence type and clinical outcome. Using this same scheme, a specific study was performed for *C. psittaci* to establish an association between sequence type and host species (Pannekoek et al., 2010). The same analysis was carried out using 10 isolates of *C. gallinacea* by Zocevic et al., (2012). However, only four out of seven housekeeping genes were successfully amplified (*eno*A, *gat*A, *gid*A and *hlf*X), probably because of sequence divergences. Surprisingly, the analysis evidenced a higher diversity between strains than previously seen for other species such as *C. psittaci* and *C. abortus*, but comparable to *C. trachomatis* and *C. pecorum*. No further associations of *C. gallinacea* diversity could be performed (i.e. host or geographic origin) (Zocevic et al., 2012). Few years later, Guo et al., (2017) developed specific primers to amplify and sequence seven housekeeping genes to perform MLST following the same schema aforementioned but with some variants, i.e. *opp*A-3, based on the sequence of *C. gallinacea* type strain 08-1274/3 and other chlamydial species. This study reinforced the observation that *C. gallinacea* exhibits substantial intra-species genetic diversity. Sequence types or allelic profiles are determined from the *Chlamydiales* MLST database (http://pubmlst.org/chlamydiales/).

Table 6. *C. psittaci* described genotypes encompassing the suggested subgroups and representatives of six new provisional genotypes (*). Adapted from Sachse et al., (2009a) and (2008). Genotypes associated to zoonotic cases are in bold, according with the review carried out by Hogerwerf et al., (2020).

Genotype	Type strain	GenBank accession number	Predominant associated species
A	VS1 6BC 84-55	AF269281.1 X56980.1 Y16561.1	Psittacine birds, chickens, turkeys, ducks, geese, guinea fowl and passerines
B	CP3	AF269265.1	Pigeons, turkeys, swans and peacocks
C	GR9	L25436.1	Ducks, chickens, turkeys and partridges
D	NJ1 9N	AF269266.1 EF375557.1	Turkeys, chickens, seagulls, pigeons, psittacine birds and guinea fowl
E	CPMN	X12647.1	Ducks, turkeys, pigeons, ostriches and nandous
F	VS225	AF269259.1	Psittacine birds and turkeys
E/B	WS/RT/E30 06-859/1 KKCP-1	AY762613.1 EU159263.1 AB284062.1	Ducks, chickens, turkeys and geese
M56	M56	AF269268.1	Muskrats and hares
WC	WC	AF269269.1	Cattle
1V*	1 V	EF028916.1	Passerine (corvids)
6N*	6 N	EF197820.1	Passerine (corvids)
Mat116*	Mat 116	AB284058.1	Psittacine birds (budgerigar)
R54*	R 54	AJ243525.1	Seabirds (skua)
CYP84*	Daruma-1981	AB284065.1	Psittacine birds
CPX0308*	CPX0308	AB284064.1	*Ciconiiformes* (storks)

Reduction of costs and time of sequencing is making it the method of choice for characterizing chlamydial strains or specimens, especially when atypical chlamydia appears. Atypical chlamydia classification mostly relies on DNA– or protein–based phylogenetic reconstructions, particularly because sometimes they are difficult to grow *in vitro*. For this reason, Pillonel et al., (2015) proposed the analysis of up to twenty protein sequences that allows a highly reliable classification of new specimens. The process to assess bacterium identity starts with the analysis of 16S rRNA and 23S rRNA gene sequences to classify it within a family inside *Chlamydiales* order. Then, four other genes (DnaA, SucA, Hyp325 and FabI) are analyzed to classify the specimens within the same genus, and finally, the analysis of five other genes (RpoN, FtsK, PepF, Adk and HemL) allows to classify the specimens within the same species, based on the percentage of sequence identity **(Figure 7)**. To date, MLST and the polyphasic approach for chlamydial classification proposed by Pillonel et al., (2015) are probably the most robust and consistent methodologies to characterize chlamydial strains.

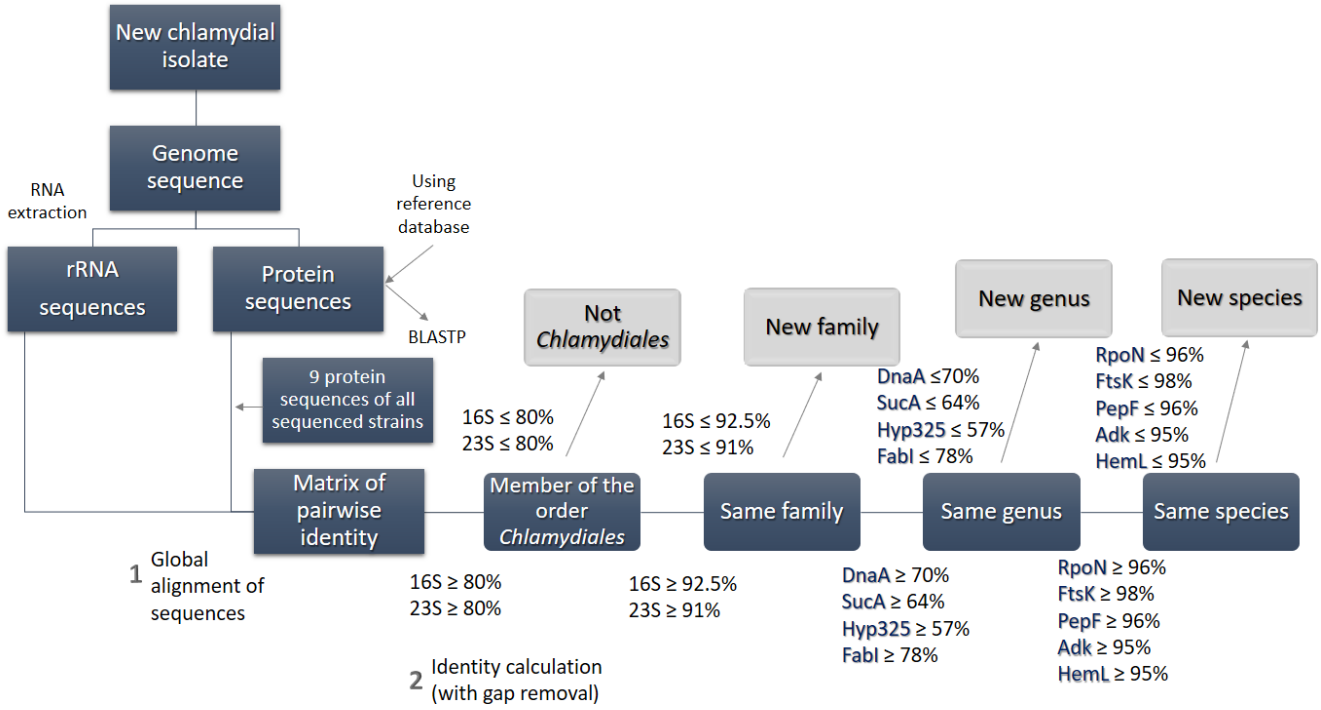

Figure 7. Chlamydial classification based on a polyphasic approach using percentage of genomic and protein sequences similarity. BLASTP is Protein-protein BLAST (basic local alignment search tool). Adapted from Pillonel et al., (2015).

1.5.5. *Preventive measures and treatment in birds*

There are no commercial vaccines available for chlamydiosis control in poultry or any other birds (OIE, 2019). New attempts have been carried out to develop a protective vaccine for birds, so far, these efforts have been done only for *C. psittaci*. Nevertheless, some of the candidate vaccines are at an *in vitro* stage of development, requiring further testing in animal models, e.g. a recombinant vaccine constructed in a herpesvirus of turkeys (HVT) expressing the chlamydial polymorphic membrane protein D (PmpD) (Liu et al., 2017). Others vaccines confer only partial protection to birds during *in vivo* assays, e.g. a recombinant vaccine constructed also in a HVT virus but with different promoter, expressing the same chlamydial PmpD protein (Liu et al., 2015).

Control and treatment of chlamydial infections in diseased animals relies on the use of antimicrobials, e.g. tetracyclines, quinolones, macrolides and even rifamycins (Bommana and Polkinghorne, 2019; Sachse et al., 2015b). However, as reported for human chlamydial infections, relapses, intermittent shedding of bacteria as well as disease progression, even after treatment with antimicrobials, have also been reported for treated animals (Borel et al., 2016). When conditions for the development of *Chlamydia* are not optimal, e.g. antimicrobial sub–treatment or exposure to β-lactam antibiotics, bacteria can enter a state of persistence (see 1.4.2 persistence section), in which it remains viable until optimal growth conditions are restored, in this case when the antimicrobial treatment is suspended.

The most used antimicrobial classes in birds to treat chlamydial infections are tetracyclines, especially oxytetracycline or its derivative doxycycline and the fluoroquinolone enrofloxacin, both with the advantage of

massive administration through drinking water or food. It should be observed that long periods of treatment are suggested, ranging from 21 to 25 days (Bommana and Polkinghorne, 2019). Historically, a 45-day treatment with antimicrobials has been recommended (Balsamo et al., 2017). For treatment of individual birds, a parental administration is also possible through intramuscular route. In fact, an experimental infection performed in psittacines, a 21–days treatment with either doxycycline or the macrolide azithromycin was effective in eliminating *C. psittaci* infection. Bacterial clearance was corroborated through PCR of the liver and spleen of the euthanized birds, although histopathological lesions, considered as residual, remained in these organs (Guzman et al., 2010). Ultimately, the elimination of the bacteria is also related to the immune system of the infected bird, therefore it is suggested retest birds using a PCR-based method 2 to 4 weeks after finishing treatment (Balsamo et al., 2017).

1.6. Chlamydial zoonotic infections from birds: only psittacosis?

Psittacosis is also called parrot fever or ornithosis. Birds are the major reservoir for *C. psittaci*. Most of the reported avian–associated psittacosis cases come from birds of the order *Psittaciformes* (parakeets, parrots, macaws, lories, cockatoos, and budgerigars), *Anseriformes* (ducks, geese), *Galliformes* (chickens, turkeys, pheasants), *Columbiformes* (pigeons and doves) and *Passeriformes* (passerine birds) (Hogerwerf et al., 2020). In fact, according with the review performed by Hogerwerf et al., (2020), poultry, i.e. turkeys, chickens and ducks, are the main *C. psittaci* source of transmission for humans according to most the number of cases reported in the literature of zoonotic infections , exceeding those related to psittacines. However, *C. psittaci* infection in humans could also occur following their exposure to other non–avian infected animals, e.g. equine fetal membranes infected with the bacterium (Chan et al., 2017).

Transmission to humans occur through the airborne route or by direct inoculation into the eyes. *C. psittaci* is shed in massive quantities by infected birds in respiratory secretions or feces, secretions or dried feces are aerosolized in the air as very fine droplets or dust particles (Chu and Durrani, 2020). Infection could be also transmitted indirectly to humans from environment contaminated by feces of infected birds, e.g. poultry houses, slaughterhouses, bird exposition centers, veterinary hospitals or breeding facilities (Hulin et al., 2016). Handling of birds and slaughter of poultry are considered the riskiest activities, as reported in most of the recent outbreaks (Dickx et al., 2010; Hulin et al., 2015; Lagae et al., 2014; Shaw et al., 2019). Additionally, veterinarians working in veterinary laboratories are at risk of exposure when post–mortem examinations are performed. The World Organization for Animal Health (WHO, 2019) recommends that necropsies and sampling of dead birds presumably infected by *C. psittaci*, as well as handling of cultures, should be done in certified class II laminar flow hoods whenever possible or with proper protective equipment, followed by appropriate decontamination procedures.

The incubation period is usually 5–14 days; however, longer incubation periods have been observed. Human infections vary from unapparent to severe systemic disease involving multiple organs. The disease is often

described as an influenza–like syndrome, which is characterized by fevers, chills, headache, and a cough. Respiratory illness could lead to interstitial pneumonia and encephalitis (Chu and Durrani, 2020).

To date, the only reliable method to prevent zoonotic cases is risk reduction strategies of people having contact with birds, either because of their work activity, travel history, hobbies, or because they own pet birds, among other possible reasons (OIE, 2019). All working activities involving handling of birds require to wear personal self–protective equipment designed to protect the respiratory tract from airborne transmission of infectious agents, as well as eye protection, specific work clothing, and to apply all additional biosecurity measures to reduce the risk. Human-to-human transmission could be possible, but it is thought to be a rare event (Balsamo et al., 2017). Nowadays disease is rarely fatal in properly treated patients, thus, it is essential that physicians be aware of zoonotic chlamydial infections enabling a proper clinical history and an early diagnosis.

There is a compendium of measures to control *C. psittaci* infections among humans and pet birds, which is regularly updated, and assembles information about psittacosis to make it available for public health officials, physicians, veterinarians, pet bird industry, and other sectors concerned (Balsamo et al., 2017). This compendium is elaborated by members of the American Veterinary Medical Association, the American Association of Avian Veterinarians, the Pet Industry Joint Advisory Council, the American Association of Zoo Veterinarians and veterinary epidemiologists, and they recommend the following measures to help efforts to prevent chlamydial transmission from birds to humans: educate persons at risk and healthcare providers about psittacosis, reduce risk of human infection when caring for diseased or exposed birds, maintain records of all bird-related trades for at least one year in order to identify possible sources of infected birds and potentially exposed people, avoid trading birds that have clinical signs consistent with avian chlamydiosis, avoid mixing birds from multiple sources, quarantine newly acquired or exposed birds and isolate diseased birds, screen birds with frequent public contact, test birds before its arrival to new facilities and before being sold, establish good husbandry practices coupled with biosecurity measures and disinfection procedures.

The zoonotic potential of the newly described chlamydial species has not been fully understood. There is a report indicating that the presence of infected flocks with *C. gallinacea* coincided with cases of atypical pneumonia in workers, however, duck flocks were also occasionally slaughtered in the same plant. Therefore an infection of poultry workers with *C. psittaci* could not be ruled out (Laroucau et al., 2009b). Since this punctual case, no zoonotic infections by *C. gallinacea* nor by other chlamydial species associated with avian infections have been reported.

1.7. Biosafety regulatory framework when working with *Chlamydia* in the laboratory

The World Health Organization (WHO) does not provide an official list with the classification of microorganisms regarding their biological safety in laboratory but provides criteria to consider for their classification. These criteria include their pathogenicity, their hazard for laboratory workers, the risk for the community, livestock and environment, as well as the availability of effective preventive measures and

treatment (WHO, 2004). Considering these aspects, among the chlamydial species of veterinary interest, the European Union (EU Parliament, 2000) and the United States (HHS-CDC-NIH, 2009) have classified only the strains of *C. psittaci* of avian origin as class 3 microorganisms, which means that they represent a high risk for individuals, although they do not ordinarily spread from one infected individual to another. Therefore, class 3 microorganisms have to be manipulated only in biosafety level 3 laboratories. The other chlamydial species, including the non-avian strains of *C. psittaci*, are classified as class 2 microorganism by all the aforementioned regulations, which means that they represent a moderate risk for individuals but it is unlikely to be a serious hazard for laboratory workers, the community, the livestock, or the environment. Mexican regulations are aligned with the dispositions aforementioned, as stipulated in articles 78 to 83 of the General health law regulations on Health Research (Reglamento de la Ley General de Salud en Materia de Investigaciones para la Salud) (DOF, 2014).

2. Mexico, a major poultry-producing country

This section will revisit the importance of Mexican poultry farming in the international and national context. Also, particularities of laying hens and chicken broilers husbandry will be reviewed. Finally, an overview of poultry health and food safety constraints in Mexico will be provided.

2.1. Mexican poultry industry in the international context

Mexico is a major poultry producing country with both large-scale intensive commercial poultry industry and a noteworthy self-consumption backyard production. Mexico is one of the leading egg and chicken-meat producing countries in the world (WATT Global Media, 2019). Egg and chicken production places Mexico as the 4th and 6th producer worldwide, respectively, this according with the average production from 2010 to 2018 data registered by the Food and Agriculture Organization of the United Nations database (FAOSTAT) (FAO, 2020), and yet it fails to meet the national demand for chicken meat and egg. **Figures 8** and **9** show the top 10 ranking countries for chicken meat and egg production, respectively, with data of the average production from 2010 to 2018, obtained from the FAOSTAT (FAO, 2020).

Sustained growth and a promissory expansion to international trade characterize the national poultry industry, representing almost 64% of the national livestock in 2018 (35% of broilers, 29% of laying hens and 0.01% of turkeys) (UNA, 2018). By 2018, the value of Mexican poultry farming was almost $7 billion USD (UNA, 2018).

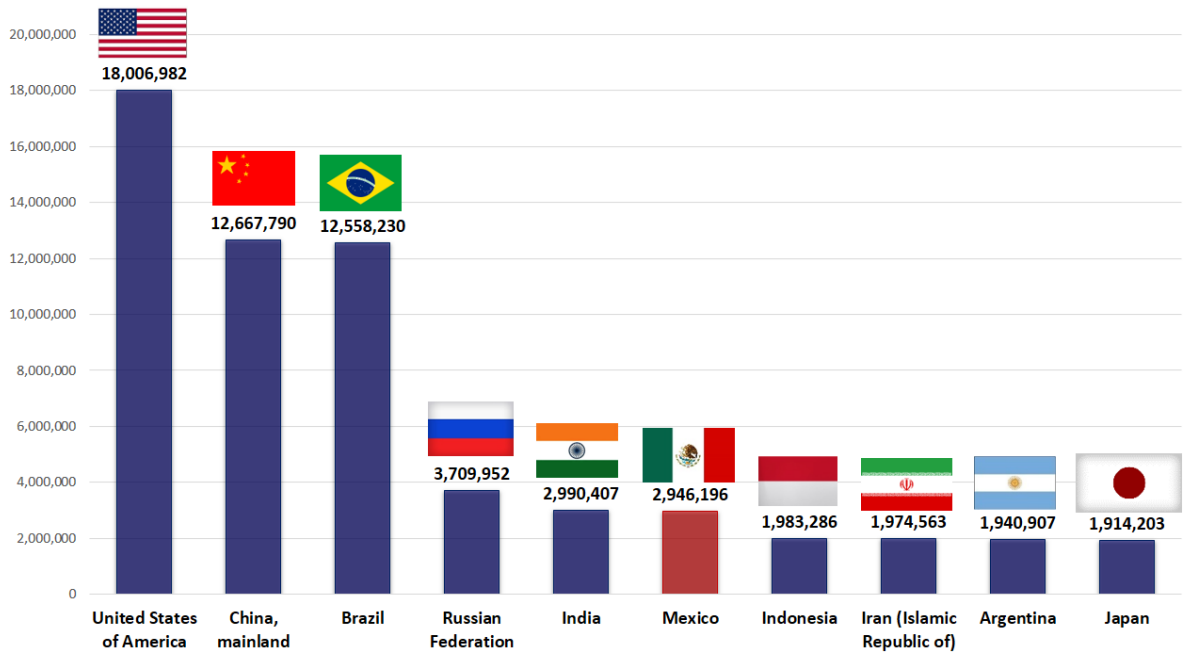

Figure 8. Top 10 ranking of countries with the highest average chicken meat production from 2010 to 2018, according with data of the Food and Agriculture Organization of the United Nations database (FAOSTAT), numbers indicate the average annual production in tones (FAO, 2020).

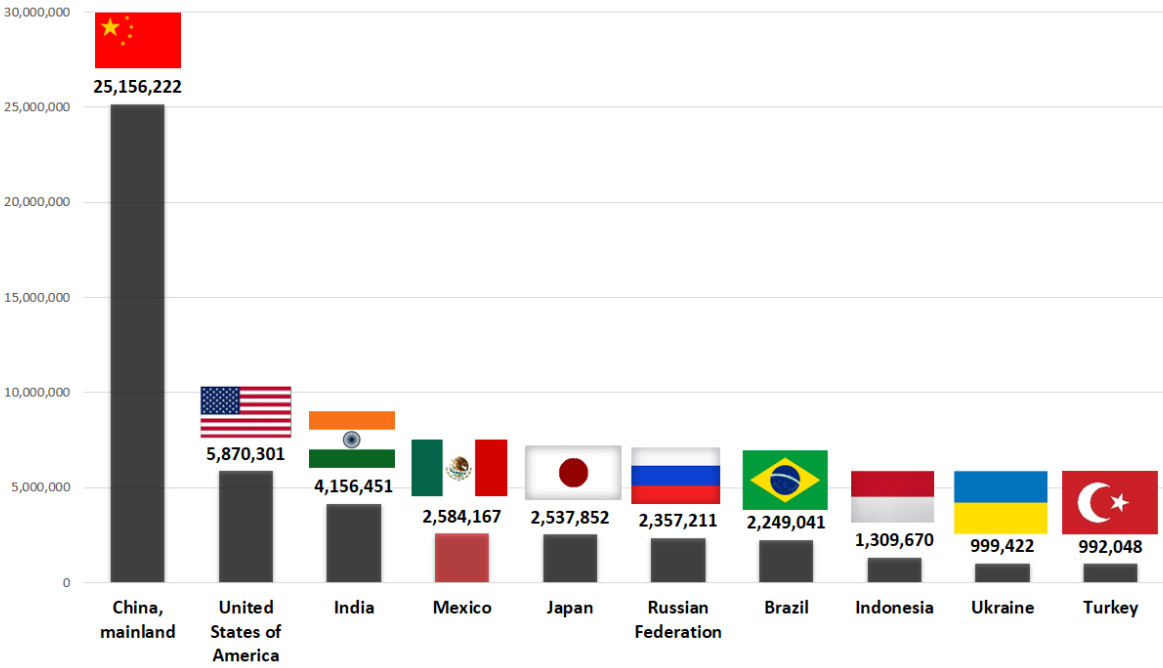

Figure 9. Top 10 ranking of countries with the highest average egg production from 2010 to 2018, according with data of the Food and Agriculture Organization of the United Nations database (FAOSTAT), numbers indicate the average annual production in tones (FAO, 2020).

2.2. Particularities of the Mexican poultry industry

Poultry products and by-products are the foremost human source of animal-origin protein in Mexico. The apparent national egg consumption is the highest in the world, with an average consumption of 23.3 kg per

person per year in 2018, while chicken consumption reaches 33 kg per capita. Poultry industry requires 16.2 million tons of balanced feed each year; 63% is fodder grain (corn and sorghum), equivalent to 10.9 million tons, while the rest are oleaginous and other inputs. Mexican animal feed industry is largely dependent on imports from the United States, although on some occasions grains have been imported from Argentina, Brazil, Uruguay, South Africa, depending on the global market. Half of the yellow corn and 90% of the oleaginous plants used to produce feed for poultry come from the United States (UNA, 2018).

An important aspect of the competitiveness of the domestic poultry industry is its high level of integration. Approximately 85% of the poultry industry is integrated both horizontally, i.e. feed mills, hatcheries and processing plants, and vertically, i.e. breeder farms, replacement pullet rearing farms and layer farms. However, direct distribution to the consumer is minimal, resulting in a high dependence on intermediaries (UNA, 2018). Poultry population in Mexico by 2019 was estimated in approximately 750 million birds, according with data from the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) (SAGARPA-SENASICA, 2019a). Detailed numbers by poultry species of the national poultry flock is presented in **Table 7**. **Figure 10** shows the composition of the Mexican poultry flock according to poultry species and purpose. We can notice that Mexican poultry industry relies mainly on laying hens and chicken broilers, representing 99% of the national poultry flock.

Table 7. Number and proportions of poultry species in Mexico in 2019 (SAGARPA-SENASICA, 2019a).

Poultry species	N° of poultry	%
Broiler chickens	460, 215, 866	61.28
Laying hens	287, 380, 328	38.27
Game fowl	1, 116, 232	0.15
Broiler turkeys	928, 429	0.12
Quails	889, 750	0.12
Broiler ducks	304, 534	0.04
Other species	79, 463	0.01
Pigeons	58, 814	0.01
Total	**750, 973, 416**	

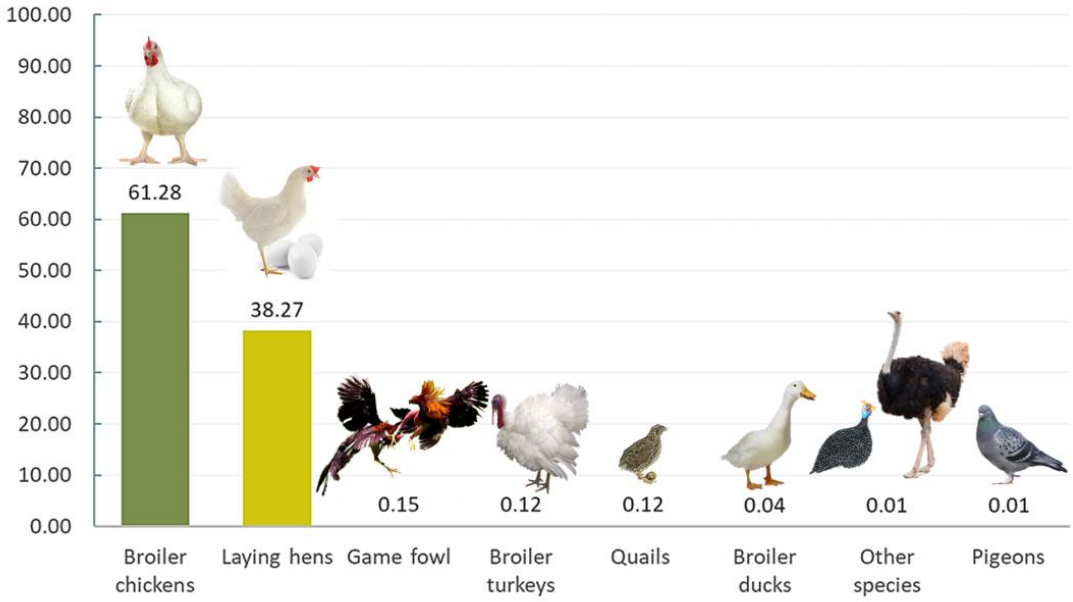

Figure 10. Composition in percentage of the Mexican poultry flock according to poultry species and purpose in 2019. Detailed information in **Table 7**. Data obtained from Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food statistics (SAGARPA) (SAGARPA-SENASICA, 2019a).

Commercial poultry farms are characterized by using specialized genetic lines, they manage an elevated bird density with high degree of confinement either with controlled environment or with open-sided housing systems (Anon, 2016). All commercially-reared poultry are kept under intensive conditions, not limited by legislation except for some restrictions concerning transport and stunning at slaughter (Bracke et al., 2019). Infrastructure for food and water massive supply, biosecurity and disease prevention measures, such as vaccination and nutrition management, are carefully managed. Commercial farms supply poultry products to most of the urban areas of Mexico (Hernandez and Parrish, 2018; UNA, 2018). Conversely, backyard farms are characterized by rearing small number of non-specialized poultry breeds, most of them indigenous breeds. Generally, they are raised loose on ground and shelter is usually provided by a rustic roofing. In backyard farms, poultry density, biosecurity measures, preventive medicine and nutrition management are related to the individual knowledge of the farmer, resulting in large variations between farms (Sims, 2006).

Cockfighting is still a popular “sport” in Mexico as well as in Southeast Asia, Philippines, and in some parts of Spain. In Mexico, cockfighting dates back to the 18th century. Globally, cockfighting is a billion dollar a year industry (Bracke et al., 2019). Nevertheless, fighting cock breeders recognize that this practice in the so-called "*palenques*" (i.e. which is a round area with wet sand in which the roosters fight, surrounded by a wooden circle that separates the roosters and tribunes, from where people observe and bet (Losada Hermenegildo et al., 2018)), is gradually diminishing and could come to an end in the near future as stated by the representative of the Mexican Commission for Fighting Cock Promotion, in response to the update of the Animal Protection Law for the State of Veracruz in 2016 (Rodríguez, 2018). Veracruz has the highest number of registered game fowl in Mexico and was the first to officially ban this practice. According with data from

this Commission, there are 50 million fighting cocks in Mexico in an industry that moves almost 200,000 USD per year. **Figure 11** shows the game fowl distribution across Mexico.

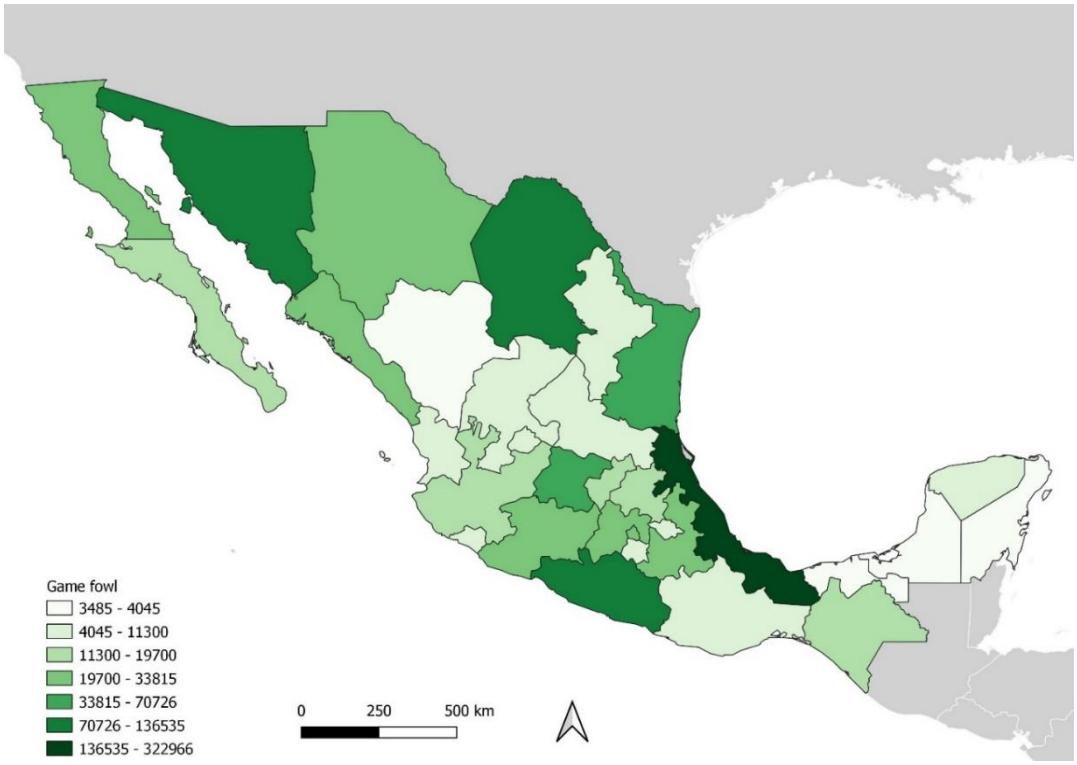

Figure 11. Game fowl distribution across federal states in Mexico. Data obtained from the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food statistics (SAGARPA-SENASICA, 2019a).

Two types of housing systems can be distinguished in commercial farms. The "controlled environment" houses are closed barns with an airflow provided by an automatic tunnel ventilation system and artificial illumination, while the "open-sided" houses are barns with open walls allowing natural ventilation, modulated by manually-operated curtains, they benefit from the temperate climate that prevails in most of the country. **Figure 12** illustrate this two types of housing systems.

In Mexico, eggs from cage-free hens, organic and free-range chicken farming, as well as other poultry products that incorporate additional quality and animal welfare criteria into their business model are incipient and have begun to increase in recent years. By the end of 2018, more companies joined the initiative to use only eggs from cage-free hens, which increases the sum to 38,000 hens per year under this system (Gutiérrez, 2018). However, a legal framework needs to be consolidated to certify farms that comply with these procedures. It is also necessary to promote the implementation of the general animal welfare law, which has been in construction. Although the low purchasing power of the average population affects the development of these production systems, there is a growing demand among some Mexican consumers. This sector of the poultry industry is expected to grow over the next few years (UNA, 2018).

Figure 12. Two major types of housing systems in Mexican commercial farms: on the left, an open-sided type of barn; on the right, a controlled environment type of barn (personal photograph collection).

2.3. Eggs and laying hen industry

Genetic lines of laying hens that are present in Mexico are Bovans (64%), Hy Line (19%) and Lohman (14%) for lightweight genetic lines, as well as Hy Sex Brown (2%) and Isa Brown (1%) for dual purpose genetic lines. White eggs account for 97% of national egg production, while brown eggs account for 3% of the national production. 75% of egg commercialization is done in bulk through wholesalers, who distribute them through mobile markets and convenience stores. Only 15% of the commercialization is through supermarkets and 10% directly to the industry (hotels, restaurants, bakeries, etc.). A small proportion of this 10% is processed and commercialized as egg products, i.e. whole eggs, egg whites, and egg yolks in frozen, refrigerated liquid, and dried forms (UNA, 2018).

The two main Mexican federal states where egg are produced are Jalisco and Puebla, accounting for 54% and 13% of the national production, respectively (UNA, 2018). In **Figure 13** is presented the laying hen density map per federal Mexican state. The leading company of egg production is Proteina Animal (PROAN) with 30 million layers per year. PROAN is the second largest egg producer company in the world. Other Mexican companies that are in the ranking of the 20 companies with the largest egg production in the world are El Calvario and Empresas Guadalupe, each company with 10 million laying hens per year. The top five companies in Mexico have a combined market share of 40% (van Horne et al., 2018).

Layers are typically kept in cages with a space allowance of about 345-400 cm²/hen, although a minimum density of 450 cm²/layer is advised in the good husbandry practices manual issued by the government (SAGARPA-SENASICA, 2016a). Recommendation of a maximum of 16 hours of light, with an intensity of 10-20 lux are also made in this manual. Beaks are trimmed in the first week (4.2 mm shortened, hot blade). With the purpose to have one extra production cycle (or even more), and to extend the laying period, forced molting is commonly practiced. To do so, hens are left without food and water for 3 to 4 days along with

artificial lighting suspension. Hens can also be sold to other farms for them to carry out the forced molting and start another cycle of egg production. Forced molting could happens 2 to 3 times depending on the national market and the price of the egg (Bracke et al., 2019). Egg production cycle is shown in **Figure 14**.

