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Koko Epiphania Barrigah-Benissan

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

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Unité de recherche Inserm U1047 : Virulence Bactérienne et Infections Chroniques

Antiseptiques et plaies du pied diabétique : Évaluation *in vitro* de l'efficacité des antiseptiques sur des modèles de biofilm des plaies chroniques

Présentée par Koko Epiphania BARRIGAH-BENISSAN

Le 15 Décembre 2022

Sous la direction du Pr Albert Sotto

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UNIVERSITÉ
DE MONTPELLIER

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Abréviations

AFNOR : Association Française de Normalisation

CEE : Communauté Économique Européenne

CMEB : Concentration Minimale Éradicatrice de Biofilm

CMI : Concentration Minimale Inhibitrice

CMIB : Concentration Minimale Inhibitrice du Biofilm

EUCAST : European Committee for Antimicrobial Susceptibility Testing

HS : Hypochlorite de Sodium

IWGDF : International Working Group on the Diabetic Foot

PHMB : Polyhexamethylene Biguanide

PRISMA : Preferred Reporting Items for Systematic Review and Meta-Analyses

PVPI : PolyVinylPyrrolidoneIodine

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Résumé

Les plaies chroniques sont un problème majeur de santé qui touche environ 2% de la population mondiale. Leur prévalence est en constante augmentation parallèlement à l'obésité et au vieillissement de la population. Les ulcères de pied chez les patients diabétiques en particulier, voient leur prévalence « exploser » au fil du temps. Le retard de cicatrisation des ulcères de pied chez les patients diabétiques est associé à la présence de biofilm qui peut favoriser la survenue d'infections au sein de ces plaies, et contribuer à l'antibiorésistance. Aussi, l'utilisation d'alternatives aux antibiotiques dans la prise en charge des plaies chroniques est activement documentée. Les antiseptiques pourraient constituer une alternative, notamment dans leur rôle éventuel dans la cicatrisation dirigée. Pourtant actuellement, il existe peu d'études permettant d'affirmer leur efficacité dans la cicatrisation des plaies chroniques et encore moins de les comparer et d'en positionner l'un d'entre eux comme traitement de référence.

Aussi, les travaux effectués pendant ce doctorat ont porté sur l'adaptation de modèles d'étude de biofilms aux antiseptiques et sur l'évaluation des antiseptiques sur du biofilm mono et bi espèces à partir de 2 microorganismes fréquemment isolés dans les ulcères de pied chez les patients diabétiques. *S. aureus* et *P. aeruginosa*. Cette évaluation s'est déclinée en 4 niveaux : d'abord sur les cellules planctoniques, ensuite sur l'inhibition de la formation du biofilm, puis sur la réduction de la charge bactérienne vivante statique d'un biofilm et enfin sur du biofilm débridé dans un milieu dynamique en flux, à l'image de ce qui est fait en pratique clinique

Ces travaux ont permis de démontrer une efficacité de l'hypochlorite de sodium comparativement à l'octénidine et au polyhexaméthylènebiguanide.

Mots clés : Biofilm, Antiseptiques, Plaies de pied diabétiques, *P. aeruginosa*, *S. aureus*,

Abstract

Chronic wounds are a severe public health challenge affecting 2% of the global population. Their prevalence is constantly increasing, along with population aging and obesity. Diabetic foot ulcer (DFU) particularly is constantly rising as the number of people with diabetes mellitus is also rising every year. Wound impairment in DFU is associated with biofilm. Biofilm induces wound infection and promotes antibiotic resistance. Therefore, instead of antibiotics, alternative therapeutic options are crucially needed in DFU treatment.

Antiseptic agents are one of the alternative options in DFU care. However, there are few studies documenting antiseptic efficiency in chronic wound healing and a lack of evidence on recommending one antiseptic over another.

This thesis work focused on biofilm models adaptation for evaluating antiseptic agents on monospecies and bispecies biofilms using clinical isolates frequently isolated from DFU: *S. aureus* and *P. aeruginosa*. Evaluation was proceeded at 3 levels. First on free planktonic bacteria, next on biofilm formation inhibition, then on living bacterial load reduction and finally on a biofilm post debridement in a dynamic flow model, like clinical practice.

These works allowed the identification of sodium hypochlorite as a relevant antibiofilm agents In DFU treatment compared to polyhexamethylenebiguanide and Octenidine.

Keywords: biofilm; Antiseptics; DFU; *P. aeruginosa*, *S. aureus*

I. PARTIE I

GENERALITES

1.1 Les plaies chroniques

La peau normale ou barrière cutanée est composée de 3 couches : l'épiderme, le derme et l'hypoderme (tissu adipeux). La barrière cutanée est le premier et principal mode de défense de l'organisme contre les diverses attaques chimiques, microbiennes, physiques et immunologiques[1].

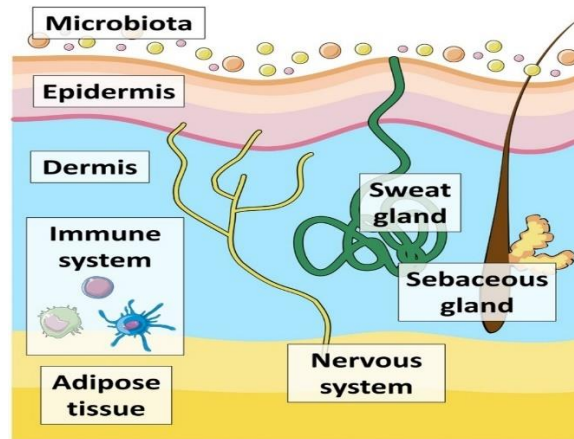


Figure 1 : Architecture de la peau. D'après Kwiecien et al ; Architecture of antimicrobial skin defense.

D'après le dictionnaire Larousse de langue française, une plaie est un nom féminin désignant une déchirure des tissus due à un accident (blessure, brûlure) ou à une intervention chirurgicale [2]. Une blessure quant à elle, est une lésion produite en un point quelconque du corps par un choc, un coup, une arme ou un corps dur quelconque[3]. Les plaies sont aussi définies comme un environnement dynamique dans lequel se produit une interaction complexe entre les tissus de l'hôte et la triade constituée de tissus morts, d'exsudat et de microorganismes[4]. Ces différentes définitions désignent la rupture de la barrière cutanée.

Le processus de guérison biologique des plaies associe chronologiquement les étapes d'hémostase, d'inflammation, de réparation tissulaire du derme, de réduction du

diamètre de la plaie, de régénération de l'épithélium et enfin de cicatrisation[5]. Dans le cadre d'une plaie dite chronique, ce processus de guérison est altéré et découle sur une plaie qui persiste au fil du temps. Le caractère chronique d'une plaie en opposition au caractère aiguë est défini par l'absence de guérison dans un délai de 4 semaines à 3 mois selon le type de plaie[5,6]. La société américaine de cicatrisation des plaies définit 4 principaux types de plaies comme chroniques[7]. Il s'agit des ulcères de pied chez les patients diabétiques, des escarres et des ulcères artériels ou veineux de jambe[7].

La prise en charge des plaies chroniques nécessite des soins récurrents, notamment la cicatrisation dirigée et des séjours hospitaliers prolongés. Il en découle une dépense de soins de santé élevée entraînant une pression financière élevée sur le système de santé[5,8] et une diminution de la qualité de vie des patients atteints de plaie chronique[9]. Les plaies chroniques concourent aussi à une augmentation de la morbidité et de la mortalité des patients qui en sont atteints[10]. Cette situation est d'autant plus préoccupante que la proportion de patients souffrant qui seront affectés par les plaies chroniques au moins une fois au cours de leur vie est en constante augmentation parallèlement à l'augmentation de l'âge de la population [5,9]. Toutefois, une prise en charge à un stade précoce des facteurs de risque peut prévenir la chronicité des plaies ou à défaut favoriser la cicatrisation[5].

1.1.1. Facteurs de risques associés aux plaies chroniques

Plusieurs facteurs de risque peuvent concourir à la chronicité d'une plaie. Il y a avant tout les facteurs socio-économiques : le climat politique, le niveau économique qui peut impacter la formation du corps médical et les pratiques médicales. La connaissance et la mise en application des dernières recommandations par les praticiens en dehors des centres spécialisés de prise en charge des plaies chroniques peuvent être insuffisantes[11,12]. Le diagnostic de plaie chronique, l'orientation vers un spécialiste

réfèrent, la prise en charge des comorbidités peuvent ne pas être optimales[13,14]. Les recommandations de prise en charge des plaies chroniques varient beaucoup d'un pays à un autre[15,16]. Ces recommandations peuvent parfois se contredire, menant à la confusion et la limitation de l'efficacité de la prise en charge [5]. Un autre type de facteurs de risque de la chronicité des plaies est lié au patient. L'impact des comorbidités (obésité, statut nutritionnel altéré, hypertension artérielle, dyslipidémie, diabète sucré, syndrome métabolique, maladies inflammatoires chroniques, insuffisance lymphatique, immunosuppression, facteurs génétiques, etc.) du patient n'est pas négligeable puisque les plaies les plus compliquées à guérir ont été rattachées à des patients avec les comorbidités les plus sévères ou les plus nombreuses[17]. De même, les croyances des patients[18] ainsi que leur adhésion au traitement est primordial pour la guérison des plaies chroniques[19]. Certains facteurs de risque sont aussi inhérents à la pathologie sous-jacente et au type de plaie chronique. Le facteur de risque le plus important de survenue d'un ulcère veineux par exemple est le large spectre de pathologies pouvant conduire à l'insuffisance veineuse[20]. Celui des ulcères artériels des membres est la maladie artérielle qui induit un apport sanguin et oxygéné réduit aux membres[5]. Les escarres quant à eux sont attribués au panel de conditions provoquant une souffrance cutanée sous l'effet de la pression, du cisaillement et du frottement exagéré de la zone atteinte[5]. Les ulcères de pied chez les patients diabétiques sont intrinsèquement liés aux complications du diabète sucré.

1.1.2. Micro-environnement des plaies chroniques

Les plaies chroniques présentent un micro-environnement particulier, favorisant autant qu'il résulte d'une altération des différentes étapes de cicatrisation.

Le pH de la peau saine est acide avec des valeurs variant entre 4 et 6[21,22]. Le pH du microenvironnement des plaies chroniques est plus alcalin (7,3 à 8,9) [23]. Cela

favoriserait la complexité des interactions bactériennes et la formation du biofilm[24]. En comparaison, le pH des plaies aiguës après une courte étape d'alcalinité, récupère des valeurs acides pendant son processus de guérison[25,26]. Ce pH alcalin impacte également la stabilité des antibiotiques et peut réduire leur efficacité [27]. Cette augmentation du pH pourrait être liée à la perte de CO₂ à travers la surface de la plaie[26], à l'activité bactérienne et à la nécrose tissulaire[28,29] ou au retrait des tissus nécrosés via le débridement des plaies[22].

L'augmentation du pH du microenvironnement des plaies chroniques entraîne une diminution du niveau d'oxygène[30]. L'activation de l'immunité est également consommatrice d'oxygène[31]. Les microorganismes du microbiote des plaies aussi[32]. L'angiogenèse des plaies chroniques est déficiente, limitant aussi l'apport en oxygène [33,34]. De plus, les pathologies sous-jacentes des plaies chroniques (problèmes circulatoires, diabète sucré, ...) nuisent à l'oxygénation des tissus [35]. L'association de ces facteurs déplète le microenvironnement des plaies chroniques en oxygène[30]. Pourtant, l'oxygène est nécessaire à la guérison des plaies[31,36].

Si l'on s'intéresse aux macrophages qui sont les cellules immunitaires les plus étudiées dans ce contexte, on remarque que leur activation est retardée, ce qui entraîne une persistance de débris cellulaires, de cellules apoptotiques et de neutrophiles[37]. Cette situation, ajoutée aux facteurs étiologiques des pathologies sous-jacentes module un état inflammatoire chronique[5]. L'étape d'inflammation est persistante jusqu'à l'étape de remodelage, ce qui empêche la guérison de la plaie[38]. Cette inflammation persistante induit une altération de la cascade inflammatoire avec une exacerbation de certains substrats comme les protéases. Le rôle des protéases est de préparer le lit de la plaie à la cicatrisation en dégradant les composants de la matrice extracellulaire de certaines cellules, pour stimuler par exemple les facteurs de croissance des cellules épidermiques, ou stimuler la migration cellulaire[39]. La

surreprésentation des protéases dans le microenvironnement des plaies chroniques induit une dégradation accrue des facteurs de croissance[40] et des matrices extracellulaires. Cela aboutit à une anomalie des interactions et des réponses cellulaires à la base de la migration, de la différenciation et de la prolifération cellulaire [39]. La différenciation cellulaire intensive nécessite un apport conséquent d'oxygène. Elle ne peut se produire dans ce microenvironnement déplété en oxygène [33,34]. La migration des cellules épithéliales nécessaire à régénération de l'épithélium est altérée[41]. Il y a une production excessive d'espèces oxygénoréactives et de protéases, qui endommagent les cellules de l'épiderme[42], altèrent la viabilité cellulaire, inhibent la migration cellulaire et contribuent à l'inflammation persistante[43].

L'environnement humide retrouvé au niveau des plaies, associé à la présence permanente des nutriments rendent ce microenvironnement idéal à la prolifération bactérienne[44]. Les bactéries présentes dans le lit des plaies chroniques interagissent les unes avec les autres[10]. Elles s'organisent majoritairement sous forme de micro-agrégats recouverts par une matrice protectrice, appelés biofilm[37]. La présence de biofilm est corrélée à un retard de cicatrisation des plaies[45]. Le biofilm participe au maintien du microenvironnement des plaies chroniques en étant un médiateur de l'inflammation chronique[46] qu'il stimule à différents niveaux de la cascade inflammatoire[47]. Sa participation au maintien de ce microenvironnement passe aussi par une sénescence accrue des cellules fibroblastiques[48]. L'accroissement de la sénescence des fibroblastes provient également de l'inflammation chronique[5]. Ces fibroblastes à sénescence accrue répondent plus lentement à la stimulation des facteurs de croissance et de fait prolifèrent plus lentement, ce qui retarde la cicatrisation[5].

Somme toute, le microenvironnement créé par la plaie participe au maintien des conditions d'altération de la cicatrisation des plaies.

1.2 Les ulcères de pied de patient diabétiques

Les ulcères de pied chez les patients diabétiques sont le type de plaies chroniques le plus fréquent chez cette population[49]. Ils constituent l'une des complications prédominantes du diabète sucré[50]. Le diabète sucré, groupe hétérogène de troubles métaboliques, est caractérisé par une hyperglycémie résultant d'une anomalie carencielle de l'insuline[50]. L'hyperglycémie diabétique est biologiquement définie par une glycémie supérieure à :

- 1,26 g/L (7 mmol/L) à deux reprises à jeun (G0), ou
- 2 g/L (11,1 mmol/L) à n'importe quel moment de la journée.

Il existe plusieurs types de diabètes sucrés. Le principal est le diabète sucré de type 2, qui représente 90% des diabètes sucrés [51]. Les 10% restant correspondent au diabète sucré de type 1, au diabète sucré gestationnel, aux diabètes sucrés secondaires à une pathologie ou à un traitement médicamenteux et au diabète sucré de type Mody[51]. Le diabète sucré est une maladie à laquelle sont associées plusieurs complications complexes, graves, insidieuses et pour une majorité, chroniques[52]. L'espérance de vie d'un patient diabétique est amputée de 5 à 10 ans[52]. Sa qualité de vie est détériorée par les complications associées au diabète.

1.2.1 Complications associées aux ulcères de pied de patients diabétiques

Les complications associées aux ulcères de pied chez les patients diabétiques peuvent être classées en 2 catégories : les complications aiguës et celles neurodégénératives.

Les complications diabétiques aiguës sont des situations d'urgence vitale[53]. Elles sont de 2 types :

- L'hypoglycémie ;
- Les 2 complications hyperglycémiques : l'acidocétose diabétique et le syndrome d'hyperglycémie hyperosmolaire[53–56].

Les complications diabétiques chroniques correspondent à :

- L'angiopathie (lésions vasculaires)[52] qui est classée en 2 sous-groupes :
 - Les microangiopathies qui touchent les capillaires :
 - De la rétine : rétinopathie diabétique pouvant déboucher sur la cécité ;
 - Des reins : néphropathie diabétique évoluant à terme en une insuffisance rénale.
 - Les macroangiopathies qui touchent les vaisseaux de plus gros calibre. L'athérosclérose accélérée, la cardiopathie ischémique et l'artériopathie oblitérante des membres inférieurs en sont un représentant majeur[57].
- La neuropathie diabétique qui a pour cause l'hyperglycémie constante qui provoque un stress oxydatif des cellules nerveuses[58]. Elle consiste en une lésion des nerfs périphériques, sensitifs, moteurs ou autonomes et est souvent associée aux angiopathies[59]. Elle se traduit cliniquement par une perte de sensibilité du patient au niveau des périphéries.