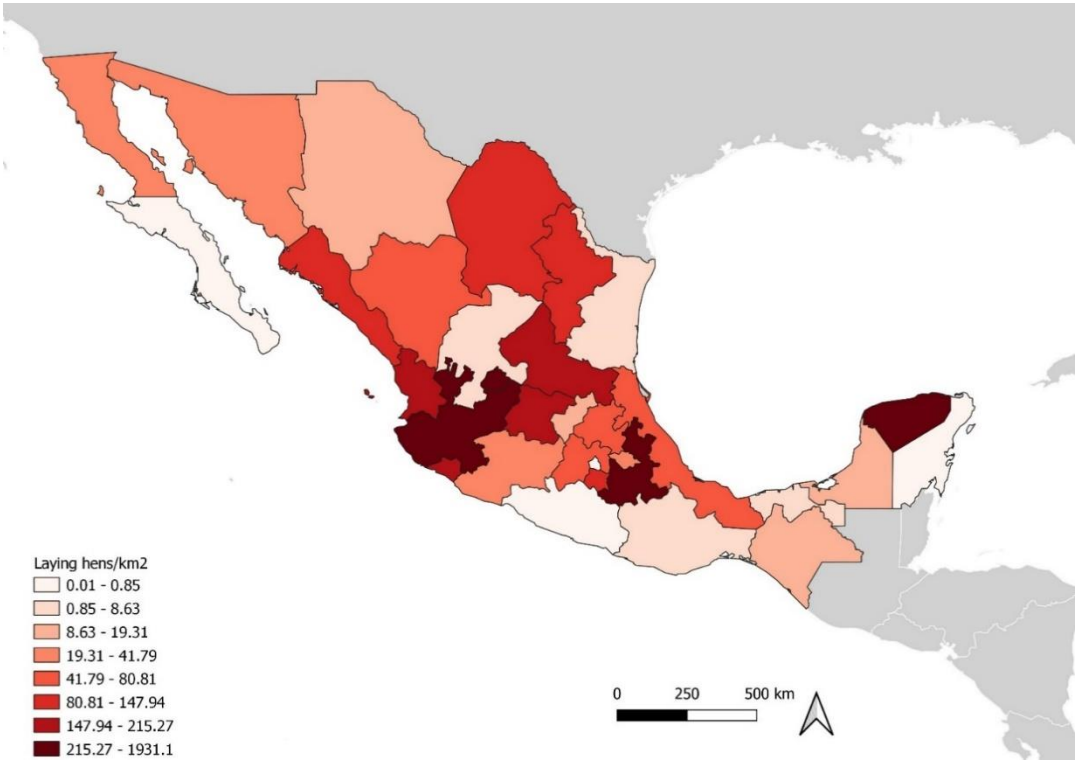

Figure 13. Laying hen density map per federal Mexican state. Data obtained from the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food statistics (SAGARPA-SENASICA, 2019a).

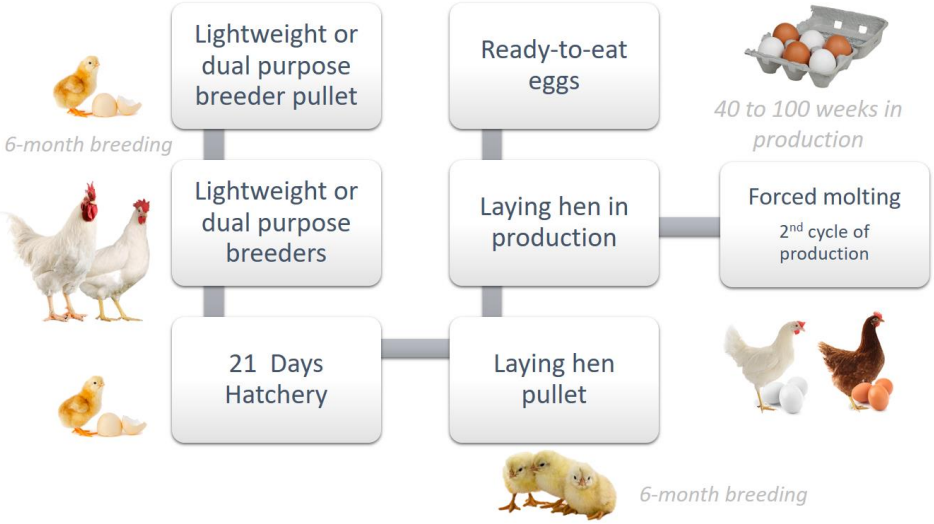

Figure 14. Egg production cycle. Adapted from (UNA, 2018).

2.4. Chicken meat and chicken broiler industry

Genetic lines of chicken broilers that are present in Mexico are Ross (48%) followed by Cobb (46%), and Hubbard (6%). Thirty-seven percent of the chickens are sold alive, 35% as roasted chicken, 12% already cut into pieces and/or with added value (i.e. nuggets, marinated or boneless chicken parts), 11% on public markets and 5% on supermarkets. Around 90% of the national apparent consumption (i.e. proxy measure for consumption) is covered by the national production and 10% is covered by imports mainly from the United States. Approximately 50% of the national chicken production is concentrated in five states, which are in decreasing order Veracruz, Aguascalientes, Queretaro, Jalisco and the bordering region of Coahuila (UNA, 2018). In **Figure 15** is shown the chicken broiler density map per federal Mexican state. The market leader company is Industrias Bachoco with 27% of the market, followed by Pilgrim's de Mexico and Tyson de Mexico. These three companies share around 50% of the market (van Horne et al., 2018).

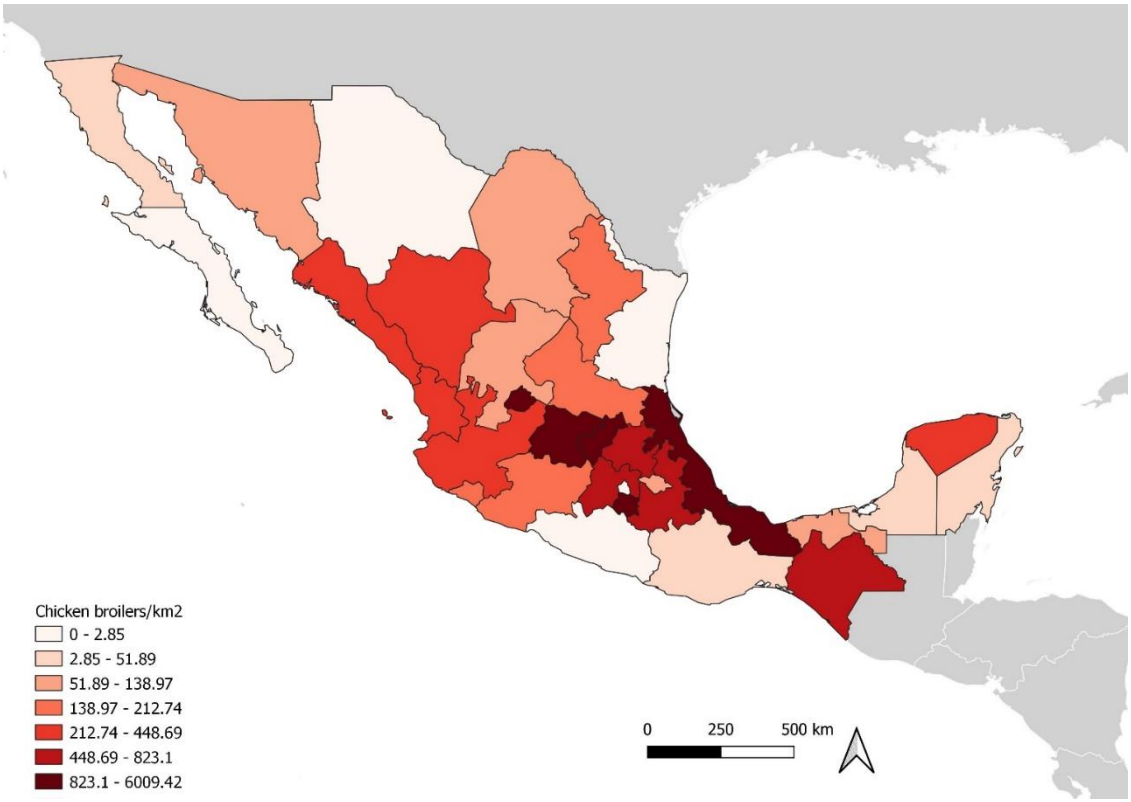

Figure 15. Chicken broiler density map per federal Mexican state. Data obtained from the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food statistics (SAGARPA-SENASICA, 2019a).

Broilers are mainly kept in single use litter on the floor. In controlled-environment type of barns, a maximum density of 36 to 39 kg per m^2, i.e. from 15 to 19 birds per m^2, depending on the final projected weight. In open-sided type of barns between 10 to 12 birds per m^2 are allowed. Final bird weight varies from 1.8 to 3 kg: about 60% of broilers are raised to reach a weight of 1.8 to 2.5 kg for processing in a slaughterhouse, while the rest are raised to reach a higher weight and to be sold as live birds. Performance rates of chicken broilers are comparable to those of the United States, however, mortality rate in chicken broiler flocks in Mexico could

reach 8 to 10% per cycle, mainly due to health issues (van Horne et al., 2018). Chicken meat production cycle is presented in **Figure 16**.

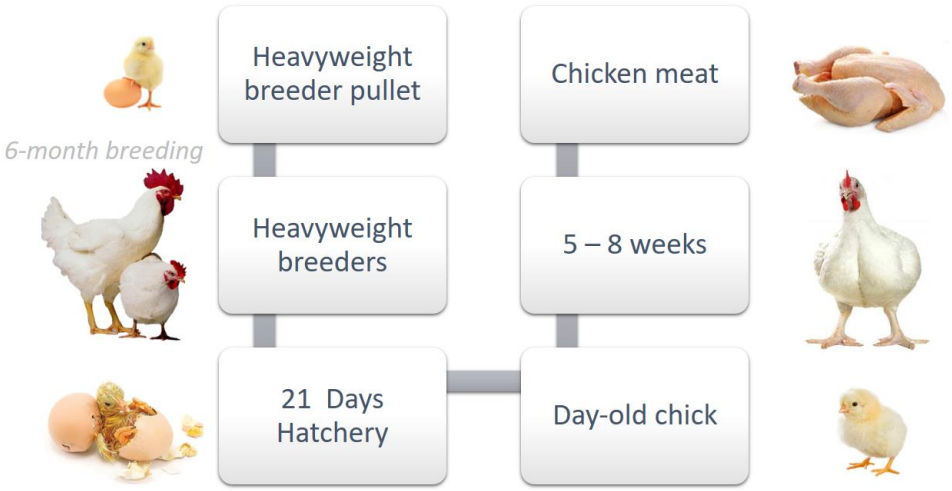

Figure 16. Chicken meat production cycle. Adapted from (UNA, 2018).

2.5. Poultry health and food-safety concerns in the poultry sector in Mexico

The World Organization for Animal Health (OIE) has established a list of notifiable diseases, in line with the Sanitary and Phytosanitary Agreement of the World Trade Organization (WTO). In this list, diseases are classified with the same degree of importance for international trade. To date, 13 avian diseases and infections are considered within this list: avian chlamydiosis, avian infectious bronchitis, avian infectious laryngotracheitis, two avian mycoplasmosis, (due to *Mycoplasma gallisepticum* and *M. synoviae*), duck virus hepatitis, fowl typhoid, infection with avian influenza viruses, infection with influenza A viruses of high pathogenicity in birds other than poultry including wild birds, infection with Newcastle disease virus, infectious bursal disease (Gumboro disease), Pullorum disease and turkey rhinotracheitis (OIE, 2020a). The animal health authorities of Mexico have issued an official list, in which all the microorganisms enlisted by the OIE are classified in three groups, according their status in the national territory and the urgency of their notification in the event of a positive case. Group 1 includes diseases that are exotic to the national territory and that due to the great impact on animal health and/or production that their presence may represent, together with the risk to public health that their dissemination implies, their detection should be notified immediately. In this group are avian influenza caused by viral subtypes H5, H7 and any other influenza virus with an intravenous pathogenicity index greater than 1.2, Newcastle disease and fowl typhoid. In Mexico there are specific legal provisions for the diagnosis and surveillance to control and eradicate these three diseases. Group 2 includes diseases that are present in the country and that, due to their significant effects on animal health and/or production, international trade, and risk to public health, their detection should be also notified immediately. In this group are included low pathogenic avian influenza viruses. Group 3 gathers those diseases considered as endemic and that represent a lower risk from an epidemiological, economic and public health point of view, as well as for national and international trade. Most diseases in this group can be controlled through good animal husbandry practices, vaccines or antimicrobial treatments. Some diseases included in this

group are aspergillosis, coccidiosis, avian mycoplasmosis, infectious bronchitis and avian chlamydiosis. This list was updated in 2016 and the last version dates from 2018 (SAGARPA-SENASICA, 2018). Before the 2016 update, avian chlamydiosis and enzootic abortion of ewes were considered as exotic diseases.

All laboratories that perform diagnostics of animal diseases in Mexico, must be licensed by the government and they are responsible for issuing reports of their diagnoses to the animal health authorities. Reporting periodicity depends on the disease group, i.e. immediate for diseases in group 1 and 2 and monthly for diseases in group 3 (SAGARPA-SENASICA, 2018).

As for psittacosis, the zoonotic infection caused by *C. psittaci*, no official diagnosis is available in the National Institute of Epidemiological Diagnosis and Reference, which is the official reference institution offering diagnostic services, issuing guidelines for all human diagnostic laboratories in the country (InDRE, 2018) . It is also in charge of compiling data of morbidity and mortality of the diseases subject to national epidemiological surveillance. Hence, no official data is available concerning zoonotic psittacosis cases in Mexico (SS, 2013).

Since 2012, Mexico is considered free of *Salmonella* Pullorum and Gallinarum serotypes (SAGARPA-SENASICA, 2012). The official surveillance campaign to detect these two serotypes has not reported a single isolation for several years of either serotype. On the other hand, Mexico is free of Newcastle disease in commercial flocks under exhaustive vaccination programs. However, sporadic outbreaks are reported each year in backyard poultry. In 2019, four outbreaks of Newcastle disease were reported in backyard poultry, in which a total of 875 birds died or were culled (OIE, 2020c). These outbreaks occurred in the federal states of Guanajuato, Jalisco, Chiapas and Sonora.

Regarding avian influenza, several outbreaks have been recorded in the country caused by two genotypes: H5N2 and H7N3, whose first identification occurred in 1994 and 2012, respectively (Afanador-Villamizar et al., 2017). After the first detection of a highly pathogenic avian influenza (HPAI) virus subtype H5N2 in Mexican commercial poultry in 1994 (Villarreal-Chávez and Rivera-Cruz, 2003), the Mexican government initiated a national campaign for its control and eradication that has been maintained and updated ever since then (SAGARPA, 2011). In 2019, 33 outbreaks of HPAI were reported, all of them caused by H7N3 genotype which resulted in the death or culling of 870,825 birds (OIE, 2013; SAGARPA-SENASICA, 2019b). Twenty-five outbreaks occurred in backyard poultry, in 8 federal states: Guanajuato, Guerrero, Hidalgo, Jalisco, Querétaro, State of Mexico, Tlaxcala and Veracruz, while 8 outbreaks occurred in commercial poultry farms in 3 federal states: Hidalgo, Jalisco and Querétaro (**Figure 17**). In the first semester of 2020 HPAI outbreaks were not reported.

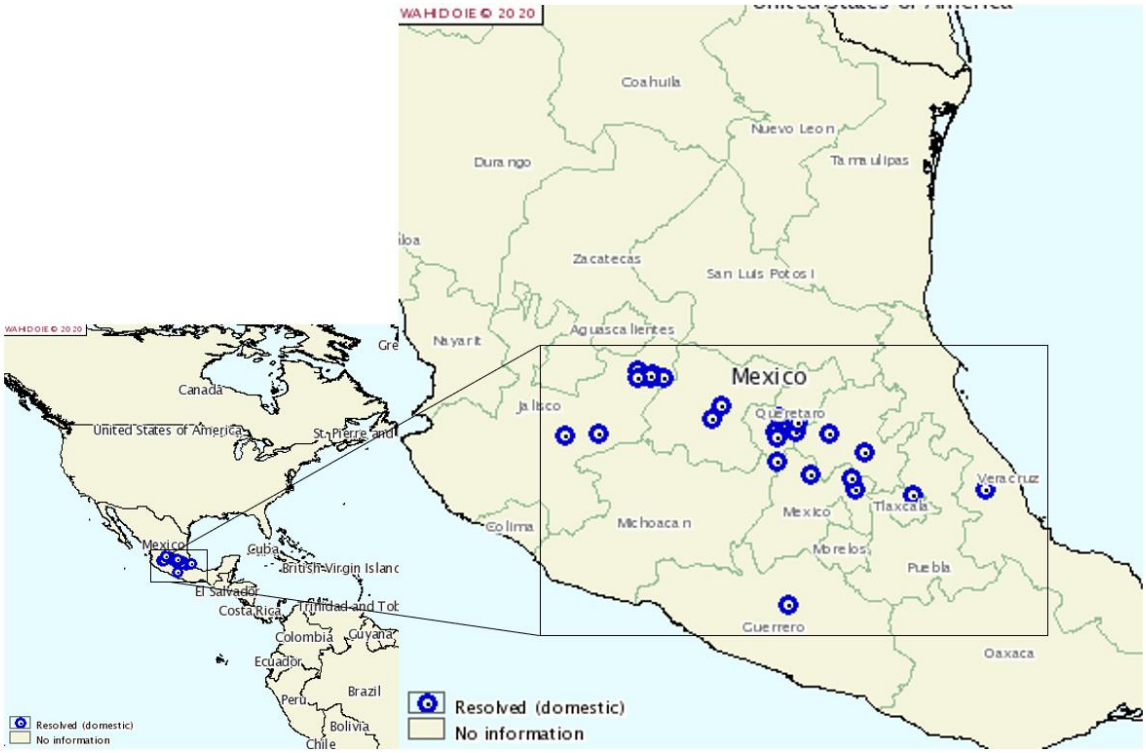

Figure 17. Location of the 33 highly pathogenic avian influenza outbreaks reported in 2019 in Mexico both in backyard and in commercial poultry, data obtained from the World Animal Health Information System (WAHIS) interface (OIE, 2020c)

Compliance monitoring of the Sanitation Standard Operating Procedures (SSOPs), Good Manufacturing Practices (GMPs), Hazard Analysis and Critical Control Points (HACCP), among other programs for quality control in poultry farms is certificated by the National Organism for the Alimentary Certifications (Organismo Nacional de Certificación Agroalimentario A.C.). Additionally, there are other voluntary certifications that can be obtained by poultry farms, some of them are: ISO 9001 (for quality control), ISO 14000 (to ensure that negative environmental impacts caused by their operations are minimized), as well as ISO 22000 (to ensure food safety) (UNA, 2018).

By 2018, 50 Federal Inspection Type (TIF, according to its Spanish acronym) accredited slaughter plants dedicated to the slaughter and processing of poultry products and by-products were present in Mexico: 28 were dedicated to slaughter and poultry processing, 14 to processing of chicken meat exclusively and eight to egg and egg products processing (UNA, 2018). TIF processing plants are distributed in 19 federal states and are subjected to permanent inspections to verify that facilities and processing comply with internationally recognized official regulations.

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3. Problem statement and objectives

Several chlamydial species can be detected in birds of which *C. psittaci*, *C. gallinacea* and *C. avium* are the most common. In poultry, numerous studies of chlamydial infections have been conducted in Europe, Asia and in America, mostly in the United States. These studies have revealed the presence of *C. psittaci* and, more recently, of *C. gallinacea*, that has been recently included as a new taxon within *Chlamydiaceae*. The presence of *C. psittaci* on farms is of particular concern due to its zoonotic potential, that may constitute a public health concern. Outbreaks of psittacosis are occasionally reported mostly among poultry workers, in particular those working in slaughterhouses. Poultry infected with *C. psittaci* are mostly asymptomatic, and most of the time the presence of the bacterium is indirectly revealed by associated human cases.

Before 2016, chlamydial infections in animals were considered as exotic in Mexico by the national animal health authorities, both in birds and small ruminants. Few punctual studies had been conducted aiming to detect the presence of *Chlamydia* in birds, mostly in pet shops, zoos and at the avian hospital of the FMVZ-UNAM. Some of them revealed the presence of *Chlamydia* spp. (Morales Luna, 2006; Rojas Martinez, 1996), while in others it was not possible to detect it (López Yelmi, 2011; Pérez Olmedo, 2018). In addition, many chlamydial infection cases have been suspected through postmortem examination and histopathological findings in birds submitted to the Laboratory for Diagnosis and Research of Avian Diseases (FMVZ-UNAM) in recent years, however these cases remained without further confirmation of the diagnosis (Ornelas-Eusebio and Ledesma-Martínez, 2017). It was not until 2015 that, with the financial support of the Secretariat of Environment and Natural Resources (SEMARNAT), a molecular test to perform the diagnosis of avian chlamydiosis was implemented and incorporated to the diagnostic catalogue of the aforementioned laboratory at the FMVZ-UNAM. This test allowed to gather the elements to report for the first time *C. psittaci* in endemic endangered psittacine birds housed in a management unit for wildlife conservation in Mexico in 2016 (Ornelas-Eusebio et al., 2016). This finding was notified to the animal health national authorities. The different reports of *Chlamydia* occurrence in Mexico may have prompted a review of animal health regulations in the country that resulted in the final reclassification in 2016 of avian chlamydiosis as an endemic disease.

A study conducted in 2016 in cooperation with ANSES showed the circulation of *C. psittaci* as well as unidentified chlamydial species in wild and companion birds in Mexico (Ornelas-Eusebio et al., 2017), but no studies had been conducted regarding chlamydial infections in poultry in Mexico. On the other hand, good husbandry and biosecurity practices, together with antimicrobial treatment in punctual cases, are considered to be the most effective mechanisms to control chlamydial infections among poultry (Balsamo et al., 2017); only few studies have provided information regarding biosecurity practices on poultry farms in Mexico (Absalón et al., 2019; Afanador-Villamizar et al., 2017; Cardenas Garcia et al., 2013; Peña Aguilar et al., 2016).

Different studies suggest that conditions that promote *in vitro* growth of a chlamydial species/specimen appear to be not applicable for the rest of them (Onorini et al., 2019; Schiller et al., 2004). Although several optimizations have been made to the chlamydial growth protocol -all aimed at increasing the infectivity rate and *in vitro* growth enhancement-, the propagation of some chlamydial strains/species remains difficult. Such difficulties are encountered for *C. gallinacea* (personal communications from several European researchers), even if few studies have achieved the *in vitro* cultivation of this new identified species (Guo et al., 2016; Hölzer et al., 2016; Laroucau et al., 2009b; You et al., 2019).

The objectives of this thesis were: (i) to detect the presence of *Chlamydiaceae* and identify the diversity of *Chlamydia* species found in poultry on commercial and backyard farms of Mexico, (ii) to identify potential associated risk factors, (iii) to characterize on-farm management and biosecurity practices and (iv) to optimize the protocol for *C. gallinacea in vitro* growth to further characterize Mexican *C. gallinacea* isolates.

CHAPTER I. EPIDEMIOLOGICAL INVESTIGATION OF *Chlamydiaceae* IN POULTRY OF MEXICO

1. Introduction

C. psittaci was reported for the first time in Mexico in psittacine birds in a management unit for wildlife conservation in 2016 (Ornelas-Eusebio et al., 2016), but no data were available regarding the presence of *Chlamydiaceae* in poultry. Similarly, few studies have been conducted on Mexican poultry farms assessing on-farm biosecurity practices implementation (Absalón et al., 2019; Afanador-Villamizar et al., 2017; Cardenas Garcia et al., 2013; Peña Aguilar et al., 2016).

As many of the reported zoonotic cases of psittacosis were traced back from poultry subclinically infected with *C. psittaci* (Durfee et al., 1975; Hedberg et al., 1989; Hogerwerf et al., 2020; Hulin et al., 2015; Laroucau et al., 2015, 2009b; Newman et al., 1992; Newman, 1989; Shaw et al., 2019; Yin et al., 2013a). And considering that there is no commercially available vaccine to prevent chlamydial infections in poultry; good husbandry and biosecurity practices, coupled with antimicrobial treatment in punctual cases, are the most effective mechanisms to control chlamydial infections among poultry (Balsamo et al., 2017). Biosecurity is the set of practices implemented with the objective of preventing the introduction and dissemination of infectious agents in an animal population (OIE, 2019a), but also to prevent potential zoonosis (FAO, 2007). It has been extensively demonstrated for poultry farms that implementing proper biosecurity practices contributes not only to the control of pathogen exposure (Abraham et al., 2020; Gibbens et al., 2001; Newell et al., 2011; Sylejmani et al., 2016; Volkova et al., 2012), but also to improved productive performance (Raasch et al., 2018; Sylejmani et al., 2016; Tablante et al., 2008), as well as to reduced antimicrobial usage (Adam et al., 2019; Chauvin et al., 2005).

Mexico is one of the leading chicken-meat and egg-producing countries worldwide, with both a large-scale intensive commercial poultry industry and substantial self-consumption backyard production (Hernandez and Parrish, 2018); moreover poultry represents 64% of national livestock (UNA, 2018). Hence, the study of poultry husbandry related issues, such as on-farm biosecurity practices, antimicrobial usage and infectious diseases affecting poultry, is essential due to the large importance of poultry in livestock of Mexico.

Therefore, the objectives of the study presented in this first chapter were to detect the presence of *Chlamydiaceae* in poultry on commercial and backyard farms of Mexico, and to identify potential associated risk factors, as well as to characterize the on-farm management and biosecurity practices.

2. Materials and methods

2.1. Sampling design for the cross-sectional study

A cross-sectional study was conducted between June 2017 and June 2018 on commercial and backyard farms in eight federal states of Mexico characterized by high poultry density. We sought to include commercial farms with different degrees of confinement (controlled environment *vs* open-sided houses), and farms raising chicken broilers and laying hens. The controlled environment houses are closed barns with airflow provided by an automatic tunnel ventilation system and artificial lighting, while open-sided houses are barns with open walls allowing natural ventilation, modulated by manually-operated curtains. Backyard farms are characterized by a small number of poultry (mainly of indigenous breeds) raised free-roaming on the ground with shelter provided by basic roofing.

Farm and poultry sample sizes were calculated to detect a between-farm prevalence in each of the three groups of farms of 20%, and a within-flock prevalence of 30% with a 95% level of confidence, considering prevalence values reported previously (Donati et al., 2018; Heijne et al., 2018; Szymańska-Czerwińska et al., 2017a) and using Epitools epidemiological calculators (Sergeant, 2017). This yielded a minimum sample size of 14 farms and 9 birds per flock. As a national database for commercial poultry farms was not available, veterinarians who provided technical support to farms located in the states where most of the Mexican poultry producers are located, were contacted during a national congress on poultry farming. Contacted backyard farmers were acquaintances of veterinary students from the Veterinary Faculty of the National Autonomous University of Mexico (UNAM). On both commercial and backyard farms, birds were randomly selected and subjected to cloacal swabbing. Two swabs were taken from each bird in parallel; the first cloacal swab was stored in 1 ml of sucrose phosphate glutamine medium (SPG, see **Annex III** for further details), while the other was stored dry. Both specimens were transported at low temperatures. Dry swabs were frozen at -20°C until processing for DNA extraction and swabs in SPG medium were stored at -80°C until processing for chlamydial growth in cell culture (see chapter II).

2.2. Weekly monitoring of chicken broilers to investigate *Chlamydiaceae* shedding

To investigate the shedding dynamics of *Chlamydiaceae* in commercial chicken broiler flocks, two flocks of chicken broilers housed in controlled-environment poultry houses were sampled using cloacal swabs during their normal breeding process. Fifteen cloacal swabs were randomly collected from chicken broilers on five occasions by the workers present in the farm. Samples were collected when there was a handling in the flock, e.g., during the chick reception, a vaccination or a vaccine boost procedure, as shown in **Figure 18.**

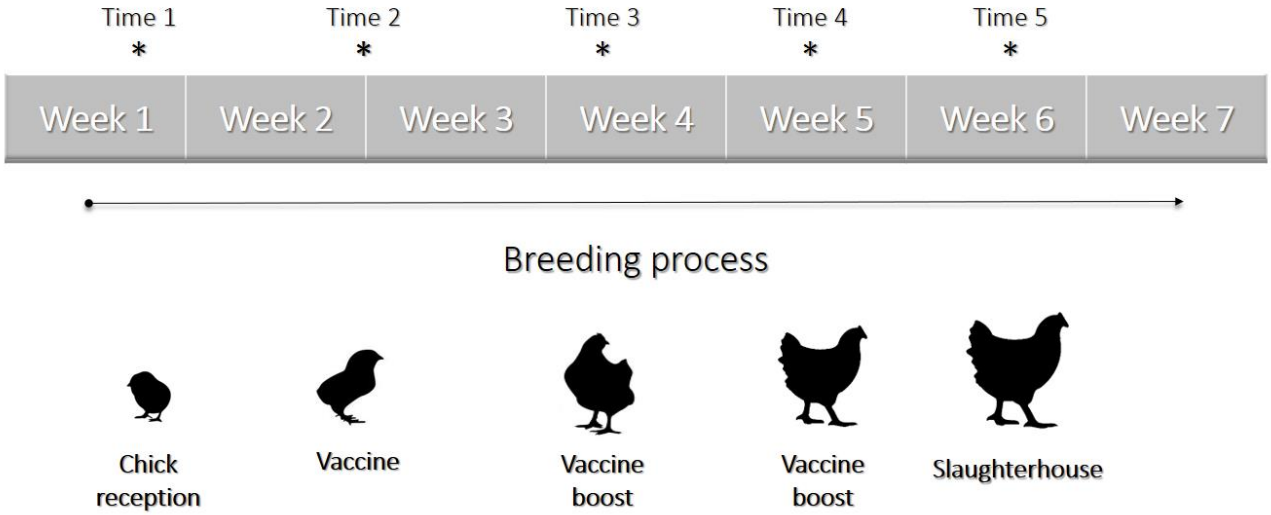


Figure 18. Overview of a chicken broiler breeding process with the chlamydial sampling interventions marked on the top to investigate the shedding dynamics of *Chlamydiaceae*. Samples were collected at weeks 1, 2, 4, 5 and 6.

2.3. Data collection

A questionnaire including 48 fill-in-the-blank and close-ended questions was designed to capture information regarding (i) farm specifications, (ii) housing type and facility description, (iii) sampled flock characteristics, (iv) flock/poultry health status and health management, (v) farm management practices, (vi) cleaning and disinfection procedures, and (vii) biosecurity practices. The questionnaire was designed in view of the manuals for good husbandry practices for chicken broilers and laying hens issued by the Mexican government (SAGARPA-SENASICA, 2016b, 2016a). There were questions applicable only to commercial farms or only to backyard farms (the full content of the questionnaire in Spanish and the specific questions administered to commercial or backyard farms are in the **Annex I**). Questionnaires were administered by the same interviewer through an on-farm semi-structured interview of the farmer in backyards, or of the poultry veterinarian or supervisor on commercial farms.

Taking into account the distances between farms, and to avoid potential pathogen introduction or dispersion, a maximum of two farms were visited per day. Additionally, the biosecurity protocol implemented for visitors by the majority of the establishments were followed. Visits were performed wearing clean clothes (most of the time provided on the farm, otherwise, a single-use coverall was worn). Systematic hand sanitization and showering, when feasible, were conducted before and after entering the farm. Interviews were conducted either with the farm manager or the veterinarian in charge of poultry health.

The last sections of the questionnaire (i.e. farm and poultry management practices, cleaning and disinfection procedures, and biosecurity measures) were open questions, allowing the respondent to give a detailed answer, especially concerning antibiotic usage. An overview of the information gathered through the questionnaire to investigate the possible risk factors associated with the presence of *Chlamydiaceae* and the on-farm biosecurity practices is shown in **Figure 19**.

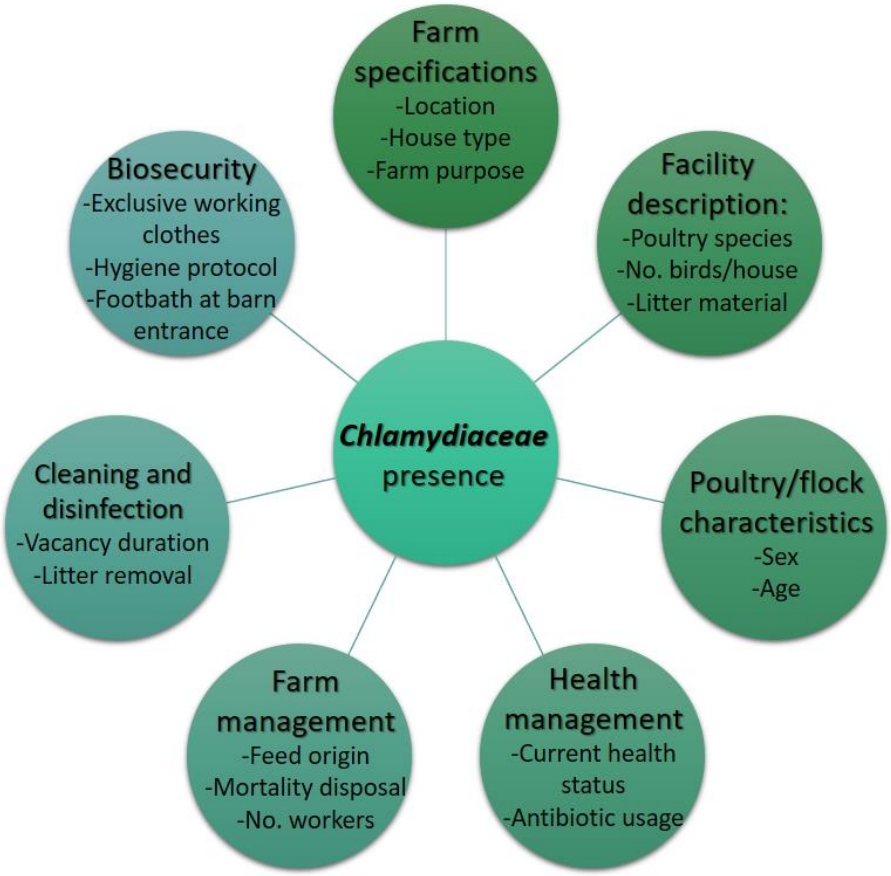


Figure 19. Overview of the information gathered to investigate the possible risk factors associated with the presence of *Chlamydiaceae* and the on-farm biosecurity practices.

Interviewees provided verbal consent before the interview was started. The research protocol for the ethical manipulation of the animals involved in the study was approved by the Institutional Subcommittee for the Care and Use of Experimental Animals of the UNAM, under registration number DC-2018/1-4. All efforts were made to ensure animal welfare during sampling. All farmers gave their consent to participate in the study. This subcommittee ensures the confidentiality and data protection of the information gathered through the questionnaires.

2.4. Laboratory analysis

All dry cloacal swabs were subjected to DNA extraction using a QIAamp DNA Mini Kit (QIAGEN, Ventura, CA, USA), following the buccal swab protocol. DNA was eluted with 200 µl of AE buffer and stored at -20°C until PCR examination. An internal extraction and PCR inhibition control DiaControlDNATM (Diagenode Diagnostics, Liège, Belgium) was systematically included in all samples, following the manufacturer's instructions.

Preliminary screening of all DNA samples was performed using a broad-range *Chlamydiaceae*-specific real-time PCR technique (rt-PCR) targeting the 23S rRNA gene (Ehricht et al., 2006). For each real-time PCR run, an only reagent/no template control and DNA from the *C. psittaci* Loth strain as a positive control were

systematically included. Samples with a cycle quantification (Cq) value ≥ 40 were considered negative. This rt-PCR method has been validated for the specific detection of all *Chlamydiaceae*. Measurements on dilution series of cell culture aliquots with defined numbers of inclusion-forming units (IFUs) indicated a detection limit in the range of 3 IFUs for this method. It is considered to be a highly sensitive and specific method.

All *Chlamydiaceae* rt-PCR-positive samples were further analyzed by chlamydial species-specific rt-PCR systems targeting the *omp*A gene for *C. psittaci* and the *eno*A gene for *C. gallinacea*, with conditions, primers and probes as previously described (Laroucau et al., 2015; Pantchev et al., 2009). DNA from the *C. psittaci* Loth strain and *C. gallinacea* 08-1274/3 strain were used as positive controls.

2.6. Statistical analysis

2.6.1. *Chlamydiaceae* prevalence

Exact between-farm and animal prevalence confidence intervals were calculated. A farm was considered to be positive if at least one animal tested positive. Between-farm and animal prevalence values were compared according to the farming system using a Fisher's exact test.

2.6.2. Risk factors: logistic regression and mixed-effect logistic regression model

Data gathered through the questionnaires were entered into a Microsoft Excel© datasheet. Considering that some variables applied only to commercial farms and others to backyard farms, two separate analyses were conducted to identify potential risk factors for the presence of *Chlamydiaceae*. As all the variables for commercial farms were farm-level predictors, logistic regression was performed to compare controlled-environment commercial farms vs open-sided commercial farms, using the farm as the epidemiological unit. For backyard farms, a mixed-effect logistic regression model (generalized linear mixed model with a binomial link) was implemented to take into account information collected either at the animal level or at the farm level (see **Annex I**). For this second model, risk factors were considered fixed effects and the farm as the random effect.

Quantitative variables were categorized taking into account physiological events and the farm type. For commercial farms, flock age was divided into three categories (≤ 20 weeks (reference category), between 20 and 45 weeks, and > 45 weeks), and number of birds per barn into two categories (≤ 30,000 (reference) and > 30,000). The duration of the vacancy period was categorized as ≤ 1 week and > 1 week (reference). For backyard farms, age was divided into two categories (≤ 6 months and > 6 months (reference)), and number of birds into two categories (≤ 40 birds (reference) and > 40 birds).

Multicollinearity diagnosis of variables was performed using the variance inflation factor (VIF) ensuring a VIF value < 2. When a pair of variables was found to be collinear, only the most biologically plausible variable was kept for further analysis.

The logistic regression model was fitted with the MASS package, and the mixed-effects logistic regression model with the lme4 package in R (R Core Team 3.6.2, 2019). An automated backward stepwise model selection procedure was performed, using the stepAIC function (MASS package), to identify the model with the smallest Akaike information criterion (AIC). Interactions between the variables that had a significant association with the outcome in the final main effect model were assessed. The area under the receiver operating characteristic (ROC) curve (AUC) was calculated as a measure of the goodness of fit of both models using the ROCR package in R.

The *Chlamydiaceae*-positive and negative farms were plotted on a choropleth map representing the number of poultry per state. Data for constructing the map were obtained from the Annual compendium of economic indicators, issued by the National Association of Poultry Farmers (UNA, 2018) and imported into ArcGIS 9.3.1 (ESRI, Redlands, CA).

2.6.3. Multivariate analysis of the commercial poultry farms

For this analysis all the variables from the questionnaire administered to commercial farms were included except age and sex of the sampled flock. Quantitative variables were transformed into qualitative variables. Different categories for the quantitative variables were established (number of birds per barn, number of barns per farm, number of workers per farm, and duration of the vacancy period). Categorical boundaries were established taking into account the quantiles as cut-off points. Number of birds per barn was divided into two categories (≤ 22,000 and > 22,000). Number of barns per farm was used to classify farms into small farms (≤6 barns) and large farms (>6 barns). Number of workers per farm was divided into two categories (≤ 3 and > 3), as well as the duration of the vacancy period before restocking (≤ 1 week and > 1 week).

A multivariate analysis of the data collected through the interview-questionnaire process was performed with R, version 3.6.2 (R Core Team 3.6.2, 2019). A multiple correspondence analysis (MCA) was performed to summarize and visualize the multidimensional dataset constructed with individuals (i.e. farms) and the categorical variables describing them. MCA is the correspondence analysis of the indicator matrix, where the rows are the respondents and the columns are the dummy variables for each of the categories of the variables. The goals of this analysis are first to study the similarities between the individuals, then to study the relationships between the variables, while assessing the associations between each of the variable categories, in order to finally associate the study of the individuals with that of the variables, with the aim of characterizing the individuals through their pattern of variables (Husson and Josse, 2018). In this way, the most important variables that contribute to explain the variations in the dataset are revealed. The cloud of individuals and variables is represented in a low-dimensional Euclidian space by maximizing the variance (inertia) of the projected cloud of points (Husson and Josse, 2018). Inertia is a measure of variance, showing the dispersion of data around their center of gravity, i.e. the dispersion of individual profiles around the average profile. In addition, eigenvalues are computed, which represent the contribution of each dimension to the total inertia,

with the highest eigenvalue in the first dimension, and decreasing gradually for the rest of the dimensions. The eigenvalue is used to select the maximum number of dimensions to be included in the MCA – a value ≤ 0.5 is not usually considered (Rodriguez-Sabate et al., 2017; Sourial et al., 2010). Graphical representations of the distances between individuals and the links between variables and their categories are also obtained. The distance between each point in the Euclidean space accounts for the variance between the points; therefore, the larger the distance, the lower the association.

In addition to the default indicator matrix, a Burt table was computed. This is the matrix of all pairwise associations between variables, including the diagonal associations between each variable and itself (Blasius and Greenacre, 2014). In this table, only the information about the relationships between categories is present, and not the information about the individuals (Husson and Josse, 2018). The advantage of the Burt table is that theoretical eigenvalues obtained from it provide a better approximation of the inertia explained by the dimensions, as they are the squares of those obtained through the analysis of the indicator matrix. Although these values are theoretical, they yield the same coordinates for individuals and variable categories as the analysis performed from the indicator matrix.

Using the dimensions with the greatest variance (inertia) generated by the MCA, the farms were classified into clusters through an agglomerative hierarchical cluster analysis (HCA) based on Ward's method, which consists in adding two groups (clusters) such that the growth of within-group inertia is minimal at each step of the algorithm. The hierarchical clustering algorithm can be visualized using a dendrogram. Within-group inertia characterizes the homogeneity of a cluster (Husson et al., 2016; Kassambara, 2017). The FactoMineR package was used to perform the MCA and HCA, and the factoextra package was used to visualize the outputs (Kassambara and Mundt, 2019; Lê et al., 2008). The optimal number of clusters was validated using the NbClust package that provides 30 indices for determining the number of clusters and proposes the best clustering scheme from the different results obtained (Charrad et al., 2014; Kassambara, 2017).

Data for constructing the poultry density map on commercial poultry farms per federal Mexican state were obtained from the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) (SAGARPA-SENASICA, 2019a), and imported into QGIS version 3.8.3.

3. Results

In total, 59 poultry farms were visited, 43 commercial and 16 backyard farms. Consequently, 59 questionnaires were filled-out. Unfortunately, biological samples from six farms (94 cloacal swabs in duplicate) were discarded due to contamination.

3.1. Descriptive analysis of the study population for the cross sectional study

A total of 37 commercial farms (586 poultry samples) were included in the cross sectional study to investigate de prevalence of *Chlamydiaceae* in poultry and associated risk factors (9 to 22 birds per farm). On commercial

farms, we decided to sample egg-laying hens and broiler flocks, as they are the more commonly raised flocks nationwide. Moreover, obtaining access to breeder flocks is more difficult in terms of biosecurity. Out of the 37 commercial farms included in the study, 14 were controlled environment farms and 23 open-sided farms (**Table 8**). The farms sampled were located in temperate, dry or tropical areas, having an altitude ranging from 0 to 2,250 m above sea level. The owners allowed access to only one barn per farm for biosecurity reasons.

With regard to backyard farms, 16 backyard farms were chosen, seeking to include different types according to the species that were raised: only chicken farms, only turkey farms, and farms with different bird species. There were no refusals to participate in the study. A total of 293 samples were collected (10 to 20 birds per farm). All the contacted backyard farmers and poultry veterinarians agreed to participate in the study.

On commercial farms, the only sampled species was chicken (*Gallus gallus)* of commercial breeds/strains. Laying hens were housed in battery cages and chicken broilers were kept on the floor in a deep litter system (litter material evenly extended on the floor to a depth of 2 to 5 cm). Reported bedding materials in the 28 broiler farms were rice hulls (21.5%), coffee hulls (42.8%), and chopped straw (35.7%). Cattle were also raised on three open-sided egg-laying farms.

On backyard farms, chickens and turkeys (*Meleagris gallopavo*) were the main sampled species. Two backyard farms housed specialized fighting cock breeds and on one farm, the only bred species was turkey. Domestic ducks (*Anas platyrhynchos*), quails (*Coturnix coturnix*) and pheasants (*Phasianidae*) were also sampled on three backyard farms. The coexistence of poultry with other domestic species such as cattle, sheep, goats or pigs was observed on eight of the 16 sampled backyard farms.

Regarding the poultry health status, respiratory clinical signs were observed on 6 out of 16 backyard farms. Observed signs included ruffled feathers, dyspnea, watery to purulent discharge from the eyes and nostrils, sneezing, and mild to severe facial and periorbital swelling. All commercial poultry flocks were apparently healthy at the time of sampling.

Antibiotic usage was reported on 28 out of the 37 commercial farms (75.7%). Antibiotic use as therapeutic treatment was reported on 8 out of the 16 backyard farms. Antibiotics were not used as growth promoters on any farm. Biosecurity practices were implemented only on commercial farms. Many of these biosecurity practices were implemented in the majority of commercial farms. The characterization of these practices is presented in section 3.6.

Feed on backyard farms was predominantly a mixture of industrialized and homemade food. A mortality management plan was reported on nine out of 16 backyard farms. Burial was the main method of disposal; otherwise dead poultry were disposed of with municipal garbage.

Table 8. General characteristics of sampled poultry farms in the epidemiological study on *Chlamydiaceae* prevalence and associated risk factors on Mexican poultry farms.

Farm type	House type	Reared poultry species	State	No. sampled farms	Farm size range	Farm purpose (No. farms)	Other domestic animals raised (No. farms)
Commercial	Controlled-environment house	Commercial chicken breeds	Chiapas	8	22000 - 25000	Broilers (8)	-
			Guanajuato	6	30000	Broilers (6)	-
	Total			**14**			
	Open-sided house	Commercial chicken breeds	Jalisco	3	20000 - 23000	Egg layers (3)	Cattle (3)
			Mexico city	3	1000 - 2000	Broilers (2) Egg layers (1)	-
			Morelos	12	16000 - 17800	Broilers (12)	-
			Puebla	5	105000 - 150000	Egg layers (5)	-
	Total			**23**			
	Total (commercial)			**37**			
Backyard		Chicken	Puebla	1	15	Egg layers (1)	-
			State of Mexico	3	50 - 150	Egg layers (1) Breeders (2)	Sheep and goats (1)
			Tlaxcala	1	180	Egg layers (1)	-
		Turkey	Tlaxcala	1	35	Breeders (1)	Sheep and pigs (1)
		Multiple poultry species	Mexico city	2	30 - 50	Breeders and layers (2)	-
			Puebla	5	30 - 60	Breeders and layers (5)	Sheep (2) Sheep and cattle (1) Sheep, cattle and pigs (1)
			State of Mexico	1	20	Breeders and layers (1)	-
			Tlaxcala	2	40 - 70	Breeders (1) Breeders and layers (1)	Sheep and cattle (1) Sheep and pigs (1)
	Total (backyard)			**16**			
Total of sampled farms				**53**			

3.2. Apparent prevalence estimation

Of the 879 birds analyzed, 104 were considered *Chlamydiaceae*-rt-PCR positive. Apparent animal prevalence was 0.4% on controlled-environment commercial farms, 5.4% on open-sided commercial farms, and 28.7% on backyard farms (**Table 9**). These apparent prevalence values were significantly different ($p<0.0001$).

Table 9. Apparent prevalence values of the epidemiological study on *Chlamydiaceae* prevalence and associated risk factors on Mexican poultry farms.

Farm type	Farm characteristic	No. pos. farms /total	Apparent between-farm prevalence (95% CI)	No. pos. birds /sampled birds	Apparent animal prevalence (95% CI)	Median within-farm apparent prevalence % [min – max]
Commercial	Controlled-environment house	1/14	7.1 (0.1 – 33.8)	1/236	0.4 (0 – 2.3)	5.0
	Open-sided house	6/23	26.1 (10.2 – 48.4)	19/350	5.4 (3.2 – 8.3)	12.5 [5 – 55]
	Total	**7/37**	**18.9 (7.9 – 35.1)**	**20/586**	**3.4 (2.0 – 5.2)**	**10.0 [5 – 55]**
Backyard	Only chicken	3/5	60.0 (14.6 – 94.7)	22/82	26.8 (17.6 – 37.7)	20.0 [10 – 80]
	Only turkey	0/1	001	0/35	0	0
	Multiple poultry species	9/10	90.0 (55.4 – 99.7)	61/176	34.6 (27.6 – 42.1)	25.0 [5 – 80]
	Total	**12/16**	**75.0 (47.6 – 92.7)**	**83/293**	**28.6 (23.5 – 34.2)**	**10.0 [5 – 80]**

Apparent between-farm prevalence values were also significantly different ($p=0.03$): 7.1% for the controlled-environment commercial farms, 26.1% for the open-sided commercial farms, and 75% for the backyard farms (**Table 9**).

Only one broiler chicken tested positive on the sole positive commercial farm with a controlled environment (**Table 9**). Within-farm apparent prevalence ranged from 5% to 55% on the commercial farms, and from 5% to 80% on the backyard farms. On the only-turkey backyard farm and on the only-male chicken backyard farm (fighting cocks), no birds tested positive. All the bird species sampled that were not chicken tested negative (4 pheasants, 4 quails and 15 ducks). **Figure 20** shows the *Chlamydiaceae*-positive and negative commercial and backyard farms in eight federal states of Mexico characterized by high poultry density.

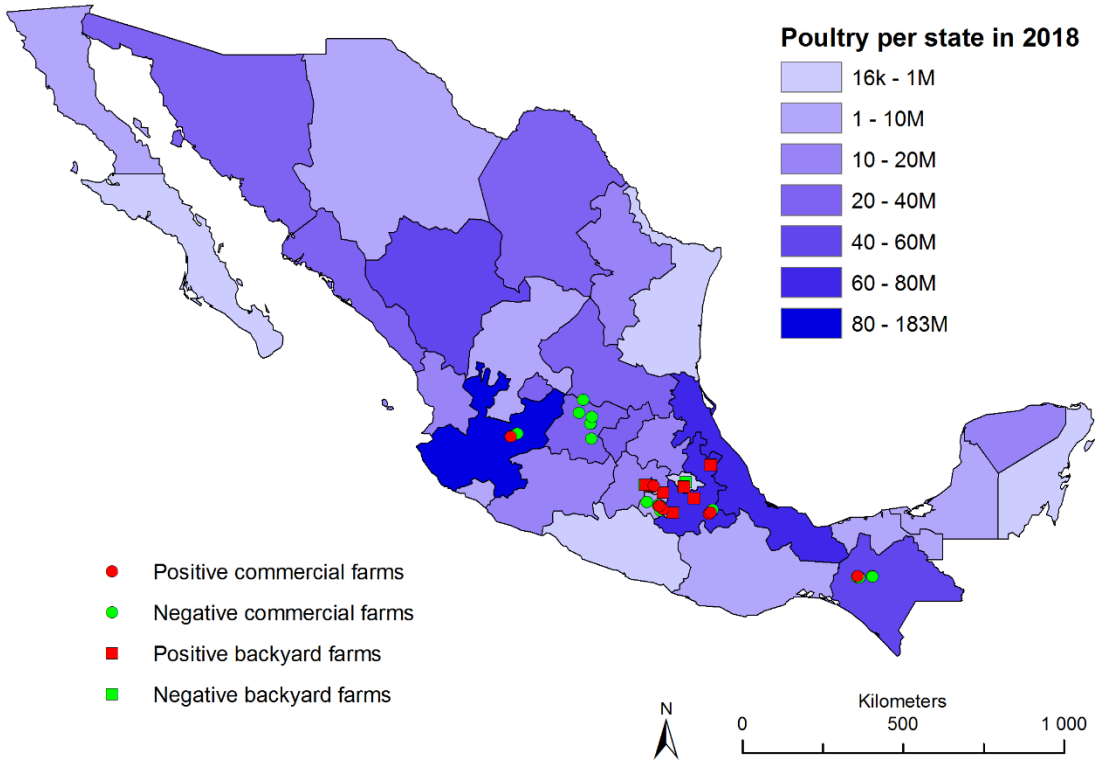


Figure 20. *Chlamydiaceae*-positive and negative farms plotted on a choropleth map representing poultry population per state in the epidemiological study on *Chlamydiaceae* prevalence and associated risk factors on Mexican poultry farms.

3.3. *Chlamydiaceae* rt-PCR results

3.3.1. Cross-sectional study

In terms of *Chlamydiaceae* excretion loads, the rt-PCR Cq values on open-sided commercial farms ranged from 27.3 to 39.9, and from 21.4 to 38.2 on backyard farms (**Table 10**). Positive birds with high excretion loads (Cq<28) were detected on an open-sided farm hosting 40 week-old egg-layers. High excretion loads were also detected on 4 backyard farms hosting multiple species (Cq<29).

3.3.2. Weekly monitoring of *Chlamydiaceae* shedding

Due to available resources, only one broiler flock of the two weekly monitored flocks was analyzed. Only one chicken broiler in the analyzed flock was shedding *Chlamydiaceae* at week 2 of the breeding process with a low level of excretion (Cq 39.6).

3.4. *Chlamydiaceae* species identified

No DNA from *C. psittaci* was detected. *C. gallinacea* was the only chlamydial species found. Of note, some poultry were *Chlamydiaceae*-positive but *C. gallinacea* negative (with *Chlamydiaceae* rt-PCR Cq values above 36) (**Table 10**).

Table 10. rt-PCR Chlamydiaceae and rt-PCR *C. gallinacea* results per poultry farm type in the epidemiological study on *Chlamydiaceae* prevalence and associated risk factors on Mexican poultry farms.

Farm type	Farm characteristic	*Chlamydiaceae* detection 23S rRNA rt-PCR		*Chlamydia gallinacea* identification *eno*A rt-PCR	
		No. pos. samples	**Mean Cq value [min - max]**	**No. pos. samples**	**Mean Cq value [min - max]**
Commercial	Controlled-environment house	1/236	39.9	0/1	-
	Open-sided house	19/350	36.9 [27.3 – 39.6]	9/19	32.5 [24.4 – 37.8]
	Total	**20/586**	**37.1 [27.4 – 39.9]**	**9/20**	**32.5 [24.4 – 37.8]**
Backyard	Only chicken	22/82	33.2 [20.7 – 39.9]	20/22	32.3 [21.4 – 37.9]
	Only turkey	0/35	-	0	-
	Multiple poultry species	61/176	34.3 [22.4 – 39.2]	56/61	32.2 [23.5 – 38.2]
	Total	**83/293**	**34.2 [20.8 – 39.9]**	**76/83**	**32.4 [21.4 – 38.2]**

3.5. Risk factor analysis

In the multivariable analysis for commercial poultry, only the flock purpose was significantly associated with the presence of *Chlamydiaceae*. Egg-laying hen flocks had 6.7 times higher odds of being infected than broilers flocks (OR=6.7 [95% CI: 1.1 – 44.3], p=0.04). The AUC was 0.7, suggesting that the model was fair at discriminating between positive and negative farms.

The final mixed-effects multivariable model obtained in backyard poultry is shown in **Table 11**. Two significant potential risk factors were associated with chlamydial infection: the lack of antibiotic use (OR=8.4 [95% CI: 1.84 – 38.49, p=0.006]) and an impaired health status (OR=8.8 [95% CI: 1.9 – 38.9, p=0.004]). There were no significant interaction terms. The high AUC obtained (0.9) indicates that the model fits the data well. An overview of the risk factors found to be associated to *Chlamydiaceae* presence on analyzed commercial and backyard poultry farms is shown in **Figure 21**.

Table 11. Results of mixed-effects logistic regression conducted to identify risk factors associated with *Chlamydiaceae* infection in Mexican backyard poultry based on backward automated stepwise selection (16 farms, 293 poultry).

Risk factors	Categories	Odds ratio	95% CI	*p*-value
Antibiotic use	No	8.4	1.8 – 38.5	0.006
	Yes, either occasional or systematic	Reference		
Health status	Current signs of respiratory disease	8.8	1.9 – 38.9	0.004
	Apparently healthy	Reference		
Flock age	Less than 6 months	0.4	0.1 – 1.1	0.074
	More than 6 months	Reference		

AUC: 0.9

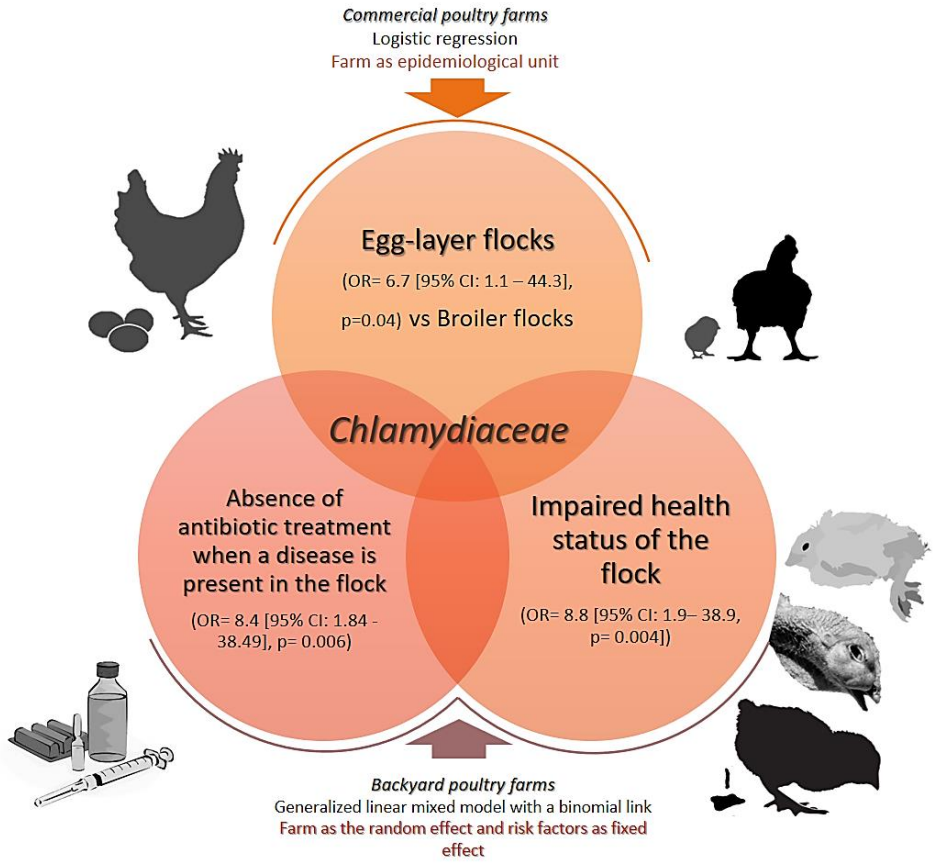


Figure 21. Overview of the risk factors found to be associated to *Chlamydiaceae* presence on analyzed commercial and backyard poultry farms.

3.6. Characterization of biosecurity practices on commercial poultry farms

This analysis was conducted using information collected on all the 43 visited commercial poultry farms. The six other farms included in this study were located in the state of Chiapas (five farms) and in the state of Veracruz (one farm). Farms in Chiapas had open-sided houses while farms in Veracruz had controlled-environment houses. The choropleth map indicating the visited commercial poultry farms is shown in **Figure 22**.

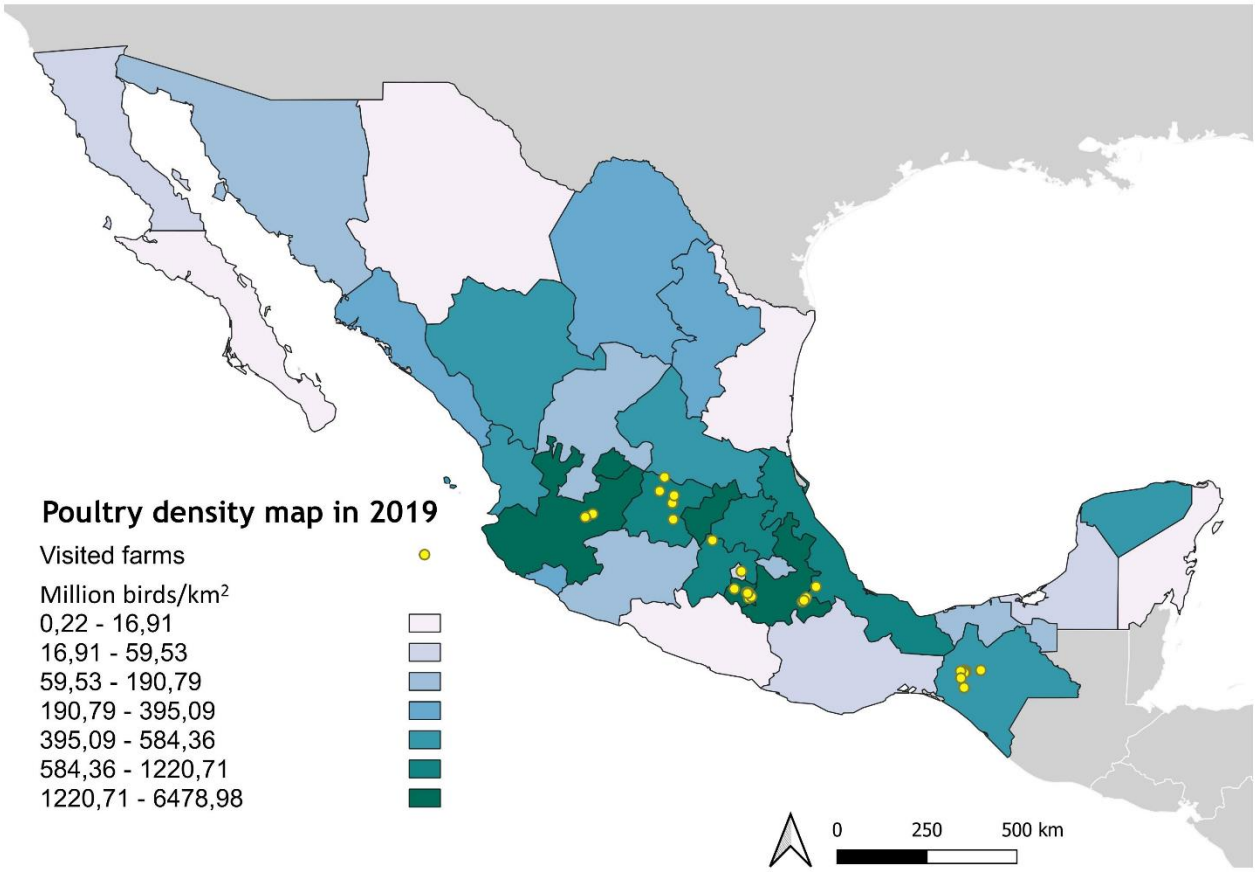


Figure 22. Poultry density map per federal Mexican state with the location of the 43 poultry farms included in the study to characterize the biosecurity practices on commercial poultry farms in Mexico.

All farms had a well-defined fenced perimeter with specific monitored access points and were restricted to authorized personnel. Feed mills were integrated by the major companies owning these poultry farms. Ten farms were specialized in laying hens and 33 farms in chicken broiler breeding. All chicken broiler farms bred males and females separately, except one farm. All-in/all-out systems were systematically applied by barn on all farms. The farm purpose was exclusive. Breeding of other poultry species on the same farm was not reported. All laying hens were housed in battery cages and all chicken broilers in barns, with the floor covered with at least 2 to 5 cm of single use litter. The most frequent bedding materials used were sawdust, coffee husk and rice hulls, with 39.4% for each of the first two materials, and 21.2% for rice hulls.

Flock size per barn on the chicken broiler and laying hen farms ranged from 1,000 to 38,000 and from 2,000 to 150,000 birds, respectively. There were between 2 and 6 barns per farm on 61% (n=20/33) of the chicken

broiler farms, and in half (n=5/10) of the laying hen farms. On 39% (n=13/33) of the chicken broiler farms and on the other half of the laying hen farms, there were between 7 and 16 barns. Most of the chicken broiler farms (63.6%, n=21/33) and 60% of the laying hen farms (n=6/10) employed at least four workers. The number of workers employed by chicken broiler and laying hen farms ranged from 2 to 8 and from 2 to 12 workers, respectively.

3.6.1. Multiple correspondence analysis

Of the 50 variables generated from the questionnaire, 19 were retained for the MCA analysis, seven of which described farm characteristics, six management practices, and five adopted on-farm biosecurity measures. The remaining 31 variables were dismissed for the following reasons: homogeneity in the response among the interviewees (13), their use to identify the farm and to describe its location (4), binary variables for which 5% or less of respondents gave the same answer (3), variables that were transformed into a new one (4), and the low pertinence of the obtained information (7). Farm type and farm purpose were introduced as supplementary (or illustrative) variables in the analysis, meaning that they had no influence on the dimension construction but they helped in result interpretation.

The MCA was performed keeping the first five dimensions covering 80.8% of the data variance with none of the remaining dimensions explaining more than 5% of the data variance (**Table 5**). Eigenvalues obtained from the Burt table showed that three dimensions already covered 89.1% of the data variance, while the rest of the dimensions explained <5%.

Table 12. Eigenvalues and proportion of explained variance for the first ten dimensions obtained from the multiple correspondence analysis conducted for 43 Mexican commercial poultry farms. Eigenvalues represent the contribution of each dimension to explain the total variability of the biosecurity practices and antimicrobial use considered in the analysis.

	Indicator matrix			**Burt matrix**		
	Eigenvalue	**Variance (%)**	**Cumulative variance (%)**	**Eigenvalue**	**Variance (%)**	**Cumulative variance (%)**
Dim 1	0.337	27.306	27.306	0.108	43.339	43.339
Dim 2	0.284	22.964	50.270	0.085	34.141	77.480
Dim 3	0.178	14.384	64.654	0.029	11.683	89.164
Dim 4	0.115	9.344	73.999	0.012	4.897	94.061
Dim 5	0.084	6.815	80.814	0.007	2.655	96.716
Dim 6	0.062	4.998	85.812	0.003	1.383	98.099
Dim 7	0.047	3.825	89.636	0.002	0.807	98.905
Dim 8	0.032	2.615	92.251	0.001	0.470	99.376
Dim 9	0.022	1.765	94.017	0.000	0.189	99.564
Dim 10	0.021	1.700	95.717	0.000	0.167	99.731

The variables more significantly related to the construction of the first dimension (p<0.001) were: (i) the mortality disposal strategy (R^2=0.67); (ii) the use of phosphonic acid derivatives as antimicrobial treatment (R^2=0.66), and (iii) the use of exclusive working clothes by staff and visitors (R^2=0.52). For the second

dimension, the variables more significantly related to its construction (p<0.001) were: (i) the use of personal protective equipment by staff and visitors (e.g. face masks, hair caps, and eye protection) (R^2=0.82), (ii) staff and visitor hygiene protocol requirement before and after entering the farm (R^2=0.53), (iii) and the use of quinolones as antimicrobial treatment (R^2=0.51).

3.6.2. Hierarchical cluster analysis

Taking into account the highest relative loss of within-group inertia, the consolidated partition of the hierarchical dendrogram evidenced three clusters (**Figure 23**). This number was validated through the simultaneous evaluation of 20 indexes available in the NbClust package. Three clusters were proposed by the majority of the indexes by an objective "voting process".