Une autre complication non négligeable de l'hyperglycémie persistante est l'immunopathie. Chez les patients diabétiques, les fonctions des cellules effectrices de l'immunité comme les phagocytes sont altérées[60].

L'association des macroangiopathies occlusives et ischémiques, de la neuropathie et de l'immunopathie provoque une complication majeure du diabète sucré, les ulcérations de pied chez les patients diabétiques[61].

Le diabète sucré est un problème majeur de santé qui touche globalement en 2021 un patient sur 10 dans le monde, soit 536 millions de personnes[62]. Les projections estimatives ne sont pas rassurantes. En 2045, 12,2 % de la population mondiale soit 783,2 millions de patients seront atteints de diabète sucré[62]. Parmi cette population de patients diabétiques, une proportion de 15 à 25%[63] voire 34%[5] sera au moins une fois au cours de sa vie touchée par des problèmes d'ulcères de pied. 65% des ulcères de pied chez les patients diabétique est persistant après 5 ans[64]. Du fait de la colonisation naturelle des plaies, du dysfonctionnement immunitaire et de la faible perfusion sanguine liés à la pathologie sous-jacente, les ulcères de pied chez les patients diabétiques sont fréquemment sujets aux infections. 50 à 80% des ulcères présenteront une infection[65]. La nature infectieuse de ces ulcères peut varier de l'hypodermite superficielle à une gangrène[61]. L'infection sévère augmente le risque d'amputation[57,65,66]. Le poids des complications infectieuses est tel que leurs préventions et traitements sont intégrés dans la classification et la prise en charge des ulcères de pied chez les patients diabétiques.

1.2.2 Classification des ulcères de pied de patients diabétiques

Le groupe de travail international sur les ulcères de pied chez les patients diabétiques (International Working Group on the Diabetic Foot (IWGDF)) a établi une classification des ulcères de pied en 4 grades selon le statut infectieux[67].

- Le grade 1 correspond à une plaie non infectée et donc sans signes d'inflammation ou de purulence.

- Le grade 2 représente les plaies avec une infection mineure définie par :
 - Au moins 2 manifestations d'inflammation (purulence, érythème, chaleur ou induration), mais sans cellulite (hypodermite) ou extension de l'érythème ou
 - Un diamètre ≤ 2 cm autour de la plaie, et une infection limitée à la peau ou aux tissus subcutanés superficiels avec une absence d'autres complications locales ou de maladies systémiques.
- Le grade 3 regroupe les plaies avec une infection modérée définie par un patient systématiquement en bonne santé mais avec au moins une des caractéristiques cliniques suivants :
 - Cellulite (hypodermite) s'étendant à plus de 2cm ;
 - Stries lymphangitiques s'étendant au-delà du fascia superficiel ;
 - Abscès des tissus profonds ;
 - Gangrène ;
 - Implication du muscle, du tendon, de l'articulation ou de l'os.
- Le grade 4 regroupe les plaies avec une infection sévère ci-après définie : Infection chez un patient avec une toxicité systémique ou une instabilité métabolique (par exemple : fièvre, frissons, tachycardie, hypotension, confusion, vomissement, hyperleucocytose, acidose, hyperglycémie sévère, azotémie)

Cette classification présente également l'avantage d'orienter la décision clinique de la prise en charge[68]. Elle permet de prédire la nécessité d'une hospitalisation[69] ainsi que le risque d'amputation[70].

1.2.3 Prise en charge des ulcères de pied de patients diabétiques

La prise en charge des plaies chroniques, au-delà du traitement de la pathologie initiale sous-jacente est structurée autour de 4 étapes principales. La première étape est le nettoyage ou irrigation de la plaie qui favorise un environnement humide, favorable à la cicatrisation[71]. La deuxième étape consiste en un débridement de la plaie. Ce débridement consiste en l'élimination du tissu mort dévitalisé ou nécrosé autour de la plaie ou dans la plaie jusqu'à exposition du tissu sain [72,73]. La troisième étape consiste en l'apposition d'un pansement adapté au type de plaie. La quatrième étape ne s'applique qu'en cas d'infection avérée. Elle consiste en l'administration d'une antibiothérapie adaptée à l'infection documentée[74].

L'étape de débridement a pour but de réduire la charge microbienne présente au niveau du lit de la plaie. L'écologie microbienne (flore, microbiote) de même que le microbiome des plaies chroniques comme les ulcères de pied chez les patients diabétiques sont des facteurs importants à prendre en compte dans leur prise en charge. Elle participe à la chronicité et à l'infection[23]. L'infection peut significativement impacter la cicatrisation et le traitement des plaies chroniques[72].

1.2.4 Écologie bactérienne des ulcères de pied de patients diabétiques

La peau humaine est naturellement colonisée par des microorganismes qui forment le microbiote cutané[75]. En effet, la peau possède une flore ou microbiote cutanée résidente et une autre, transitoire. Le microbiote résident représente l'ensemble des micro-organismes naturellement implantés sur et dans la couche épidermique au niveau de la couche cornée et des follicules pileux. Elle joue un double rôle protecteur : empêcher l'implantation de microorganismes exogènes et participer à l'équilibre biochimique de cet écosystème[76]. La flore transitoire représente l'ensemble des micro-

organismes présents transitoirement au niveau des couches superficielles de l'épiderme. Ces micro-organismes sont issus des contacts de la peau avec son environnement (surfaces inertes, autres individus, etc.)[76]. De ces microbiotes très diversifiés font partie des pathogènes opportunistes et des bactéries anaérobies dont la présence a été corrélée avec une chronicité des plaies[77,78]. On trouve par ordre décroissant d'abondance numéraire, les genres *Staphylococcus* (avec une prédominance de *S. hominis* et *S. capitis*), *Corynebacteria*, *Propionibacteria*, *Pseudomonas*, *Micrococcus*, *Enhydrobacter*, et *Kocuria*[79]. Les plaies offrent une opportunité aux microorganismes environnementaux et ceux du microbiote de la peau de passer la barrière anatomique cutanée pour se nicher dans les tissus sous-jacents qui présentent des conditions de développement optimales[49,80]. L'analyse de l'écologie microbienne des plaies chroniques montre une dysbiose du microbiome de la peau qui favoriserait les complications infectieuses[81]. Même si le genre *Staphylococcus* est encore majoritaire au niveau du microbiote des plaies chroniques, *Staphylococcus aureus* constitue l'espèce bactérienne prédominante retrouvée dans les cultures de plaies chroniques, particulièrement les ulcères chez les patients diabétiques[9,10,23]. Parmi les espèces fréquemment isolées figurent *Pseudomonas*, *Corynebacterium spp.*, *Streptococcus spp.*, *Enterobacteriaceae*, *Anaerococcus spp.*, et *Enterococcus spp.* [66,82,83]. La présence d'espèces fongiques au niveau du microbiote des plaies chroniques est aussi rapportée dans la littérature avec une majorité du genre *Candida* (*C. albicans*, *C. parapsilosis*)[84,85]. Une perte de diversité du microbiote a également été démontrée au niveau du microbiote des plaies chroniques avec une médiane de 5 espèces [77] dont une à 2 prédominantes[86]. Certaines espèces présentent des particularités. L'augmentation de *P. aeruginosa* dans le microbiote est associée à un allongement du délai de guérison des plaies[87]. La chronicité d'une plaie est également significativement corrélée aux anaérobies facultatives[79].

Toutefois, la difficulté d'une plaie à cicatriser ne saurait être rattachée à un microorganisme en particulier[88]. Elle est modulée par les interactions bactériennes de l'ensemble des microorganismes du microbiote des plaies chroniques[89]. Ces interactions modulent la virulence des différents microorganismes pour affaiblir le système immunitaire[10].

La complexité de ces interactions est favorisée par le microenvironnement des plaies chroniques mais surtout par l'organisation structurale des microorganismes en biofilm qui favorise leur persistance et la chronicité de la plaie[10,90].

1.3 Le Biofilm

Le biofilm est un mode de développement des microorganismes[91]. Le biofilm serait présent dans l'environnement depuis 3,4 milliards d'années[92]. Il est considéré comme une stratégie de survie [44,91] et d'adaptation des microorganismes aux conditions nutritionnelles et environnementales[91,93,94]. Les microorganismes peuvent se développer sous forme planctonique(cellule isolée et libre) ou sous forme sessile (agrégats)[91]. Quand les conditions de développement deviennent hostiles, les microorganismes se développent sous forme de biofilm. Ils adhèrent entre eux et accessoirement à une interface, forment des micro-agrégats. Il est communément admis que ces agrégats ne sont pas composés d'une seule espèce mais de plusieurs espèces organisées sous forme de consortium[91]. Ils produisent en complément une matrice extracellulaire polymérique protectrice[44,91,95–97]. Cette matrice est considérée comme la « maison des cellules du biofilm »[98]. Elle est la composante principale à hauteur de 50 à 90% des biofilms[99]. Elle encapsule les microorganismes et elle établit leur conformation structurale[91].

1.3.1 Développement du biofilm

Le développement des microorganismes sous forme de biofilm peut schématiquement se décrire comme axé autour de 5 principales étapes. Ces étapes ne sont ni chronologiques, ni individuelles. Elles peuvent se chevaucher, faisant de la formation du biofilm, un mécanisme complexe et dynamique [96]. Ces 5 étapes sont :

1. Adsorption,
2. Adhésion,
3. Formation de micro-colonies ou agrégats,
4. Maturation,
5. Dispersion.

L'étape d'adsorption d'une bactérie planctonique à une surface se produit sous l'effet de forces gravitationnelles[100] et d'interactions spécifiques ou non spécifiques entre la surface et la bactérie[101]. Ces interactions sont médiées par des appendices de locomotion : les flagelles et les pili[96]. Ces interactions permettent à la bactérie d'explorer et d'analyser la surface d'adsorption [102], le microenvironnement local de cette surface [103,104] et de s'y adapter à travers une série d'attachement et de détachement successifs[105,106].

Pendant l'étape d'adhésion, l'attachement de la cellule bactérienne à la surface se renforce et se stabilise [107] jusqu'à l'atteinte d'un état d'irréversibilité[106]. Ces deux premières étapes sont déterminantes pour la poursuite du cycle de formation du biofilm[108]. C'est pendant ces étapes que se produit l'adaptation des bactéries avec la cascade de modifications métaboliques [109], biochimiques et régulatrices de la virulence (pour permettre la modulation de la réaction immunitaire de l'hôte) [96,110], nécessaire à un développement en mode biofilm[96].

La troisième étape, qui est celle de croissance et de division cellulaire aboutit à la formation de micro-colonies ou agrégats[91]. Après l'établissement d'une forte adhésion, les cellules bactériennes prolifèrent et se divisent par fission binaire [97]ou division asymétrique[102,111]. La prolifération cellulaire s'amplifie et la surface est colonisée. Il s'en suit une activation de messagers secondaires et une sécrétion initiale de la matrice extracellulaire[112]. Cette matrice initiale fait office de support structurel. Elle permet la communication cellulaire indispensable à la stabilisation des agrégats et à l'organisation du biofilm[113,114]. Une fois une première couche initiale de biofilm formée, plusieurs autres couches s'y rajoutent par l'accumulation des cellules, contribuant à la densité et la conformation 3D du biofilm[115].

La quatrième étape de développement du biofilm est la maturation. Cette étape est modulée par la communication de cellule à cellule encore appelée « Quorum sensing »[116]. Durant cette étape, la structure du biofilm est stabilisée. Les agrégats augmentent de taille et deviennent des macro-agrégats ou « tours »[117]. Le biofilm mature est constitué de macro-agrégats séparés par un réseau de canaux bien défini[96]. Il est majoritairement polymicrobien[96]. Les interactions entre les différentes espèces formant le biofilm sont estimées être en faveur d'une coopération synergique[118]. Cette coopération modulerait la densité du biofilm, la résistance aux antimicrobiens et les facteurs de virulence[119,120]. Elle modulerait également la distribution spatiale inter-espèce (ségrégation[121], mixité[122] ou stratification spatiale[122,123]).

Après l'étape de maturation, le détachement ou la dispersion de portions de biofilm induirait une perte structurelle partielle[96]. Le détachement est un processus passif en réponse à un stress mécanique ou pressionnel[124]. La dispersion est un mécanisme actif interne bien régulé au sein du biofilm[125]. L'impulsion de dispersion peut provenir d'une altération du microenvironnement ou d'un signal interne du biofilm en cas de nécessité de remodelage structurel[96]. Des cellules individuelles ou

des agrégats peuvent être libérées du biofilm à cette étape[126]. Ils sont disponibles pour un nouveau cycle de formation de biofilm.

Les étapes de développement du biofilm apparaissent donc cycliques, complexes et dynamiques. C'est de là que provient toute la difficulté à établir des modèles d'étude pouvant refléter cette complexité.

1.3.2 Modèles d'étude des biofilms

Plusieurs modèles d'étude du biofilm existent actuellement. On peut les classer en 2 types selon l'utilisation de matériel vivant ou non.

Les modèles *in vivo* sont des modèles utilisant des êtres vivants animaux. La formation de biofilm sur les plaies est très rapide, ce qui empêcherait la réalisation d'études pertinentes sur les interactions entre l'organisme humaine et le biofilm[44]. Les modèles vivants sont variés allant de la mouche (*Drosophila melanogaster*)[127] au porc [128] en passant par le lapin[129] ou le rat[130]. Néanmoins, malgré leur onérosité, la transposition des résultats obtenus avec ces modèles à l'humain est très relative, puisque les systèmes immunitaires sont différents[44]. Les modèles *in vivo* reproduisent difficilement le microenvironnement des plaies chroniques[131]. Ils portent souvent sur des animaux sains, sont onéreux, non reproductibles et les études sont longues[96]. Actuellement, aucun modèle *in vivo* d'étude du biofilm n'est en mesure de mimer la combinaison des caractéristiques de la peau humaine, du processus de guérison et de la réponse immunitaire[30].

Les modèles *in vitro* sont des modèles qui n'utilisent pas de sujet vivant (humain ou animal). Ce sont les modèles les plus simples et les moins onéreux [96]. Les modèles *in vitro* sont de 2 types. Les modèles statiques ou fermés sont les premiers modèles qui ont historiquement été utilisés pour étudier le biofilm. Les premiers modèles *in vitro*

statique décrits datent de 1935[132]. Comparativement aux modèles *in vivo*, ils sont moins complexes, moins coûteux, de réalisation plus rapide et reproductibles[44]. L'apport nutritionnel est limité dans les modèles *in vitro* statiques. Il y a également une accumulation de déchets métaboliques[133]. L'absence de modulation de flux dans ces modèles ne permet pas de mimer le flux des fluides vasculaires dans une plaie. La mise en place des modèles *in vitro* statique d'étude du biofilm demande peu de matériel spécialisé[96]. Ils permettent d'étudier certaines étapes ou caractéristiques spécifiques du biofilm, en particulier les premières étapes de formation. Le Biofilm ring Test (BRT)[®] par exemple, permet d'étudier la capacité d'adhésion des souches et par extension la capacité de formation du biofilm (Figure 2)[134].