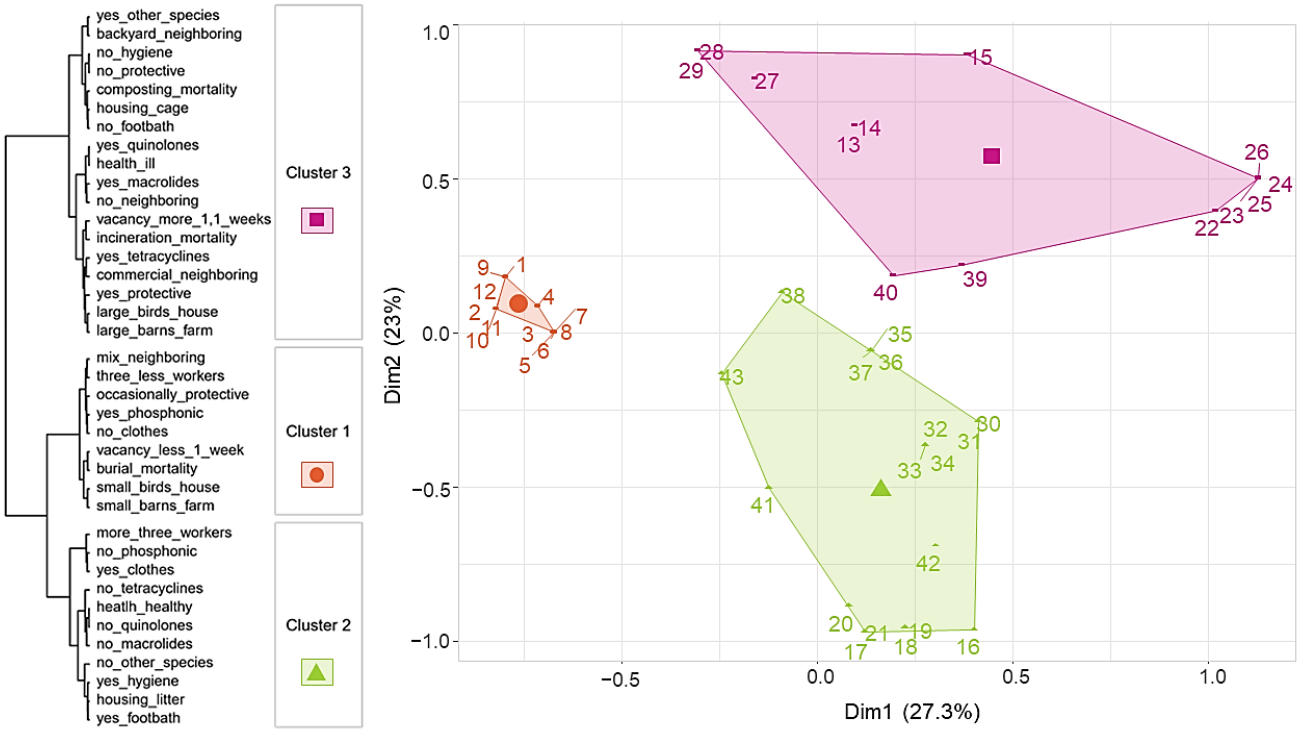


Figure 23. Projection of the 43 Mexican commercial poultry farms included in the study to characterize the biosecurity practices on commercial poultry farms in Mexico. All farms fell inside one of the three clusters identified through the HCA and they were plotted in the first two dimensions of the Euclidean space. The dendrogram shows the categories of variables that most characterize the farms within each cluster.

The biosecurity practices most significantly linked with the cluster partition (p<0.001) were: (i) use of personal protective equipment by staff and visitors (e.g. face masks, hair caps, and eye protection); (ii) compulsory staff and visitor hygiene protocol before and after entering the farm; (iii) staff and visitor use of exclusive working clothes, (iv) footbath presence at barn entrance, and (v) mortality disposal method. Other variables contributed to the characterization of each of the three clusters with p-values < 0.05.

The detailed biosecurity practices and farm characteristics observed by cluster are shown in **Table 13**. All 12 farms within cluster 1 raised chicken broilers in open-sided type barns and were classified in the smallest category of birds per barn (≤ 22,000 birds/barn) and barns per farm (≤ 6 barns/farm). Staff and visitors on these

farms did not wear exclusive working clothes and used personal protective equipment only occasionally. The only mortality disposal system used on these farms was burial, and the vacancy period of poultry premises for hygiene and sanitation purposes was one week or even less. Eight farms within this cluster had neighboring commercial and backyard farms within a distance of 3 km or less, while four had no neighboring poultry farms. The use of footbaths at the entrance of each barn and the compulsory nature of the hygiene protocol before and after entering the poultry living area for staff and visitors were also significant characteristics describing all the farms within this cluster.

Table 13. Frequency of biosecurity practices, antimicrobial usage and farm characteristics observed by cluster obtained from the multivariate analysis conducted on 43 commercial poultry farms in Mexico. Percentages indicate the proportion of farms included in the study representing this category and were grouped into each cluster. Significance of the link between the variable category and the cluster is expressed according to p-values (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001). Categories that stood out within each cluster are highlighted in bold.

Variable	**Category**	**Cluster 1** n=12 farms (%)		**Cluster 2** n=18 farms (%)		**Cluster 3** n=13 farms (%)		**Overall** n=43 farms (%)
Farm characteristics								
House type	Open-sided	**12 (100)**	**	4 (22)	***	**12 (92)**	*	28 (65)
	Controlled environment	0	**	**14 (78)**	***	1 (8)	*	15 (35)
Farm purpose	Broilers	**12 (100)**	*	**17 (94)**	*	4 (31)	***	33 (77)
	Egg-laying hens	0	*	1 (6)	*	**9 (69)**	***	10 (23)
No. of birds per barn	Small (≤ 22,000)	**12 (100)**	***	6 (33)	*	6 (46)		24 (56)
	Large (> 22,000)	0	***	**12 (67)**	*	7 (54)		19 (44)
No. of barns per farm	Small (≤ 6)	**12 (100)**	***	5 (28)	***	8 (62)		25 (58)
	Large (> 6)	0	***	**13 (72)**	***	5 (38)		18 (42)
No. of workers	≤ 3	6 (50)		3 (17)	*	7 (54)		16 (37)
	> 3	6 (50)		**15 (83)**	*	6 (46)		27 (63)
Housing system	Litter	**12 (100)**	*	**18 (100)**	**	4 (31)	***	34 (79)
	Cage	0	*	0	**	**9 (69)**	***	9 (21)
Neighboring farms < 3 km reported	Commercial	0	**	**7 (39)**		6 (46)		13 (31)
	Backyard	0		1 (6)		3 (23)		4 (9)
	Both	**8 (67)**	*	4 (22)		4 (31)		16 (37)
	None	4 (33)		6 (33)		**0**	*	10 (23)
Biosecurity measures								

Vacancy period	≤ 1 week	**12 (100)**	***	9 (50)		2 (15)	**	23 (53)
	> 1week	0	***	9 (50)		**11 (85)**	**	20 (47)
Staff and visitor hygiene protocol before and after entering the farm	Compulsory	**12 (100)**	**	**18 (100)**	***	1 (8)	***	31 (72)
	Optional or inexistent	0	**	0	***	**12 (92)**	***	12 (28)
Footbath at barn entrance	Yes	**12 (100)**	*	**18 (100)**	**	4 (31)	***	34 (79)
	No	0	*	0	**	**9 (69)**	***	9 (21)
Use of exclusive farm clothes	Yes	0	***	**18 (100)**	***	6 (46)		24 (56)
	No	**12 (100)**	***	0	***	7 (54)		19 (44)
Personal protective equipment	Yes	0	**	**14 (78)**	***	0	**	14 (33)
	No	0	**	0	***	**13 (100)**	***	13 (30)
	Occasionally	**12 (100)**	***	4 (22)		0	***	16 (37)
Farm management practices								
Health status of the flocks	Healthy	12 (100)		**11 (61)**	**	12 (92)		35 (81)
	Ill	0		7 (39)		1 (8)		8 (19)
Breeding of other domestic species	Yes	0		0		**4 (31)**	**	4 (9)
	No	12 (100)		18 (100)		9 (69)	**	39 (91)
Mortality disposal	Burial	**12 (100)**	***	7 (39)		3 (23)	*	22 (51)
	Incineration	0	**	**11 (61)**	**	4 (31)		15 (35)
	Composting	0		0	*	**6 (46)**	***	6 (14)
Antimicrobial usage								
Phosphonic acid derivatives	Yes	**12 (100)**	***	**0**	***	3 (23)		15 (35)
	No	0		18 (100)		10 (77)		28 (65)
Tetracyclines	Yes	**0**	**	6 (33)		**7 (54)**	*	13 (30)
	No	12 (100)		12 (67)		6 (46)		30 (70)
Macrolides	Yes	3 (25)		6 (33)		2 (15)		11 (36)
	No	9 (75)		12 (67)		11 (85)		32 (74)
Quinolones	Yes	**0**	*	**8 (44)**	**	1 (8)		9 (21)
	No	12 (100)		10 (56)		12 (92)		34 (79)

Cluster 2 was composed mainly of specialized chicken broiler farms (n=17/18) in controlled-environment type barns (n=14/18), and encompassed the majority of farms categorized as the largest in terms of both number of barns per farm (n=13/18), and number of birds per barn (n=12/18). Staff and visitors at all farms within cluster 2 had to follow a mandatory hygiene protocol before and after entering the poultry living area, they were required to wear exclusive working clothes, and the presence of footbaths at the entrance of each barn was constant. The systematic use of personal protective equipment was reported on the majority of farms (n=14/18). The primary mortality disposal method on farms within this cluster was incineration (n=11/18), followed by burial (n=7/18).

Cluster 3 mainly included farms with open-sided type barns (n=12/13) specialized in laying hens (n=9/13). The farm size was not significantly associated with this cluster; however, most of the farms (n=7/13) were large in terms of number of birds per barn. On most of the farms, the staff were not required to follow a hygiene protocol to access the poultry living area (n=12/13), nor to use personal protective equipment (n=13/13). Likewise, there were no footbaths at the entrance of each barn in the majority of farms within this cluster (n=9/13). The most significantly associated mortality disposal method was composting (n=6/13) p-value < 0.001, even though burial (n=3/13) was also significantly associated with farms in this cluster (p<0.05). The few studied farms on which the presence of other domestic species (cattle) was reported fell into cluster 3. A long barn vacancy period (≥ 1 week) was significantly linked to farms within this cluster (n=11/13); the maximum vacancy period reported was 22 days.

Among the variables with less than 5% variability or for which the answers were homogeneous, and therefore excluded from the MCA, it is worth mentioning the following: problems with rodent control mentioned only in one out of the 43 farms. On two farms, members of the staff stated that they were not aware of potential zoonotic diseases associated with poultry, while training on continuous biosecurity and poultry disease prevention for staff was common across the rest of the analyzed farms; a hand washing facility and/or hand sanitizer availability at the entrance of each barn was absent on almost all farms (n=41/43); however, handwashing facilities were in the clean room at the general entrance to each farm. Exhaustive cleaning and disinfection procedures during the vacancy period were reported on all farms. Several disinfectant products were mentioned, with organic acids the most extensively used.

The use of antimicrobials as growth promoters was not reported on any farm. Antimicrobial usage was more extensive within farms belonging to clusters 1 and 3, with 100% (n=12/12) and 85% (n=11/13), respectively, while only 45% (n=8/18) of farms from cluster 2 reported its usage. Four antimicrobial classes were reported to be used on the farms (**Table 13**), in decreasing order: phosphonic acid derivatives (n=15/31), tetracyclines (n=13/31), macrolides (n=11/31), and quinolones (n=9/31). On some farms, the use of more than one antimicrobial class was reported: three in cluster 1, six in cluster 2, and one in cluster 3.

Either the usage or the non-usage of certain antimicrobial classes on the farms was significantly associated with farms within each cluster. The prevailing antimicrobial used on all farms within cluster 1 (n=12/12) was a phosphonic acid derivative (fosfomycin). In addition, three of these farms also reported the use of tylosin, a macrolide antibiotic. Thus, the potential interaction of fosfomycin and tylosin in flocks on these 3 farms was possible. The lack of use of tetracyclines and quinolones as antibiotics was significantly associated with the farms belonging to cluster 1. Quinolones were significantly associated with farms using antimicrobials (n=8) within cluster 2. On six of these farms, tetracyclines and macrolides were also given. Thus, the potential interaction of tetracyclines, quinolones and macrolides in flocks within these six farms was possible. Conversely, the lack of use of phosphonic acid derivatives as antimicrobials was significantly associated with farms belonging to this cluster. Tetracyclines were the antimicrobial class reported to be used on seven farms within cluster 3, and the only antibiotic class whose use was significantly associated with them. Additionally, on three out of these 11 farms, a phosphonic acid derivative antimicrobial was used, while one used macrolides and one quinolones. On one out of the 11 farms belonging to cluster 3 that used antimicrobials, the use of tetracyclines and quinolones was reported; thus, the interaction of these antimicrobials in flocks on this farm was possible.

4. Discussion

4.1. *Chlamydia gallinacea,* the only chlamydial species identified in this study

Chlamydia gallinacea was the only chlamydial species identified in poultry in our study. This finding is consistent with previous studies showing that this species is endemic in chickens (Guo et al., 2016; Hulin et al., 2015; Taylor-Brown and Polkinghorne, 2017). In a research study conducted in chicken flocks sampled between 2009 and 2011 in France, Greece, Slovenia, Croatia and China, most of the chlamydia-positive chickens were infected by *C. gallinacea* (Zocevic et al., 2012). In another study conducted in China, *C. gallinacea* represented 63.8% of all *Chlamydiaceae*-positive sampled birds (Guo et al., 2016). Similarly, in studies carried out in Poland, the Netherlands and Italy, 65.5%, 96% and 100% of the *Chlamydiaceae*-positive flocks were *C. gallinacea*-positive, respectively (Donati et al., 2018; Heijne et al., 2018; Szymańska-Czerwińska et al., 2017a).

4.2. Prevalence values obtained in this study compared with other studies

Chlamydiaceae prevalence values found on commercial farms in our study (7.1% on the controlled-environment commercial farms and 26.1% on open-sided commercial farms) were close to the between-farm prevalence value found in Polish poultry (15.9%), but were lower than the reported between-farm prevalence values on Dutch commercial poultry farms (47%) (Heijne et al., 2018; Szymańska-Czerwińska et al., 2017a). However, the overall between-farm prevalence on backyard farms (75%) was higher than the backyard farm prevalence reported in studies conducted in the United States (12.4%, n=66/531), Italy (15%, n=24/160), and China (16.7%, n=384/2,300) (Donati et al., 2018; Guo et al., 2016; Li et al., 2017).

The higher *Chlamydiaceae* prevalence values for backyard farms compared to those found for commercial farms in our study was an expected result, since it has been demonstrated that stringent biosecurity measures, cleaning and disinfection practices, preventive medicine and nutrition management reduce the risk of introduction or onward transmission of pathogens (Hulin et al., 2015; Sims, 2006; Vorimore et al., 2015). The significant increase of animal prevalence in birds housed under less confined conditions (controlled environment *vs* open-sided poultry houses *vs* backyard) indicates that the environment could be a source of *C. gallinacea* contamination, as has been suggested in previous studies (Hulin et al., 2015; Vorimore et al., 2015). In fact, Laroucau *et al*., 2009 found that five poultry flocks coming from a single *C. gallinacea*-positive breeder flock, but housed in different farms, were infected with different strains of *C. gallinacea* based on partial molecular characterization.

4.3. Health status and lack of antibiotic usage as risk factors associated to *Chlamydiaceae* presence in backyard poultry

Impaired health status was significantly associated with *C. gallinacea* presence on backyard farms. Co-infections with other pathogens such as *Mycoplasma gallisepticum*, *Ornithobacterium rhinotracheale*, avian influenza virus, and *Aspergillus fumigatus* might exacerbate *C. gallinacea* infection, as has already been reported for *C. psittaci* (Chu et al., 2016, 2017; Gaede et al., 2008; Darrell R Kapczynski et al., 2013). However, *C. gallinacea* was also detected on backyard farms where birds were clinically healthy. This is in line with previous studies in which no clinical disease was directly associated with *C. gallinacea* infection (Donati et al., 2018; Guo et al., 2016; Heijne et al., 2018; Taylor-Brown and Polkinghorne, 2017). Nonetheless, Guo *et al.* (2016) were able to show the presence of this bacterium in oropharyngeal and cloacal swabs, as well as in the blood, lungs, heart, liver, trachea, kidneys, pancreas and spleen of naturally infected chickens, suggesting that this bacterium is not only a commensal, but a pathogen with moderate virulence (Guo et al., 2016).

Lack of antibiotic use as therapeutic treatment was significantly associated with *C. gallinacea* occurrence in backyards. It has been reported that some antibiotics (mainly oxytetracycline) can clear *C. psittaci* and *C. gallinacea* infections (Gaede et al., 2008). The use of antibiotics on most of the sampled commercial farms in our study may also explain the lower proportion of chlamydia in commercial chickens.

Interestingly, we did not find any association between *Chlamydiaceae* infection and the presence of other domestic animals (cattle, sheep, pigs and goats), unlike findings of a previous study where the presence of horses was associated with *C. gallinacea* infection (Heijne et al., 2018). Likewise, it has been suggested that cattle may play a role in *C. gallinacea* epidemiology since it was the main chlamydial species found in vaginal swabs, whole blood, and milk from cows (M. Li et al., 2016).

4.4. Flock purpose as the only risk factor associated with *Chlamydiaceae* presence on commercial farms

On commercial farms, the only risk factor identified that could explain the higher proportion of infection in open-sided *vs* controlled environment commercial farms was the flock purpose. Layer flocks were 6.6 times more at risk of being infected than broiler flocks (p=0.04). This risk factor might be explained by the longer exposure of egg-laying hens or particular management/biosecurity practices on layer farms. For example, the longer exposure of egg-laying hens to the source of infection prompted by a longer life period (up to 90 weeks old *vs* 7 weeks old for broilers), coupled with the fact that they are raised in battery cages, allowing higher animal density per barn. This type of facility increases the amount of stool and waste generated, creating a need for constant removal. This stool removing activity generates high amounts of dust that could sediment in the feeders. It has been shown that barn dust might be a vector for microorganisms and toxins (David et al., 2015), and specifically it could be the source of chlamydial infection, as previously described by Hoffmann *et al.* (2015) in a study carried out on pig farms. This finding is supported by data showing efficient transmission of *C. gallinacea* by the fecal-oral route (You et al., 2019). Moreover, weekly monitoring that we conducted in one controlled commercial broiler flock also supports the low chlamydial infection rate found in commercial broilers: 15 randomly selected broiler birds were sampled weekly for 5 weeks (prior to slaughter), and only one 2-week-old broiler tested positive for *C. gallinacea*.

Remarkably, even though flock age was not found to be a risk factor, the only commercial poultry flock having high *Chlamydiaceae* excretion loads was a 40-week-old egg laying hen flock (with birds reaching a Cq value of 24.4). This age is within the age category found to be at higher risk of *C. gallinaceae* infection in the study conducted on layer farms in the Netherlands (Heijne et al., 2018). This age is also close to the peak of egg production, which might affect the immune system and could favor the reactivation of chlamydial shedding. Other authors have also found that *C. gallinacea* excretion is higher as the age of the chicken breeder flock increases: less than 10% of infected poultry when they are under 8 weeks old, and 100% when they are between 20 and 53 weeks old (Laroucau et al., 2009b; Zocevic et al., 2012).

The fact that only the flock purpose was found to be a risk factor associated with the presence of *Chlamydiaceae* encouraged us to perform a multivariate analysis of farm management and biosecurity practices. This analysis allow us to further characterize those practices that may predispose laying hens to the presence of *Chlamydia* and other avian pathogens; as it was illustrated by the highly pathogenic avian influenza (HPAI) virus subtype H7N3 outbreak that occurred for the first time in 2012 on commercial poultry farms in Mexico (D. R. Kapczynski et al., 2013). The first outbreak was detected in laying hen farms in a region with high poultry density, then it spread within a few months to broilers, breeders and backyard farms (Lu et al., 2014).

4.5. Multivariate analysis to characterize the on-farm management and biosecurity practices on commercial poultry farms in Mexico

This study provided an overview of the critical on-farm biosecurity practices that are most likely not to be implemented or to be breached (if they have been implemented). Significant variations in the application of biosecurity practices were observed across the farm clusters identified in our study. This finding is consistent with the results of previous studies showing that the on-farm application of biosecurity measures tends to be variable and could often be intermittent (Racicot et al., 2011; Tanquilut et al., 2020), both on chicken broiler farms (Gibbens et al., 2001; Gifford et al., 1987; Tablante et al., 2008) and laying hen farms (Durr et al., 2016; Lestari et al., 2011; Ssematimba et al., 2013). We conducted a multidimensional exploratory analysis considering that evaluation of biosecurity practices is measured by a large number of variables. As many of these variables may be correlated, this methodology makes it possible to uncover the relationships among categorical variables within and between farms, to ultimately find patterns (Husson et al., 2016; Sourial et al., 2010). This information may not otherwise be discovered through a pairwise analysis (Sourial et al., 2010). The subsequent hierarchical clustering analysis conducted allowed us to objectively group the farms according to these previously identified patterns. This approach was adopted instead of describing the biosecurity practices implemented through specific farm characteristics, such as degree of confinement (open-sided barns *vs* controlled-environment barns), farm size or farm purpose (broilers *vs* layers).

4.6. Biosecurity practices most significantly associated with farm classification into three clusters

Five biosecurity practices were identified as the most significantly associated with farm classification into three clusters. Three of these practices were related to measures concerning directly the staff or visitors (appropriate use of personal protective equipment, hygiene protocol before and after entering the farm, use of exclusive working clothes), while the last two were related to general farm facilities (i.e. footbath presence at barn entrance) and poultry mortality disposal methods. Previous studies have established that the implementation of and compliance with biosecurity measures regarding personnel are crucial to prevent the transmission of pathogens into a flock (Gifford et al., 1987; Nespeca et al., 1997; Racicot et al., 2011; Ssematimba et al., 2013; Tablante et al., 2008; Volkova et al., 2012). In a study performed on poultry farms in the Netherlands, it was found that non-adherence by personnel to the hygiene protocols, and not wearing exclusive working clothes before entering the poultry living area, represented the highest transmission pathways of pathogens for poultry from an external source (Ssematimba et al., 2013). In fact, if staff or visitors have contact with infected birds and/or their feces and/or contaminated material, they could become the main source of contamination, within and between farms. The use of personal protective equipment is not intrinsically a biosecurity practice, but an occupational safety recommendation, as poultry workers are at increased risk of respiratory exposure to dust, particulate feathers, and atmospheric contaminants including ammonia and hydrogen sulfide during certain handling activities (Kearney et al., 2014; Saksrithai and King, 2018). Its usage is not mandatory for staff nor for visitors in accordance with Mexican law. However, according

to Dorea et al., (2010), the mandatory usage of personal protective equipment, mainly for farm visitors, emerged as a response to address the threat of introduction of pathogens either by veterinarians who ensure technical support to the farms or by farmers. In our study, the use of personal protective equipment was observed systematically on farms within cluster 2, and occasionally on some farms within cluster 1, the two clusters with better biosecurity practices, whereas its use was inexistent on farms within cluster 3. Furthermore, in a study performed in Latin-American poultry workers conducted in poultry processing plants in the United States, Arcury et al., (2012) found that the use of personal protective equipment, coupled with receiving constant training on biosecurity, were significantly associated with a positive work safety climate, especially among employees in this sector.

Poultry farming faces a major environmental challenge associated with waste generation, its adequate treatment and disposal. As a result, methods considering both the environmental impact and safe waste disposal should be prioritized (Blake, 2004). There are several methods and technologies for handling carcasses, each with its pros and cons. Burial is one of the least acceptable methods mainly due to environmental issues, e.g. the potential risk of ground water pollution due to adsorption of pollutants by the soil. Incineration is recognized as one of the biologically safest methods, while composting is becoming increasingly adopted as it has been successfully used for emergency disposal of carcasses (Blake, 2004; Wilkinson, 2007). The only method for waste disposal used on farms from cluster 1 in our study was burial, which may represent a health risk. For instance, Tablante et al., (2008) found that farms on which carcasses were not properly buried – resulting in scavenging by other animals – experienced recurring infectious laryngotracheitis outbreaks. In contrast, the majority of farms within cluster 2 opted for incineration. Even though its implementation is initially expensive and facility maintenance should be permanent, it is the safest disposal method as it does not attract scavengers or pests, and its residues can be safely disposed of without causing water quality problems (Blake, 2004). The main method for carcass disposal on farms within cluster 3 was composting. When this is performed properly, pathogens are efficiently eliminated and the resulting material can be used in further agricultural processes (Benson et al., 2008; Cornell University, 2009). Farms within cluster 1 should improve their waste disposal methods, as in many cases they have enough space to perform composting; this could be an affordable, easy-to-implement solution.

Remarkably, in only 5% of the farms included in our study, a formal protocol for hand washing before and after entering each barn was described. This finding is in accordance with results of the study carried out by Racicot et al., (2011) on chicken broiler and laying hen farms, in which they found that one of the most frequent breaches by staff was related to hand sanitizing. This is important because poorly sanitized hands can act as an efficient mechanism to spread pathogens within and between farms (Racicot et al. 2013). Furthermore, Racicot et al., (2011) also observed that waterless alcohol-based gel for hand sanitizing was better accepted by poultry workers. However, its use should not replace hand washing with soap when visible organic material is present on hands (i.e. moderate to high contamination), because dirt significantly interferes with the microbicidal activity of handrubs (Muñoz-Figueroa and Ojo, 2018; Racicot et al., 2013). Several formulations

and presentations are available for handrubs. Racicot et al. (2013) found that there was no difference in effectiveness between products and protocols only when the initial level of bacterial contamination was low; hence, prior hygiene of hands is essential in these cases (Racicot et al., 2013). Similarly, Wilkinson et al., (2018) found no difference regarding antibacterial efficacy attributable to isopropanol- (IPA) *vs* ethanol (EtOH)-based formulations. However, in their study performed with 20 volunteers, EtOH-based handrubs in liquid or foam presentations were more comfortable for use because they dry faster than gel presentations (Wilkinson *et al.* 2018). This was further confirmed by Greenaway et al. who found that a 1.5 mL handrub dose yielded the most acceptable cost-effect result (Greenaway et al., 2018). The WHO recommends the use of these alcohol-based handrubs in resource-limited or remote areas with lack of accessibility to sinks or other facilities for hand hygiene. This is a method for promoting hand hygiene compliance, by making the process faster and more convenient for workers (World Health Orgaization, 2009). Therefore, we propose the implementation of handrub dispensers at each barn entrance as an immediate alternative to correct this biosecurity breach.

4.7. On-farm biosecurity practices are not similar for chicken broiler and laying hen farms

It has been proposed that in general, most of the breaches in biosecurity practices are similar for laying hens and chicken broiler farms (Ssematimba et al., 2013). However, we did find a difference by using the clustering approach. Overall, farms from cluster 3, in which laying hen farms predominated, were more prone to breach biosecurity practices that had previously been identified as risk factors associated with low pathogenic outbreaks of influenza virus subtype H5N2 in laying hen farms in Japan (Nishiguchi et al., 2007). These practices were the inexistence of or vague implementation of hygiene protocols before entering the poultry living area, no footbath at each barn entrance, and not using exclusive working clothes, thus coinciding with our findings. Interestingly, the first case of HPAI virus subtype H7N3 in Mexican poultry occurred on laying farms (D. R. Kapczynski et al., 2013). Moreover, the presence of animal species other than poultry e.g. cattle, observed only on farms within cluster 3, might be a relevant factor for avian influenza introduction and dissemination into the poultry premises, as shown in a previous study (Ssematimba et al., 2013). Cattle presence can generate additional personnel movements and activities related to cattle rearing (e.g. extra farm visits, feed-related activities) and, more importantly, they could have a potential role as pathogen carriers. Kalthoff et al., (2008) showed that cattle experimentally infected with an avian influenza virus can actually seroconvert and become asymptomatic shedders of the virus. Conversely, more stringent and exhaustive biosecurity protocols were in place on farms from cluster 2, followed by farms from cluster 1, both clusters mostly encompassing chicken broiler farms. This finding is in agreement with the results of a study carried out by Scott et al., (2018) on Australian poultry farms, in which they observed that more demanding biosecurity measures were practiced on chicken broiler farms than on laying hen farms. The authors of this study also found that footbaths where absent at each barn entrance on all laying hen farms, a breach that we also observed. It would be interesting to investigate the occurrence of avian influenza or/and other important poultry pathogen outbreaks on Mexican poultry farms to compare their frequencies according to the farm purpose.

4.8. Antimicrobial usage on commercial farms

No use of antimicrobials for growth promotion was reported on any farm, which is in alignment with national and international measures implemented to prevent antimicrobial resistance (Maron et al., 2013). Four antimicrobial classes were reported to be used for treatment on 31 of the 43 visited farms: tetracyclines, quinolones, macrolides and phosphonic acid derivatives. According to the list of antimicrobial agents of veterinary importance issued by the OIE, the classes of antimicrobials used on the farms included in our study are approved for use in food-producing animals (OIE, 2019b). The WHO established a list of critically important antimicrobials for human medicine, whose scope is to classify those antimicrobials that are also used in veterinary medicine (World Health Organization, 2018). According to this list, of the four classes of antimicrobials used on the farms included in our study, tetracyclines are highly important for human medicine, and phosphonic acid derivatives are critically important, while quinolones and macrolides have the highest priority. WHO recommends that all antimicrobials should be used prudently in veterinary medicine, especially those classified as critically important and with the highest priority. In Mexico, there has been a list of antimicrobials allowed in veterinary medicine since 2012 (SAGARPA, 2012). However, this list does not classify the antimicrobial classes in relation to the risk to public health posed by their use in animals, leaving the therapeutic choice to the discretion of the veterinarian providing technical support to the farm. Conversely, the United States Food and Drug Administration (FDA) together with their veterinary authorities, have provided guidelines that include a list of antimicrobials approved for its use exclusively in poultry, and classified according to their importance in human medicine – these guidelines are also intended to aid veterinarians in their therapeutic decision-making (AAAP-AVMA, 2017). In parallel, the European Medicines Agency (EMA) provides a categorization of antimicrobials for use in animals, reserving only some of them for use in food-producing animals (EMA, 2019). Both guidelines, American and European, are in accordance with the list of critically important antimicrobials for human medicine established by the WHO. However, the classification scale of some antimicrobials could be more stringent on either list in addressing their particular needs, e.g. the phosphonic acid derivative fosfomicyn is banned for use in veterinary medicine in Europe, and is not included in the antimicrobial schema for poultry in the United States.

The proportion of farms using antimicrobials differed by cluster; the more stringent the biosecurity measures on farms within a cluster, the fewer the farms that used antimicrobials. Specifically, antibiotics were used on only 45% of farms within cluster 2 *vs* 85% on farms within cluster 3, reaching even 100% on farms within cluster 1. In a study conducted on 60 German pig farms, Raasch et al., (2018) confirmed that the improvement of biosecurity measures is a feasible strategy to reduce antimicrobial usage at the herd level. Similarly, Chauvin et al., (2005) observed that compliance with biosecurity practices was associated with a lower antimicrobial consumption level, after quantifying the consumption level of antibiotics in 246 turkey broiler flocks. Furthermore, we found that the more breaches there were to on-farm biosecurity practices, the more likely it was to observe the use of antibiotics critical for human health. To illustrate this, fosfomycin was the most widely used antibiotic among farms reporting antimicrobial use in our study. Its use was extensive on farms within clusters 1 and 3, the two clusters of farms in which less stringent biosecurity measures were practiced,

while the lack of its use was significantly associated with farms belonging to cluster 2. The antimicrobial class whose use was significantly associated with farms within cluster 2 was the tetracycline group, which is classified as highly but not critically important by the WHO. Fosfomycin is used to treat infections caused mainly by *E. coli* and *Salmonella* spp. in poultry, but to ensure its efficacy on susceptible bacteria, it must be used at specific concentrations under a specific schema (Gutierrez et al., 2010; Pérez et al., 2014). A 10-year longitudinal study of uropathogenic *E. coli* strains (UPEC) in humans in Mexico, identified these strains as the leading cause of urinary infections (Lagunas-Rangel, 2018). Moreover, rates of multidrug-resistant UPEC have significantly increased over time, reaching more than 60% of isolated strains, complicating their treatment, and leading to severe complications such as cystitis, pyelonephritis and urosepsis (Morales-Espinosa et al., 2016). Fosfomycin is used mainly for the treatment of urinary tract infection in humans, with bacterial resistance arising readily *in vitro* (Greenwood et al., 2012). In Mexico, fosfomycin represents the last-resort antimicrobial therapeutic alternative (Ortega Martell et al., 2019). Therefore, we suggest to add to the Mexican manuals of good husbandry practices, a classification of the antimicrobial classes that are used in poultry aligned with WHO criteria. The aim would be to guide field veterinarians towards more judicious therapeutic choices and to restrict the usage of medically important antimicrobials only to specific situations. The use of such critical antimicrobials for humans in veterinary medicine is highly undesirable, especially in food-producing animals. This is because antimicrobial-resistant bacteria could develop in livestock and then spread to the environment through their feces or waste from processing plants. Human exposure to food or water contaminated by antimicrobial-resistant bacteria has been found to be the most common and efficient transmission route (Laxminarayan et al., 2013).

4.9. Limitations of the study

Our study has some limitations. First, a single cross-sectional survey may not allow for the detection of *C. psittaci*. Indeed, Hulin *et al*. (2015) were able to detect only one *C. psittaci*-positive flock among 129 non-duck sampled flocks over a 1-year sampling period, while *C. gallinacea* was detected in most of the sampled flocks at all time points of the year (Hulin et al., 2015). This was also the case in a 7-month follow-up study of naturally infected backyard chickens in China, in which *C. gallinacea* was detected at all-time points, while other chlamydial species were observed only transiently (Guo et al., 2016). Similarly, in Italy, *C. psittaci* was found only at the second sampling time (Donati et al., 2018).

Second, the representativeness of the farms included in the survey could be questioned, as they could not be randomly selected using a list of farms or farmers. Since such a list was not available, the approach we used to include the commercial farms was the only way to obtain access to the main Mexican poultry producers. The commercial farms were selected in terms of location (covering the most densely poultry populated states) and farm purpose (encompassing broiler and laying hen farms, the two most numerous nationwide farm purposes), and not for ease of sampling. Obtaining consent to visit the commercial poultry farms and to perform the on-farm interviews was difficult since allowing access was itself a biosecurity risk, especially when visiting several farms at different geographical locations over a fixed period of time. Moreover, the Mexican animal

health authorities have issued standardized breeding recommendations (SAGARPA-SENASICA, 2016b, 2016a) that were applied on the sampled commercial farms. We believe that the farms included in our study, which were already following these breeding recommendations for the best practices on poultry husbandry (SAGARPA-SENASICA, 2016b, 2016a), may be representative of the homogeneous large-scale poultry farming sector in Mexico. Therefore, the results we found could be applicable to Mexican commercial farms raising broilers and egg-layers that follow these standardized breeding recommendations. As only open-sided egg-laying farms were included, controlled-environment egg-layer farms should also be assessed in future studies. Regarding the backyard farm sample, it could be considered a convenience sample. Nevertheless, as we sought to include different kinds of backyard farms, it is likely that the breeding conditions recorded in our study cover much of the management practices implemented on Mexican backyard farms. As farmers included in the study were not aware of *Chlamydiaceae* infection and all of them agreed to participate in the study, there is no reason to think that there was a selection bias towards farms with greater or lower degrees of infection.

Third, the large between-prevalence confidence intervals obtained in our study indicate that the sample size was small to provide more precision. Due to the small sample size, the lack of power to detect effects of other risk factors cannot be excluded. Finally, compliance with on-farm biosecurity measures could be questionable. Racicot et al., (2011) and Delpont et al., (2018) found that discrepancies between the implemented biosecurity measures and their actual practice are more frequent that one may expect, leading to a decrease in their effectiveness with the associated risks in terms of pathogen exposure and transmission. To take these possible discrepancies into account, our study design included on-farm visits and personal interviews to administer the questionnaire. We consider that this approach gave us the opportunity to gather complementary information through an open dialogue with the interviewees, with the understanding and reassurance that this was not an audit nor an official inspection, but an independent, anonymous study aiming to gather knowledge and assist the poultry sector. Only certain practices could be observed directly, but the bias of an external observer may have played a role. However, we assumed that since the studied farms belonged to large, well-integrated poultry companies, the implementation of and compliance with biosecurity measures would tend to be higher. In a study involving 921 Australian poultry farms, East, (2007) showed that the implementation rates of biosecurity practices were higher in integrated companies than on independently owned farms. In addition, a non-negligible number of variables representing the implementation of major biosecurity practices (16 out of 50) were dismissed from the analysis due to the lack of variability and homogeneity in the responses given by the interviewees. This fact can be interpreted as a positive consequence of the extensive implementation of these biosecurity practices on these poultry farms. For example, the existence of a perimeter fence, the implementation of a logbook, the use of the all-in/all-out system, the ban on breeding two poultry species or zootechnical purposes simultaneously in the same facilities, and the establishment of a vacancy period, have been implemented on all the visited farms. This is similar to the findings of East, (2007), where the farms owned by a major company were compliant with all the major biosecurity practices evaluated. In future studies, a scoring system could be used to overcome this homogeneity and more accurately assess the degree of compliance and not just the presence or absence of a given practice.

4.10. Conclusions

We have shown that *C. gallinacea* is present on both commercial and backyard poultry in Mexico; therefore, *Chlamydiaceae* diagnosis in poultry should no longer be neglected. Its association with laying hens on commercial farms points to the need for further investigations regarding its impact on egg quality, production performance, as well as its potential pathogenicity. Remarkably, *C. psittaci* was not detected on any of the sampled Mexican farms. However, considering the sporadic nature of *C. psittaci* outbreaks in poultry and in slaughterhouse workers reported in numerous European countries and the United States (Anderson et al., 1978; CDC, 2018; Gaede et al., 2008; Hedberg et al., 1989; Irons et al., 1951; Laroucau et al., 2015; Newman et al., 1992; Salisch et al., 1996; Shivaprasad et al., 2015), the awareness of psittacosis should be maintained.

Our study provides an exploratory analysis of patterns of on-farm biosecurity practices across the different groups of poultry farms in Mexico identified through our analysis. This could be helpful to field veterinarians or farmers to understand how to guide strategies to reinforce staff training, as well as for on-farm implementation and compliance of biosecurity practices, prioritizing those identified as critical in our analysis. This study also offers information characterizing antimicrobial usage in the poultry industry, and thereby contributes to the national need for information on this subject. These data may help to consolidate a national strategy to improve the use of antimicrobials and contain antimicrobial resistance. We hope that our results could also be useful to other poultry industries with similar conditions outside Mexico. Further studies investigating the effectiveness of the official provisions issued in the last few years should be conducted, to follow up on trends in on-farm biosecurity practices and antimicrobial usage in the Mexican poultry industry.

CHAPTER II. COMPARISON OF GROWTH CHARACTERISTICS OF *Chlamydia gallinacea* IN DIFFERENT EXPERIMENTAL TRIALS

1. Introduction

When attempting to grow chlamydiae *in vitro* using cellular models, it is essential to provide suitable conditions for the attachment of chlamydial elementary bodies to the host cell membrane, as well as to create a favorable intracellular environment for bacterial development (Johnson and Hobson, 1976; Weiss and Dressler, 1960). *In vitro*, infection rate and chlamydial growth can be improved by chemical or physical treatments of cell cultures, e.g. by altering the electrostatic charge of the host cell membrane to enhance bounding of chlamydial elementary bodies (treating cell cultures with the cationic polymer DEAE-dextran) (Kuo et al., 1972), by optimizing the incubation temperature (Onorini et al., 2019), by performing centrifugation assisted infection (Allan and Pearce, 1979; Prain and Pearce, 1985), or by impairing (irradiating or pretreating cell cultures with 5-iodo-2-deoxyuridine) (Gordon and Quan, 1965; Wentworth and Alexander, 1974), or depressing the eukaryotic cell metabolism (adding cycloheximide to infection medium) (Ripa and Mardh, 1977; Wanninger et al., 2016; Wills et al., 1984).

Cell lines have shown to play a key role to optimize chlamydial growth protocols. For example, to grow the human respiratory pathogen *C. pneumoniae*, Wong et al., (1992) compared several cell lines, mostly originating from the human respiratory tract, with the HeLa 229 cell line (human cervical cancer) normally used to propagate *C. trachomatis*. This study showed that H292 (human mucoepidermoid pulmonary carcinoma) and HEp-2 cell lines (human epithelial carcinoma) were more efficient to grow *C. pneumoniae* by yielding higher number of inclusions. These two cell lines also had the advantage of remaining viable for longer periods after changing the growth medium, therefore allowing new reinfections with the same inoculum, and hence giving an increased number of inclusions per specimen (Wong et al., 1992). The impact of the cell line used for chlamydial growth has been also assessed for various chlamydial species. Schiller et al., (2004) found that Vero cell line (African green monkey kidney cells) provided the highest inclusion numbers for all evaluated chlamydial strains coming from different animal hosts, although inclusions observed in Caco-2 cell line (human colon adenocarcinoma) were bigger.

All these studies suggest that conditions that promote *in vitro* growth of a chlamydial species/specimen appear to be not applicable for the rest of them. Despite improvements made to the chlamydial growth protocol aiming to increase the infectivity rate and to enhance *in vitro* growth, the propagation of some chlamydial strains/species remains difficult, e.g. *C. psittaci* strains isolated from cattle and pigs, *C. pecorum* strains from intestinal tract of ruminants (Sachse et al., 2009b), *C. suis* strains from field samples of swine (Schiller et al., 2004), *C. pneumoniae* strains from human clinical respiratory samples (Kuo and Grayston, 1988; Wong et al., 1992). Difficulties are described as restrictions either in cultivating a strain from a specimen, or in propagating a strain during passages, after a first successful isolation.

Such difficulties are encountered for *C. gallinacea*, even if few studies have so far focused on the *in vitro* cultivation of this new species. First isolates of *C. gallinacea* (08-1274/3, /13, /19, /21, /22, /23) were obtained from cloacal swabs collected from different chicken flocks in France, after inoculation of chicken embryos (Laroucau et al., 2009b). One of these isolates (08-1274/3) was later propagated in cell culture using BGM cell line to study its ultrastructural and molecular characterization. This strain was designated as the reference strain of *C. gallinacea* (Hölzer et al., 2016). In the course of studies conducted to deeply characterize new field isolates of *C. gallinacea*, it was observed that these isolates were gradually losing their infectivity through the subsequent passages in chicken embryos or in BGM cells; the more passages are performed, the fewer bacteria grow (unpublished results). Similar observations have been made in other research laboratories (Dr. Marloes Heijne of the Wageningen University and Dr. Daisy Vanrompay of the Ghent University, personal communications). Moreover, the Leibniz Institute, German collection of microorganisms and cell cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DMZS) does not sell the strain anymore and has similarly stated that they have had difficulties in propagating the *C. gallinacea* strain that they keep.

On the other hand, during a study conducted in China, four isolates of *C. gallinacea* (JX-1 to 4) were successfully isolated in HEp-2 cells from oral and cloacal swabs collected from chickens (Guo et al., 2016). Infection with these strains from China in chicken embryos via yolk sac resulted in 80% embryonic mortality, from 8 to 10 days post infection. Strain JX-1 was further propagated in chicken embryos via yolk sac to sequence the whole bacterial genome (Guo et al., 2017), and to perform an experimental study to asses *C. gallinacea* transmission routes (You et al., 2019). No further information about passages conducted on this strain is available. To date, only full-genome sequences of strains 08-1274/3 and JX-1 of *C. gallinacea* are available (Guo et al., 2017; Hölzer et al., 2016).

Taking into account that *in vitro* growth of *C. gallinacea* tends to be fastidious, and in order to perform further genetic characterization that help to answer the unknowns that still surround this chlamydial species, the study presented in this second chapter aimed (i) to compare the growth characteristics of the reference *C. gallinacea* strain 08-1274/3 (08DC63) under three different experimental infection protocols, and (ii) to implement a proposed alternative protocol to grow *C. gallinacea*-positive field specimens.

2. Materials and methods

2.1. Chlamydial strains and specimens

Two chlamydial reference strains were used in this study. To perform the experimental infection protocols, we used the *C. gallinacea* strain 08-1274/3, isolated from a cloacal swab collected from a chicken showing no clinical signs of disease on a poultry farm in France (Laroucau et al., 2009b). A cryotube with the cell culture containing 7.3 x 10^7 inclusion forming units per mL (IFU/mL) (500 µL) of the reference strain of *C. gallinacea*, suspended in SPG medium and preserved at -80°C, was provided by the Friedrich-Loeffler-Institut (FLI) in Germany. This vial corresponded to a 10^{th} passage on BGM cells of the original strain cultivated onto vitellus

membrane. This strain was named 08-1274/3 (08DC63). We used the *C. psittaci* Loth strain, isolated from a pigeon with systemic infection in Netherlands (F. Dekking, Hygiene Institut, Amsterdam), as a control for cell permissiveness to chlamydial infections.

The retained protocol was implemented on eight *C. gallinacea*-positive field specimens. Specimens from Mexico were collected during the study presented in **chapter I**. Specimens from Italy were sent by the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta as part of a collaborative project searching to optimize *C. gallinacea* growth in cell culture. Specimens were stored in SPG medium, transported at low temperatures and then frozen at -80°C until they were shipped in dry ice to the laboratory (ANSES). Once in the laboratory, specimens were kept at -80°C. Details of these specimens are provided in **Table 14**.

Table 14. Overview of *C. gallinacea*-positive field specimens subjected to *in vitro* culture.

Origin	Date of collection	Specimen identification	Type of specimen and host	Cq *Chlamydiaceae* rt-PCR	Cq *C. gallinacea* rt-PCR
Mexico	May, 2018	18-2470/Ameca-1	Cloacal swab, chicken	21.6	21.6
Mexico	May, 2018	18-2470/Ameca-8	Cloacal swab, chicken	20.8	21.4
Mexico	February, 2018	18-2470/Pue6-6	Cloacal swab, chicken	22.4	23.6
Mexico	February, 2018	18-2470/Pue6-7	Cloacal swab, chicken	25.1	26.0
Italy	May, 2019	20-2327/41645-5	Cloacal swab, chicken	29.6	19.3
Italy	May, 2019	20-2327/44638-9	Cloacal swab, chicken	29.6	18.2
Italy	July, 2019	20-2327/60260-3	Cloacal swab, chicken	28	27.6
Italy	August, 2019	20-2327/67320-6	Cloacal swab, chicken	21.1	20.9

2.2. Cell culture systems and media

The cell line used in this study as the reference for chlamydial growth was BGM (Buffalo Green Monkey epithelial kidney cells). Two additional cell lines were tested to compare the growth of *C. gallinacea*: DF-1 and Caco-2. Each cell culture system required a different growing media supplemented with different components mentioned in **Table 15**. All cell lines were tested to confirm the absence of *Mycoplasma* spp. (protocol described in **Annex II**).

To perform chlamydial infections, cells were seeded in 12 mm round glass coverslips contained in polystyrene bijou vessels (Sterilin, Thermo Scientific™ 129AX/1, France), and synchronously grown to confluence by the day of inoculation under conditions and growth media indicated for each cell line as specified in **Table 15**. The cell maintenance and subculturing protocol is detailed in **Annex III**.

Table 15. Specifications of growth media, cell maintenance and subculture for the three cell lines included in this study.

Cell line	Growth media and supplements	Cell incubation temperature and CO_2 conditions used	Maintenance frequency per week	Cell dissociation process for subculture	Density at the seeding
BGM	Minimum Essential Medium [MEM, Lonza] + 5% Fetal Calf Serum + 1% L-glutamine	37 °C ± 1°C 5% CO_2	Twice	Trypsinization	1-6 x 10^6/ mL
DF-1	Dulbecco's Modified Eagle Medium (Gluc/Pyr/Sodium Bicarbonate) [DMEM D654, Sigma-Aldrich] + 10% Fetal Calf Serum + 1% L-glutamine	39 °C ± 1°C 5% CO_2	Twice	Trypsinization	1-6 x 10^6/ mL
Caco-2	Dulbecco's Modified Eagle Medium (Gluc/Pyr) [DMEM D654, Thermo Scientific] + 15% Fetal Calf Serum + 1% L-glutamine + 1% Non-Essential Aminoacids 100X + 0.5% Penicillin-Streptomycin (10,000 U/mL)	37 °C ± 1°C 5% CO_2	Once + change of growth medium twice/week	Trypsinization	2.5 x 10^5/ mL

2.2.1. BGM, Buffalo Green Monkey epithelial kidney cells

BGM is a continuous epithelial cell line originated from kidney tissue of the African green monkey (*Cercopithecus aethiops*) (Barron et al., 1970). This cell line was originally initiated at the Buffalo Children's Hospital, Buffalo, New York, U.S.A. in 1962. It has been extensively used for isolation of enterovirus, paramyxovrius (mumps and measles virus), herpesvirus and *Chlamydiaceae* (Barron et al., 1970; Hobson et al., 1982; Sachse et al., 2003). This cell line has a broad spectrum of sensitivity to diverse host-dependent microorganisms. BGM cells exhibits contact inhibition without piling of cells or changing its morphology after reaching confluence **(Figure 24)** (Hobson et al., 1982; Johnston and Siegel, 1992).

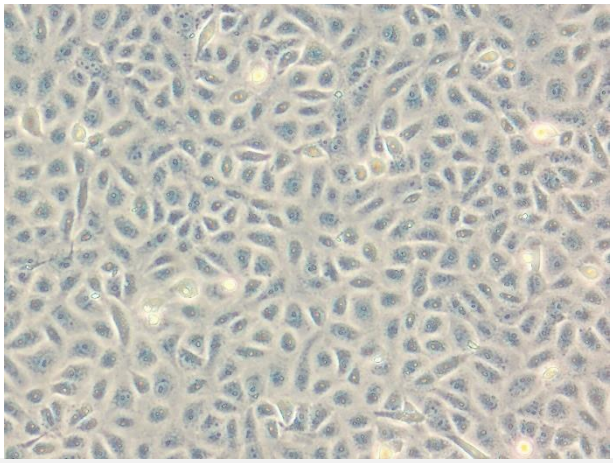


Figure 24. Normal morphology of BGM epithelial cell line in Minimum Essential Medium (MEM) (20X).

2.2.2. DF-1, chicken fibroblast spontaneously transformed, UMNSAH/ [DF1] (ATCC® CRL12203™)

DF-1 (named after the founder, Douglas Foster) is a continuous cell line derived from spontaneously transformed fibroblast of a 10-day-incubation chicken embryo (*Gallus gallus*). DF-1 cells are free of endogenous sequences related to avian sarcoma and leukosis virus group (Himly et al., 1998). This cell line exhibits a normal fibroblastic morphology (**Figure 25**). It has been described that three mitochondrial-encoded genes (ATPase 8/6, 16S rRNA and cytochrome b) are overexpressed in this cell line (Kim et al., 2001). The increased mitochondrial respiratory functions give these cells a higher division rate than other primary or immortal chicken embryo fibroblast (Kim et al., 2001). DF-1 cells do not exhibit contact inhibition, growing cells tend to stack forming ordered multilayers without changing its morphology. It has been described that DF-1 cells present an attenuated innate immune response (interferon signaling pathway) in comparison with primary chicken embryo fibroblast (Giotis et al., 2017). Since its establishment as a continuous cell line, DF-1 has been used as a model to study important avian viral and bacterial pathogens such as influenza virus (Luo et al., 2018), Newcastle disease virus (Ren et al., 2019), poxvirus (Giotis et al., 2019), flavivirus (Zhang et al., 2019), avian pathogenic *Escherichia coli* (D. Li et al., 2019) and *Mycoplasma gallisepticum* (Yu et al., 2019).

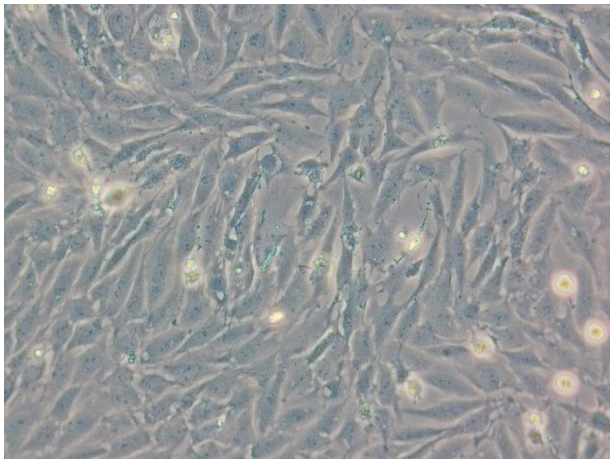


Figure 25. Normal morphology of DF-1 fibroblastic cell line in Dulbecco's Modified Eagle Medium (DMEM) (20X).

2.2.3. Caco-2, human epithelial-like colon adenocarcinoma cells [Caco2] (ATCC® HTB37™)

Jørgen Fogh established Caco-2 cell line in 1974 (named after its origin, caucasian colon adenocarcinoma, at the Memorial Sloan-Kettering Cancer Center, New York) (Rousset, 1986). It is a human intestinal epithelial-like cell line derived from a colon adenocarcinoma **(Figure 26)**. In culture, Caco-2 cells differentiate spontaneously forming polarized monolayers of mature intestinal cells (enterocytes) (Natoli et al., 2012). The differentiation process start at day 7 after seeding, and it is completed within 20 days (Natoli et al., 2012; Rousset, 1986). Caco-2 cell line is widely used across the pharmaceutical industry as an *in vitro* model of human small intestinal mucosa to predict the absorption of orally administered drugs. It has been proposed as a reliable model for studies related to intestinal cell function (Natoli et al., 2012). Caco-2 cells have low rates

of glucose consumption and lactic acid production and accumulate high quantities of glycogen (Rousset et al., 1985).

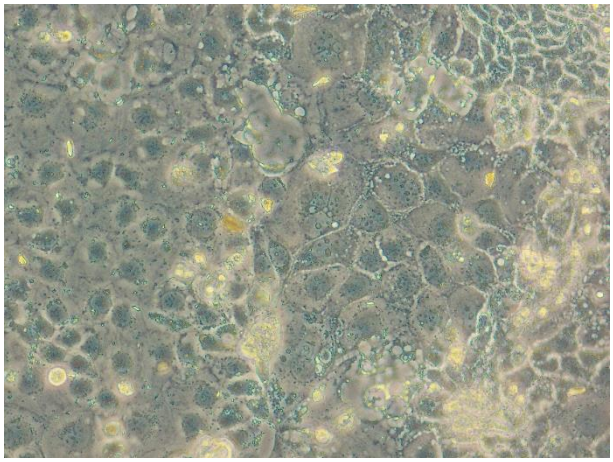


Figure 26. Normal morphology of Caco-2 epithelial-like cell line seeded at high cell density in Dulbecco's Modified Eagle Medium (DMEM) (20X).

2.3. Experimental infections

To prepare inocula, original cryotubes containing the infected cells with one of the two reference strains, or tubes containing field specimens (all preserved at -80°C), were rapidly thawed at 37°C and then sonicated (2 cycles of ten 8-seconds strikes with 0.2 seconds pause between each strike, at an amplitude of 80%).

For the reference strains, ten-fold dilutions of each strain were performed in SPG medium. For each reference strain, there were in total four inocula, i.e. undiluted strain, dilution 10^{-3}, dilution 10^{-4} and dilution 10^{-5} (**Figure 27**).

2.3.1. Preliminary step to corroborate permissibility of cell lines to chlamydial infection

The cell line DF-1 had not been previously used for the growth of *Chlamydia*. As a preliminary step, and to corroborate the permissibility of the DF-1 and Caco-2 cell lines to chlamydial infections under our working conditions, confluent monolayers of DF-1 and Caco-2 cell lines (the new cell lines to be tested) were infected with the four inocula prepared with the *C. psittaci* strain Loth (i.e. undiluted strain and dilutions 10^{-3}, 10^{-4} and 10^{-5}).

2.3.2. Experimental infection to optimize *C. gallinacea* growth protocol using the reference strain 08-1274/3 (08DC63)

Three different experimental infection protocols were implemented to compare *C. gallinacea* growth, named experimental infection protocols 1 to 3 (E1 to E3). In each experimental infection protocol, different parameters were compared: cell culture system (E1), incubation temperature (E2) and single *vs* daily centrifugation assisted infections (E3).

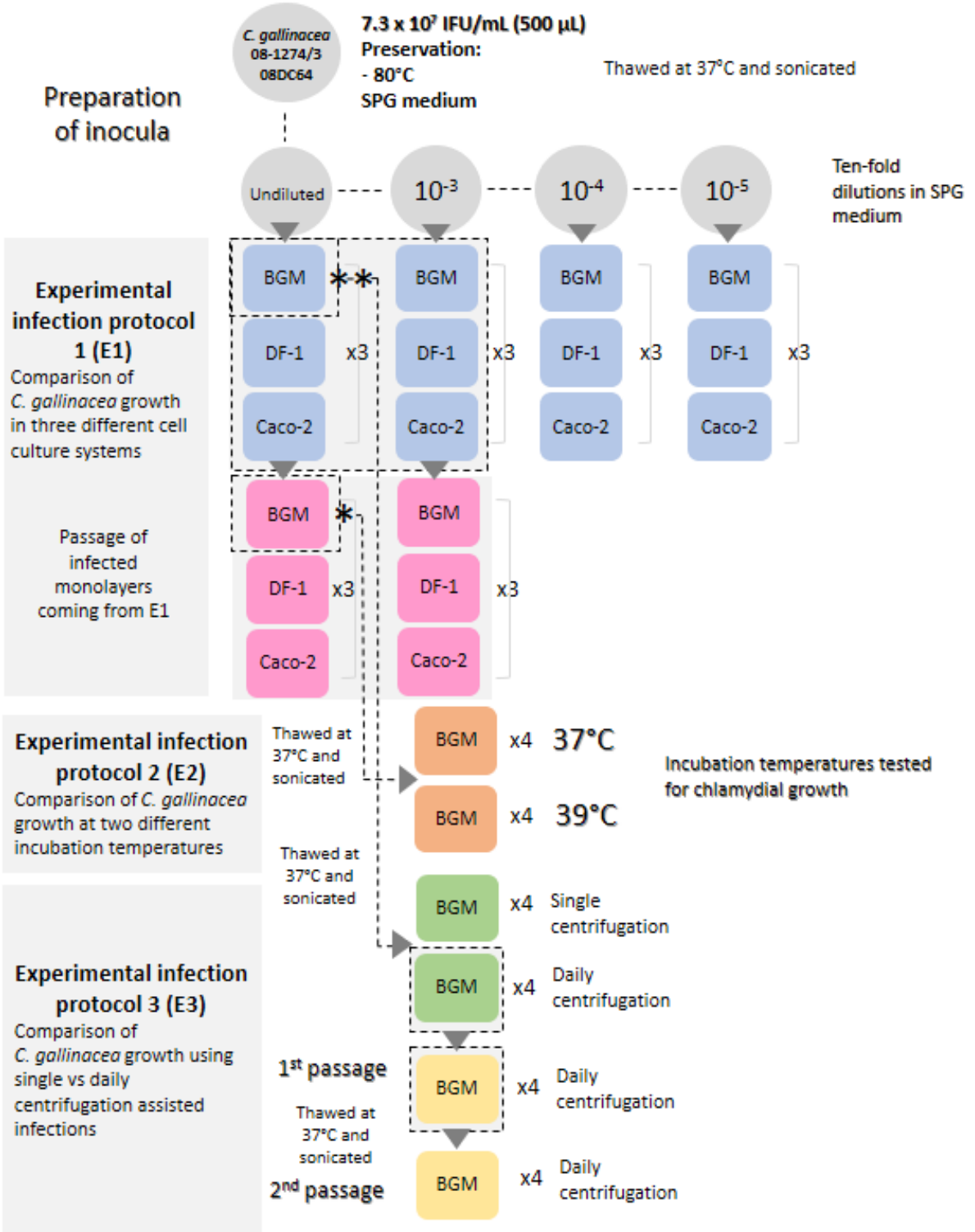


Figure 27. Overview of experimental infection protocols carried out with the different parameters compared to optimize *C. gallinacea* growth. The reference strain of *C. gallinacea* 08-1274/3 (08DC63) was used. Once inoculated, cells were subjected to the standard chlamydial growth protocol described by Sachse et al., (2003). (*), (**): inoculum used for experimental infection protocols 2 and 3, respectively (**Table 16**).

For all experimental infection protocols, cells were grown to confluence in round glass coverslips contained in bijou vessels as previously described in 2.2. Inocula used in each experimental infection protocol are summarized in **Figure 27**. After inoculation, cell monolayers were subjected to the **standard chlamydial growth protocol** described by Sachse et al., (2003) (**Annex IV**). Briefly, after inoculation cell monolayers

were centrifuged at 37°C for 1 hour at 3000 *g*. Following the centrifugation-assisted infection, infected monolayers were incubated 2 hours at 37°C in 5% CO_2. After the 2-hour incubation period, growth medium was replaced with the *Chlamydia* infection medium used in the standard protocol, i.e. UltraMDCK serum-free, chemically defined [Lonza], supplemented with 1% MEM Non-Essential Amino Acids, 1% MEM Vitamins and antibiotics/antifungals (25 µg/mL of vancomycin, 10 µg/mL of gentamycin, 2.5 µg/mL of Amphotericin B and 25 U/mL of nystatin). *Chlamydia* infection medium was renewed after 24 hours. Then, infected cells were incubated at 37°C with no CO_2 (**Figure 5**). For each experimental infection protocol, one group of infected cells followed the standard chlamydial growth protocol while in the other group a specific parameter was modified (i.e. different incubation temperature or daily centrifugations), as shown in **Table 16**.

After 48 h or 72 h pi, one coverslip with infected cells of each compared parameter were fixed with methanol, and stained using direct immunofluorescence. For some experimental infection protocol, up to two passages of the infected monolayers were made to analyze the growth of *C. gallinacea* through the number of passages.

Table 16. Experimental infection protocols implemented with the different parameters compared to optimize *C. gallinacea* growth. Different inocula from the reference strain 08-1274/3 (08DC64) of *C. gallinacea* were used in all protocols (**Figure 27**). Undiluted strain had 7.3 x 10^7 IFU/mL.

Parameter modified in each experimental infection protocol	Cell line	Incubation temperature used for infected monolayers	Centrifugation assisted infection protocol	Inocula
Different cell culture system (E1)	BGM *vs* DF-1 *vs* Caco-2	37°	Single	Undiluted strain + dilutions (10^{-3}, 10^{-4}, 10^{-5})
Different incubation temperatures (E2)	BGM	37°C *vs* 39°C	Single	Tube coming from the 1st passage of the undiluted strain obtained in E1 (*)
Different centrifugation frequency (E3)	BGM	37°	Single *vs* Daily	Tube coming from the 1st inoculation of the undiluted strain obtained in E1 (**)

2.3.3. Infections with *C. gallinacea* PCR-positive specimens

BGM cells were grown to confluence in round glass coverslips contained in bijou vessels as previously described in **2.2** (detailed in **Annex III**). Different volumes of the thawed and sonicated specimens coming from Mexico and Italy (described in **Table 14**) were inoculated in at least four bijou vessels per specimen (depending on the total volume of the specimen) according to the following schema: at least one bijou tube with 30 µL of the specimen, two bijou tubes with 100 µL of the specimen and at least one bijou tube with 300 µL of the specimen. If the volume of the sample was enough, the tubes inoculated with 30 µL and 300 µL were duplicated.

After inoculation, cell monolayers were subjected to one of the two **chlamydial growth protocols**. The **standard chlamydial growth protocol** described by Sachse et al., (2003) was followed for the specimens from Mexico, while a proposed **alternative chlamydial growth protocol** modified from Donati et al., (2010) (**Annex IV**) was implemented for the specimens from Italy. Briefly and for both protocols, after inoculation, cell monolayers were centrifuged at 37°C for one hour at 3000 *g*. Following the centrifugation-assisted infection, infected monolayers were incubated two hours at 37°C in 5% CO_2. After the two-hour incubation period, growth medium was replaced with the *Chlamydia* infection medium which varied depending the chlamydial growth protocol followed. For the **standard protocol**, *Chlamydia* infection medium and subsequent steps information is provided in **2.3.2**. While for the **alternative protocol**, *Chlamydia* infection medium consisted in Minimum Essential Medium [MEM, Lonza], supplemented with 20% Fetal Calf Serum (FCS), 1% L-glutamine, 3.33 mg/mL D-glucose (2 g/600 mL), 1 mg/mL cycloheximide and antibiotics/antifungals (25 µg/mL of vancomycin, 10 µg/mL of gentamycin, 2.5 µg/mL of Amphotericin B and 25 U/mL of nystatin). After media replacement, infected cells were incubated at 37°C with no CO_2 for the standard protocol or at 37°C with 5% CO_2 for the alternative protocol. *Chlamydia* infection medium was renewed 24 h pi in the standard protocol. For the alternative protocol, 24 h pi a centrifugation was performed under the aforementioned conditions, and 2 h after centrifugation, medium was then renewed. This additional centrifugation step was performed also at 48 and 72 h pi but the medium was not renewed. The schematic overview of both chlamydial growth protocols used is shown in **Figure 28** and detailed in (**Annex IV**).

Between 48 and 96 h pi, a coverslip with cells inoculated with 100 µL of each specimen was fixed with methanol and stained with direct immunofluorescence. For some isolates, up to four passages of the infected monolayers were made to analyze the growth of *C. gallinacea* through the number of passages.

2.4. Direct immunofluorescence detection of *Chlamydiae*

After incubation periods between 48 to 192 h, coverslips with infected cells were fixed with methanol, and stained using the direct immunofluorescence IMAGEN™ *Chlamydia* kit (Oxoid, UK), containing a FITC-labeled anti-*Chlamydia* LPS antibody. A specimen was considered positive when bright green fluorescent spots within the cellular cytoplasm, with a morphology evocative of chlamydial inclusions were observed. If a decrease in fluorescent reaction after two passages was observed, the infection was considered unsuccessful. The stained cells were visualized using an immunofluorescence or a confocal microscope. Evans blue was used as counterstain for cells (in red color).

2.5. Chlamydial identification and quantification through real-time PCR

Inocula or supernatants of the chlamydial-infected cell cultures to be tested with real-time PCR (rt-PCR) were subjected to DNA extraction using the QIAamp DNA minikit™ (QIAGEN, Ventura, CA) following the manufacturer protocol. A broad-range *Chlamydiaceae*-specific real time-PCR targeting the 23S rRNA gene was used, as previously described by Ehricht et al., (2006). Species identification was done with *C. gallinacea*

and *C. psittaci* specific rt-PCR systems targeting the *eno*A and *omp*A genes, respectively (Laroucau et al., 2015; Pantchev et al., 2009).