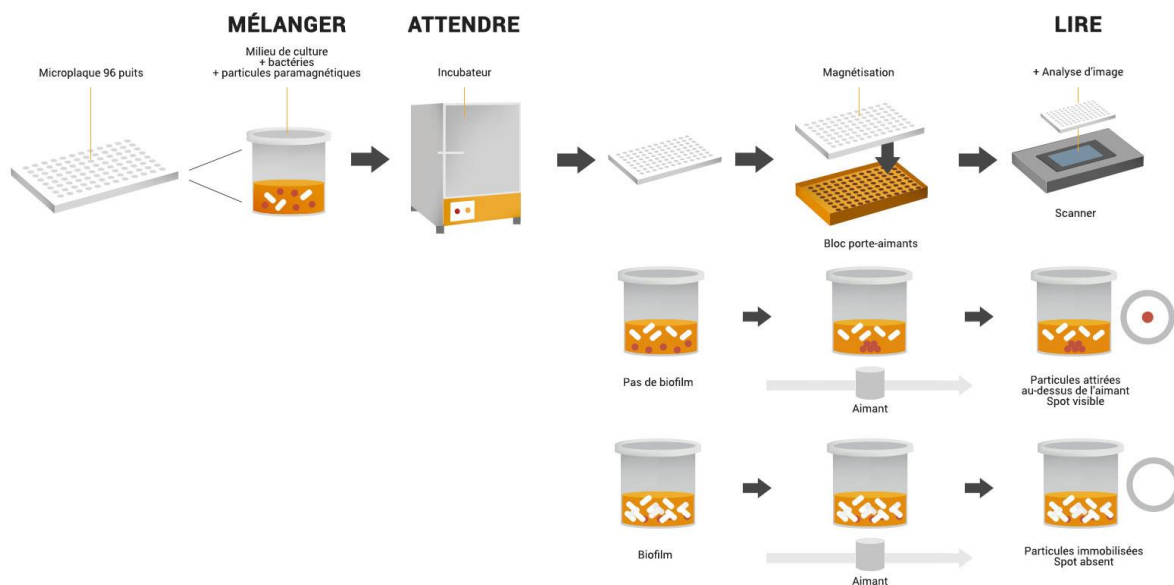


Figure 2 : Principe du Biofilm Ring test[®]

Même si chacun des modèles *in vitro* a ses limites propres, certaines limites sont communes. Globalement la simplicité des modèles *in vitro* statiques d'étude de biofilm les limite à l'étude d'une des étapes de la formation du biofilm. Une autre des limites communes des modèles *in vitro* statiques est l'absence des interactions avec l'hôte et l'inadéquation avec le microenvironnement des plaies chroniques[44,96]. Néanmoins à

cause de ces limitations, les modèles d'étude du biofilm ont peu à peu évolué vers des modèles dynamiques ou ouverts plus complexes. Ces modèles dynamiques, plus récents permettent un apport continu de nutriments, un flux, et des forces de pression ajustables. Même si leur mise en place est plus longue et requiert des équipements spécialisés, ils permettent de reproduire au mieux certaines conditions retrouvées en pratique clinique[131].

1.3.3 Biofilm et ulcères de pied de patients diabétiques

Les infections chroniques sont associés dans 50 à 90% à des biofilms [5,39,45,96,135]. C'est le cas des ulcères de pied chez les patients diabétiques[45].

Plusieurs facteurs concourent à privilégier le développement sous forme de biofilm des microorganismes des ulcères de pied chez les patients diabétiques[136], et par extension, leur chronicité. On peut citer entre autres la diversité du microbiote et le microenvironnement favorable au développement du biofilm[96,136,137]. En outre, le biofilm utilise certaines stratégies pour assurer sa persistance et par la même, la chronicité des plaies [96].

Le développement sous forme de biofilm favorise les interactions bactériennes via le quorum sensing[96]. Les bactéries sessiles à l'intérieur du biofilm peuvent promouvoir une coopération métabolique et une régulation de leurs gènes en faveur de la survie des différentes espèces[136]. Ainsi, un biofilm polymicrobien composé de *P. aeruginosa*, *Enterococcus faecalis*, *Acinetobacter baumannii*, et *S. aureus* s'est révélé beaucoup plus dense et plus difficile à éradiquer que du biofilm mono espèce de ces souches[138].

Le profil transcriptomique de certaines souches de *P. aeruginosa* isolées d'ulcères de pied chez les patients diabétiques est modifié. Les gènes de résistance aux

antibiotiques incluant ceux codants pour les pompes d'efflux sont surexprimés[139]. Il en est de même pour des souches de *S. aureus* qui ont exprimé des changements dans plusieurs voies métaboliques en plus de l'augmentation des gènes codant pour leur virulence[140]. Grâce à la coopération métabolique et la régulation des gènes entre les différents microorganismes le constituant, le biofilm polymicrobien a une capacité remarquable d'adaptation dans différents environnements[136]. Plus généralement, l'expression des gènes de virulence ainsi que les modifications métaboliques contribuent à la persistance du biofilm dans les plaies chroniques[139].

Le biofilm module et échappe au système immunitaire de l'hôte. Il a la capacité de déclencher l'apoptose (mort cellulaire programmée) des tissus humains et des cellules immunitaires[141]. Le biofilm participe à l'inflammation chronique du microenvironnement des plaies chroniques en augmentant la sécrétion des cytokines pro-inflammatoires[142] et des protéases[96]. Cette inflammation est à l'origine d'une dégradation cellulaire accrue qui sert de substrat au cycle de formation du biofilm [96].

Le rôle du biofilm dans le maintien du microenvironnement des plaies chroniques ne se limite pas à l'inflammation. Les bactéries du biofilm consomment aussi de l'oxygène, contribuant ainsi à la déplétion oxygénique qui favorise ce microenvironnement[27,32].

Les bactéries se développant sous forme de biofilm auraient la capacité de ralentir leur développement métabolique, pour augmenter leur survie entraînant ainsi la chronicité du biofilm[30,136]. Aussi, la localisation spatiale en profondeur du biofilm dans une plaie peut affecter sa survie en empêchant l'action des traitements antibiofilm qui auraient du mal à l'atteindre[143].

La matrice extra-cellulaire du biofilm des plaies chroniques a été décrite comme contenant non seulement des composants des microorganismes (exopolysaccharides, protéines, ADN extra-cellulaire, lipides...) mais aussi des composants de l'hôte comme

de la fibrine ou du collagène[144,145]. La matrice extracellulaire peut moduler le microenvironnement pour faciliter l'adaptation des microorganismes à l'intérieur du biofilm[145]. Elle protège également les microorganismes assemblés sous forme de biofilm contre les attaques : celles des prédateurs , les attaques immunitaires des hôtes en cas d'infection[146–148] ou celle des antibiotiques[149,150]. Cette matrice est également supposée jouer un rôle dans la formation du réseau de communication et de signalement du biofilm[151,152].

Les données rattachant le biofilm à la chronicité des plaies sont de plus en plus probantes, motivant les stratégies de prise en charge à intégrer des stratégies antibiofilm[5,7,46,47,71,135]. Ce rôle prépondérant du biofilm dans la chronicité des ulcères du pied chez les patients diabétiques est encore plus préoccupant quand les ulcères s'infectent. L'identification des microorganismes par la culture ne permet pas d'établir le diagnostic d'infection puisque la peau et les plaies sont naturellement colonisées par des microorganismes[153]. La distinction entre colonisation et infection est dès lors délicate, provoquant une utilisation excessive d'antibiotiques chez les patients atteints de plaies chroniques[154]. Cette pratique a fortement contribué à la problématique grandissante d'antibiorésistance[155]. Aussi, dans l'idée de palier l'antibiorésistance et limiter l'utilisation des antibiotiques, plusieurs stratégies alternatives de prise en charge des ulcères de pied chez les patients diabétiques sont envisagées[156,157]. Les antiseptiques sont l'une de ces stratégies[158]. Le rôle des antiseptiques dans la prise en charge des ulcères de pied chez les patients diabétiques n'est pas censé se limiter au traitement des infections.

1.4 Antiseptiques

D'après l'association française de normalisation (AFNOR), les antiseptiques sont des produits ou procédés utilisés pour l'antiseptie dans des conditions définies. Si le produit ou le procédé est sélectif, ceci doit être précisé. Ainsi un antiseptique ayant une action limitée aux champignons est désigné comme antiseptique à action fongicide[161]. Toujours d'après l'AFNOR, l'antiseptie est une opération au résultat momentané permettant, au niveau des tissus vivants, dans la limite de leur tolérance, d'éliminer ou de tuer les micro-organismes, et/ou d'inactiver les virus, en fonction des objectifs fixés. Le résultat de cette opération est limité aux micro-organismes et/ou virus présents au moment de l'opération[161].

Réglementairement, les antiseptiques sont des médicaments (Article L-511-1 du code de la santé publique) et possèdent une Autorisation de Mise sur le Marché (AMM). Certaines substances avec une activité antiseptique rentrent dans le cadre réglementaire des dispositifs médicaux (directives 90/385/CEE relative aux dispositifs médicaux implantables actifs et 93/42/CEE du 14 juin 1993 relative aux dispositifs médicaux).

Selon les définitions européennes ci-dessus, les antiseptiques sont des médicaments. Les dérivés d'argent ne possèdent pas d'AMM dans cette indication et par conséquent ne sont pas des agents antiseptiques[162].

1.4.1 Classification et mode d'action des antiseptiques

Il existe plusieurs familles d'antiseptiques dont les principales utilisées sont :

- La famille des alcools (60 à 90°)
- Les dérivés halogénés avec comme principaux composés utilisés :
 - Hypochlorite de Sodium

- PolyVinylPyrrolidoneIodine (PVPI)
- Les biguanides comme
 - La chlorhexidine
 - Polyhexamethylene Biguanide (PHMB)
- Les ammoniums quaternaires comme
 - L'octénidine
 - Le chlorure ou bromure de benzalkonium
- Les oxydants comme l'eau oxygénée ou le peroxyde d'hydrogène
- Les carbanilides comme l'hexamidine

Ces différentes familles ont différentes activités sur les bactéries comme résumé dans le tableau 1.

Tableau 1 : Exemples de spectre d'activité antimicrobienne pour les principales classes d'antiseptiques dans des conditions d'utilisation prolongée et/ou itérative sur la peau en prenant en compte la concentration du principe actif et le temps de contact d'après le CCLIN Sud-ouest.

Familles d'antiseptiques*	Bactéries végétatives						Fungi	
	Gram +		Gram -		CMN**			
Alcool 70°	BC +	SL	BC +	SL	BC +	FC +	SL	
Halogénés								
PVP-I 10%	BC +++	SL	BC +++	SL	BC ++	FC +++	SL	
D. chlorés 0,5%	BC +++	SL	BC +++	SL	BC ++	FC +++	SL	
Biguanides								
Chlorhexidine 2%	BC +++	SL	BC +++	SI	AN	FC ++	SI	
Ammoniums quaternaires	BC ++	SL	BC ++	SI	AN	FC +	SI	
Oxydants								
Eau oxygénée	BC +	SL	BC +	SL	AN	FC +	SL	

BC : Bactéricide

+++ élevée

++ moyenne

+ faible

AN : Activité nulle

FC : Fongicide

SL : Spectre Large

SI : Spectre Incomplet

* les exemples proposés pour chacune des familles ne sont pas exhaustifs et se réfèrent aux études disponibles.

** CMN : Actinobactéries soit *Corynebacterium*, *Mycobacterium*, *Nocardia*

Plusieurs modes d'actions sont décrits pour l'activité antimicrobienne des antiseptiques. L'activité du PHMB et de l'octénidine par exemple passe par une fixation sur la membrane. Concernant le PHMB, cette fixation est suivie d'une perturbation de l'intégrité de la membrane bactérienne avec une fuite d'enzymes et de cytokines, une condensation chromosomique, l'arrêt de la réplication cellulaire et une entrave aux mécanismes de réparation de l'ADN [163,164]. La finalité est une mort cellulaire par nécrose[165,166]. L'octénidine de son côté après fixation sur la membrane, la désintègre aussi, entraîne une perte de matériel génétique et la lyse cellulaire[167].

Un autre mode d'action décrit est la dégradation des protéines. C'est celui utilisé par la PVPI. La PVPI interagit avec des sites d'insaturation des lipides et oxyde les groupes sulfhydryle. Elle entraîne une dégradation protéique qui interfère avec la formation des groupements d'hydrogènes de l'ADN et des acides aminés des microorganismes. La résultante est l'instabilité du matériel nucléaire et la lyse cellulaire[168].

Un troisième mode d'action est l'oxydation. Il est retrouvé avec le peroxyde d'hydrogène. Ce composé qui est naturellement retrouvé dans les processus de cicatrisation des plaies[169], génère des espèces oxygénoréactives comme les radicaux hydroxyles qui provoquent une peroxydation lipidique qui endommagent l'ADN, induisant une mort cellulaire[169].

Certains antiseptiques associent plusieurs mécanismes d'action. C'est le cas de l'hypochlorite de sodium qui combine la fixation à la membrane, la formation d'espèces oxygénoréactives et la dégradation protéique [170].

1.4.2 Évaluation clinique de l'utilisation des antiseptiques dans la prise en charge des plaies chroniques

Actuellement, malgré plusieurs recommandations qui les positionnent dans cette indication, [8,158,162,171,172], il n'existe pas de consensus européen pour l'utilisation des antiseptiques dans la prise en charge des plaies chroniques. Les antiseptiques ne sont pas recommandés voire sont contre-indiqués dans cette indication (Figure 3). Le sérum physiologique reste le soluté principal recommandé pour l'irrigation des plaies chroniques[74].

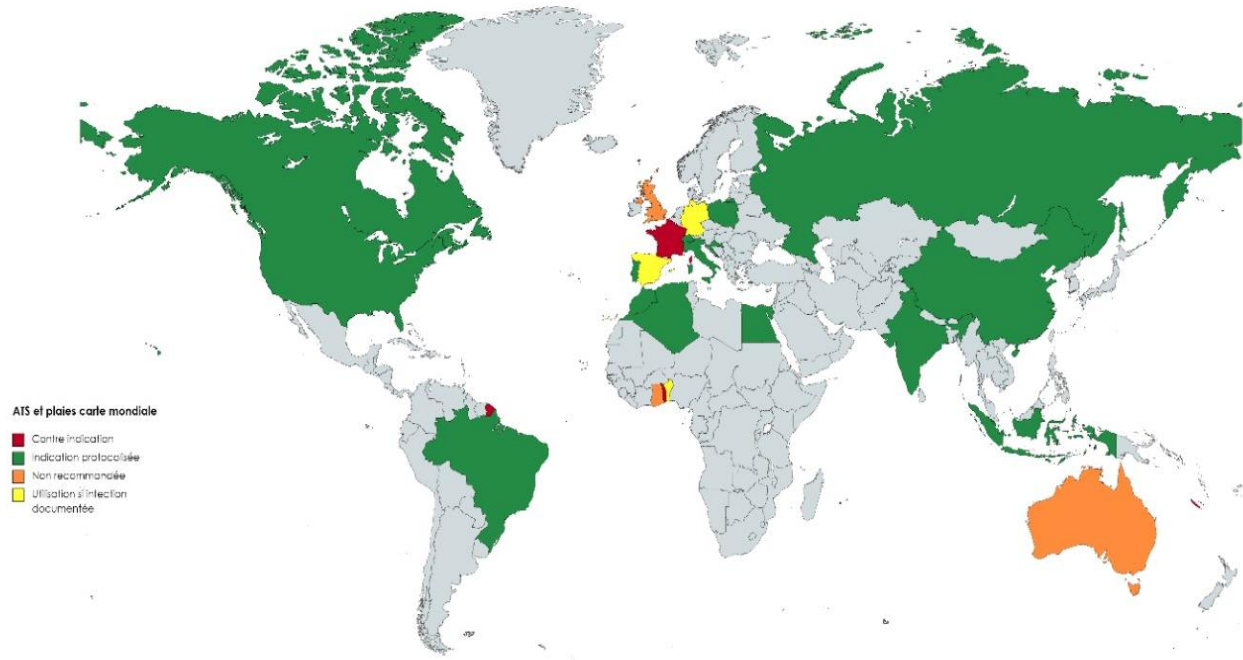


Figure 3 : État des lieux des différentes recommandations concernant l'indication des antiseptiques dans la prise en charge des plaies chroniques. Réalisé avec mapchart.net

Les antiseptiques peuvent être utilisés à l'étape de nettoyage de la plaie pour maintenir le lit de la plaie humide[173]. Ils peuvent aussi compléter le débridement mécanique des plaies chroniques[159]. En effet, le débridement mécanique des plaies chroniques est documenté incomplet pour éradiquer le biofilm, avec 10 à 15% de

biofilm résiduel post débridement [160]. Du fait de leur spectre d'activité, ils peuvent aussi participer à limiter les infections des plaies chroniques.

Plusieurs solutions antiseptiques sont d'ailleurs positionnées comme des composés antibiofilm et des solutions d'irrigation dans la prise en charge des plaies chroniques. Il s'agit notamment de la PVPI, de l'hypochlorite de sodium, du PHMB et de l'octénidine [173–175]. De plus, la littérature est de plus en plus abondante sur l'utilisation des antiseptiques dans la prise en charge des plaies chroniques, notamment à l'international[176–193]. Il nous apparaissait donc important d'évaluer la littérature à ce sujet pour trier les informations et ne retenir que les études cliniques de qualité.

Publication scientifique N°1

**Antiseptic agents for chronic wounds: A
systematic review**

Koko Barrigah-Benissan, Jérôme Ory, Albert Sotto, Florian Salipante,
Jean-Philippe Lavigne, And Paul Loubet

Publication dans « Antibiotics » en mars 2022

Publication scientifique N°1 : Contexte et résumé introductif

Ce travail était introductif du sujet de recherche de la thèse. Les antiseptiques sont contre-indiqués dans la prise en charge des plaies chroniques en France[74]. Cependant, des données internationales mettent en avant leurs avantages pour la cicatrisation des plaies, leur effet antimicrobien, antibiofilm et irrigant des plaies chroniques[158,162,171,172,194]. Aussi, il était nécessaire de faire un état des lieux des données pertinentes de la littérature disponibles sur le sujet. Nous avons donc réalisé une revue systématique de la littérature portant sur toutes les études cliniques ayant évalué l'efficacité des antiseptiques sur les plaies chroniques[74].

La méthodologie de cette revue a été établie en nous basant sur les recommandations du groupe Cochrane[195] et sur la méthodologie PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses)[196]. Une suite de mots clés a été définie pour identifier les articles publiés sur les études cliniques évaluant les 4 antiseptiques recommandés dans la prise en charge des plaies chroniques, à savoir hypochlorite de sodium, PVPI, PHMB et octénidine[171]. Les articles et études ciblés étaient ceux qui ont évalué la guérison clinique des plaies chroniques avec une utilisation de solutions antiseptiques, en comparaison à d'autres solutions antiseptiques ou de sérum physiologique. Nous avons défini la guérison clinique des plaies comme la cicatrisation complète. La population ciblée était les patients de plus de 18ans avec une plaie chronique (ulcère des pieds chez les patients diabétiques, escarres de décubitus, ulcères veineux ou artériels de la jambe). Les plaies de brûlure, les plaies de site opératoire, d'armes à feu ont été exclues au vu de leur prise en charge particulière. En cas d'absence de guérison, nous avons cherché la vitesse de réduction du diamètre des

plaies. Nous avons exclu les études où l'utilisation des antiseptiques était concomitante à celle d'autres traitements ou thérapies avec un effet soupçonné ou avéré dans la guérison des plaies chroniques. Il en fut de même pour les articles rapportant des études où les antiseptiques ou le sérum physiologique n'étaient pas la seule différence entre les 2 groupes de patients comparés dans l'analyse. Accessoirement, nous avons relevé la présence d'effets indésirables comme la douleur à l'utilisation, l'allergie, l'infection des plaies, la charge bactérienne au niveau de la plaie, l'apparition d'odeur. 4 bases de données ont été scrutées : PubMed, Cochrane, google scholar et Embase. Nous avons extrait tous les articles récupérés sur les différentes bases données. Il s'en est suivi une sélection sur 3 critères : le titre, le résumé et la lecture globale. Un contrôle de la sélection a été effectué par un deuxième auteur. Un comité de validation des articles constitué de 3 auteurs a été formé pour la validation et la discussion des articles et études à intégrer dans la revue à la suite de ce contrôle. Nous avons évalué le risque de biais avec l'outil Rob checklist de la deuxième version du guide Cochrane pour les revues systématiques. La qualité des informations rapportées quant à elle, a été évaluée avec le logiciel du système de graduation GRADE (Grading of Recommendations Assessment Development and Evaluation) [197].






Sur 838 études identifiées, 6 portant sur 725 patients ont été retenues, jugées pertinentes et incluses dans l'étude. 3 études portaient sur la PVPI[182,183,185], 2 sur le PHMB[186,198] et une sur l'octénidine[184]. Aucune étude clinique respectant les exigences des critères de sélection n'a pu être extraite sur l'hypochlorite de sodium. De même, aucune étude clinique de qualité n'a comparé les différents antiseptiques entre eux. Les antiseptiques étaient comparés au sérum physiologique dans toutes les études incluses. Les différences de population ont empêché la comparaison par une méta-analyse des antiseptiques entre eux. Des 3 antiseptiques évalués parmi les articles extraits, l'iode était le seul à montrer une activité supérieure au sérum physiologique.

Aucun des 3 antiseptiques évalués n'a présenté plus d'effets indésirables que le sérum physiologique. Aucune des études n'a évalué la présence de biofilm. Les 2 études qui ont évalué le risque infectieux se sont focalisées sur la charge bactérienne. Les études se sont étendues sur 4 à 24 semaines. Elles portaient sur plusieurs types de plaies : les ulcères de pied chez les patient diabétiques, les ulcères veineux de la jambe, et les ulcères artériels de la jambe. Toutes les études étaient multicentriques. Elles se sont déroulées dans 55 centres hospitaliers à travers 8 pays : La France, la Corée du Sud, l'Inde, la Hongrie, le Royaume-Uni, les Etats-Unis, le Canada et L'Italie. Toutes les études ont évalué la vitesse de guérison clinique à travers la vitesse de réduction du diamètre des plaies mais seulement 3 d'entre elles ont rapporté la guérison complète. 4 études ont évalué les effets indésirables. Le risque de biais était élevé pour une étude et incertain sur les 5 autres. Le domaine majeur de source de biais était le secret de l'information sur l'objectif de l'étude. La qualité globale des informations rapportées par l'étude était modérée pour l'iode, élevée pour le PHMB et basse pour l'octénidine.

En conclusion, des 838 études identifiées par nos méthodes de recherche, seules 6 ont été jugées pertinentes selon nos critères de sélection. Cette revue révèle le gap dans la littérature sur l'utilisation des antiseptiques dans la prise en charge des plaies chroniques. Tout cela malgré l'existence de recommandations qui mettent en avant leur utilisation en pratique clinique. Seule l'iode a montré une supériorité face au sérum physiologique dans la guérison des plaies chroniques. Aussi, il est impossible à l'heure actuelle de recommander un antiseptique en première intention dans la prise en charge des plaies chroniques. De meilleures études cliniques portant sur la cicatrisation complète des plaies chroniques avec plusieurs bras de différents antiseptiques sont nécessaires pour tirer cette conclusion.

Review

Antiseptic Agents for Chronic Wounds: A Systematic Review

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Abstract: In many parts of the world, antiseptic agents remain non-indicated in chronic wound care. In the current context of bacterial resistance to antibiotics and the development of new-generation antiseptic agents, wound antiseptics represents an asset for the prevention of wound infection. We aimed to evaluate four common antiseptic agents in chronic wound care complete healing. The review protocol was based on the Cochrane Handbook for Systematic Reviews of Intervention and devised in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) statement guidelines. Five databases and three clinical trials registries were searched from inception to 30 June 2021 without language restrictions. We included randomised trials evaluating the efficacy of antiseptic agents in chronic wound care in adults. Interventions considered were those using antiseptics for cleansing or within a dressing. Risk of bias was assessed using the bias excel tool provided by the Bristol Academy. Evidence quality was assessed using Grading of Recommendation Assessment, Development and Evaluation (GRADE) criteria. Of 838 studies, 6 were finally included, with a total of 725 patients. The included studies assessed iodine (cadexomer or povidone iodine) ($n = 3$), polyhexanide ($n = 2$), and octenidine ($n = 1$). Limited evidence suggested a better wound healing completion with iodine compared to saline (two randomised controlled trials (RCT), 195 patients, pooled RR 1.85 (95%CI (1.27 to 2.69)), moderate-quality evidence). There was not enough evidence to suggest a difference in wound healing using octenidine or polyhexamide. None of the antiseptic agents influenced adverse event occurrence compared to saline.

Keywords: antiseptic agents; efficiency; iodine; systematic review; wound healing; wound infection



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1. Introduction

Chronic wounds are wounds failing to proceed through the normal phases of healing in an orderly and timely manner. The definition of time without complete or partial healing differs across countries, ranging from 4 weeks to 3 months [1]. The Wound Healing Society defines four types of chronic wounds: diabetic foot ulcers (DFU), vascular ulcers (containing venous and arterial ulcers), and pressure ulcers (PU) [2].

Infection is a common complication of chronic wounds. Historically, the research on wound infection control and improvement was focused on reducing the “pathogen burden”. However, quantitative consideration of microbial load is insufficient for assessing wound improvement or wound risk of infection [3,4]. Microorganisms in a chronic wound are co-aggregated together within a protective extracellular matrix, constituting a biofilm. This biofilm conformation induces a dramatically increased tolerance to host immune defences and a greater resistance to antimicrobials [5]. Biofilm delays wound healing by

inducing an ineffective host inflammatory response and damaging host tissues [6]. For this reason, a management of the biofilm is more relevant for the treatment of chronic wound [7]. In 2011, Dissemond et al. classified wounds into four categories, depending on wound bed clinical and microbiological situation: (1) contaminated or colonised wounds without risk of infection; (2) colonised wounds at risk (WAR) or critically colonised wounds; (3) wounds with local infection; and (4) systemic infections and infected wounds. The authors suggested the use of antiseptic agents for wounds from the second category alongside other treatments [8].

Chronic wound care commences with wound bed preparation via (i) wound cleansing to create a wet or moist environment, favourable to healing. (ii) Wound debridement via removal of devitalised, contaminated tissue from within or adjacent to a wound, until surrounding healthy tissue is exposed [9,10]. Debridement can be mechanical (sharp debridement, surgical), enzymatical, or bio-surgical (e.g., maggot therapy) [11–15]. Negative pressure wound therapy has also been used for bacterial decontamination and wound bed preparation [16]. (iii) Application of a suitable dressing, according to the type of wound. (iv) Antibiotic treatment, exclusively for infected wounds. Other therapies are beneficial to specific wounds: compression therapy is required for venous leg ulcers (VLU) [17]; arterial revascularisation, offloading foot ulcers, and diabetes control are essential in DFU [18,19]; and skin assessment and care, offloading and pressure redistribution, dressings [19,20], and structured educational program are useful for all types of chronic wounds.

In some countries, tap water or saline remain the only recommended agents for wound cleansing. Antisepsis is a common, yet controversial, wound cleansing method. Some studies consider debridement alone insufficient to reduce the biofilm that delays wound healing and suggest antiseptics to delay biofilm reformation and reduce the risk of infection [7,20]. Antiseptic agents may complement the debridement process and control infection.

The primary mode of action of antiseptics can be pharmacological, metabolic, and/or immunological [21]. For the purpose of this review, antiseptic agents are defined as medication that can prevent the growth or destroy microorganisms in or on a living tissue. Following this definition of medication, antiseptic agents must pass through a drug authorisation procedure with a medicine agency [22]. The main antiseptic agents used in chronic wound care are halogenated compounds, alcohol-based agents, biguanides (e.g., polyhexanide also called polyhexamethylenebiguanide or PHMB, chlorhexidine), and quaternary ammoniums (e.g., octenidine). Halogenated compounds include subfamilies such as the iodine/iodophor agents (e.g., povidone iodine, cadexomer iodine) and chlorous agents (hypochlorite, hypochlorous acid) [21,22]. Alternative therapeutics (e.g., honey, silver), while been antimicrobial agents are not antiseptic agents as they did not go through an authorisation procedure for this purpose [23]. They therefore are not part of the antiseptic agents' classification. International guidelines recommend against the routine use of topical antiseptics to manage infected chronic wounds [24–27].

The emergence and diffusion of multidrug resistant bacteria and a better formulation of antiseptics with less side effects has renewed consideration of antisepsis. No resistance or adaptation has yet been observed in antiseptic agents with unspecific effects such as iodine agents, polyhexanide, octenidine, or oxidising agents (e.g., hypochlorous acid) [21]. In countries where antiseptic agents are a part of chronic wound care management protocol, there is no consensus on the best antiseptic agents for chronic wound care. Our study aimed to assess the evidence of four common antiseptic agents on chronic wound healing.

2. Results

2.1. Characteristics of the Included Studies

We identified 838 studies including 61 registered clinical trials through database searching and 31 through manual searching. Of these studies, 149 duplicates were removed, and a further 613 studies were excluded by title or abstract. Three studies could not be retrieved and they were withdrawn from the database. We fully screened 73 studies, leaving 6 studies eligible for inclusion (Figure 1) [28–33]. A total of 17 studies were excluded after

full screening: 23 studies met a population exclusion criterion (10 studies were not clinical trials and 13 studies included non-chronic or infected wounds); 21 out of 23 were identified through databases and registers searching and the last 2 were identified via other methods. A total of 18 studies met an intervention exclusion criterion (non-antiseptic agents or unbalanced methodology; 16 were identified by databases or register searching and the last 2 by other methods). Nine studies met a comparison exclusion criterion (no control group), 11 studies were uncompleted or prematurely ended, and six studies were ongoing. The six included studies were published from 1989 to 2020 and included 725 patients [28–33]. No unpublished studies were included.

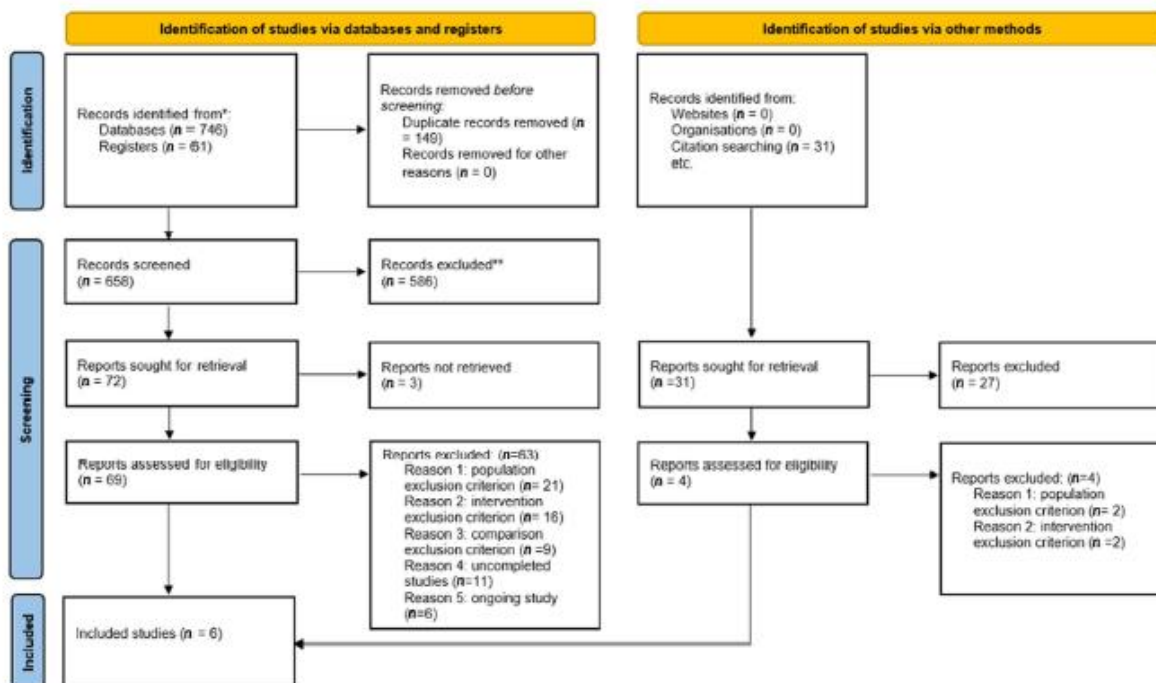


Figure 1. Study flow diagram.

The included studies are listed in Table S1. No studies assessing chlorous agents met our eligibility criteria. Saline was the comparator in all studies. None of the studies compared two or more antiseptic agents. Three studies evaluated the efficacy of iodine (cadexomer iodine or polyvidone iodine) [28,31,32], two studies evaluated polyhexanide [30,33], and one last study evaluated octenidine [29]. Five studies had two comparative groups of patients, and one had three comparative groups [32]. All studies were multicentre, taking place in 55 centres, both inpatient and outpatient settings across South Korea, India, France, Hungary, the UK, the USA, Germany, Canada, and Italy. Different types of chronic wounds were studied: one including DFU only [28]; one including VLU only [29]; two including PU only [30,31]; one including DFU, VLU, and PU [32]; and the last including chronic wounds without precision of the type of the wound [33]. The minimum duration used to define chronic wounds ranged from 4 weeks [28,29] to 3 months [31], although others did not state the definition, but gave the mean duration of the wounds [32], or merely described wounds as “chronic” [33], or only gave the duration of the comorbidity [30]. Study duration ranged from 4 weeks [30,33] to 24 weeks [31].

The seven domains of risk of bias were assessed following the Cochrane recommendation of 2011 [34] and are presented in Table S2. The most common source of bias was outcome assessment blinding (see Table S2). The Risk of Bias tool (2019) released by the

Cochrane collaboration was also used for the risk of bias assessment and presented in Table S3. The overall risk of bias among included studies was rated with some concerns for five studies [28–30,32,33] and high for one [31] (Table S3). A plot of the percentage of risk of bias assessments per domain is presented in Figure S1, as recommended by the Cochrane collaboration tool for risk of bias assessment in randomised trials (2019 version).

Three studies reported the primary outcome (wound healing) as the proportion of patients with complete wound healing at 4 and 8 weeks [29], at 12 weeks [32], and as healing time from wound size [29]. All studies assessed wound healing rate as wound size reduction by planimetry measurement. Three studies assessed pain as a secondary outcome with various pain scales (e.g., verbal rating scales, visual analogue scales) [30,31,33]. Two studies evaluated bacterial bioburden reduction [31,33]. Four studies reported adverse events (AEs) [28,29,31,32]. No AEs were noted in the last two studies [30,33]. Table 1 reports the summary of findings for the primary outcome and Table S4 the different outcomes reported among the studies.