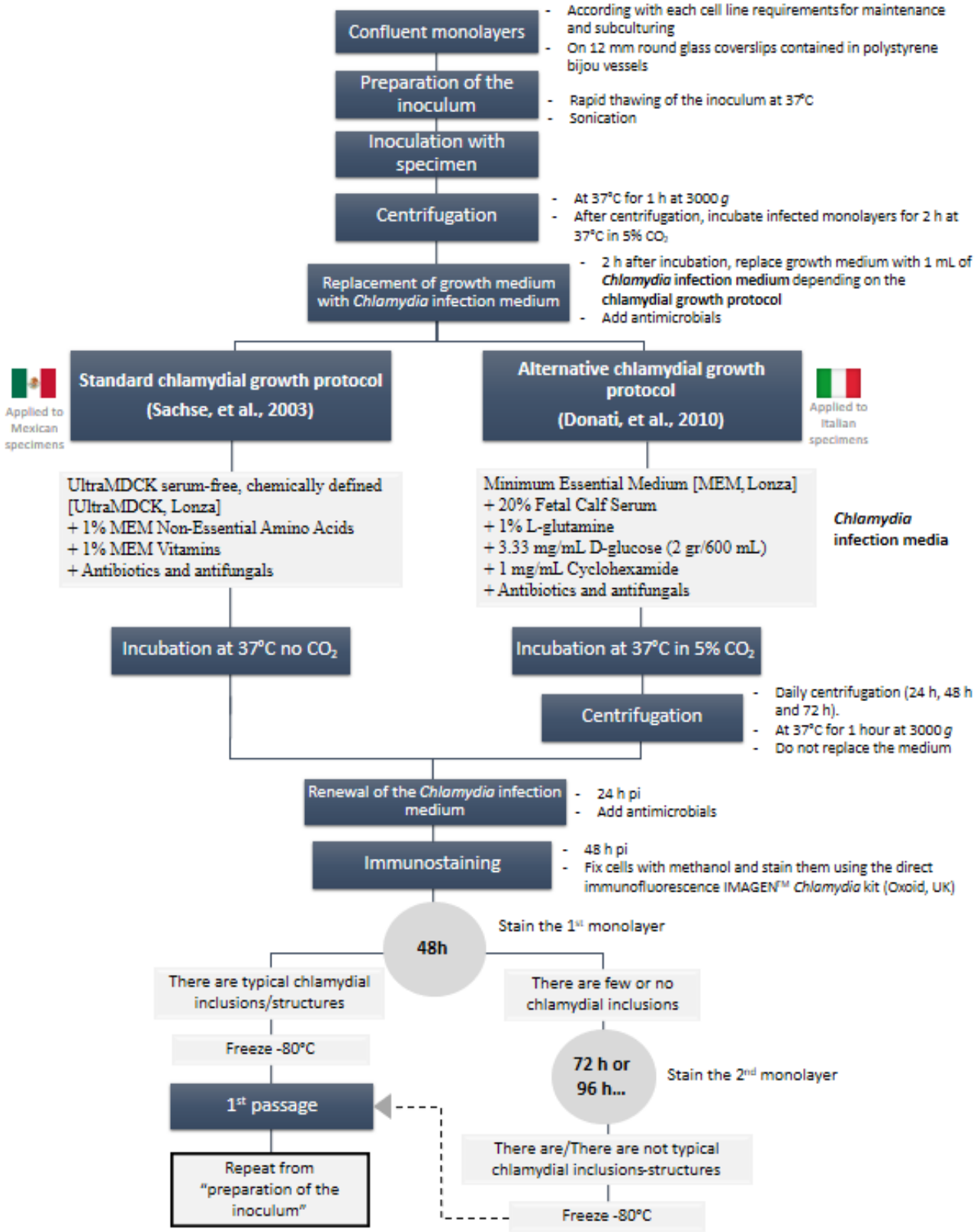


Figure 28. Schematic overview of the two implemented chlamydial growth protocols. The standard chlamydial growth protocol was described by Sachse et al., (2003). The proposed alternative chlamydial growth protocol was modified from Donati et al., (2010).

3. Results and discussion

3.1. C. *gallinacea* growth protocol optimization

Overall, typical chlamydial inclusions were rarely visible. Therefore, *C. gallinacea* growth could not be quantitatively measured or compared using classical approaches, i.e. number and size of inclusions as commonly presented in the literature.

3.1.1. Comparison of *C. gallinacea* growth in three different cell culture systems (E1)

Immunostainings performed at 48 h pi of infected DF-1 and Caco-2 cell monolayers with different concentrations of the *C. psittaci* reference strain (i.e. undiluted and dilutions 10^{-3}, 10^{-4} and 10^{-5}), revealed typical chlamydial inclusions in all of them (microphotographs not shown). Hence, both cell lines showed to be permissive for *C. psittaci* infection under the conditions used for this study. This is the first study reporting the permissiveness of the DF-1 cell line for the growth of *Chlamydiaceae*.

Immunostainings performed at 48 h pi of the three cell lines (DF-1, Caco-2 and BGM) infected with the undiluted reference strain of *C. gallinacea* and the three dilutions (10^{-3}, 10^{-4} and 10^{-5}), showed that all cell lines allowed the growth of *C. gallinacea*. Countless large, well-delimited, bright green typical chlamydial inclusions, occupying almost the entire cell cytoplasm were observed in cells infected with the undiluted *C. gallinacea* reference strain (7.3 x 10^7 IFU/mL) at 48 h pi (**Figure 29A**). However, only few inclusion-like chlamydial structures were observed in the three cell lines (DF-1, Caco-2 and BGM) infected with the 10^{-3} dilution of the reference strain at 48 h pi (**photos not taken**). No chlamydial inclusions were observed in cells infected with dilutions 10^{-4} and 10^{-5} of the *C. gallinacea* reference strain. Therefore, only one passage was performed from the infected cells with the undiluted reference strain and the dilution 10^{-3}. No difference in *C. gallinacea* growth was observed between the three cell lines when this passage was performed (**Figure 29B**).

Regarding the size of the chlamydial inclusions in the three cell lines, slight differences were observed only for the first inoculation of the undiluted strain (**Figure 29A**). *C. gallinacea* inclusions in Caco-2 seem to be larger than those observed in the other two cell lines (real sizes were not measured), as observed in previous studies conducted using this cell line for *C. suis* infection (De Puysseleyr et al., 2017; Schiller et al., 2004), and *C. trachomatis* infection (Lantos et al., 2018). *C. gallinacea* infection in our study was perceived to occur in several overlapping layers. This is because Caco-2 and also DF-1 cells do not present contact inhibition during their growth and they continue to multiply once the growth medium is replaced by the *Chlamydia* infection medium, which is intended to slow down the cellular metabolism (UltraMDCK medium, for this experimental infection). We also observed that overlapping layers were exfoliated during the immunostaining, which was revealed by holes in the cell monolayers corresponding to detached cells, as shown in **Figure 29A**. Although multiple layers can lead to multiple chlamydial infections, washing steps performed during

immunostaining induce cell layer exfoliation. Therefore, altogether, using these cell lines did not provide the expected improvement in *C. gallinacea* growth.

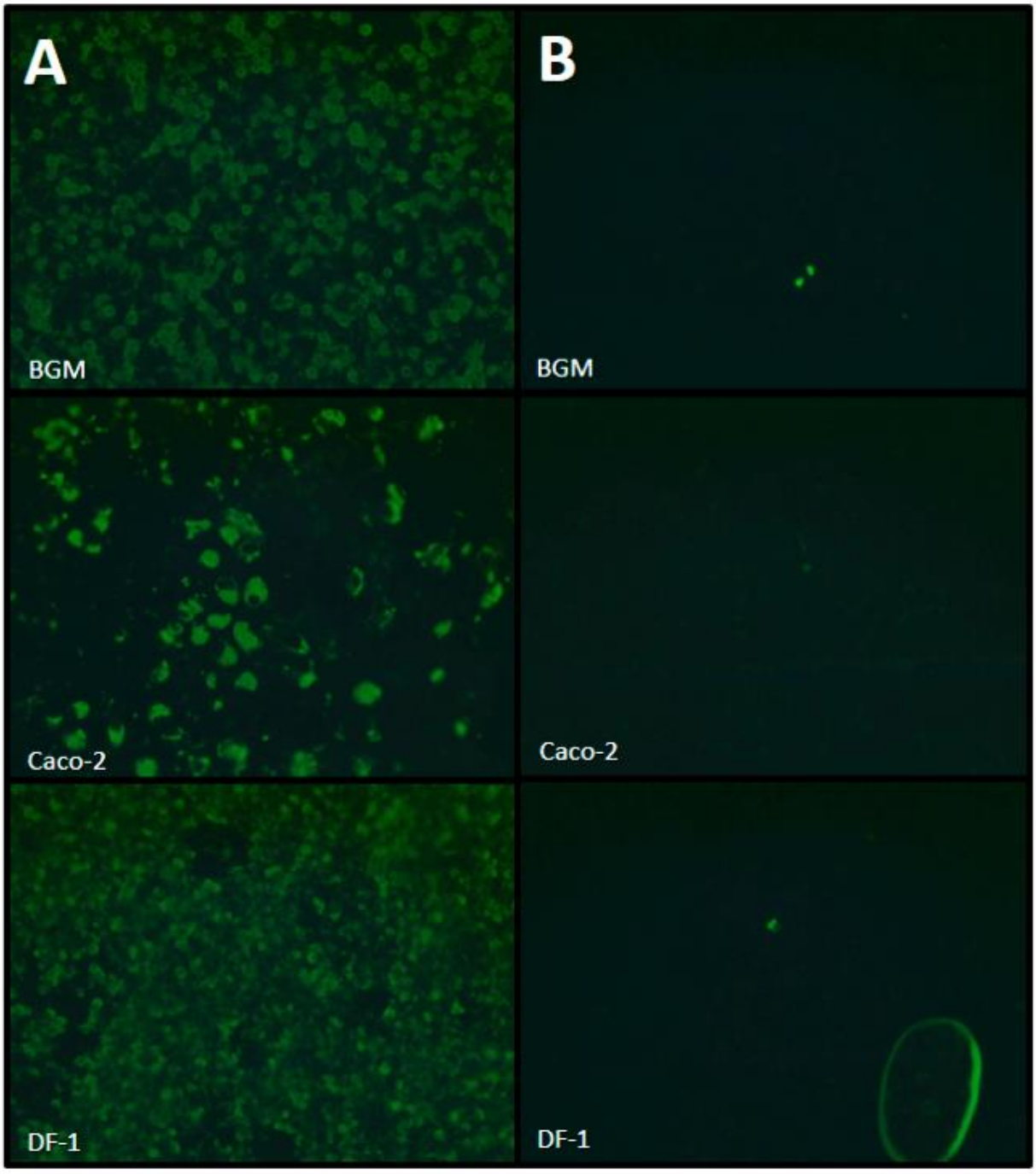


Figure 29. A) Microphotographs of the infected BGM, Caco-2 and DF-1 cells with the undiluted reference strain 08-1274/3 (08DC63) of *C. gallinacea*. Immunostainings were performed at 48 h pi (20X). **B)** Microphotographs of the BGM, Caco-2 and DF-1 cells infected with the passage of the dilution 10^{-3} of the *C. gallinacea* reference strain 08-1274/3 (08DC63). Immunostainings were performed at 48 h pi (20X).

According with the study conducted by Guo et al., (2016), *C. gallinacea* can cause persistent infection in the chicken gut. Therefore, we selected Caco-2 cells because it had been reported that they exhibit a significantly increased inclusion number and sizes when they were infected with *C. suis* and *C. pecorum* strains; both

obtained from subclinical infections (intestinal content) of swine and sheep, respectively (Schiller et al., 2004). Moreover, Caco-2 cells have been proposed as a reliable model for studies related to intestinal cell functions (Natoli et al., 2012). Additionally, we sought to include other permanent cell lines derived from chickens to provide *C. gallinacea* with the most similar conditions to those it might found in its natural host (birds of the order *Galliformes*), e.g. temperature and cell receptors. This is why we included DF-1 cells (fibroblasts from chicken embryo) in our study. We planned to include also the CLEC213 cells (epithelial cell line derived from lung explants of hens (Soubieux et al., 2011)), however we were unable to establish their culture in our laboratory.

It had been observed in previous studies that, depending on the species, chlamydial growth could be better in certain type of cells (Croy et al., 1975; De Puysseleyr et al., 2017; Schiller et al., 2004; Wong et al., 1992). For this reason, chlamydial growth protocol should be tailored not only for chlamydial species, but also for specimens from different host origin, in order to improve the odds to succeed in isolating and growing *Chlamydia*. For example, in the study performed by Schiller et al., (2004), the growth of several chlamydial species (i.e. *C. suis*, *C. pecorum*, *C. abortus* and *C. psittaci*) was compared in different cell lines (i.e. Vero, Calu, HEC and Caco-2) obtaining significant differences. They concluded that no single cell line outperformed the others in terms of *Chlamydia* growth for all chlamydial species. In our study, few differences were observed between the three cell lines when they were infected with the reference strain of *C. gallinacea*. However, BGM cells have additional advantages beyond its robustness and sensitivity to chlamydial infections. BGM cells have been selected as a model to chlamydial growth across several studies over the years. One reason being that the count of chlamydial-inclusions in BGM cells was higher in comparison to other cell lines such as McCoy (Hobson et al., 1982) or HeLa 229 (Wong et al., 1992). Other reasons are their easy-step subculturing procedure and their altered appearance when they are infected by *Chlamydia*, producing large easy-to-see inclusions (cells increase their size by 3 or 4 times). Likewise, we observed that BGM cells infected with the undiluted *C. gallinacea* reference strain increased their size, producing large-easy to see inclusions.

Inclusions in BGM cells are visible even after several days of infection, whereas in other cell lines such as McCoy, this is not the case. It has been hypothesized that this difference is due to detachment of the McCoy cell monolayer from the surface to which they are attached over time, even when they are not infected, and to a greater extent after being exposed to an infection (Johnston and Siegel, 1992; Wills et al., 1984). Cellular detachment was observed for DF-1 and Caco-2 cells tested in our study. This detachment could be due to the accelerated growing rate of these two cell lines (compared to BGM cells) as noticed in other studies (Kim et al., 2001; Natoli et al., 2012), coupled with the fact that they do not present contact inhibition and keep growing, forming multiple layers (Johnston and Siegel, 1992). Accelerated cell growth could constitute an advantage for some chlamydial specimens/ species as it has been proposed that the faster the cellular multiplication occurs, the faster the chlamydial inclusions can be seen (Wills et al., 1984). Indeed, Wills et al., (1984) reported that when inoculating human conjunctival samples, chlamydial inclusions were observed earlier on McCoy cells (18h pi) than on BGM cells. Likewise, this accelerated cellular growth could be helpful

for the propagation of low concentrated chlamydial field specimens. However, as changes in cells due to cellular aging occur more rapidly (Chen et al., 2013), fast-growing cell lines could be inappropriate for slow-growing *Chlamydia*.

Under our experimental conditions, BGM cells appear to be more resistant than DF-1 and Caco-2, as they remain viable longer without changing the culture medium once they have been infected. Our results confirm that the BGM cell line is more suitable as standard cell culture system, since once cells are infected it is not necessary to perform additional manipulations that could lead to cross-contamination when simultaneous infections are carried out. Therefore, we decided to keep the BGM cell line for the next infection protocols.

3.1.2. Comparison of *C. gallinacea* growth in two different incubation temperatures (E2)

As it has been suggested that some chlamydial species might grow better in temperatures similar to those that could be found in their natural hosts, we decided to study the impact of the incubation temperature on the *C. gallinacea* growth. Indeed, the formation of mature inclusions of *C. poikilothermis*, a chlamydial species obtained from snakes (a poikilothermic animal), was enhanced at 28°C rather than at 37°C, the standard temperature for chlamydial growth (Onorini et al., 2019). As the natural temperature of birds ranges between 38.5° to 43°C (Prinzinger et al., 1991), we decided to perform a test with an incubation at 39°C in order not to exceed the temperature limit set for BGM cells (Barron et al., 1970).

The first infection performed in BGM cells with the undiluted *C. gallinacea* reference strain (derived from E1) was used as inoculum for this experimental infection (**Figure 27**). *C. gallinacea* infected BGM cells were incubated for up to 4 days (96 h) at 37°C and 39°C. Immunostainings were performed at 48, 72 and 96 h pi. Few inclusion-like, irregularly sized, bright green chlamydial structures were observed in the cytoplasm of infected cells at both temperatures. As infections for this experiment were performed using a first passage as inoculum (derived from E1) fewer than expected chlamydial inclusions were observed. In fact, this experiment corresponded to the second passage of the original tube provided by the Friedrich-Loeffler-Institut. No difference in *C. gallinacea* growth was observed between the two different temperatures (37° *vs* 39°C) over the evaluated time of infection (48 h *vs* 72 h *vs* 96 h) (**Figure 30**). However, as we did not quantitatively measure chlamydial growth, we cannot conclude with certainty on the impact of temperature on of *C. gallinacea* growth. The variation in incubation temperature has been the subject of several studies. Onorini et al., (2019) found that the viability of eight strains of eight different chlamydial species (*C. trachomatis*, *C. pneumoniae*, *C psittaci*, *C. abortus*, *C. suis*, *C. pecorum*, *C. serpentis* and *C. poikilothermis*) was maintained at 28°C, but their infectivity was significantly higher when incubated at 37°C, compared with any other temperature tested. Up to now, incubation temperatures between 35°-37°C have shown to be the optimum for most of *Chlamydiaceae* strains from endothermic animals (i.e. birds and mammals) (Donati et al., 2010; Kuo and Grayston, 1988; Onorini et al., 2019; Wong et al., 1992).

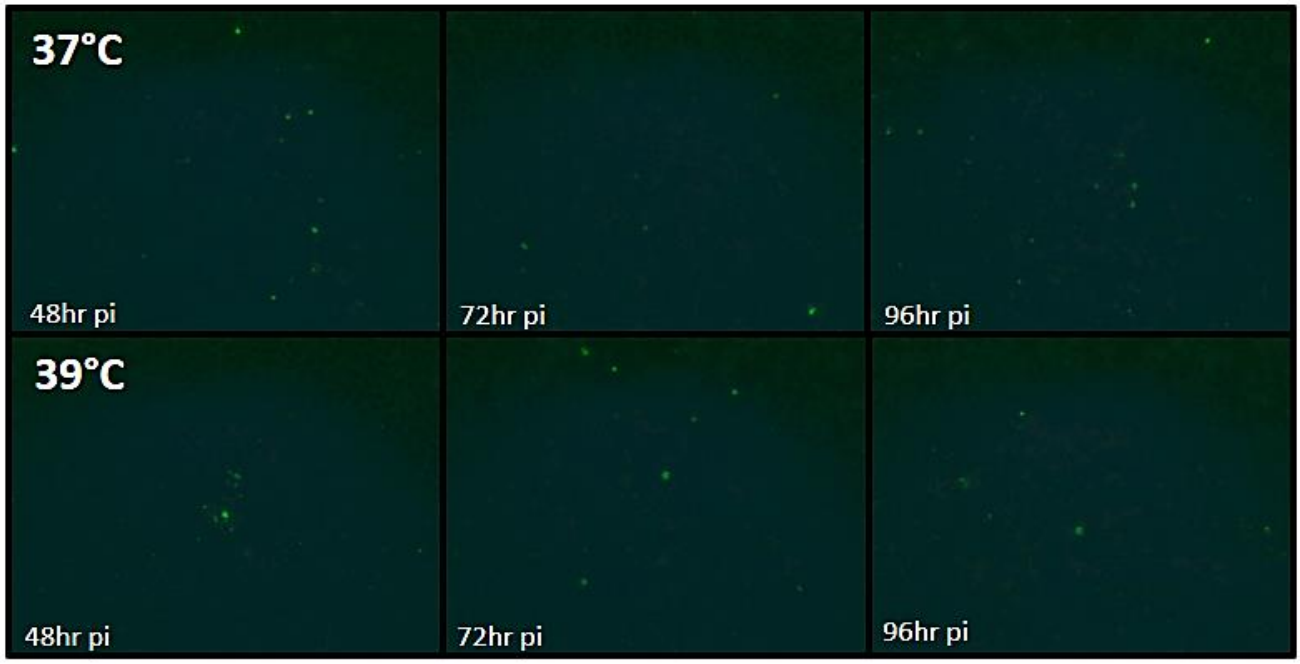


Figure 30. Microphotographs of infected BGM cells incubated at 37° and 39°C. Cells were inoculated with the 1st passage of the undiluted reference strain 08-1274/3 (08DC63) of *C. gallinacea*. Immunostainings were performed at 48, 72 and 96 h pi (20X).

The mechanism by which higher incubation temperatures (33-37° *vs* 28°C) may promote the growth of *Chlamydia* is not completely understood (Onorini et al., 2019). It can be hypothesized that higher temperatures may emulate the natural temperature of the host from which the specimen originates, and thereby create an optimal environment that enhances the infectivity of the bacterium (Kuo and Grayston, 1988). It should be noted that each cell line has its own optimal temperature limits, so if they are exposed to incubation temperatures that exceed these limits for a prolonged period, undesirable changes due to heat stress, e.g. decreased cellular viability and cell death, might hinder chlamydial growth (Kapila et al., 2016). We did not notice such changes on BGM cells incubated at 39°C.

From our results, it is evident that *C. gallinacea* infectivity decreases with time and number of passages, which was the initial reason why we decided to set up this study. Therefore, since we did not perceive dramatic differences in the growth of the reference strain of *C. gallinacea* and, in order not to alter other variables by raising the temperature to 39°C, we decided to maintain 37°C as the incubation temperature to perform isolations from field samples.

3.1.3. Comparison of *C. gallinacea* growth using single *vs* daily centrifugation assisted infections (E3)

The mechanism by which the use of centrifugation assisted infections could enhance *in vitro* chlamydial growth is not well elucidated. It has been hypothesized that centrifugation could produce cell surface structural changes that favors certain cell entry pathways, but also that it promotes the attachment of chlamydial elementary bodies to host cells, thus enhancing productive infections (Allan and Pearce, 1979; Prain and

Pearce, 1985; Weiss and Dressler, 1960), both theories are not mutually exclusive and can occur simultaneously. Daily centrifugation has already been implemented to improve the *in vitro* growth of *C. pneumoniae* (Dr. Manuela Donati, Bologna University, personal communication). Therefore, we decided to test the effect of including a daily centrifugation step to our protocol in order to optimize the growth of *C. gallinacea*.

The first infection performed in BGM cells with the undiluted *C. gallinacea* reference strain (derived from E1) was used as inoculum for this experimental infection (**Figure 27**). Once infected, BGM cells were incubated for up to 7 days (168 h) at 37°C using single *vs* daily centrifugation assisted infections. Immunostainings were performed at 48, 72, 96 and 168 h pi. Countless inclusion-like, irregularly sized, bright green chlamydial structures could be observed in the cytoplasm of infected cells. No difference in *C. gallinacea* growth was observed between the two centrifugation assisted infection protocols during the course of the infection (up to 168 h) (**Figure 31**). At the time immunostainings were performed, supernatants were collected and analyzed by rt-PCR to obtain a relative quantification of the bacteria present in the extracellular medium. Cq of supernatants are shown in **Figure 31**. Differences less than 3.5 Cq values were observed between the two tested conditions, at different sampling times, corresponding to less than 1 log of difference.

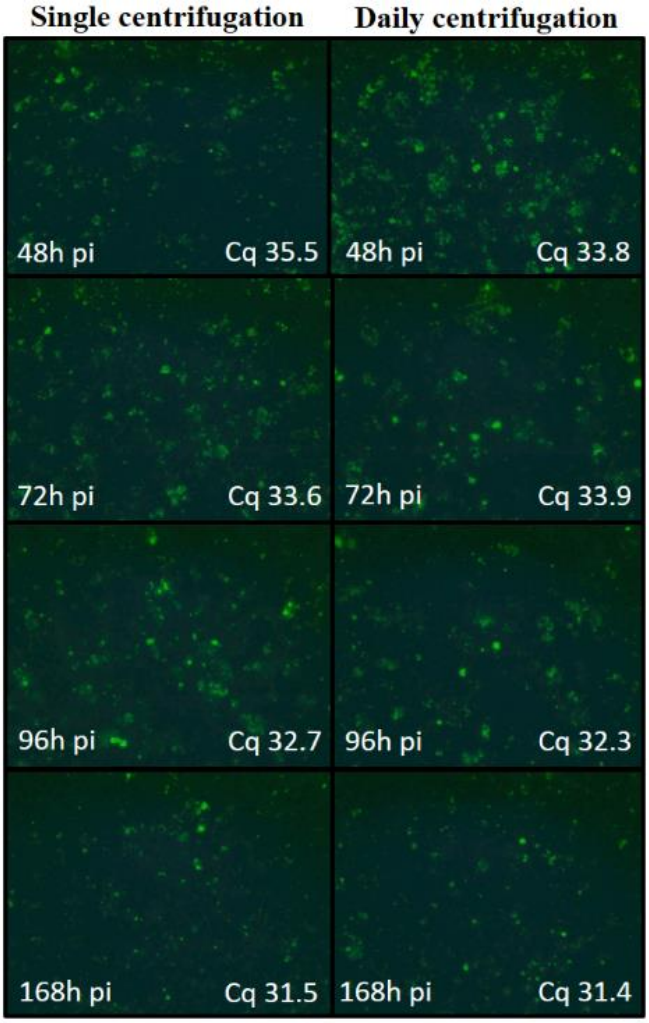


Figure 31. Microphotographs of the infected BGM cells using single *vs* daily centrifugation assisted infections. Cells were inoculated with the first infection performed with the undiluted *C. gallinacea* reference strain 18-1274/3 (08DC63).

Immunostainings were performed at 48, 72 and 96 h pi (20X). Countless inclusion-like, irregularly sized, bright green chlamydial structures could be observed in the cytoplasm of infected cells. Cq of supernatants are shown.

Centrifugation-assisted infections have been included over the years as an easy-to-implement technique in the laboratory to enhance chlamydial infections in cell culture, instead of laying in spontaneous infection that might occurs when static incubation is performed. Some studies have proposed that centrifugation could facilitate bacterial entry to the host cell by increasing adsorption of *Chlamydia* to cells (Johnson and Hobson, 1976; Prain and Pearce, 1985). For this reasons and considering that no negative impact of a daily centrifugation was observed, we decided to keep this additional step, without renewal of the infection medium.

3.2. Application of the standard and an alternative chlamydial growth protocol to *C. gallinacea*–positive field samples

3.2.1. Application of the standard protocol to field specimens from Mexico

C. gallinacea-positive specimens from cloacal swabs of chickens from Mexico (described in **Table 14**) were subjected to chlamydial isolation in cell culture using the standard chlamydial growth protocol. First immunostainings performed between 48 and 72 h pi revealed countless inclusion-like, irregularly sized, bright green chlamydial structures in the cytoplasm of the cells infected with all Mexican specimens. When performing the 1st passage of all isolates, immunostaining at 48 h showed no improvement of the chlamydial inclusion-like structures for any of them (**Figure 32/A** *vs* **Figure 33/A**, respectively). Instead, inclusion-like chlamydial structures tended to decrease in size and number as can be observed in **Figures 32/A**, **33/A** and **34/A**. Up to four passages were performed of some isolates, however, no positive evolution of chlamydial growth could be perceived for any of them (microphotographs not shown).

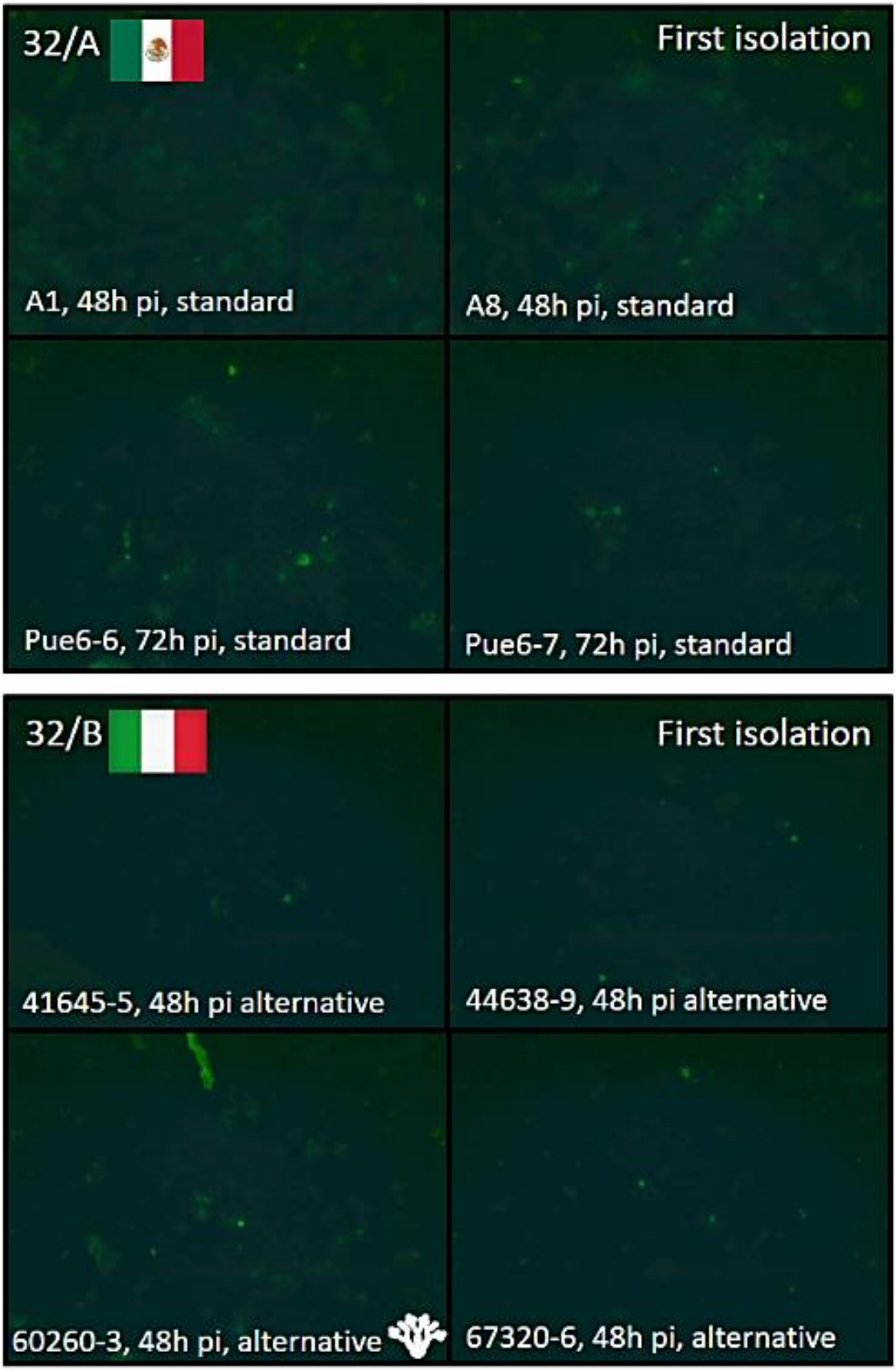
Results from specimens from Mexico were consistent with previous unpublished results obtained in the French reference laboratory for animal chlamydiosis (ANSES, Maisons-Alfort) and those reported by Dr. Marloes Heijne from the Department of Bacteriology and Epidemiology of the Wageningen University, and Dr. Daisy Vanrompay of the Ghent University (personal communications), when working with *C. gallinacea* specimens. These unpublished results together with results obtained from Mexican specimens, stressed the necessity to optimize the *C. gallinacea* growth protocol which was the objective of 3.1 and was later implemented on Italian specimens (**Figure 28**).

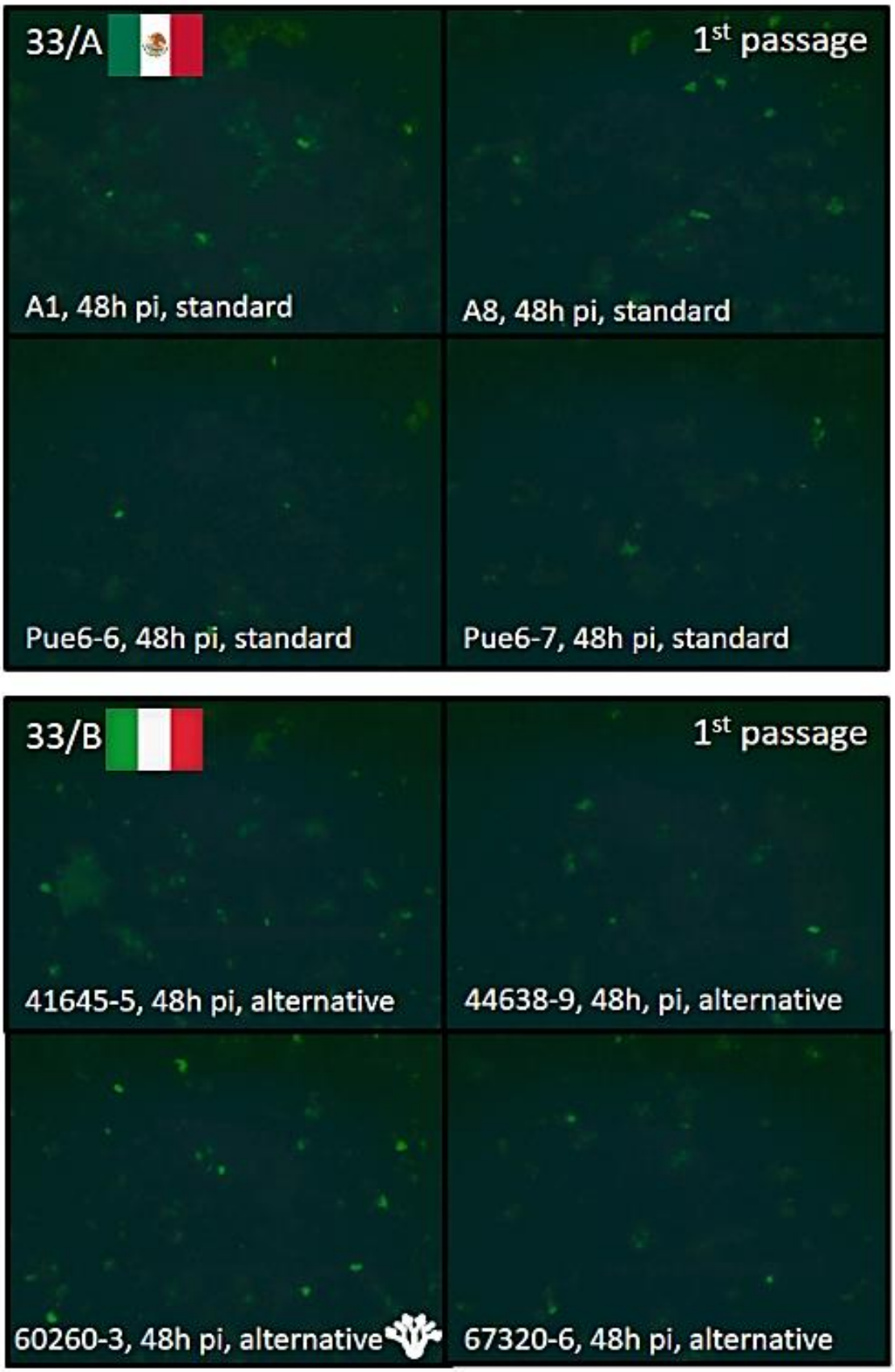
3.2.2. Implementation of an alternative chlamydial growth protocol to field specimens from Italy

As a result of the optimization trials conducted in order to improve the growth of *C. gallinacea* previously presented (3.1), we decided to keep (i) BGM cells as the reference cell line, (ii) an incubation temperature of 37°C once the cells had been infected, and (iii) to perform daily centrifugations to promote the infectivity rate of the inocula. Additionally, we decided to test a new *Chlamydia* infection medium that had been referenced

in the literature (Donati et al., 2010), i.e. EMEM supplemented with glucose, L-glutamine, cycloheximide, higher concentration of FCS compared with the cell growth medium used for BGM cells (5% *vs* 20%), and an incubation with 5% CO_2 after infection This set of parameters were used in an alternative protocol for chlamydial growth. This proposed alternative protocol was applied to four *C. gallinacea*-positive specimens from cloacal swabs of chickens from Italy (presented in **Table 14**).