2.2. Iodine vs. Saline (3 Randomised Controlled Trials (RCT), 260 Patients)

Three studies, with a total of 260 patients, compared iodine to saline [28,31,32]. In 1984, Holloway et al. studied the efficacy of cadexomer iodine in 3 months venous stasis ulcers over 24 weeks [31]. The study was rated at high risk of bias. In 2018, Raju et al. also studied the efficacy of cadexomer iodine in the treatment of various 1 month chronic ulcers over 12 weeks [32]. In 2019, Gwak et al. studied the efficacy of an 8 weeks treatment of povidone iodine in patients with DFU with a mean duration of 7 weeks [28]. The risk of bias was unclear for these studies.

Two studies assessed the primary outcome as the proportion of patients with complete wound healing (Table S1) [28,32]. Gwak et al. observed no significant difference in the two groups at 8 weeks (44.4% vs. 44.1%; $p = 0.978$) [28], whereas Raju et al. found a significant complete wound healing for patients treated with two different formulations of cadexomer iodine compared to saline at 12 weeks (61.9% vs. 20%; $p < 0.001$) [32]. The pooled data showed that iodine has a higher percentage of patients achieving complete wound healing (RR 1.85 (95%CI (1.27 to 2.69), $n = 2$ studies, moderate quality evidence) (Table 2). In the last study, the endpoint was the time to complete healing (Table S1) [31]. The authors found no significant difference between the two groups (31.0 days \pm 14.1 for povidone iodine vs. 33.3 \pm 12.6 for saline; $p = 6.54$).

Table 1. Description of included studies.

Study	Country	Date	Setting	Number of Participants	Diagnosis	Main Outcome (Scale Details)	Duration of the Study	Intervention	Comparator
Gwak et al., 2020 [28]	Korea	March 2016–September 2017	Multicentre 10 sites	71	DFU ≥ 1 cm ² post debridement and no clinical signs of infection	Proportion of patients with complete wound healing	8 weeks	PVP-I 44.4% (16/36)	Saline 44.1% (15/35)
Raju et al., 2019 [32]	India	March 2016–March 2017	Multicentre 15 sites	124	Chronic ulcers (single VLU, DFU, PU with adequate arterial blood supply)	Proportion of patients with complete wound healing	12 weeks	Cadexomer iodine ointment: 65.9% (27/41) Cadexomer iodine powder: 58.1% (25/43)	Saline 20% (8/40)
Bellingeri et al., 2016 [30]	Italy	June 2010–December 2013	Multicentre 6 sites	289	PU less than 80 cm ²	Wound improvement measured by BWAT scale tool	4 weeks	PHMB	Saline
Vanscheidt et al., 2011 [29]	Germany, France, Hungary, UK	November 2007–December 2009	Multicentre 15 sites	126	Chronic venous ulcer locally infected	Time to complete wound healing Proportion of patients with complete wound healing	12 weeks	Octenidine 92 days 30.6% (15/49)	Saline 87 days 32.0% (16/50)
Sibbald et al., 2011 [33]	Canada	February 2008–April 2009	Multicentre 2 sites	40	Chronic wounds > 1 cm ²	Healing rate	4 weeks	PHMB 35% reduction in wound surface	Saline 28% reduction in wound surface
Holloway et al., 1989 [31]	USA	NG	Multicentre 3 sites	75	At least a venous stasis ulcer present for a minimum of 3 months	Healing rate	24 weeks	Cadexomer iodine 0.95 cm ² per week	Saline 0.41 cm ² per week

BWAT, Bates Jensen Wound Assessment Tool; DFU, diabetic foot ulcer; NG, not given; PHMB, polyhexamethylenebiguanide; PU, pressure ulcer; PVP-I, povidone-iodine; VLU, venous leg ulcer.

Table 2. Summary of findings table with comparison between iodine and saline solution in chronic wound care.

Comparison One: Iodine Compared to Normal Saline for Chronic Wound Care					
Patient or Population: Chronic Wound Care					
Intervention: Iodine					
Comparison: Normal Saline					
Outcomes	Anticipated Absolute Effects * (95% CI)		Relative Effect (95% CI)	№ of Participants (Studies)	Certainty of the Evidence (GRADE)
	Risk with Normal Saline	Risk with Iodine			
Proportion of patients with complete wound healing assessed with: visual assessment follow up: range 8 weeks to 12 weeks	307 per 1000	567 per 1000 (390 to 824)	RR 1.8478 (1.2706 to 2.6874)	195 (2 RCTs)	⊕⊕⊕⊖ MODERATE ^a
Adverse events assessed with: report follow-up: range 8 weeks to 24 weeks	115 per 1000	166 per 1000 (89 to 308)	RR 1.440 (0.774 to 2.676)	270 (3 RCTs)	⊕⊕⊕⊖ MODERATE ^a
Ulcer healing rate (healing rate) assessed with: planimetry follow-up: range 8 weeks to 24 weeks	Raju et al. [32] found a significantly ($p < 0.001$) higher percentage of reduction for both formulations of iodine (94.3% and 90.4%) compared to saline (67.8%). Holloway et al. [31] found a rate reported to baseline of 0.04 ± 0.01 cm ² /week/cm ² for cadexomer iodine and 0.03 ± 0.01 cm ² /week/cm ² for saline. There was no significant difference ($p = 0.079$). Gwak et al. [28] presented the healing rate with three different visual displays showing the percentage change rate for the length, the width, and the area. They found no difference.			270 (3 RCTs)	⊕○○○ VERY LOW ^{b,c,d}
Pain evaluation (Pain) assessed with: mean rate of change follow up: mean 24 weeks	The mean rate of change in pain scores were -2.44 ± 0.4 for cadexomer iodine and -2.47 ± 0.3 with saline with a $p = 0.96$.			(1 RCT)	⊕⊕○○ LOW ^{d,e}

GRADE Working Group grades of evidence

⊕⊕⊕⊕ **High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect.

⊕⊕⊕ **Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.

⊕⊕ **Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.

⊕ **Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.

* The risk in the intervention group (and 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and 95% CI). CI: confidence interval; RR: risk ratio; RCT: randomised controlled trial. (a) Missing outcome data, around 30% of patient losses during follow-up. (b) One study with a high risk of bias due to methodology risk and both studies at risk to outcome reporting. (c) Different results in the two studies. (d) Selective outcome report. (e) Study with an overall high risk of bias.

Among the three studies, two reported the healing rate (Table 2) [31,32]. Raju et al. presented the healing rate as reduction percentage in ulcer size from baseline to 12 weeks. They observed a significant reduction on wounds treated with both formulations of iodine (94.3% and 90.4%) compared to saline (67.8%) ($p < 0.001$) [32]. Holloway et al. defined the healing rate as the ulcer size reduction per week per baseline size. They noted no significant difference between patients treated with cadexomer iodine versus saline ($0.04 \text{ cm}^2/\text{week}/\text{cm}^2 \pm 0.01$ vs. 0.03 ± 0.01 , respectively; $p = 0.079$) [31]. Finally, Gwak et al. reported three indirect markers of healing rate: the percentage rate of change of wound length, width, and area [28]. They found no difference between the two treatments. The heterogeneity of the data prevented pooling. The quality of the evidence was very low.

Pain was only evaluated in one study [31]. No statistical difference was observed between the two treatments (cadexomer iodine versus saline) ($p = 0.96$) (Table 2). The quality of the evidence was low.

Finally, no studies evaluated the bacterial bioburden.

All three studies reported AEs [28,31,32]. The pooled data showed no significant difference between AE incidence in the iodine group compared to the saline group (RR 1.44 (95%CI 0.77 to 2.68), $n = 3$ studies, moderate quality evidence) (Table 2).

2.3. Polyhexanide Compared to Saline (2 RCT, 334 Patients)

Two studies, with a total of 334 patients, compared polyhexanide to saline [30,33], both with unclear risk of bias. In 2011, Sibbald et al. studied the efficacy of polyhexanide solution in patients with chronic wounds over 4 weeks [33]. In 2016, Bellingeri et al. studied the efficacy of polyhexanide solution in patients with PUs or mixed aetiologies of chronic ulcers, over 4 weeks [30]. Neither study reported wound healing, and thus the primary outcome could not be evaluated.

The healing rate is described in Table 3 [30,33]. Bellingeri et al. evaluated healing rate as wound improvement on the 13 item BWAT scale (Bates Jensen Wound Assessment Tool) [30]. They observed a significantly improved healing rate in the polyhexanide group ($p = 0.072$), and a significantly better score at 4 weeks compared to the first week in the experimental group ($p = 0.025$). Sibbald et al. calculated the percentage decrease of the wound surface area by planimetry measurement and comparison [33]. They noted no significant difference between the median reduction of the wound surface in the polyhexanide group versus the saline group (35% vs. 28%; $p = 0.85$). Due to the heterogeneity of the measurement tools, we could not pool the data. The quality of the evidence was low.

Table 3. Summary of findings table with comparison between polyhexanide and saline solution in chronic wound care.

Comparison Two: Polyhexanide Compared to Saline for Chronic Wound Care					
Patient or Population: Chronic Wound Care					
Intervention: Polyhexanide					
Comparison: Saline					
Outcomes	Anticipated Absolute Effects * (95% CI)		Relative Effect (95% CI)	N of Participants (Studies)	Certainty of the Evidence (GRADE)
	Risk with Saline	Risk with Polyhexanide			
Wound healing follow-up: mean 4 weeks Not measured	Not reported			(0 studies)	-
Adverse events assessed with: report follow-up: mean 4 days	12 per 1000	2 per 1000 (0 to 50)	RR 0.2024 (0.0098 to 4.1813)	334 (2 RCTs)	⊕⊕○○ LOW ^{a,b}
Healing rate assessed with: planimetry follow-up: median 4 weeks	Bellingeri et al. [30] found a significantly better progression of wounds in the polyhexanide group ($p = 0.0025$) using the BWAT wound assessment scale. Sibbald et al. [33] found no significant difference ($p = 0.85$) between the two groups by comparing wound surface reduction (35% vs. 28%).			334 (2 RCTs)	⊕⊕○○ LOW ^{a,b}
Pain assessment assessed with: Pain scales follow-up: mean 4 weeks	Bellingeri et al. [30] found similar pain scores with no significant difference in the two groups (average score = 3 with minimal or no change during follow up). Sibbald et al. [33] reported significant pain reduction in the polyhexanide group compared to the saline control group (73.1% vs. 38.1%; $p = 0.02$).			(2 RCTs)	⊕⊕○○ LOW ^{a,b}

GRADE Working Group grades of evidence

⊕⊕⊕⊕ **High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect.

⊕⊕⊕ **Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.

⊕⊕ **Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.

⊕ **Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.

* The risk in the intervention group (and 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and 95% CI). CI: confidence interval; RR: risk ratio; RCT: randomised controlled trial. (a) Two studies with different results. (b) Very few participants in one study.

Concerning pain, Bellingeri et al. observed no significant difference between groups [30], whereas Sibbald et al. reported a significant pain reduction in the polyhexanide group compared to the saline group (73.1% vs. 38.1%, $p = 0.02$) [33]. We graded the quality of the evidence low.

Finally, Sibbald et al. assessed the reduction of the bacterial bioburden [33]. They noted polymicrobial microorganisms in 5.3% of the polyhexanide group wounds versus 33% in the control group ($p = 0.016$).

Bellingeri et al. reported no AEs in either group [30], but Sibbald et al. recorded two AEs as infection in the saline control group [33]. The pooled data showed a significant difference between the incidence of AEs in the polyhexanide group compared to the saline group (RR 0.2 (95%CI (0.01 to 4.18), $n = 2$ studies, low-quality evidence) (Table 3).

2.4. Octenidine Compared to Saline (1 RCT, 126 Patients)

One study, with 126 patients, compared octenidine to saline [29]. Vanscheidt et al. assessed the efficacy of octenidine in patients with locally infected chronic VLU that was at least 1 month old and who had had no previous or concomitant drug therapy for 12 weeks. Their study was categorised at unclear risk of bias.

Wound healing was assessed as time to complete wound closure and the proportion of patients with complete wound closure (Table S1). The time to complete wound healing was not significantly different between the groups (92 days for octenidine vs. 87 days for saline; $p = 0.952$) (Table 4). Accordingly, the proportion of patients with complete wound healing was similar (30.6% vs. 32%; $p = 0.882$). Interestingly, Vanscheidt et al. reported a significant proportion of healing for patients with ulcers larger than 6 cm² and older than 6 months in the octenidine group versus the saline group (33.3% vs. 0%, respectively $p=0.022$) [29]. We graded the quality of the evidence as high.

Table 4. Summary of findings table with comparison between octenidine and saline solution in chronic wound care.

Comparison Three: Octenidine Compared to Saline for Chronic Wound Care					
Patient or Population: Chronic Wound Care					
Intervention: Octenidine					
Comparison: Saline					
Outcomes	Anticipated Absolute Effects * (95% CI)		Relative Effect (95% CI)	N ^o of Participants (Studies)	Certainty of the Evidence (GRADE)
	Risk with Saline	Risk with Octenidine			
Wound healing assessed with: Proportion of patients with complete wound healing follow-up: mean 12 weeks	242 per 1000	250 per 1000 (136 to 461)	RR 1.0313 (0.5595 to 1.9009)	126 (1 RCT)	⊕⊕⊕⊕ HIGH
Adverse events assessed with: AE report follow-up: mean 12 weeks	317 per 1000	178 per 1000 (90 to 351)	RR 0.5614 (0.2844 to 1.1081)	120 (1 RCT)	⊕⊕⊕⊕ HIGH
Healing rate assessed with: planimetry follow-up: mean 12 weeks	No difference in the healing rate of the patients in the octenidine group, compared to patients in the saline group (37.9% vs. 40.3%; $p = 0.769$) [29].			(1 RCT)	⊕⊕⊕⊕ HIGH
Pain assessment—not measured	Not reported			-	-

GRADE Working Group grades of evidence

⊕⊕⊕⊕ **High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect.

⊕⊕⊕ **Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.

⊕⊕ **Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect.

⊕ **Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect.

* The risk in the intervention group (and 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and 95% CI). CI: confidence interval; RR: risk ratio; RCT: randomised controlled trial.

No difference in the healing rate was noted in the octenidine group compared to the saline group (37.9% vs. 40.3%, $p = 0.769$). The octenidine group achieved better results from 5 weeks. We graded the quality of the evidence high.

The study did not assess pain or bacterial bioburden.

AEs were reported in 10 patients in the octenidine group and 19 patients in the saline group, without significant difference between the groups [29]. We graded the quality of the evidence high.

3. Discussion

We reviewed the RCT evidence for the use of antiseptic agents in chronic wound care in adult patients. Although saline is the main recommended product used in chronic wound cleansing, numerous clinical studies described the benefit of antiseptic agents in this situation [7,17,21,35–37]. A limited number of studies are available on the efficacy of antiseptic agents on chronic wound healing. More limited studies are available on the efficacy of antiseptics on pain. The trials are small, clinically heterogeneous, without clearly defined outcomes, and at high or unclear risk of bias. Of the 838 RCT identified, only 6 studies were included, representing a total of 725 patients.

Our review established a better wound healing with iodine compared to saline (2 RCT, 195 patients, RR 1.85 (1.27 to 2.69)), although the quality of the evidence was moderate. In contrast, no statistical efficacy of octenidine on healing rate (compared to saline) was seen with a high-quality evidence grade (1 RCT, 126 patients RR 1.03 (0.56 to 1.90)). Interestingly, none of the antiseptic agents influenced AE occurrence compared to saline. Notably, over half of the clinical trials have never been published. Most studies had unclear risk of bias, as previously described [38,39].

Of the 838 studies, most of them did not evaluate clinical signs of infection, and mainly focused on bacterial load reduction, a measure long deemed unsuitable [2–4]. Furthermore, the six included studies ignored the effect of biofilm in delaying the healing process. Of the two studies assessing microbiological impact on infection, none assessed biofilm reduction [30,31,33]. For future research, we suggest the use of dynamic models mimicking the wound environment instead of the traditional quantitative microbial load in *in vitro* studies [40]. This includes non-static models and the consideration of multispecies biofilm reduction over a clinically relevant time (>1 month).