As observed for Mexican specimens using the standard protocol, first immunostainings performed at 48 h pi revealed countless inclusion-like, irregularly sized, bright green chlamydial structures in the cytoplasm of cells infected with all Italian specimens (**Figure 32/B**). Two passages were carried out for all the isolates from Italy. Immunostainings of 1st passages were performed at 48 h pi revealing almost imperceptible differences in the growth of *C. gallinacea* for three of the four isolates (41645-5, 44638-9 and 67320-6) (**Figure 33/B**). Remarkably, from the 1st passage, one of the four isolates from Italy (60260-3) showed a distinctive evolution of chlamydial growth; few medium-sized well-delimited, bright green chlamydial inclusions were observed at 48 h pi (**Figure 33/B/60260-3**). For the 2nd passage of this isolate, several large well-delimited, bright green typical chlamydial inclusions, occupying almost the entire cell cytoplasm were observed at 48 h pi (**Figure 34/B/60260-3**). While for the other three isolates from Italy (41645-5, 44638-9 and 67320-6), the growth of *C. gallinacea* was comparable to that observed for the isolates from Mexico. Since the growth of the isolate 60260-3 of *C. gallinacea* was exceptional, we confirmed through species-specific rt-PCR that (i) it was indeed an isolate of *C. gallinacea* and (ii) that there was no *C. psittaci* present in the specimen.


32/A
First isolation
A1, 48h pi, standard
A8, 48h pi, standard
Pue6-6, 72h pi, standard
Pue6-7, 72h pi, standard
32/B
First isolation
41645-5, 48h pi alternative
44638-9, 48h pi alternative
60260-3, 48h pi, alternative
67320-6, 48h pi, alternative


33/A
1st passage
A1, 48h pi, standard
A8, 48h pi, standard
Pue6-6, 48h pi, standard
Pue6-7, 48h pi, standard
33/B
1st passage
41645-5, 48h pi, alternative
44638-9, 48h, pi, alternative
60260-3, 48h pi, alternative
67320-6, 48h pi, alternative

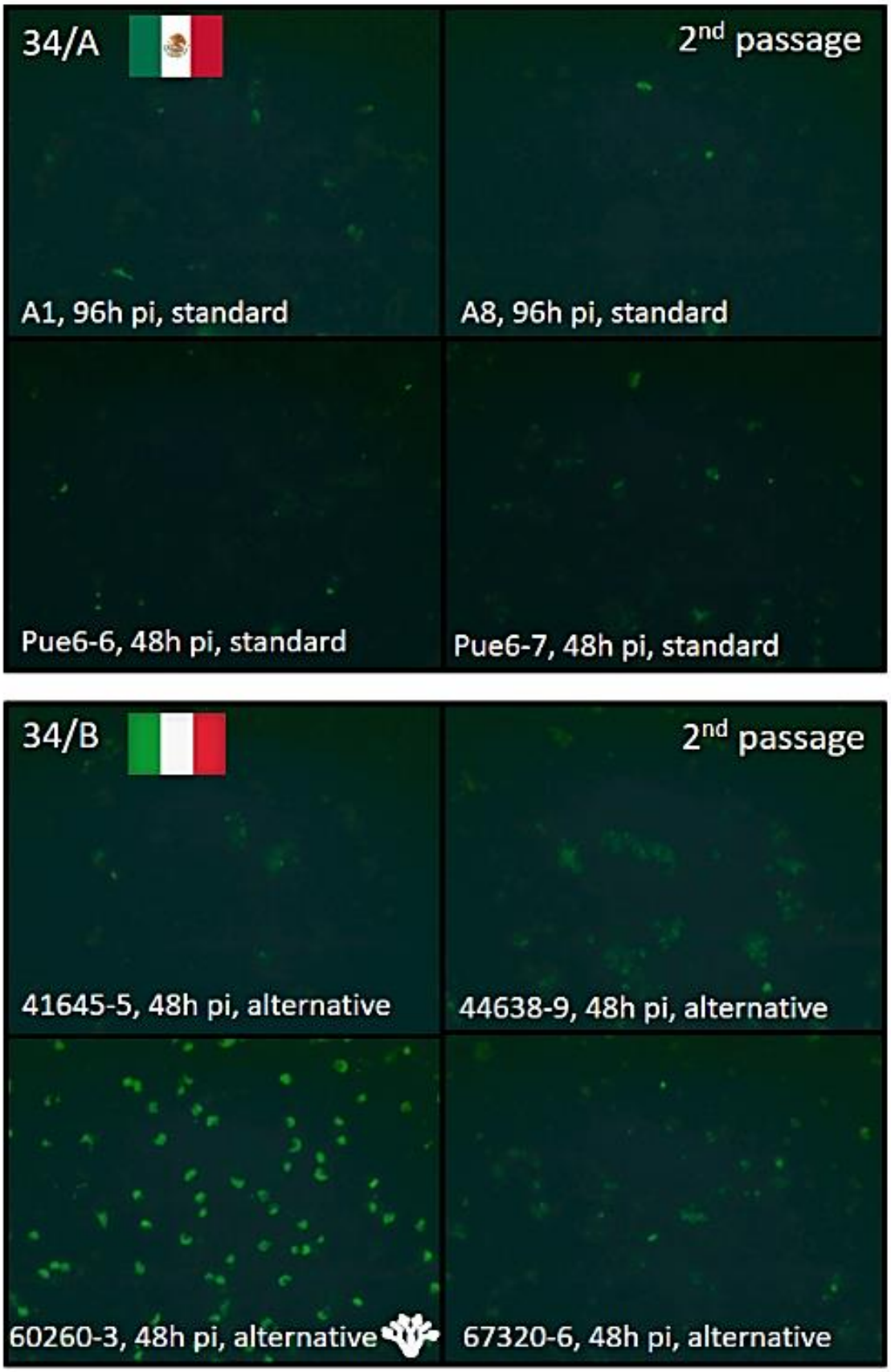


Figure 32-34. Microphotographs of the infected BGM cells inoculated with the C. gallinacea field isolates from Mexico (**Figures 32-34, A**) and Italy (**Figures 32-34**, **B**). Microphotographs correspond to the first isolation (**Figure 32**, **A/B**), the 1st (**Figure 33, A/B**) and the 2nd (**Figure 34, A/B**) passages. Immunostainings were performed between 48 to 96 h pi (20X) (best pictures were kept and shown in these figures). Isolation of *Chlamydia* was performed using the standard or

the alternative growth protocol (as indicated in each photo). The fungus-like structure indicates that there was a fungal contamination from the origin.

It should be mentioned that the isolate 60260-3 from Italy presented an initial contamination detected within the first 24 h pi. The contamination was mainly fungal (fungus-like structures, white in color similar to mycelia) but also with other bacteria. After making the passages and renewing every 24 h the *Chlamydia* infection medium (with antibiotics and antifungals), the bacterial contamination disappeared but the fungus remained until the second passage, as shown in **Figure 35**. Four-fold concentration of the antifungals added to the *Chlamydia* infection medium (10 µg/mL of Amphotericin B and 100 U/mL of nystatin) were insufficient to control its proliferation.

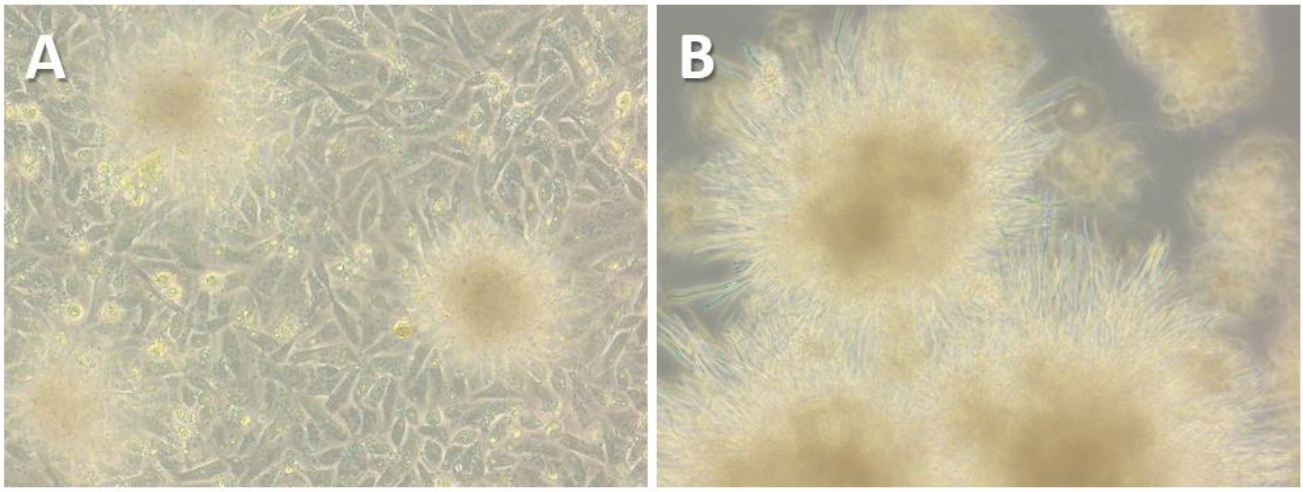


Figure 35. Microphotographs showing the fungus-like structures contaminating the confluent BGM cells seeded in a T25 flask, infected with the specimen 60260-3 of *C. gallinacea* (**A**). Photo taken 24 h pi (20X). (**B**) The fungus isolated in *Chlamydia* cultivation medium (for the alternative protocol), 18 h pi at 37°C with 5% CO_2. Medium consisted in EMEM + 1% L-glutamine + D-glucose (3.33 mg/mL) + cycloheximide (1 mg/mL) + 20% of FCS (20X).

3.2.3. New insights

Results obtained from the growth of *C. gallinacea*-positive specimens, especially for the exceptional growth of an Italian isolate (60260-3), made us reflect about the implementation of the alternative chlamydial growth protocol. Questions that should be explored are listed hereafter.

Did the time of sample storage affect the bacteria growth?

At the time of launching infections in cell culture of the field specimens, between seven months and a year had elapsed since their collection. It has been pointed out that the longer the storage time, the less success in *Chlamydia* growth (Onorini et al., 2019). Specimens from each country had about the same storage time between them, including the *C. gallinacea* field specimen from Italy (60260-3) for which a distinctive growth was obtained. Therefore, we consider unlikely that the storage time could have played a determining role in the chlamydial growth of the rest of the Italian specimens. Coincidentally, this isolated Italian had strong fungal

contamination from the outset. We believe that supplements added to the *Chlamydia* infection medium (i.e. glucose, L-glutamine, extra FCS) used in the alternative growth protocol, coupled with the incubation parameters (37°C, 5% CO_2), could have been favorable for the growth of the fungus and other bacteria, which could have somehow enhanced *C. gallinacea* growth.

What is the identity of the fungus present in the Italian specimen 60260-3?

Supernatant of the 2nd passage of the isolate 60260-3 from Italy was taken and grown in blood agar. The pure culture of the fungus was subjected to identification through matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) service at the École nationale vétérinaire d'Alfort (Dynamic research team). The fungus was identified as *Trichosporon asahii*.

Trichosporon spp. are yeast-like anamorphic organisms which are ubiquitous in nature. So far, 50 species of *Trichosporon* have been described, 16 of them have clinical relevance. These yeast-like microorganism can be found in soil, decomposing wood, rivers, lakes, seawater, scarab beetles, bird droppings, bats, and cattle (Colombo et al., 2011). In humans, these fungal species occasionally are part of the gastrointestinal and oral cavity microbiota and can transiently colonize the respiratory tract and skin (Cho et al., 2015). *T. asahii* is considered, together with *T. mucoides* and *T. asteroides*, as opportunistic microorganisms capable of causing invasive systemic infections in humans (Colombo et al., 2011). Next generation sequencing revealed that up to 125 fungal genera could be find in the gastrointestinal tract of chickens, however, *Microascus* sp., *Trichosporon* spp. and *Aspergillus* spp. account for over 80% of the total fungal population diversity, being *Trichosporon asahii* the predominant species among *Trichosporon* genus (Robinson et al., 2020). Moreover, mycological examination of microbiota present in the skin of 500 chickens housed in different housing systems, revealed that the genus *Trichosporon* is present across the different housing systems, ranking third in frequency (Gründer et al., 2005).

Does Trichosporon asahii have an impact on the exceptional growth of the isolate 60260-3 from Italy?

To verify this hypothesis, we tried to grow the bacteria without the presence of the fungus. Since the amount of antifungals added was not sufficient to eliminate the fungus, and resistance to Amphotericin B in *Trichosporon asahii* had already been reported (Colombo et al., 2011), we decided to use filtration to eliminate the fungus. To do so, BGM cells were grown to the confluence in a T25 flask. Cells were infected with the 2nd passage of the isolate 60260-3 from Italy using the alternative chlamydial growth protocol (thus, this infection corresponds to the 3rd passage of this Italian isolate). At 24 h pi, the severe contamination persisted with macroscopic fungus-like structures, white in color similar to mycelia. At 48 h pi, immunostaining was performed and countless well-formed typical chlamydial inclusions were observed (**Figure 36/A**). Immunostaining was visualized using confocal laser scanning microscopy (**Figure 36/B**). The infected confluent monolayer of BGM was harvested (1 mL SPG medium), and divided in two aliquots: 500 µL were frozen directly and 500 µL were filtered through a 0.45 µm membrane. A confluent monolayer of BGM cells was inoculated with the filtered 500 µL (corresponding to the 4th passage of this Italian isolate), and few

inclusion-like, irregularly sized, bright green chlamydial structures could be observed at 48 h pi (**Figure 36/C**). The fungus was not found.

No conclusion could be drawn from this preliminary study, aimed at eliminating the fungus by filtration. The first hypothesis that arises further the growth absence of the Italian isolate 60260-3 of *C. gallinacea* after the fungus removal by filtration, is that the fungus clogged the filter and prevented *Chlamydia* from passing through. An alternative hypothesis could be that the majority of the chlamydial elementary bodies (EB) bounded to the yeast cell wall, thus, both the yeasts and bacteria remained trapped in the filter. It has been shown that EB of *C. trachomatis* could firmly bind to the cell wall of *Candida albicans* (a common yeast in the oral and vaginal microbiota of humans), thus decreasing chlamydial infectivity (Kruppa et al., 2019). Both scenarios are not mutually exclusive.

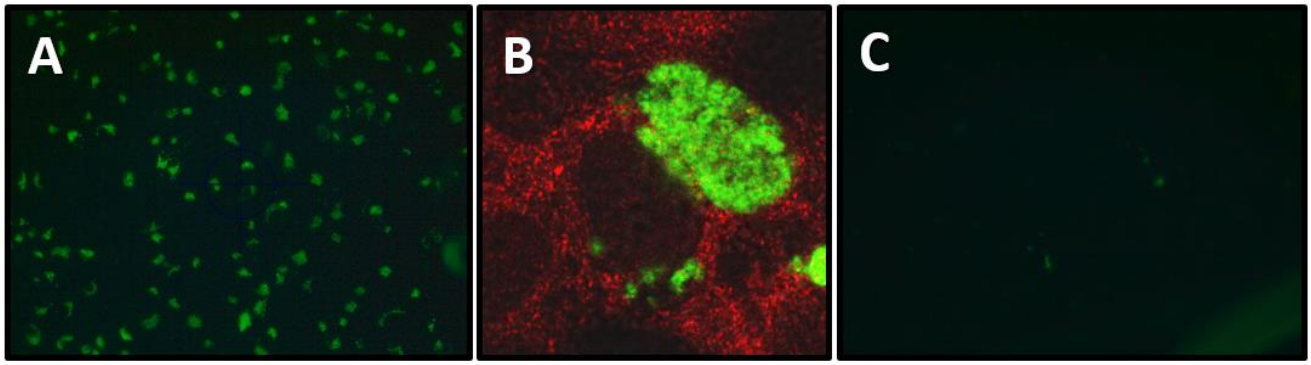


Figure 36. Microphotograph of a confluent monolayer of BGM cells infected with the 2nd passage of the isolate 60260-3 of *C. gallinacea* from Italy (48 h pi, 20X). **B:** Confocal laser scanning microphotograph of a BGM cell infected with the isolate 60260-3 of *C. gallinacea* from Italy. The infected monolayer was fixed with methanol 48 h pi and stained using the direct immunofluorescence IMAGEN™ *Chlamydia* kit (Oxoid, UK) containing a FITC-labeled anti-LPS antibody specific for *Chlamydiaceae* and Evans blue, as counterstain for cells (red color) (60X). A typical chlamydial inclusion could be observed in bright green. **C:** Microphotograph of a confluent monolayer of BGM cells inoculated with the 0.45 µm filtered supernatant of the 2nd passage of the Italian isolate 60260-3. Few inclusion-like, irregularly sized, bright green chlamydial structures could be observed at 48 h pi (20X).

In our study, the presence of this fungus in the culture of this atypically growing *C. gallinacea* isolate, raises the question of the impact of this fungus could have on the growth of this isolate. Synergies have already been described when there is a coinfection between two microorganisms, in particular for *Chlamydia*. Indeed, there is evidence that coinfections can be beneficial to one or both pathogens (Hament et al., 1999; Sid et al., 2015) or, conversely, prior infection by one pathogen can prevent the growth of the other one (Kinnula et al., 2017). Natural occurring coinfections between *Chlamydia* and other viral and bacterial pathogens have been reported. For example, in a longitudinal survey of respiratory pathogens in broiler chicken flocks, coinfection between *Ornithobacterium rhinotracheale* and *C. psittaci* was found. Broilers showed clinical signs of disease such as conjunctivitis and dyspnea, when normally *C. psittaci* infections in chickens are asymptomatic (De Boeck et al., 2015). Mixed infections with fowlpox virus and *C. psittaci* have also been reported in commercial laying hens. This report took place during an outbreak of fowlpox virus in Poland that lasted longer than usual (several months) with a higher than expected mortality rate (20%). In this outbreak, clinical signs suggested fowlpox,

that was later confirmed through histopathological examination of tissues, revealing unusual changes. Therefore, they proceed to carry out ultrastructural examination of tissues. Electromicrsocopy, performed from mucous epithelial tissue, revealed the presence of intracellular inclusions filled with chlamydial particles that were compatible with chlamydial elementary bodies. This study suggests that infection by fowlpox virus and the associated proliferative lesions occurred first. Then, the intense cell damaging observed could have weakened the host cell membranes enabling infection by a second pathogen, i.e. *C. psittaci* (Karpińska et al., 2014). Similarly, Gaede et al., (2008) reported a severe respiratory disease outbreak in a mixed poultry flock (chickens and ducks) associated with human cases of psittacosis. *C. psittaci* was found in poultry, which were simultaneously infected with *Mycoplasma gallisepticum* (a primary poultry pathogen). In a similar way, To et al., (2014) described a novel psittacine adenovirus identified during a human psittacosis outbreak. Sixteen mealy parrots (*Amazona farinosa*) imported to Hong Kong from Guyana were quarantined, two of them died while 14 developed clinical signs of disease and therefore were euthanized. Postmortem examination revealed severe lesions compatible with those caused by *C. psittaci*. Using PCR, several common viral pathogens were investigated as well as *C. psittaci*. The presence of *C. psittaci* was confirmed, and the novel adenovirus was identified. Interestingly, bacterial load positively correlated with adenovirus viral load in the lungs of affected parrots.

Furthermore, experimental studies have been conducted to assess the effect of the interaction of various common avian pathogens with chlamydiae. For example, in an experimental study conducted in SPF chickens co-infected with avian influenza virus (subtype H9N9), *O. rhinotracheale*, *Aspergillus fumigatus* and *C. psittaci*, the highest mortality rates and the most severe damage to the air sacs and lungs of the chickens were obtained only when all these pathogens interacted together (Chu et al., 2017). Likewise, in other experimental study it was shown that prior infection with *Bacillus cereus* could facilitate infection with *C. psittaci*, thus aggravating the gizzard erosion and ulceration syndrome in SPF chickens, which was accompanied by severe respiratory distress, high chlamydial loads in the lungs and severe lesions in respiratory organs (Zuo et al., 2020).

Trichosporon asahii or its metabolites could interfere with the growth of chlamydia at several levels. For example, by activating genes in *Chlamydia*, by providing nutrients necessary for its growth (e.g. energy molecules and/or co-factors), or by facilitating its penetration into the cell. It has been described that among the major virulence factors of the genus *Trichosporon* figures the ability to produce and secrete enzymes (e.g. proteases, phospholipases) for scavenging nutrients from the environment or to facilitate the invasion process to different tissues within the host (Colombo et al., 2011), and the expression of glucuronoxylomannan (GXM) in their cell walls composed by mannose (60%), xylose (24%), glucose (8%) and glucuronic acid (Zimbres et al., 2018). *Trichosporon* releases GXM into the supernatant when is cultivated *in vitro* and could be found in the serum and/or cerebrospinal fluid of patients with disseminated trichosporonosis (Heslop et al., 2011; Karashima et al., 2002).

Are there any particularities at the genetic level of the Italian isolate 60260-3?

The Italian isolate 60260-3 has not yet been finely characterized. So far, only seven housekeeping genes have been sequenced with the proposed MLST scheme for *Chlamydia* with specific primers suggested for *C. gallinacea* by Guo et al., (2017). Sequences have been compared with other strains of *C. gallinacea*, positioning it within the group of *C. gallinacea*, without particularities (data not shown). Whole genome sequencing of the strain must be conducted in order to analyze particular regions of the genome which might suggest differences in virulence of other strains of *C. gallinacea*, i.e. the plasticity zone, *pmp* genes, inclusion proteins, etc.

How can we explain the fact that as the passages are made the strains/isolates of C. gallinacea are lost?

A couple of hypotheses can be proposed, (i) a poor conservation of the strain/isolate at -80°C (strains/isolates are affected after long periods of freezing, which has been proposed by Onorini et al., (2019) for other chlamydial strains), although the same method of conservation for the other chlamydiae is used. Could it be that *C. gallinacea* is a particularly susceptible to freezing species with respect to the other chlamydia (e.g. by a different composition of its cell wall); (ii) in the course of the passages the original inoculum is diluted, consequently important metabolites or compounds necessary for the infectivity of *C. gallinacea* could be lost. This could explain that inclusions can be seen in the first isolation from a field specimen, however these inclusions are lost with each passage. Are there any essential elements that are lost or diluted in the course of the passages?

This question emerges also with the fact that reference strain was successfully propagated up to 10 passages at the FLI in Germany on BGM cell line. The protocol followed at the ANSES is identical, however the batch of each product changes over time (e.g. the FCS). We presume that slight variations in the concentration of certain nutrients in the FCS, such as proteins, vitamins, minerals, might occur, and this somehow gets critical for the grow of *C. gallinacea*.

3.3. Limitations of our study

There are several limitations to our study intended to improve the growth of *C. gallinacea*. First, since typical chlamydial inclusions were only rarely observed in our experimental infections, *C. gallinacea* growth could not be quantitatively measured using classical approaches (i.e. number and size of inclusions). This limitation has already been addressed in studies focusing on other chlamydial species. For example, in the study conducted by Schiller et al., (2004), clear chlamydial inclusions of *C. suis* could only rarely be observed when infected cells were chemically treated with 5-iodo-2-deoxyuridine or with the cationic polymer DEAE-dextran; observing “inclusion-like” chlamydial structures instead. These “inclusion-like” chlamydial structures could be estimated semi-quantitatively, giving a qualification of “more” or “less” when these inclusion-like structures were compared with cycloheximide-treated cells as a reference method to grow *C. suis*. As we did not have a reference method in which the growth of *C. gallinacea* is optimal, we could not implement this kind of comparative measurement. In another study, high-content microscopy was implemented as another approach to quantify the growth of different strains of *C. suis* in different cell lines and growth media (De

Puysseleyr et al., 2017). This technique is an automated microscope-based cell screening allowing to measure slight morphological cellular changes, typically, through one or more fluorescent dyes (Buchser et al., 2014). By using this technique, De Puysseleyr et al., (2017) obtained three automated measurements to compare *C. suis* growth: mean spot area (i.e. inclusion size), mean spot number (i.e. inclusion number per cell), and mean spot occupancy per cell (interpreted as overall replication). As a result, De Puysseleyr et al., (2017) proposed that all these three parameters should be considered for a complete assessment of the infection status, as they provide useful information that otherwise would not be considered when evaluating difficult to grow *Chlamydia*. We suggest to use this technique in further studies assessing *C. gallinacea* growth.

The second limitation is not having included the HEp-2 cell line, which had been pointed out by several studies as a cell line whose performance stands out from the rest when working with *Chlamydia* species of difficult growth (Croy et al., 1975; Roblin et al., 1992; Wong et al., 1992), even from environmental *Chlamydiae* (Sampo et al., 2014). Interestingly, the study in which the strain JX-1 was first isolated (the second strain of *C. gallinacea* whose entire genome has been sequenced), HEp-2 cell line was used (Guo et al., 2016). We suggest to include HEp-2 cell line in future studies addressing *C. gallinacea* growth. It may also be useful to include chicken enterocytes to compare the growth of *C. gallinacea*. To our knowledge, there is no permanent cell line originating from the chicken gut, although there are some protocols to standardize the management of primary cells with this origin (Kaiser et al., 2017; Rath et al., 2018).

Finally, given the limitation of our study design, consisting of the impossibility to initiate all experimental infection with the same inocula (i.e. aliquots of the same dilution of the reference strain), results of E2 and E3 could not be replicable, as we used for both experiments inocula derived from E1. E2 (3.1.2) and E3 (3.1.3) were not planned from the beginning. At the time we were carrying out the first experimental infection, the study of Onorini et al., (2019) in which different centrifugation and incubation temperatures for several chlamydial species were tested, was published at the time we were carrying out the first experimental infection (E1). This final limitation is related with the first one, because as we did not have a reference method to grow *C. gallinacea* (and we account with only 500 µL of the reference strain), we could not grow the bacterium to have enough inoculum throughout the experimental infections. However, we searched to conduct each experimental infection using the same inoculum within each one of them. It is important to note that it was precisely this final limitation that led us to apply the alternative protocol (arising from the results obtained from E1-E3) to unique field samples.

4. Conclusions

The different experimental infection protocols tested in this study to optimize the growth of *C. gallinacea* did not yield an optimized protocol for the cultivation of this species. Similarly, the two protocols applied to field specimens did not allow adequate propagation of these isolates to produce sufficient quantities to study them in more detail, with the exception of one strain. Indeed, we identified that there were individual differences in

the growth of *C. gallinacea*-positive specimens. These differences are not related to the chlamydial load in the specimen, nor to the time and conditions of storage, (because Italian specimens were similarly handled and they had similar bacterial load and storage time). We believe that differences could be associated with (i) the implementation of the alternative protocol for *Chlamydia* growth, which might have provided the optimal conditions for the development of a microenvironment that promoted the growth of other microorganisms (such as *T. asahii*), or (ii) to the fact that we had to deal with a resistant yeast, while for other samples the antibiotic/antifungal cocktail was sufficient to eliminate microorganisms from the beginning. In any case, the presence of other microorganisms in the specimen might eventually influenced the growth of *C. gallinacea*. We presume that perhaps this phenomenon could occur *in vivo*. There is no doubt that further research is essential to address whether differences in the growth of this *C. gallinacea* isolate 60260-3 from Italy are at genetic level (by sequencing the entire genome of the strain), or rather the difference could lie at the transcriptional level, i.e. the way how particular genes are regulated. It should be kept in mind that genomes of the two so-far sequenced *C. gallinacea* strains (08-1274/3 and JX-1, from France and China, respectively), are 99.4% similar (Guo et al., 2017); and that generally speaking, genomes of the different chlamydial species are highly conserved.

C. gallinacea, a recently described species, still has several unknowns regarding its pathogenicity, mechanisms of transmission, zoonotic potential, etc. Strain isolation is still necessary to investigate the relationship of the bacterium with the host, carry out fundamental microbiological studies, development of diagnostic tests and not only to perform genetic characterization. So far, we still do not have an optimal protocol to grow *C. gallinacea*. More than conclusions, several questions arise from this study: What made the difference in the growth of the various isolates of *C. gallinacea* with respect to the growth of the isolate 60260-3 from Italy? Could the medium used and the incubation parameters have had any influence on the conditions necessary for this marked differential growth? Should we keep the proposed alternative protocol for chlamydial growth as the preferred one for *C. gallinacea*-positive specimens? Could the presence of the fungus, directly or through its metabolites, have provided the necessary substrates or conditions for *C. gallinacea* growth? In any case, interaction between different microorganisms within a host is the rule and not the exception. Microbial communication and coinfections could answer other questions regarding chlamydial infections, such as disease outcome, tissue tropism, host preference and zoonotic potential.

FINAL CONCLUSION AND PERSPECTIVES

This thesis work represents the basis of research on chlamydial infections in poultry in Mexico, while enriching the knowledge of on-farm management and biosecurity practices implemented in one of the largest poultry producing countries. However, as expected, new questions emerged from this study. **The presence of *Chlamydiaceae* in poultry was evidenced both on commercial as on backyard Mexican farms**, and ***C. gallinacea* was the only chlamydial species identified**. Although this result is consistent with previous surveys suggesting that this chlamydial species is the most frequent in chickens (Guo et al., 2016; Hulin et al., 2015; Taylor-Brown and Polkinghorne, 2017); the conducted cross-sectional study may have not allowed the detection of *C. psittaci*, hence, its presence cannot be ruled out. Moreover, *C. psittaci* is often detected in poultry flocks following investigations carried out after psittacosis cases are identified, frequently involving processing plant workers (Hogerwerf et al., 2020), as chlamydial infections in poultry are usually subclinical. Therefore, in the future, it would be advisable to carry out a study in Mexico characterizing the presence of *Chlamydiaceae* in poultry at slaughterhouses at various time points. The interest in carrying out an investigation in slaughterhouses lies in the fact that a large number of poultry flocks from different farms and geographical origins are gather in a short period of time. A serological survey among workers could be conducted also to diagnose previous exposures to *C. psittaci*. Additionally, it would be interesting to inquire into the clinical records of slaughter plant workers with cases of respiratory disease or pneumonia to assess the magnitude of exposure of this population, considering that because of their work activity, they are at risk of contracting psittacosis. Even though the presence of *C. psittaci* was not detected within the sampled panel in our study, awareness of psittacosis in Mexico should be maintained, especially since it has been shown that *C. psittaci* can be present in captive and pet birds in Mexico (Ornelas-Eusebio et al., 2017, 2016). It could also be of interest to investigate respiratory diseases among people who work in close contact with birds in captivity, e.g. veterinarians, pet bird owners and breeders, pet shop, zoo and wildlife employees, perhaps by conducting serological surveys. But above all, physicians in Mexico should be aware about the presence of *C. psittaci* in birds and keep it in mind when facing atypical pneumonias.

Furthermore, it was possible to identify that **apparent prevalence of *Chlamydiaceae*-positive poultry increased inversely to the level of confinement** (controlled environment *vs* open-sided poultry houses *vs* backyard), thus suggesting that the environment could be a source of *C. gallinacea* contamination. This result is in agreement with previous studies identifying that environmental contamination with *C. psittaci* was significantly correlated with chlamydial shedding by poultry (Hulin et al., 2016), and that excretion was more important when poultry were reared in open range conditions on farms (Vorimore et al., 2015). Taken together, previous and current studies suggest that the environment may be the most likely source of contamination for poultry, considering that the oral/fecal route of transmission is the principal for *C. psittaci* (Thierry et al., 2016) and *C. gallinacea* (You et al., 2019). Since soil and water have been identified as the most likely reservoirs of

Chlamydia in nature (Collingro et al., 2020; Taylor-Brown et al., 2015b), it would be interesting to examine the water and litter material before placing it in contact with commercial poultry.