Although most guidelines recommend against the use of antiseptic agents [2,4,25,27], a recent consensus suggested using hypochlorite and polyhexanide in chronic wound care [21]. We found no study demonstrating a significant effect of hypochlorite on the healing of chronic wounds. We could not assess the efficacy of polyhexanide due to the heterogeneity of outcomes between studies. However, this consensus included other types of non-healing wounds such as post-surgical or burn wounds and made no distinction between WAR score categories (colonised and infected wounds). Finally, it also included non-randomised trials, which provide lower evidence than RCTs and different systematic bias are encountered [41]. The main limitation of this guideline is the extrapolation of recommendations from various studied wounds to specific chronic wound care. Another key problem is the attribution of effect to antiseptic agents when antibiotics were systematically used in case of infected wounds.

Following the diverse interpretations of study results in recommendations, future investigations in primary research must focus on value to patients and healthcare professionals, particularly treatment choice. The design of future trials should be driven by high-priority questions. Moreover, good practice guidelines must be followed at each step (e.g., design, implementation, reporting). Assessment of complete wound healing instead of wound healing rate would be more relevant, and time to complete wound healing should be reported as the main endpoint. Future research should be controlled at least against saline, and preferably with another or multiple other antiseptic agents. Another fruitful area of research would be the impact of antiseptic agents according to wound size. Two of our included studies on two different antiseptic agents reported increased healing rate for

wounds larger than 6 cm² versus smaller wounds [29,31]. Further good quality evidence studies may aid decision making about the use of topical antiseptics in the management of chronic wounds.

The main limitation of this review was the great heterogeneity in study designs, methodologies, and outcomes. Overall, the six studies included in this review were too heterogeneous for pooling. We could not compare the four antiseptic agents with one another. Sensitivity analysis could not be performed due to the low number of results nor could subgroup analysis by age or type of chronic wound.

Despite its limitations, this review assesses the quality of the available data on the four selected antiseptic agents on chronic wound healing, using a well-known and robust methodology. It focuses the topic on chronic wound, reducing the bias of specific cares required for the other types of wounds.

The relative effects of topical antiseptic treatment on chronic wounds are unclear. There is insufficient evidence to determine the superiority of one antiseptic agent over the others. We could not assess the effect of hypochlorous agents on chronic wound healing. Moderate evidence suggests an improvement of wound healing with iodine compared to saline. Currently, there is not enough evidence to recommend one antiseptic agent over another in this clinical situation.

4. Materials and Methods

4.1. Selection Criteria and Search Strategy

The study is registered at PROSPERO (CRD42020213494).

The review protocol was based on the Cochrane Handbook for Systematic Reviews of Intervention (version 6.2) [42] and devised in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) statement guidelines [43]. A meta-analysis was initially planned. Due to heterogeneity in included studies, a systematic review with a summary of effect estimates was performed.

Published and unpublished RCT were eligible. Two reviewers (K.B.B., J.O.) independently screened titles and abstracts to determine eligibility and assessed the full text of retained articles. Disagreements were solved by discussion or arbitration by three independent reviewers (A.S., P.L., J.P.L.). Exclusion reasons were recorded. This study followed this PICO strategy:

1. **Population:** Our population was adult patients (≥ 18 years) with chronic wounds as previously defined [2]. We included studies from primary, secondary, and tertiary clinical settings. We included different types of chronic wounds (leg ulcer, DFU, PU, eschar). We excluded studies containing patients with wounds requiring specific care (acute wounds, burn wounds, systemic infected wound, postsurgical wounds).
2. **Interventions** of interest were those including antiseptics as cleansing method or within a dressing with at least weekly application.
3. **Comparative** regimens included saline solution or another antiseptic. We anticipated that interventions would consist of povidone-iodine, hypochlorite or hypochlorous acid, iodine, polyhexanide, and octenidine in the form of creams, ointments, powders, sprays, or impregnated into dressings. We included intervention schedules applying concurrent therapies (e.g., negative pressure wound therapy) if the therapy was common across study arms. We excluded (i) interventions where the antiseptic agent was not the only systematic difference between treatment groups; (ii) physical and biological therapies with antimicrobial properties, such as heat or larval therapy; (iii) studies evaluating topical antiseptics in prevention of chronic wounds or those using antiseptics as preparation for surgical treatment of ulcers; (iv) studies evaluating non-recommended antiseptics in chronic wound care and those evaluating antiseptic agents alongside antibiotic agents.
4. **Outcome:** The primary outcome was wound healing, evaluated as the proportion of patients with complete healing during follow-up and/or the time to complete wound healing (analysed using survival, time-to-event approaches). An adjustment

for relevant covariates such as baseline wound area or duration were ideally used to evaluate the outcome. The secondary outcomes were healing rate (described as changes or rate of change in wound size, with adjustment for baseline size); mean pain scores; bacterial bioburden reduction; and AEs, including infection.

We searched PubMed (NLM database), MEDLINE (OvidSP), Web of Science (Thomson Reuters), Google Scholar, and Cochrane library databases, as well as 3 clinical trial registries (ClinicalTrials.gov (www.clinicaltrials.gov, accessed on 17 January 2022)), EU Clinical Trials Register (<https://www.clinicaltrialsregister.eu>, accessed on 17 January 2022), and World Health Organisation (WHO) International Clinical Trial Registry Platform (<https://apps.who.int/trialsearch/>, accessed on 17 January 2022) up to 30 June 2021 without restrictions for language, study status, date of publication, or country, using a MeSH terms string chain (Figure 1). Furthermore, we searched the reference lists of reviewed studies for relevant studies. The search strategy for all databases is presented in Appendix A.1.

4.2. Data Collection and Analysis

One reviewer (K.B.B.) performed data extraction and quality assessment for the included studies, validated by a second author (J.O.). Disagreements were resolved by arbitration by three independent review authors (A.S., P.L., J.P.L.). We contacted study authors for additional data if necessary. We performed data extraction using a standardised sheet, as recommended by the Cochrane Collaboration's handbook for systematic review (trial authors, year of publication, patient population characteristics, duration of follow-up, trial design, measured outcomes, including assessment methods, objectives, results, country where trials were performed, number of participants randomly assigned to each treatment group, clinical setting, detail of interventions in each group, details of comparators in each group, source of funding, number of withdrawals, outcomes). Data are presented in Table S2.

4.3. Risk of Bias and Certainty of the Evidence

Two reviewers (K.B.B., J.O.) independently assessed the risk of bias of eligible studies; any disagreements were resolved by arbitration by three independent review authors (A.S., P.L., J.P.L.). Risk of bias was assessed using the bias excel tool (RoB 2 checklist, 2019) [44] as recommended by the Cochrane Handbook for Systematic Reviews of Intervention (version 6.2) [42]. The overall bias risk was rated as low, moderate, high, or unclear (also some concerns). The 7 domains of bias were also assessed for each trial, following the recommendation of the Cochrane collaboration, 2011 [34].

The overall quality of evidence of included studies was assessed using Grading of Recommendations Assessment, Development and Evaluation (GRADE) as recommended by the Cochrane Handbook for Systematic Reviews of Intervention (version 6.2) [44]. Summary tables for each antiseptic were produced with GRADEPro software considering five outcomes: complete wound healing (healing rate or proportion of patients with complete wound healing), rate of change in wound size, pain assessment, bacterial bioburden reduction, and AEs. We calculated the risk ratio (RR) for dichotomous outcomes (wound healing, AEs, infection) with 95% confidence interval (CI).

4.4. Role of the Funding Source

There was no funding source for this study.

5. Conclusions

Iodine compounds showed a better effect on chronic wound healing compared to saline. Octenidine and polyhexanide did not show any difference in this healing compared to saline. Currently, there is not enough evidence to recommend one antiseptic over another in this clinical situation. Future clinical trials assessing antiseptic agents in chronic wounds management should pay attention to include several antiseptic agents in their trial for comparison, respecting the double-blind trial and with a well-defined study population.

They should establish the main efficiency criteria as complete wound healing and not only wound size reduction. A sub-group analysis based on the size of the wound would be relevant to the matter. Finally, following good practice guidelines is mandatory in every step of trials in order to avoid the numerous biases found in the assessed studies.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/antibiotics11030350/s1>, Figure S1: Plot of the percentage of risk of bias assessments at each level of risk of bias per domain. Table S1: Summary of the included studies. Table S2: Risk of bias assessment among included studies. Table S3: Review authors' judgements about each risk of bias item for each included study. Table S4: Different outcomes reported among the studies.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A Appendix

Appendix A.1 Medline and Cochrane Library Search

Our search strategy basis was to focus on the four main antiseptic agents recommended or used in practice: chlorous compounds, octenidine, iodophor compounds, and polyhexanide. Four different searches were conducted then combined:

- (((((chronic wound) OR diabetic foot) OR leg ulcer) OR pressure ulcer) OR eschar) AND (((iodine) OR povidone iodine OR betadine) OR cadexomer iodine).
- (((((chronic wound) OR diabetic foot) OR leg ulcer) OR pressure ulcer) OR eschar) AND (((hypochlorite) OR hypochlorous) OR Dakin) OR Javel).
- (((((chronic wound) OR diabetic foot) OR leg ulcer) OR pressure ulcer) OR eschar) AND (((PHMB) OR polyhexanide) OR Polyhexamethylene biguanide) OR Polyhexamethylene).
- (((((chronic wound) OR diabetic foot) OR leg ulcer) OR pressure ulcer) OR eschar) AND (Octenidine).

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1.4.3 Évaluation *in vitro* des antiseptiques

Au vu de l'absence de données cliniques permettant d'évaluer l'efficacité des antiseptiques dans la prise en charge des plaies chroniques, il apparaît crucial de se tourner vers les données d'études fondamentales. Ces données proviennent de protocoles de recherche basés sur des modèles de plaies pour cette évaluation. Les modèles *in vitro* de biofilm sont ceux qui ont le plus ciblé le micro-environnement des plaies chroniques[44,96]. Ils apparaissent ainsi comme une bonne alternative pour évaluer la place des antiseptiques dans la prise en charge des plaies chroniques.

Plusieurs travaux récents se sont intéressés aux modèles de plaies et de biofilm pour l'évaluation des composés antimicrobiens[44,88,96,131,199–201]. Ils ont mis en lumière plusieurs limites des modèles expérimentaux actuels qui empêchent la transposition des résultats obtenus avec ces modèles à la pratique clinique[199]. Ces limites portaient sur plusieurs points clé: le milieu utilisé, le nombre d'espèces incluses dans la formation du biofilm, la dynamique des nutriments, la maturation du biofilm et la surface utilisée pour la formation du biofilm.

En effet, le consensus actuel sur le microenvironnement des plaies chroniques comme les ulcères de pied chez les patients diabétiques est que le lit de la plaie est composé de tissus endommagés, de plasma et de cellules rouges[44,144,202]. Une majorité des milieux d'étude comportent présentement des composants non retrouvés dans ce microenvironnement comme l'agar, des protéines de soja, la caséine. Ces substances peuvent interagir avec les composés évalués[203–205]. Les milieux d'étude actuels paraissent donc inadaptés à l'étude des plaies.

De plus, le biofilm de plaies est majoritairement polymicrobien [45,131,143,206] avec *S. aureus* et *P. aeruginosa* étant les deux espèces majoritairement retrouvées. Plusieurs modèles d'étude actuel sont encore monomicrobiens[44,96,131].

Cliniquement, les plaies ne sont pas un environnement statique avec une quantité limitée de substrats nutritifs pour les microorganismes, ni un système fermé avec des forces de compressions. Ces sont des environnements dynamiques avec un apport continu de nutriments sous forme d'exsudats ou de cellules mortes et un léger drainage en fonction de la quantité d'exsudat[44,204]. Il est aussi estimé que l'apport continu et graduel des nutriments permettrait la création de gradients sous formes de plusieurs microenvironnements à l'intérieur du microenvironnement des plaies, ce qui faciliterait la formation d'un biofilm polymicrobien[131].

Une autre limite est la surface d'étude modélisée. Plusieurs modèles actuels utilisent majoritairement des matériaux non humains comme le verre, le silicone, le métal, le polystyrène etc., sans substances provenant du microenvironnement des plaies chroniques[131].

En addition, plusieurs modèles portent sur la recherche de la CMEB qui est la concentration minimale d'éradication du biofilm. Cependant, le microbiote cutané joue un rôle non négligeable dans la cicatrisation des plaies. Il apparaît donc dommageable de l'éradiquer totalement et de délayer la colonisation des plaies. Un paramètre plus intéressant serait la réduction de la charge bactérienne vivante par similitude avec le débridement, puisque le débridement reste indispensable dans la prise en charge des ulcères de pied chez les patients diabétiques. Un autre paramètre pertinent serait la réduction significative et la stabilisation du biofilm.

Un autre problème majeur dans les études de biofilm de plaie chronique est le manque de modèle standardisé pour l'évaluation des composés antimicrobiens. Si l'on

s'intéresse au modèle de Concentration Minimale Inhibitrice (CMI) par exemple, très peu de modèles suivent la méthode standardisée recommandée de l'EUCAST pour évaluer la CMI[137]. De ce fait, même si quelques données existent sur la capacité d'inhibition de la croissance bactérienne par les antiseptiques, la divergence des modèles d'étude utilisés empêche la comparabilité des données.

Plusieurs modèles d'évaluation des propriétés antimicrobiennes des composés portent également, sur du biofilm « jeune » de quelques heures. Toutefois, le biofilm présent cliniquement est mature. Les études de biofilm devraient donc porter également sur du biofilm mature [194].

Enfin, une analyse de la littérature actuelle des études portant sur les modèles *in vitro* d'étude du biofilm et des plaies chroniques fait ressortir le manque d'études portant sur des souches cliniques[207]. Les expérimentations portent majoritairement sur des souches de laboratoire, ce qui limite également la transposition à la clinique.

Malgré toutes ces limites, les modèles *in vitro* d'étude de biofilm restent des modèles pertinents, rapides et peu coûteux d'évaluation de simples interactions (comme par exemple les étapes d'adhésion lors de la formation du biofilm), de l'impact de changement du microenvironnement ou encore l'évaluation rapide de composés antimicrobiens[131]. Il appartient aux futures études de les adapter et de les standardiser pour mimer au mieux le microenvironnement des plaies chroniques et innover des améliorations pour remédier à ces limites.

C'est dans cet ordre d'idées que s'est développé ce projet de thèse avec pour ambition de répondre aux manques de la littérature sur l'évaluation *in vitro* des antiseptiques dans la prise en charge des ulcères de pied chez les patients diabétiques à travers des modèles de biofilm adaptés aux plaies chroniques.

Objectifs généraux des travaux effectués

Les travaux effectués pendant ce doctorat ont pour objectif de répondre en partie aux interrogations concernant l'efficacité des antiseptiques dans la prise en charge des ulcères de pied chez les patients diabétiques. Les principaux enjeux étaient :

- Identifier les antiseptiques pertinents utilisables dans la prise en charge des plaies chroniques
- Déterminer les principales concentrations d'activité des antiseptiques en utilisant une méthodologie standardisée internationale (CMI, EUCAST)
- Identifier les modèles pertinents *in vitro* d'étude du biofilm qui soient représentatifs du microenvironnement des plaies chroniques. Les modèles utilisés devraient également porter sur du biofilm mature, des souches cliniques et des techniques de visualisation appropriées du biofilm.
- Adapter les modèles identifiés pour évaluer l'effet antibiofilm des antiseptiques sur
 - du biofilm monoespèce
 - du biofilm bi espèces
- Comparer les différents antiseptiques entre eux en termes d'efficacité sur la prévention de la formation du biofilm, la réduction d'un biofilm mature et l'impact sur la charge bactérienne.

II. Partie 2 :

EVALUATION DES ANTISEPTIQUES SUR DU BIOFILM MONOESPECE

Publication scientifique N°2

Antibiofilm properties of antiseptic agents used on *Pseudomonas aeruginosa* isolated from diabetic foot ulcers

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en septembre 2022

Contexte et résumé introductif : Publication scientifique N°2

L'ulcération des membres inférieurs chez les patients atteints de diabète sucré est un problème majeur de santé publique. Il est estimé toucher 15 à 25% des patients atteints de diabète sucré au moins une fois au cours de leur vie[6]. Les ulcères de pied chez les patients diabétiques sont naturellement colonisés par des microorganismes dans le lit de la plaie[10]. En plus de cette colonisation, dans environ 60 à 90% des cas, ces microorganismes sont organisés sous forme de biofilm[45,96,206]. Le biofilm est une structure composée d'agrégats de microorganismes entourés d'une capsule protectrice polysaccharidique, qui est estimée favoriser la chronicité des plaies[91,208]. Dans cette idée, les stratégies de prise en charge des ulcères de pied chez les patients diabétiques prennent en compte l'élimination du biofilm[135]. Les antiseptiques font partie de ces stratégies. Bien qu'ils soient de plus en plus mis en avant dans les recommandations de prise en charge des ulcères de pied chez le patient diabétique[162,171,172], il n'existe actuellement pas de consensus au niveau européen concernant leur utilisation. Dans la littérature, 4 agents antiseptiques sont fréquemment cités dans les recommandations de prise en charge des plaies chroniques. Il s'agit de l'hypochlorite de sodium (HS), de la polyvinylpyrrolidoneiodine (PVPI), du polyhexaméthylènebiguanide (PHMB) et de l'octénidine [74,158,162,171,172].

L'objectif de ce travail était d'évaluer les propriétés antibiofilm de 4 antiseptiques fréquemment recommandés dans la prise en charge des ulcères de pied chez les patients atteints de diabète sucré.

Ces premiers travaux ont porté sur des souches de *Pseudomonas aeruginosa*. Cette bactérie est en effet, le pathogène modèle d'étude du biofilm le plus répandu[131] mais également le deuxième le plus fréquemment isolé dans le lit des plaies d'ulcères de pied

chez les patients diabétiques[123,199,209] et les biofilms de *P. aeruginosa* posent de graves problèmes aux patients immunodéprimés[95]. Nous avons utilisé une souche de laboratoire PAO1, ainsi que 3 souches cliniques isolées de débridement d'infections de grade 3 d'ulcères de pied chez les patients diabétiques (PAC1, PAC2 et PAC4). Nous avons dans un premier temps évalué les CMI des antiseptiques sur les souches planctoniques, en utilisant les méthodes standardisées recommandées par l'EUCAST[210]. La capacité des souches cliniques à former du biofilm a été établie avec le BRT® en amont des expérimentations avec les modèles de biofilm. Nous avons utilisé plusieurs modèles de biofilm pour compenser les limites individuelles des différents modèles et avoir une vision exhaustive de l'efficacité de ces antiseptiques à plusieurs étapes importantes de la formation du biofilm. Pour mimer l'étape de formation du biofilm, nous avons utilisé l'Antibiofilmogram® (ATBF®). Basé sur le principe du BRT®, l'ATBF® permet d'évaluer l'impact des antimicrobiens sur la capacité d'adhésion des microorganismes à une surface et par extension leur capacité de formation de biofilm. En pratique, les antiseptiques sont utilisés pour compléter le débridement clinique qui est la principale mesure de réduction de la charge bactérienne du biofilm. Le débridement permet de réduire la charge bactérienne du biofilm de 85 à 90%[7]. Nous avons donc ensuite utilisé un modèle de quantification de la charge bactérienne vivante du biofilm pour estimer l'efficacité des antiseptiques dans la réduction d'un log de 10 de la charge bactérienne vivante d'un biofilm mature de 72h. Nous avons grâce à ce modèle déterminé la concentration d'antiseptique qui permet la réduction de cette charge bactérienne d'un log 10. Enfin pour mimer au mieux les conditions cliniques des ulcères de pied chez les patients diabétiques, nous avons utilisé le modèle dynamique du Bioflux® pour estimer l'activité des antiseptiques sur un biofilm de 72h en conditions dynamiques microfluidiques. La plupart des modèles *in vitro* d'étude du biofilm ont pour limite d'utiliser des milieux d'étude non adaptés à

l'étude des plaies[131]. Pour pallier ce problème, nous avons utilisé le milieu plaie d'acronyme anglais Chronic Wound-like Media (CWM®). Ce milieu mime le microenvironnement des plaies chroniques. Pour représenter au mieux la prise en charge clinique des ulcères de pied chez les patients diabétiques, nous avons mis en place un protocole de débridement automatisé *in vitro* qui permet comme le débridement clinique de réduire le biofilm formé de 80 à 90%.

Avec le modèle d'ATBFG®, les 4 antiseptiques ont démontré une capacité à inhiber la formation du biofilm à des concentrations largement inférieures à la concentration de la solution commerciale. Dans l'ordre, les dilutions requises étaient de 1:8, 1:20, 1:400 et 1:400 pour le HS, la PVPI, Le PHMB et l'octenedine, respectivement. Dans le modèle de quantification de la charge bactérienne vivante, des concentrations faibles ont également induit une réduction de log₁₀ de la charge bactérienne. Ici, 1:3, 1:8, 1:100 and 1:50 pour le HS, la PVPI, Le PHMB et l'octenedine, respectivement. Au cours des expérimentations, le CWM® a confirmé son effet de mime du microenvironnement des plaies avec une formation de biofilm accélérée et un biofilm plus dense comparément au milieu contrôle. Des concentrations d'antiseptique plus élevées furent donc requises pour le même effet de réduction de la charge bactérienne d'un log₁₀ à l'exception de l'hypochlorite de sodium. Les dilutions furent de 1:3 pour le HS, 1:50 pour le PVPI et 1:40 pour le PHMB et l'octénidine. Dans le modèle dynamique, la PVPI n'a pas pu être évaluée du fait de sa couleur très foncée qui a empêché les analyses microscopiques. L'octénidine a présenté un effet souche dépendant. Le PHMB et le HS ont été les antiseptiques les plus efficaces pour réduire significativement le biofilm débridé.

En conclusion, l'hypochlorite de sodium et le PHMB semblent être de bons antiseptiques pour la réduction du biofilm dans la prise en charge des ulcères de pied

chez les patients diabétiques. Des études complémentaires, idéalement cliniques sont néanmoins requises pour confirmer cet effet.



Article

Antibiofilm Properties of Antiseptic Agents Used on *Pseudomonas aeruginosa* Isolated from Diabetic Foot Ulcers

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Abstract: In diabetic foot ulcers (DFUs), biofilm formation is a major challenge that promotes wound chronicity and delays healing. Antiseptics have been proposed to combat biofilms in the management of DFUs. However, there is limited evidence on the activity of these agents against biofilms, and there are questions as to which agents have the best efficiency. Here, we evaluated the antibiofilm activity of sodium hypochlorite, polyvinylpyrrolidoneIodine (PVPI), polyhexamethylenebiguanide (PHMB) and octenidine against *Pseudomonas aeruginosa* strains using static and dynamic systems in a chronic-wound-like medium (CWM) that mimics the chronic wound environment. Using Antibiofilmogram[®], a technology assessing the ability of antiseptics to reduce the initial phase of biofilm formation, we observed the significant activity of antiseptics against biofilm formation by *P. aeruginosa* (at 1:40 to 1:8 dilutions). Moreover, 1:100 to 1:3 dilutions of the different antiseptics reduced mature biofilms formed after 72 h by 10-log, although higher concentrations were needed in CWM (1:40 to 1:2). Finally, in the BioFlux200TM model, after biofilm debridement, sodium hypochlorite and PHMB were the most effective antiseptics. In conclusion, our study showed that among the four antiseptics tested, sodium hypochlorite demonstrated the best antibiofilm activity against *P. aeruginosa* biofilms and represents an alternative in the management of DFUs.

Keywords: antibiofilm; Antibiofilmogram[®]; antiseptic; biofilm; chronic wound; *Pseudomonas aeruginosa*; PHMB; PVPI; octenidine; sodium hypochlorite

1. Introduction

Diabetic foot ulcers (DFUs) in people with diabetes mellitus are a major public health problem. Its prevalence is high, affecting 15–25% of patients with diabetes at least once in their lifetime [1,2]. An infected DFU is a major complication; it is the main reason for diabetes-related hospitalizations and a major cause of amputation [1,3]. DFUs are frequently colonized and then infected by microorganisms organized in biofilms inside the wound bed [4–6]. The biofilm is a structure of microorganism aggregates that participates in the chronicity of the lesion and delays wound healing [6]. The production of an extracellular polymeric substance (EPS) matrix [7] by microorganisms is essential for their adaptation to nutritional and environmental conditions [8]. It also facilitates bacterial protection against the hostile environment [6].

Pseudomonas aeruginosa is a ubiquitous and opportunistic pathogen with a prominent role in many infectious diseases, such as nosocomial urinary tract infections, cystic fibrosis infections or chronic wounds, such as DFU infections [9]. In DFUs, this microorganism is particularly prevalent [10], notably in warm countries, and plays a key role in biofilm organization [9,11,12]. The management of biofilms on DFUs is essential [13] and consists of eliminating the biofilm and disrupting the structural stability of the EPS through extensive

debridement [14]. However, debridement is not sufficient to remove all of the microorganisms present in the wound bed [15,16]. Several additional antibiofilm strategies have been developed to complement the classical management of DFUs to eradicate bacterial biofilms and improve the healing process [17].

Antiseptics are one of the antimicrobial agents that can be used in the antibiofilm DFU strategy due to their anti-infective benefits [13,18–20]. Although the International Working Group on the Diabetic Foot (IWGDF) recommends against using topical antiseptics in DFUs [1], there is evidence that some antiseptics reduce the microbial load of the wounds [21,22]. This reinforces the need for studies to evaluate the value of antiseptics in chronic wounds.

Many biofilm models have been developed to study antimicrobial susceptibility (e.g., in vitro static, in vitro dynamic or even in vivo models) [23,24]. However, none are suitable for evaluating this activity while taking into account the wound microenvironment [23,25]. Most in vitro models generally lack the interaction of the biofilm with the host cells and are not representative of the wound microenvironment [24], whereas in vivo models are subject to inter-individual variation, are expensive and also fail to reproduce the wound microenvironment [24,25]. Recently, we developed a new in vitro medium, the Chronic Wound Medium (CWM), that mimics the microenvironment encountered in chronic wounds [17]. Using this medium, *P. aeruginosa* formed a static biofilm faster and decreased its virulence in a *Caenorhabditis elegans* model after culture in CWM [26]. Moreover, we integrated this medium into a microfluidic system, the BioFlux™ 200, a new and emerging technology adapted to study dynamic biofilm formation and the antibiofilm effects of different compounds [26].

Herein, we assessed the antibiofilm properties of four antiseptic agents (sodium hypochlorite, polyvinylpyrrolidone iodine (PVPI), polyhexamethylene biguanide (PHMB) and octenidine) on clinical *P. aeruginosa* strains isolated from infected DFUs using two in vitro static models and one dynamic model.

2. Results

2.1. Evaluation of the Susceptibility of *P. aeruginosa* Strains to Antiseptics

We evaluated the susceptibility of four strains of *P. aeruginosa* (PAO1 and three clinical strains, PAC1, PAC2 and PAC4) to four compounds (sodium hypochlorite, PHMB, PVPI and octenidine). The MIC and partial biofilm Minimum Inhibitory Concentration (MICb) values of the antiseptics are displayed with their concentrations (10^{-3} g·L⁻¹) and ratios in commercial solutions (V:V) (Table 1). MIC and MICb values were obtained in six replicates for each antiseptic agent for each strain. The MIC values were 1660×10^{-3} g·L⁻¹ (1:3 dilution factor) for sodium hypochlorite, $12,500 \times 10^{-3}$ g·L⁻¹ (1:8 dilution factor) for PVPI, 15.6×10^{-3} g·L⁻¹ (1:64 dilution factor) for PHMB and 7.8×10^{-3} g·L⁻¹ (1:64 dilution factor) for octenidine. All MIC values were lower than the commercial concentrations, regardless of the antiseptic agent. The same trend was observed for MICb values (625×10^{-3} g·L⁻¹ (1:8 dilution factor) for sodium hypochlorite; 5000×10^{-3} g·L⁻¹ (1:20 dilution factor) for PVPI; 25×10^{-3} g·L⁻¹ (1:40 dilution factor) for PHMB; and 12.5×10^{-3} g·L⁻¹ (1:40 dilution factor) for octenidine).

Table 1. Minimum Inhibitory Concentration (MIC) and partial biofilm Minimum Inhibitory Concentration (MICb) values (in 10^{-3} g·L⁻¹) and dilution ratios (in V:V) determined for the reference strain PAO1 and three clinical *P. aeruginosa* strains (PAC1, PAC2 and PAC4) against four antiseptic solutions.

	Sodium Hypochlorite				PVPI				PHMB				Octenidine			
	MIC	V:V	MICb	V:V	MIC	V:V	MICb	V:V	MIC	V:V	MICb	V:V	MIC	V:V	MICb	V:V
PAO1	1660	1:3	625	1:8	12,500	1:8	5000	1:20	15.6	1:64	25	1:40	7.8	1:64	12.5	1:40
PAC1	1660	1:3	625	1:8	12,500	1:8	5000	1:20	15.6	1:64	25	1:40	7.8	1:64	12.5	1:40
PAC2	1660	1:3	625	1:8	12,500	1:8	5000	1:20	15.6	1:64	25	1:40	7.8	1:64	12.5	1:40
PAC4	1660	1:3	625	1:8	12,500	1:8	5000	1:20	15.6	1:64	25	1:40	7.8	1:64	12.5	1:40

Two antiseptic profiles were observed. The first profile, including sodium hypochlorite and PVPI, had MIC_b values that were lower than the MIC values, whereas the second one, including PHMB and octenidine, presented MIC_b values higher than the MIC values.

2.2. Effects of Antiseptics on the Biofilm Living Bacterial Load

We first quantified the bacterial loads of mature *P. aeruginosa* biofilms at 72 h [27]. The strains were cultivated in the reference medium (BHI) and CWM. The results are presented in Figure 1 and Table S1 (see supplementary materials). No statistical difference in the mean living bacterial load was noted when the bacteria were cultivated in the BHI medium ($p = \text{not significant, NS}$). Similarly, no difference was observed when bacteria were cultivated in CWM ($p = \text{NS}$). Interestingly, the mean load value distribution was significantly higher when *P. aeruginosa* strains were cultivated in CWM compared to BHI ($p < 0.01$), demonstrating that CWM conditions favor biofilm formation.

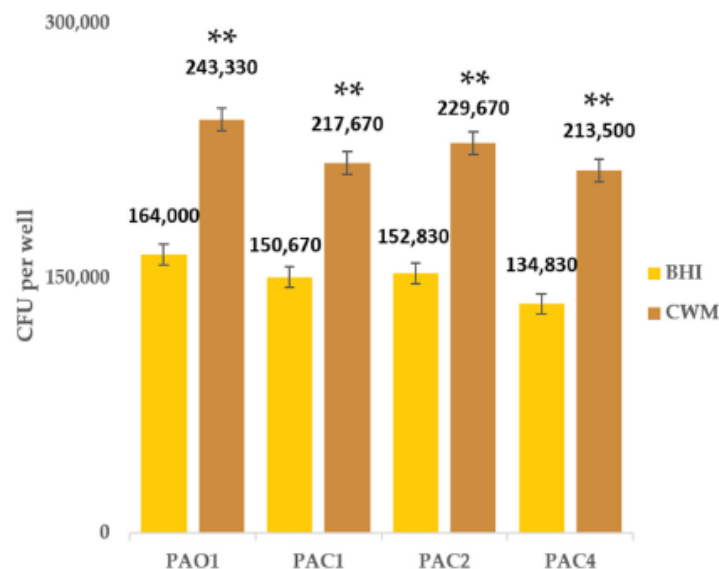


Figure 1. Mean living bacterial load after 72 h biofilm formation by *P. aeruginosa* strains (the reference strain PAO1 and three clinical strains, PAC1, PAC2 and PAC4) cultivated in two media, BHI and CWM. The average CFU per well was calculated from six independent experiments and determined by an automatic counting system. Results are presented as the mean \pm standard deviation. Statistics were performed using a Wilcoxon Rank-Sum test using the R[®] software in its 4.0.2 version to compare the difference in the mean bacterial load of each strain in two culture media. **, $p < 0.01$.

We next evaluated the activity of antiseptic agents on the living bacterial load (Table 2). In practice, antiseptic agents are used to complement the bacterial offloading action of debridement by 10 to 15% [15]. We then determined the antiseptic concentrations needed to obtain a 10-log reduction in the living bacterial load of the studied *P. aeruginosa* strains. The same sodium hypochlorite concentration was needed, regardless of the studied strain and the culture medium used. This concentration was equivalent to the MIC previously determined. For the three other antiseptic agents, higher concentrations were needed to obtain a 10-log reduction in the living bacterial load in CWM compared to BHI. No variations were noted between strains, except for PAC4, for which the concentrations of PHMB and octenidine were higher than those used for the other strains. Interestingly, the concentration of PHMB was lower than the MIC values obtained with the studied strains, whereas the concentration of octenidine was higher.

Table 2. Antiseptic concentrations (in 10^{-3} g·L $^{-1}$) and dilution ratios (in V:V) needed to obtain a 10-log reduction in the living bacterial load of *P. aeruginosa* strains (the reference strain PAO1 and three clinical strains, PAC1, PAC2 and PAC4) cultivated in two media, BHI and CWM.