In the present study, **risk factors associated with the presence of *Chlamydiaceae* in poultry were also investigated**; the analysis was performed separately for backyard and commercial poultry, resulting in two and one risk factors associated to *Chlamydiaceae* presence, respectively. **The first risk factor in backyard poultry associated with *Chlamydiaceae* presence was an impaired health status of poultry**. Although this result contrasts with previous studies in which *Chlamydiaceae*-positive poultry, and more specifically, *C. gallinacea*-positive poultry (the only *Chlamydiaceae* species found in this thesis work), were clinically healthy (Donati et al., 2018; Guo et al., 2016; Hulin et al., 2015; Li et al., 2017), it has also been mentioned that *C. gallinacea* could have a negative effect on the productivity of broilers by reducing their daily weight gain (Guo et al., 2016). The association we found contributes to open the debate on the role that this chlamydial species may have in poultry. Further research should be conducted on the pathogenicity of *C. gallinacea*, taking into account that co-infections with chlamydial agents and other common poultry pathogens have been reported, and that these co-infections may aggravate the clinical outcome in both naturally occurring (De Boeck et al., 2015; Karpińska et al., 2014; To et al., 2014) and experimental infections (Chu et al., 2017; Zuo et al., 2020). **The second risk factor found to be associated with *Chlamydiaceae* presence in backyard poultry was the lack of antibiotic usage**. In this regard, it has been hypothesized that the global trend of decreasing antimicrobial use in food-producing animals (Van Boeckel et al., 2015), especially in poultry farming, could lead to an increase of chlamydial infections (more importantly of *C. psittaci*) in poultry, thus potentially exposing poultry workers (Sachse et al., 2015b). This could be an additional reason to be aware of chlamydial infections in poultry. **On the other hand, laying hens were more at risk of being *Chlamydiaceae*-infected than broiler flocks on commercial farms**. This finding highlights the need to perform further studies to help to understand the implication that chlamydial infections could have on the health, welfare and productive performance of laying hens (e.g. number and weight of eggs laid, feed conversion, egg quality, etc.). As to date, no studies have explored the implication that the presence of *Chlamydiaceae*, and more specifically of *C. gallinacea*, could have on laying hens, considering that its prevalence may be as high as 47% (Heijne et al., 2018). All things considered, chlamydial diagnosis in poultry in Mexico should be taken into account. In order to generate more data regarding the role of *Chlamydiaceae* in poultry husbandry, it would be interesting to conduct a study under field conditions in poultry flocks having low productivity or health problems, which could certainly be of interest for poultry farmers.

Regarding the results of the multivariate analysis**, significant variations in the implementation of biosecurity practices were observed across the three clusters of commercial farms identified through this study**, and **five biosecurity practices were identified as the most significantly associated with farm classification**. Moreover, this analysis allowed to further characterize those practices that may predispose laying hens to the presence of *Chlamydia* and other avian pathogens. Indeed, the cluster of farms in which laying hen farms predominated (cluster 3), were more prone to not implement biosecurity practices that had

been previously identified as risk factors associated with low pathogenic outbreaks of influenza virus (Nishiguchi et al., 2007). Remarkably, three of these biosecurity practices coincide with the five practices that we have identified as the most critical for classification of farms within a different cluster in this study. We made recommendations for each of these five critical practices. The obtained results could be helpful for field veterinarians and poultry farmers to understand how to guide strategies to reinforce on-farm implementation of biosecurity practices, prioritizing those identified as critical. It should be noted that our study also offers information characterizing antimicrobial usage on commercial poultry farms, thus contributing to the national need for information on this subject. Further studies investigating the effectiveness of the official provisions issued in the last few years should be conducted to follow up on trends in on-farm biosecurity practices and antimicrobial usage in the Mexican poultry industry. It would also be worth presenting this analysis at a meeting that brings together poultry farmers and veterinarians specialized in poultry science in Mexico.

Last but not least, the interest to finely characterize the *Chlamydia gallinacea* found in our study, triggered the implementation of experimental trials in the laboratory aiming to **optimize the growth protocol for *C. gallinacea*, which, although not conclusive, led to interesting clues that opens new research pathways**. In fact, an enhanced growth of one *C. gallinacea*-positive Italian field specimen was identified, and this differential growth was beyond the chlamydial load present in the specimen, and the time and conditions of storage. Many hypotheses can be formulated regarding this remarkable difference in the growth of this particular isolate, which was heavily contaminated from the outset with an antifungal resistant fungus. The fungus co-cultivated simultaneously from this *C. gallinacea*-positive specimen was identified as *Trichosporon asahii*, which is abundant in the gut mycobiome of chickens (Robinson et al., 2020). Whether the fungus has helped the bacteria to grow by providing nutrients necessary for its growth (e.g. energy molecules and/or co-factors) or by facilitating its penetration into the cell (e.g. enzymes such as proteases or phospholipases), is the immediate question to be solved. However other hypothesis should be explored considering possible differences at the genetic level (which can be assessed with the ongoing whole genome sequencing of this Italian isolate), or at the transcriptional level, i.e. the way how particular genes are regulated (a whole new line of research). In any case, if this remarkable enhanced growth can be extrapolated to an *in vivo* phenomenon, it could shed light on the pathogenic potential of this newly described species as so far no pathology has been associated with its presence in poultry. In fact, it has been hypothesized that the apparent lack of pathogenicity of *C. gallinacea* could be related to its poor ability to grow *in vitro*, as has been suggested for other microorganisms such as *Aspergillus* spp. (Amarsaikhan et al., 2014; Paisley et al., 2005), and even certain genotypes of *C. trachomatis* (Nogueira et al., 2017). Moreover, the co-cultivation of the bacterium with the fungus opens the door to explore the specificity of chlamydial infections from an evolutionary point of view: to date there is no explanation regarding the host specificity of the different chlamydial species. In this regard, a question that should certainly be explored, is the co-adaptation of the different *Chlamydia* species to the host microbiome, which could allow the different *Chlamydia* species to colonize and survive in different hosts.

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ANNEX I. Content of the questionnaire

Content of the questionnaire administered to the commercial and backyard farmers participating in the cross-sectional study on *Chlamydiaceae* prevalence and associated risk factors on Mexican poultry farms

Supplementary Table 1. Content of the questionnaire administered to the commercial farmers participating in the cross-sectional study on *Chlamydiaceae* prevalence and associated risk factors on Mexican poultry farms.

Section	Variables considered	Possible outcomes
Farm specifications	Location	Mexican state
	House type	Environmentally controlled house
		Open-sided house
	Farm purpose	Layers
		Broilers
	Bred poultry species	Species name
Housing type and facility description	Number of barns per farm	Numeric
	Number of poultry per flock (barn)	Numeric
	Accommodation type	Litter
		Cage
	Litter material	Rice hulls
		Coffee hulls
		Chopped straw
	Which other domestic animals?	Cattle
		Sheep
		Goats
		Pigs
	Are there other poultry species bred on the farm?	Yes
		No
	Which poultry species?	Turkeys
		Chickens
		Ducks
		Quails
		Pheasants
Sampled flock characteristics	Sex	Male
		Female
	Bird age of sampled flock	Numeric
Flock health status and management	Current health status (sampled flock)	With clinical signs of disease
		Apparently healthy
	Description of clinical signs	Respiratory, digestive or other
	Antibiotic use	Yes: occasional or systematic usage
		No: no usage
	Antibiotic description	Type and mode of use
	Vaccination schedule	Detailed scheme provided by the farmer
Farm management practices	Feed origin	Vertically integrated feed mill plant
		Independent feed mill plant

	Number of workers on the farm	Numeric
	Coexistence with other domestic animals	Yes No
	Which other domestic animals?	Cattle Sheep Goats Pigs
	Mortality management plan	Yes No (municipal garbage)
	Which mortality management system?	Incineration Burial Composting
Cleaning and disinfection procedures	Litter removal at each ending flock	Yes No (or rare)
	Existence of disinfection procedures	Yes No
	Vacancy practice	Yes, each ending flock No (or rare)
	Vacancy duration	< 1 week ≥ 1 week
Biosecurity practices	Staff and visitor hygiene protocol before entering the farm	Yes No
	Mandatory use of exclusive clothing for work	Yes No
	Use of protective equipment (e.g. face mask)	Yes No
	Poultry movements into and outside the farm before ending flock (all-in all-out system)	Yes No
	Pest control (rodents and arthropods)	Yes No
	Staff continuous training on biosecurity	Yes No
	Zoonosis awareness	Yes No
	Perimeter fence	Yes No
	Footbath at barn entrance	Yes No
	Hand washing before enter poultry living area	Yes No
	Neighboring farms < 3 km reported	Commercial Backyard Both None

Supplementary Table 2. Content of the questionnaire administered to the backyard farmers participating in the cross-sectional study on *Chlamydiaceae* prevalence and associated risk factors on Mexican poultry-farms.

Section	Variables considered	Possible outcomes
Farm specifications	Location	Mexican state
	Farm purpose	Breeders Layers
	Which poultry species are bred on the farm? (multiple choice)	Chickens Ducks Pheasants Quails Turkeys
	Are there specialized poultry breeds?	Yes or No
Facility description	Number of poultry per farm	Numeric
	Accommodation	Soil Litter
	Bedding	Sawdust Chopped straw
	Coexistence with other domestic species?	Yes or No
	Which other domestic species?	Cattle Sheep Goats Pigs
Sampled poultry characteristics	Poultry species	Chickens Ducks Pheasants Quails Turkeys
	Sex	Male or Female
	Age	Numeric
Poultry health status and farm health management	Current poultry health status	With clinical signs of disease Apparently healthy
	Description of clinical signs	Respiratory, digestive or other
	Antibiotic use	Yes: occasional or systematic usage No usage
	Antibiotic description	Type and mode of use
Farm management practices	Feed origin	Homemade feed Industrialized feed Mixed
	Mortality management plan	Yes No (municipal garbage)
	Mortality disposal	Incineration Burial
Cleaning and disinfection procedures	Litter removal	Yes or No (or rare)
	Existence of cleaning procedures	Yes or No

ANNEX II. PCR – *Mycoplasma* spp. contamination test for cell culture

Sample preparation

1. Heat 50 µL of supernatant, 5 min at 95°C.
2. Perform a rapid centrifugation.

Primers	Primer sequence
MGSO - F	TGC ACC ATC TGT CAC TCT GTT AAC CTC
GPO-3 - R	GGG AGC AAA CAG GAT TAG ATA CCC T

* Set the primers at 10 µM (Example: Vf = 100 µL; 10 µL primers + 90 µL water).

PCR reaction mixture preparation

	Initial concentration	Final concentration	Mix (µL) for 1
10X Ex Taq Buffer	10X	1X	2.5
dNTP 2,5 mM each	2,5 mM	200 µM	2
Primer forward	10 µM	0,5 µM	1
Primer reverse	10 µM	0,5 µM	1
Takara Ex Taq	5 units/µL		0.2
Water			13.3
		Mix final volume	20
		Final volume /tube	25

3. Transfer 20 µL of mix per tube.
4. Add 5 µL of DNA (supernatant) per tube.
5. Add the positive and negative controls.

Stage	Temperature	Time	Cycles
Heat Lid	110°C		
Initial denaturation	94°C	2 min	1 cycle
Denaturation	94°C	30 sec	35 cycles
Hybridization	55°C	30 sec	
Elongation	72°C	1 min	
Elongation finale	72°C	5 min	1 cycle

6. If the sample is positive, the expected size of the amplicon is 270 bp.

ANNEX III. Protocol for cell culture maintenance and subculturing

Cell protocol standardize for BGM cell line BGM (Buffalo Green Monkey epithelial kidney cells)

Notes before start:

- All work requiring sterile conditions has to be carried out in the laminar flow fume hood.
- Avoid tearing the cellular carpet and avoid foam.
- Adjust the temperature of all solutions so that they have the same temperature as the cell culture (preheat at 37°C).
- The work area and non-sterile work equipment (racks, bottles, etc.) must be disinfected with 70% alcohol before starting work.

Materials

- Laminar flow hood, incubator at 37°C ± 1°C and 5% ± 1% CO_2, Freezer ≤-76°C, microscope (Zeiss Axiovert), centrifuge X1R(Thermo); refrigerator 4°C ± 2°C, freezer at ≤-18°C.
- Malassez cell; Pipettes 1ml, 2ml. 5 ml, 10 ml, 10-1000 precision pipettes; matching pipette tips; cryotubes.
- Cell culture tubes with inserted slide (Sterillin), cell culture vials (T25), 25 cm^2, cell culture vials (T75), 75 cm^2.

Culture medium

500 mL Eagle's Minimum Essential Medium (EMEM) with Earle salts, without L-glutamine
+ 5 mL L-glutamine 200 mmol (see below) with a final concentration of 2 mmol/ml
+ 5% Foetal Calf Serum
****Keep tightly closed in the refrigerator, consume within 4 weeks after the addition of the additives.

L-glutamine

2.922 gr of L-glutamine (cell culture tested) in 100 ml of distilled water
Dissolve, filter sterilize, aliquot and freeze at -20°C.

Trypsin - EDTA solution (pH - between 7.2-7.3)

NaCl 8.0 gr/ KCL 0.8 gr/ Glucose 1.0 gr/ $NaHCO_3$ 0.58 gr/ Trypsin 0.5 gr/ EDTA 0.2 gr/
Distilled water adjusted to 1000 mL
Add the products in order until completely dissolved, adjust the pH and filter by sterilization 0.2µm.
Label, aliquot and store at -20°C.

PBS (pH 7.2-7.4) without Ca and Mg ions (buffer for washing cells prior to the trypsination step only

NaCl 8.0 gr/ KCl 0.2 gr/ Na_2HPO_4 x 12 H_2O 2,31 gr/ KH_2PO_4 0,2 gr/
Distilled water adjusted to 1000 mL
Add the products in order until completely dissolved, adjust the pH and filter by sterilization 0.2µm.
Label, aliquot and store at -20°C.

PBS (pH 7.2-7.4) with Ca et Mg ions (buffer for washing cells and making dilutions)

NaCl 8,0 gr/ KCl 0,2 gr/ Na_2HPO_4 x 12 H_2O 2,31 gr/ KH_2PO_4 0,2 gr/ $MgCl_2$ 0,0468 gr/ $CaCl_2$ x 2 H_2O 0,132 gr
Eau distillée ajustée à 1000 ml
Distilled water adjusted to 1000 mL
Add the products in order until completely dissolved, adjust the pH and filter by sterilization 0.2µm.
Label, aliquot and store in the refrigerator.

Cell freezing medium

40% MEM/ 40% Fetal Calf Serum/ 20% DMSO

Protocol for culture in cell culture flasks (T25)[1]

1. Aspirate the culture medium.

[1] Numbers in brackets are for T75 flasks

2. Wash with PBS 1X (free of Ca and Mg ions), 5 mL (10 mL) to remove metabolic products.
3. Aspirate PBS.
4. Dispense 2 mL (5mL) of trypsin - EDTA solution onto the cell monolayer and incubate 5-10 min at 37°C.[2,3]
5. Occasionally shake and pat the box.
   Macroscopic visual inspection: the cells flow with the liquid into the bottom of the box.
   Microscopic visual inspection: most of the cells float in the liquid.
6. Add 2 mL (5 mL) of cell culture medium.
   The serum contained in the medium immediately neutralizes the effect of trypsin and partially neutralizes the cytotoxic effect of EDTA.
7. Resuspend the cells with a pipette and place them in a 15mL Falcon tube.
8. Centrifuge the suspension for 12 min at 1500 rpm at 18°C.
9. Immediately aspirate the supernatant carefully
   Risk of cell damage with EDTA**.** The presence of EDTA also complicates the re-attachment of cells during passage.
10. Add 2 ml (4 ml) of culture medium to re-suspend the cell pellet.
11. Add the volume of cells and medium needed according to the dilution to be calculated in advance.
12. Shake.
13. Incubate at 37°C and 5% CO_2.
14. Open the caps to ensure gas exchange.
15. The cell mat is reformed after 3-4 days.

The possibilities for further processing are: *Chlamydia* infection or subculturing

Culture in tubes with glass coverslip

1. Prepare a cell suspension with a cell density of $1x10^5$/mL
2. Add 1 mL / Tube
3. Make sure to have a homogenous mixture so that the cells settle quickly.
4. Leave the caps slightly open to ensure gas exchange.
5. Incubation at 37°C and 5% CO_2.
6. 2-3 days after the cell monolayer should be confluent (microscopic control)
   ***Possibilities for further processing are: Chlamydia infection

Change of medium

The change of medium is necessary if:

1. The appearance of the cells and/or the pH induces discoloration of the culture medium or the cell monolayer is not confluent.
2. Aspirate the medium
3. Re-apply the culture medium, being careful not to touch the cell monolayer.
4. Incubate at 37°C and 5% CO_2.
5. After 1-2 days cell monolayer should be confluent.
6. Flasks with a confluent monolayers and the closed cap can be kept at room temperature for 7 days.

Cryopreservation

Use MEM medium with 10% SVF for cell culture.

- Perform the subculturing procedure as previously indicated
- Adjust the cell density between 1 and 6 x 10^6 / mL.
- Add 0.9 mL of cell suspension with 0.9 mL of freezing medium (with DMSO)

[2] Time information is indicative.
[3] Cell exposure time to trypsin depends on the age of the cells and the age of the trypsin - EDTA

- Fill the cryotubes and place them in the Nalgene 1°C/min freezer box.
- Place the box 2h in the refrigerator.
- Penetration of DMSO into the cell (antifreeze)
- Put the box overnight at -80°C, the next day remove the tubes from the Nalgene box and store them in a normal cryobox at -80°C.

Thawing of cells.
Place a water bath at 37°C to thaw the cell tube.
After thawing, immediately place the contents of the cryotube in a T25 flask containing approximately 5 ml of MEM medium with 10% SVF previously heated to 18°C.
Incubation at 37°C and 5% CO_2.
After 3-4 hours, check the fixation of the cells under the microscope.
Change the medium.
Next change of medium after 18-24 hrs.
Do the same for the 1st passage.
Sterility tests
Check regularly the sterility of cell passage
Take 100-200 µL of cell suspension are spread on blood agar, 3-4 days at 37°C and then culture for another 3-4 days at room temperature.
Test for *Mycoplasma* before freezing and before re-culture.

To count cells:
Place 100µL of cell suspension in a tube and add 900µL of PBS. Agitation (vortex)
Fill the malassez cell with 20µL (1 square = 0.01µL):

Formula to determine the number of cells/mL:

$$No.of\ cells/mL = \frac{\sum of\ cells\ X\ 10^5}{No.of\ quadrants\ counted}$$

Example :

$$No.of\ cells/mL = \frac{88\ X\ 10^5}{8} = 11\ X\ 10^5$$

Formula for calculating cell volume for a selected concentration

$$Volume\ of\ cells\ for\ a\ desired\ concentration = \frac{Desired\ No.of\ cells\ X\ total\ volume}{Calculated\ cell\ concentration}$$

Example:

$$Volume\ of\ cells\ for\ a\ desired\ concentration = \frac{(1.0\ X\ 10^5)\ X\ 5}{11.0\ X\ 10^5} = 0.45\ mL$$

Add the culture medium in a flask (e.g. 4.55 mL = difference from 5 mL).
Add the calculated volume of the suspension (0.45 mL).

ANNEX IV. Chlamydial growth protocol in cell culture

Notes before starting:

- All work requiring sterile conditions must be performed in the laminar flow cabinet
- Avoid damaging the cell monolayer and create foaming.
- Adjust the temperature of all the solutions so that they have the same temperature as the cell culture (~37°C).
- Laboratory equipment have to be cleaned and disinfected with 70% alcohol or a disinfectant solution.

1. Materials

a) Laminar flow cabinet
b) Incubator 37 °C ± 1°C, 5% ± 1% CO2
c) Ultrafreezer ≤ -76 ° C
d) Fluorescence microscope
e) Centrifuge
f) Sonicator
g) Fridge 4°C ± 2 ° C
h) Freezer ≤-18 ° C
i) Pipettes 1ml, 2ml, 5 ml, 10 ml,
j) Precision pipettes & pipette tips 10-1000
k) Cryotubes
l) 7 ml sterilin polystyrene containers (bijou tubes) with removable glass coverslip (Thermo Scientific, Cat. No. 129AX/1)
m) Cell culture flasks of 25 and 75 cm2

2. Cell line BGM

It is a continuous epithelial cell line originated from kidney tissue of the African green monkey (*Cercopithecus aethiops*) (Barron et al., 1970).

3. Sonication protocol

Sonication amplitude 80 %, 0.8 seconds 10 cycles and 0.2 seconds pause between each cycle. Twice.

4. Supplementary reagents

4.1. L-Glutamine

2.922 g of L-glutamine (cell culture grade) dissolved in 100 ml of distilled water
* Dissolve, sterilize the solution by filtration 0.2µm, divide into aliquots and freeze at -20 °C.

4.2. Trypsin solution - EDTA (pH - entre 7.2-7.3)

NaCl 8.0 g KCL 0.8 g Glucose 1.0 g $NaHCO_3$ 0.58g Trypsin 0.5 g EDTA 0.2 g

* Mix the reagents in order from left to right until its complete dissolution in distilled water adjusting the volume at 1000 ml, adjust pH level and sterilize by filtration 0.2µm. Divide into aliquots and freeze at -20 °C.

Alternative: Trypsin/EDTA (1X) Solution, Lonza, Cat. No. BE17-161E.

4.3. PBS (pH 7.2 - 7.4) without Ca and Mg ions.

→ Only as washing buffer. Cell-monolayer washing with PBS is needed to remove the serum of medium so that trypsin will be able to hydrolyze peptide bonds.

NaCl 8.0 g KCl 0.2 g Na_2HPO_4 x 12 H_2O 2.31 g KH_2PO_4 0.2 g

* Mix the reagents in order from left to right until its complete dissolution in distilled water adjusting the volume at 1000 ml, adjust pH level and sterilize by filtration 0.2µm. Divide into aliquots and refrigerate at 4°C.

4.4. PBS (pH 7.2 – 7.4) with Ca and Mg ions.

→ Used as cell-monolayer washing-buffer and perform dilutions.

NaCl 8.0 g KCl 0.2 g Na_2HPO_4 x 12 H_2O 2.31 g KH_2PO_4 0.2 g $MgCl_2$ 0.0468 g $CaCl_2$ x 2 H_2O 0.132 g

* Mix the reagents in order from left to right until its complete dissolution in distilled water adjusting the volume at 1000 ml, adjust pH level and sterilize by filtration 0.2µm. Divide into aliquots and refrigerate at 4°C.

4.5. SPG

→ Used as chlamydial transport and cryopreservation medium.

Saccharose 74.60g KH_2PO_4 0.52g K_2HPO_4 1.25g L-glut 0.92 g Albumine Bovine/FractionV 1.00 g

* Mix the reagents in order from left to right until its complete dissolution in distilled water adjusting the volume at 1000 ml, with the exception of the albumin that has to be added after achieving the final volume of 1000 ml with the rest of ingredients. Adjust pH level and sterilize by filtration 0.2µm. Divide into aliquots and freeze at -20 °C.

** Stock up on without adding antibiotics or antifungal compounds – add them to the fresh medium and use within 24 hours.

5. Antimicrobials for SPG

5.1. Gentamicine

400 mg in 10 ml of distilled water → final concentration in the medium must be 40 µg/ml.

5.2. Nystatine

45 mg in 5 ml of distilled water → final concentration in the medium must be 25 U/ml.

5.3. Vancomycine HCL

250 mg in 10 ml of PBS → final concentration in the medium must be 25µg/ml

*** Mix the stock solutions of these antimicrobials and divide into 500 µl aliquots. Freeze at -20 °C.

*** Add one of these 500 µl aliquots into 50 ml of medium to obtain the suitable final concentration.

6. Other antimicrobials (penicillin / streptomycin) for the treatment of pig feces:

6.1. Penicillin G

1,000,000 UI in 10 ml of distilled water → final concentration in the medium must be 500 UI / ml

6.2. Streptomycin

1g in 10 ml of distilled water → final concentration in the medium must be 500 µg / ml

*** Mix the stock solutions of these antimicrobials and divide into 250 µl aliquots. Freeze at -20 °C.

*** Add one of these 250 µl aliquots into 100 ml of medium to obtain the suitable final concentration.

7. Antimicrobials for culture media

7.1. Amphotericin B solution

25mg in 10 ml of distilled water. Final concentration in the medium 2.5 µg / ml

7.2. Gentamycin solution

100 mg in 10 ml of distilled water. Final concentration in the medium 10 µg / ml

7.3. Stock solution of Vancomycin HCL

250 mg in 10 ml of distilled water. Final concentration in the medium 25 µg / ml

*** Mix the stock solutions of these antimicrobials and divide into 300 µl aliquots. Freeze at -20 °C.
*** Add one of these 300 µl aliquots into 100 ml of medium to obtain the suitable final concentration.

8. Cycloheximide

1000 mg (1 gr) of cycloheximide (Sigma, Cat. No. 01810-1G) in 100 ml of EMEM medium. Dilute 1 ml of this stock solution in 9 ml of EMEM medium (without supplements) to obtain the desired concentration of 1 mg/ml. Freeze at -20°C in 0.6 mL aliquots.
Or
Solubilize 10 mg of cycloheximide in 10 mL of EMEM and freeze at -20°C in 0.6 mL aliquots.

*** It is not recommended to weight the powder as it is considered hazardous and it must be handled with all the suggested safety precautions provided by manufacturer.
*** Cycloheximide is an antibiotic produced by *Strptomyces* sp. Its main biological activity is translation inhibition in eukaryotes resulting in cell growth arrest and cell death depending on the concentration. Inhibits protein biosynthesis in eukaryotic cells by binding with the 80S ribosome. (Sigma-Aldrich)

9. Cultivation media

9.1. Cell growth medium for BGM cell line

Ingredients	Final concentration	Final volume to add	Product reference
Minimum Essential Medium – Eagle with Earle's BSS		500 ml	Lonza, Cat. No. 12-125F
Fetal calf serum	5%	7.5 ml	
L-glutamine 200 mM	1 % [2mmol/ml]	0.5 ml	Lonza, Cat. No. 17-605E

*After supplements addition, preserve in the fridge at 4°C ± 2 ° C and consume within 4 weeks

Important note:

For chlamydial cultivation, prepare the cultivation media as described in the previous section (7). Then, add the antimicrobials cocktail previously aliquoted. Consume within a 24-hour period.

9.2. Chlamydiae cultivation medium (a): Ultra MDCK (Ozyme)

Ingredients	Final concentration	Product reference
Ultra MDCK	1 volume	Ozyme, Cat. No. 0000
Non-essential aminoacids	1%	Sigma, Cat. No. 0000
MEM Vitamines	1%	Sigma, Cat. No. 0000

*** Ultra MDCK is an animal-protein free, serum-free medium developed for the long-term growth of Madin Darby Canine Kidney (MDCK) and related cells. The cells, in an attachment culture, can be subcultured directly into EX-CELL MDCK from serum-supplemented media without adaptation. Cell densities and doubling times achieved under serum-free conditions are comparable to those achieved in a serum-supplemented culture. (Sigma-Aldrich)

9.3. Chlamydiae cultivation medium (b): EMEM (Lonza)*

Ingredients	Final concentration	Final volume to add	Product reference

Minimum Essential Medium – Eagle with Earle's BSS		500 ml	Lonza, Cat. No. 12-125F
Fetal calf serum	20%	100 ml	
L-glutamine 200 mM	1 % [2mmol/ml]	5 ml	Lonza, Cat. No. 17-605E
Glucose	3.33 mg/ml	2 gr	Sigma, Cat. No. 0000
Cyclohexamide	1µg / ml	0.6 ml	Sigma, Cat. No. 0000

* For *C. gallinacea*

10. Protocol

10.1. Preparation of host cells

Prepare the cell cultures flasks and/or the bijou tubes with a confluent cell monolayer.

10.2. Sample preservation and sample treatment

All samples must be refrigerated since they are taken.
Samples should be transported to the laboratory as soon as possible at refrigeration temperature (4°C). If samples cannot be sent within the first 24 hrs to the laboratory, they should be freezed at -76°C.

10.2.1. Organs

- Weigh about 1g of organ and grind it in a mortar with sand
- Mix with approximately 10 ml of transport medium (SP)
- Transfer to 15 ml centrifuge tubes
- Sonicate the organ mix three times under the standard protocol
- Centrifuge at 500 g at 18°C for 15 min
- Use the supernatant for the cell culture inoculation.
- The inoculum has to be used as quick as possible. It can be preserved at refrigeration temperature overnight.
- Preventive note: It is necessary to make 1/10 dilution of the organ mix as they could be toxic to the cell culture.

10.2.2. Swabs

- Swab (1) swab for chlamydial isolation is transferred to a sterile tube containing 2 ml of transport medium SPG.
- Swab (2) swab for chlamydial detection by PCR is transferred to a sterile tube containing the lysis mix according to the extraction procedure.
- Swab 1 for isolation has to be sonicated according to the standard protocol.
- Mix thoroughly by pipetting up and down and inoculate the cell culture as quick as possible. Otherwise the sample can be kept overnight at refrigeration temperature.

10.2.3. Feces

- Weigh 1 gr approximately of feces and grind it in a mortar with sand
- Mix with approximately 10% of transport medium (SP)
- Centrifuge at 500 g at 18°C for 15 min
- Use the supernatant for the cell culture inoculation.
- The inoculum has to be used as quick as possible. It can be preserved at refrigeration temperature overnight.
- Preventive note: Feces samples are hevily contaminated, it is necessary to add antibiotics. It coul be necessary to filter the sample before the inoculation (0.45 µm pore size filter).

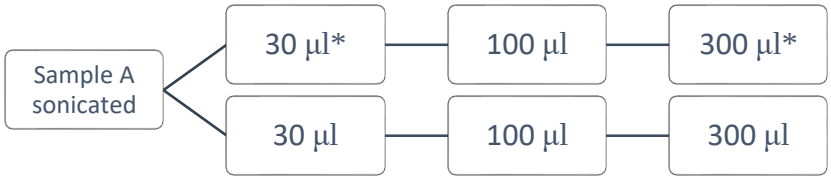
- If they are pork feces samples, it has been found useful to add penicillin G and streptomycin incubating the sample at room temperature during 4 h before inoculation.

10.3. Inoculation

Notes before starting:

a) Prepare the chlamydial cultivation medium as previously mentioned in 9.2 or 9.3 section.
b) Before the cell culture inoculation proceeding, samples must be prior analyzed by PCR to confirm the bacterial load.

Diagram for the first approach:



* Facultative. Depending on the sample volume.

- At least one bijou tube has to be inoculated with 30µl of the sample supernatant. Two bijou tubes have to be inoculated with 100µl of the sample supernatant and at least one bijou tube with 300µl of the sample supernatant.
- Once inoculated, bijou tubes have to be centrifuged at 3,000 x g at 37° for 60 min if they are only bijou tubes, or 2,000 x g at 37° for 60 minutes if they are cell culture flasks.
- Incubate the tubes for 2h at 37 ° C with 5 % CO2, slightly open cap.
- Aspirate the inoculum.
- A washing step could be added, with 1 ml of PBS. Aspirate the PBS.
- Add 1ml of the chlamydial cultivation medium previously prepared and warmed at 37°C.
- Close the cap if the cultivation medium is Ultra MDCK. If there is EMEM medium, incubate with 5 % CO2, slightly open cap.
- Incubate at 37°C.
- Change the cultivation medium 18h after with fresh medium prepared as previously described.
- Contamination of samples could appear at any time, addition of antibiotics is critical.
- After 48h: Evaluate the color and transparency of the supernatant. Evaluate the integrity of the cell monolayer under optical microscope.
- At 48h post infection, first chlamydial identification could be performed from the bijou tube with 100 µl of the inoculum.

Depending on the chlamydial identification analysis:

10.4. Positive test

A passage from the tube with the largest infecting dose could be performed in order to multiply the infection.

- A) Freeze the rest bijou tubes as they are at -80°C overnight (minimum), then, when the bijou tubes with a confluent monolayer are prepared, thawed the bijou tube with the largest infecting dose and proceed with the sonication as previously indicated.
- B) If they are already prepared bijou tubes with a confluent monolayer, direct sonication of the bijou tube could be performed.
- Mix thoroughly by pipetting up and down the cell suspension.
- Use 200µl of the cell suspension and inoculate five bijou tubes or adjust the volume according to the IF results.

- Proceed with the inoculation as previously described.

A PCR test could be carried out in parallel (taking 200µl of the cell suspension).

- If the culture is pure then the strain could be stored.
- When it is possible to count between 5 and 10 inclusions at 200X magnification, it is possible to make a 25cm² flask passage inoculated with 250µl of a jewel tube (this volume could be adjusted).

10.5. Negative test

A passage from the tube with the largest infecting dose could be performed in order to multiply the infection.

- Sonicate the bijou tube (twice) and mix thoroughly by pipetting up and down the cell suspension.
- Use 200µl of the cell suspension and inoculate five bijou tubes
- Proceed with the inoculation as previously described.
- Change the cultivation medium 18h after with fresh medium prepared as previously described.
- After two passages without a positive result, sample is considered negative.

11. Controls

In all culture experiments a positive and negative controls should be carried out in parallel. The infection dose of the chlamydial strain must be previously defined. Infection dose recommended 2.4×10^3 EBE (strain Chlamydia psittaci C 1/97) and a negative control.

ANNEX V. Chlamydial detection by direct immunofluorescence

Materials
Fluorescence microscope (Zeiss Fluovert).
Glass slide, glue (Entellan - Merck); 25 µL precision pipettes, tweezers, cover slides.

Reagents
Acetone, methanol
PBS
Chlamydia IMAGEN Kit (Oxoid)

Sample Preparation
To fix drops or organ impression smears:

- Allow the sample to air dry on a glass slide.
- Fix for 10 min with acetone and allow to dry.

For coverslips in track bottles:

- Aspirate the cell culture medium from the sample.
- Fix with 1 mL of methanol for 10 min.
- Never allow the cells to dry before fixation, there could be a rupture of the inclusions.
- Remove the glass slide with tweezers, allow the methanol to evaporate.
- Glue the glass lamella on a slide with a drop of glue (Entellan).
- Ensure the cell layer must be on top.

Immunostaining

- Add 20 µl immunofluorescence reagent pre-diluted 1:5 in PBS.
- Incubate in a humidity chamber for 15 min at 37°C.
- Rinse glass slides with PBS.
- Roughly dry the glass slides on blotting paper.
- Add a drop of the mounting medium from the IMAGEN kit.
- Covering with a coverglass.
- Observe the mounting with an epi-fluorescence microscope, magnification 20X to 40X (200 to 400).

Results and control

- In all culture experiments a positive control should be performed with a cell culture tube containing a defined infectious dose and a negative control.
- Positive inclusions are mainly apple green, about 300 nm in diameter, surrounded by dark red counter-stained cells.