	Sodium Hypochlorite				PVPI				PHMB				Octenidine			
	BHI	V:V	CWM	V:V	BHI	V:V	CWM	V:V	BHI	V:V	CWM	V:V	BHI	V:V	CWM	V:V
PAO1	1660	1:3	1660	1:3	12,500	1:8	50,000	1:2	10	1:100	20	1:50	10	1:50	12.5	1:40
PAC1	1660	1:3	1660	1:3	12,500	1:8	50,000	1:2	10	1:100	20	1:50	10	1:50	12.5	1:40
PAC2	1660	1:3	1660	1:3	12,500	1:8	50,000	1:2	10	1:100	20	1:50	10	1:50	12.5	1:40
PAC4	1660	1:3	1660	1:3	12,500	1:8	50,000	1:2	10	1:100	25	1:40	10	1:50	25	1:20

2.3. Antiseptic Efficiency after In Vitro Automatized Debridement on a Pre-formed *P. aeruginosa* Biofilm

2.3.1. Evaluation of *P. aeruginosa* Biofilm Formation under Flow Conditions

To evaluate the potential of biofilm formation by the four strains, we determined the percentage of biofilm formed in the BioFlux™ 200 system in the control BHI medium and in CWM [26].

All strains were able to form biofilms and to remain attached under shear force (Table 3). The kinetics of biofilm formation of the reference strain PAO1 showed no difference in the percentage of biofilm formation after 24 h between the two media ($p = NS$), whereas these percentages were significantly different at 48 h ($p < 0.1$) and at 72 h ($p < 0.01$). All three clinical strains demonstrated significantly higher percentages of biofilm formation when bacteria were cultivated in CWM compared to BHI, regardless of the time point (Table 3).

Table 3. Kinetics of biofilm formation by *P. aeruginosa* strains in the BioFlux™ system. The percentages of biofilm formation were determined for the reference strain PAO1 and three clinical strains, PAC2, PAC3 and PAC4, at 24 h, 48 h and 72 h post-incubation in BHI and CWM after six independent experiments. Results are presented as the mean \pm standard deviation. Statistics were performed using a *t*-test in GraphPad Prism version 9.2 to compare the percentage of biofilm formed in BHI and CWM for each time point.

	Percentages of Biofilm Formation								
	At 24 h			At 48 h			At 72 h		
	BHI	CWM	<i>p</i>	BHI	CWM	<i>p</i>	BHI	CWM	<i>p</i>
PAO1	12% \pm 0.3	13% \pm 0.5	NS	38% \pm 0.1	53% \pm 0.3	<0.1	70% \pm 0.1	99% \pm 0.2	<0.01
PAC1	11% \pm 0.4	25% \pm 0.5	<0.01	45% \pm 0.5	64% \pm 0.3	<0.01	69% \pm 0.5	99% \pm 0.2	<0.01
PAC2	14% \pm 0.5	17% \pm 0.3	<0.1	42% \pm 0.4	59% \pm 0.5	<0.01	70% \pm 0.2	98% \pm 0.2	<0.01
PAC4	12% \pm 0.3	15% \pm 0.2	<0.1	42% \pm 0.5	62% \pm 0.2	<0.01	75% \pm 0.5	99% \pm 0.1	<0.01

2.3.2. Antiseptic Efficiency on a Pre-formed *P. aeruginosa* Biofilm under Flow Conditions

To mimic the management of chronic wounds and evaluate antiseptic action in a constituted biofilm, we developed a method for the in vitro automatized debridement of the biofilm inside the BioFlux™ system, as previously validated [26]. After in vitro automatized debridement, we administered the four antiseptic agents at the commercialized concentrations on pre-formed biofilms of all strains in BHI and CWM. The results are presented in Figure 2 and detailed in Table S2 (Supplementary Data).

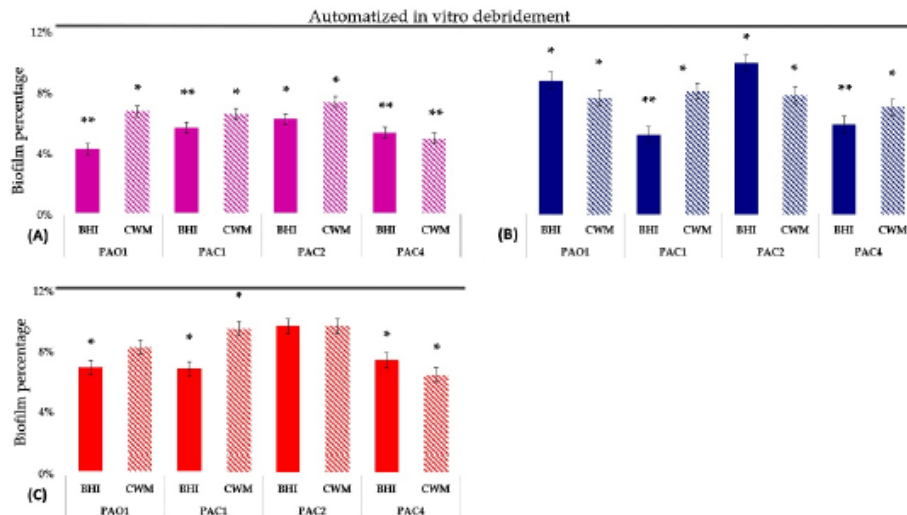


Figure 2. Percentage of biofilm reduction in pre-formed *P. aeruginosa* biofilms using commercialized concentrations of three antiseptics (sodium hypochlorite (A, pink), PHMB (B, blue) and octenidine (C, red)) in BHI and CWM. In vitro automatized debridement left a mean of 12% of biofilm in the microfluidic channel before antiseptic treatment after 72 h of culture. The mean percentage of biofilm after in vitro automatized debridement and before antiseptic treatment is represented by the dashed line. Samples were tested in six independent experiments. Results are presented as the mean \pm standard deviation. Statistics were performed using a t-test using the R[®] software version 4.0.2 to compare the efficiency of antiseptics on biofilms after in vitro automatized debridement. *, $p < 0.1$; **, $p < 0.01$.

After in vitro automatized debridement of the biofilm formed inside the BioFluxTM system, a mean of 12% (see Supplementary Data, Table S2) of bacteria remained in biofilms and were not completely removed to mimic clinical data that estimated the effectiveness of debridement at around 85–90% [16]. We administered the antiseptics at commercial concentrations without dilution. We were unable to test the activity of PVPI, as its color interfered with the MetaVueTM software (Molecular Devices, Sunnyvale, CA, USA).

The use of sodium hypochlorite and PHMB significantly reduced the pre-formed biofilm, regardless of the strain and the medium used ($p < 0.01$) (Figure 2). The residual biofilm percentage was reduced to 5.98% in BHI and 6.8% in CWM for sodium hypochlorite and 8.5% in BHI and 8.54% in CWM for PHMB (see Supplementary Data, Table S2). However, the antiseptic efficiency was better when bacteria were cultivated in BHI than in CWM. The results were further improved when we evaluated the octenidine action, which showed strain-dependent efficiency. The use of this antiseptic significantly reduced the pre-formed biofilms of PAO1, PAC1 and PAC4 cultivated in BHI and PAC1 and PAC4 cultivated in CWM ($p < 0.01$). However, no significant effects were observed when octenidine was administered after the in vitro automatized debridement of the biofilm formed by the PAC2 strain (in CWM and BHI) and PAO1 (in CWM only) ($p = NS$).

3. Discussion

The diagnosis of chronic wound infections remains problematic, leading to the inappropriate use of antibiotics and a high prevalence of multidrug-resistant bacteria carriage [28]. Moreover, commensal and pathogenic bacteria are organized in biofilms, maintaining the chronicity of the lesion and delaying wound healing [7]. New alternative solutions must be developed and evaluated. Our study aimed to assess the antibiofilm properties of four antiseptic agents (sodium hypochlorite, PVPI, PHMB and octenidine) against *P. aeruginosa*

strains, a species frequently isolated in chronic wounds [10] and classified as a strong biofilm producer [29,30]. Moreover, we studied this activity in a medium mimicking the environment encountered in chronic wounds to better evaluate the antiseptic effects.

The MIC values of the four antiseptic agents against our panel of four *P. aeruginosa* strains were always lower than initial commercialized concentrations. Previous studies have evaluated the MIC values of antiseptics on *P. aeruginosa* strains (see Supplementary Data, Table S3).

For sodium hypochlorite, the MIC values obtained in our study (1666 mg·L⁻¹ and 1:3 dilution) are 3- to 5-fold higher than the ones previously obtained [31–35]. This variation may be related to the differences in the studied strains and in the media used, as previously noted [36]. We observed the same trend using PVPI. The MIC was 12,500 mg·L⁻¹ (1:8 dilution factor), higher than the values found in previous studies [37–40]. However, although the values obtained were high, their dilution ratios were low, varying between 0.04% and 5% (1:2 to 1:250 dilution factor) [37–40]. Moreover, we noted no difference in the MIC values of the other antiseptics compared with previous studies, suggesting that the BHI medium was not solely responsible for the results obtained with the two first antiseptics. The MIC values of PHMB and octenidine were 15.6 mg·L⁻¹ and 7.8 mg·L⁻¹, respectively, in accordance with previous studies [33,37,40–43]. These findings support weaker concentrations compared to the commercialized solutions routinely used.

Classically, most studies on antiseptic efficiency determined the Minimal Biofilm Eradication Concentration (MBEC) values to evaluate the potential of antiseptics to eradicate mature and established biofilms. Here, we adapted the Antibiofilmogram[®] test to obtain the MIC_b and assess the antiseptic impact on the biofilm formation kinetics [44,45]. The principle was to use a diagnostic tool derived from the BioFilm Ring Test[®] (BioFilm Control, St Beauzire, France). This test studies early biofilm formation by bacteria. We adapted this method to assess the ability of antiseptics to inhibit early biofilm formation [44]. We determined the MIC_b, reflecting the minimum concentration of antiseptic agents that inhibits biofilm formation. Our MIC_b values were lower than commercial concentrations, with dilution ratios ranging from 1:8 for sodium hypochlorite to 1:40 for PHMB and octenidine. The comparison between MIC_b and MIC values revealed two profiles of antiseptic agents: one profile (for sodium hypochlorite and PVPI) with MIC_b values higher than MIC values, suggesting that biofilm formation was inhibited at lower concentrations than those needed to inhibit bacterial growth. The second profile (for PHMB and octenidine) had MIC_b values lower than MIC values, suggesting that biofilm formation was inhibited by concentrations higher than those needed to inhibit bacterial growth, and a delayed antibiofilm formation effect. This effect is certainly related to faster biofilm formation upon exposure to antiseptic agents rather than a defense mechanism. Thus, higher octenidine and PHMB concentrations are needed to inhibit biofilm formation.

Our results thus highlighted two profiles of the studied antiseptic agents. Sodium hypochlorite and PVPI can complement wound debridement by preventing new biofilm formation without altering bacterial growth. PHMB and octenidine can also be interesting in complementing debridement procedures by coupling antibiofilm formation properties with bacterial growth inhibition properties. For both profiles, at commercial concentrations, all tested antiseptics induced bacterial growth inhibition and biofilm formation inhibition. These findings shine a light on the antimicrobial effects of the antiseptic agents in the early phases of biofilm formation.

We also assessed the effect of the selected antiseptics on the living bacterial load of mature biofilms, which are predominant in chronic wounds [7]. First, we observed that the biofilms formed after 72 h were denser and heavier, and the bacterial load was higher when *P. aeruginosa* strains were cultivated in CWM compared to BHI, as previously reported [26]. This effect was confirmed in the dynamic flow model, BioFluxTM 200, where we also noted the influence of CWM on the development of biofilm formation. Altogether, we demonstrated the influence of the environment on biofilm formation. The decreased antimicrobial activity of antiseptics has been documented in the presence of organic matter,

such as the blood included in CWM [46,47]. Due to the denser biofilm matrix and the effect of the medium mimicking the wound environment, a 10-log higher antiseptic concentration (PVPI, PHMB and octenidine) was necessary to reduce the living bacterial load. Sodium hypochlorite was an exception, with no difference in the MICs needed to obtain this 10-log reduction in biofilms of *P. aeruginosa* cultivated in BHI or CWM. This result could be related to the fact that this antiseptic agent is less affected by the organic load [48].

In a biofilm, antiseptic penetration is reduced with a decreased growth rate of persistent cells [49]. We thus developed a protocol to test the efficiency of antiseptics after in vitro automatized debridement, mimicking a key step in the management of chronic wounds [26]. We observed the efficiency of sodium hypochlorite and PHMB and the strain-dependent activity of octenidine. In clinical practice, 85% of biofilm bacterial load offloading is obtained through debridement, with 10 to 15% of residual biofilm. Our results indicate that sodium hypochlorite can significantly complement wound debridement and reduce this percentage to around 6%, and PHMB can reduce it to 8%. This residual biofilm allows residual microbiota, which participates in DFU healing [50,51]. In addition, this biofilm reduction was effective 72 h after the antiseptic addition. Previous studies demonstrated that adhesion to surfaces for biofilm formation was accelerated in *P. aeruginosa* following their release from biofilms, which happens through debridement [52]. This study highlighted that sodium hypochlorite and PHMB prevented this faster biofilm formation. These results could be due to the mechanism of action of the antiseptic agents. Indeed, for octenidine, this mechanism is directly linked to its interaction with the bacterial membrane [53]. It is possible that the EPS plays a role in biofilm survival and reduces the ability of octenidine to reach the membrane of *P. aeruginosa*. Octenidine activity was particularly reduced when the antiseptic was used against bacteria cultivated in CWM, which enhanced the biofilm density and thickness. Moreover, *P. aeruginosa* was previously reported to adapt to octenidine by membrane remodeling and the production of efflux pumps [54]. Investigating these mechanisms in biofilm models would be a fruitful area for further work. Although the PHMB effect was also linked to its interaction with the bacterial membrane [55], the commercial solution used in our study also included betaine, a compound that disrupts EPS proteins [56], explaining the antibiofilm properties of PHMB. Finally, sodium hypochlorite has multiple mechanisms of action (reactive oxygen species, protein degradation and oxidizing action) with passive diffusion inside the microorganisms [48], suggesting that this antiseptic is an optimal candidate for eliminating bacterial biofilms associated with chronic wounds. Overall, our results reinforce the need to use a dynamic in vitro system and media mimicking the conditions encountered in the clinical situation.

4. Materials and Methods

4.1. Bacterial Strains, Culture Conditions and Antiseptic Agents

The bacteria and media used in this study are listed in Table 4

CWM is a previously described and patented in vitro media model that reproduces the chronic wound environment [17,26]. CWM has a fixed pH of 8, as seen in non-healing wounds [26]. Its composition includes Bolton broth, heat-inactivated human serum, debris of human keratinocytes and hemolyzed human blood.

Four previously characterized *P. aeruginosa* strains were used. PAO1, a reference strain [57,58], and three clinical strains (PAC1 [17], PAC2 [52] and PAC4 [52]) isolated from Grade 3 [59] diabetic foot ulcers of patients at the University Hospital of Nîmes.

Bacteria were grown overnight in bacterial culture tubes with shaking at 200 rpm in aerobic conditions at 37 °C in brain heart infusion (BHI, Sigma-Aldrich, Saint-Quentin-Fallavier, France) broth or CWM (European patent application EP21305337) [17].

Four antiseptic agents were tested for tolerance and available data on their in vitro or clinical efficiency on chronic wounds: (i) sodium hypochlorite, 5 g·L⁻¹ (Dakin®, Cooper, France), (ii) PVPI or Povidone iodine, 100 g·L⁻¹ (Betadine®, Mylan Medical, France), (iii) PHMB, 1 g·L⁻¹ (Prontosan® 0.1% undecylenamidopropyl-betaine and 0.1% PHMB

in aqueous solution; B. Braun Melsungen AG) and (iv) octenidine 0.5 g·L⁻¹ (Octenelin® wound irrigation solution, Schuelke und Mayr, Norderstedt, Germany).

Table 4. Bacterial strains and media used in this study.

Strain	Characteristics	References
PAO1	Reference strain	[57,58]
PAC 1	<i>Pseudomonas aeruginosa</i> clinical strain isolated from a Grade 3 DFI * (patient n°1)	[17]
PAC 2	<i>Pseudomonas aeruginosa</i> clinical strain isolated from a Grade 3 DFI * (patient n°2)	[52]
PAC 4	<i>Pseudomonas aeruginosa</i> clinical strain isolated from a Grade 3 DFI * (patient n°3)	[52]
Media		
BHI	Brain heart infusion	Sigma-Aldrich
LB	Luria–Bertani broth	Sigma-Aldrich
CWM	Patented medium mimicking in vivo conditions encountered in chronic wounds	[17,26]

* DFI, diabetic foot infection.

4.2. Susceptibility Testing Assays

Antimicrobial susceptibility testing (MIC) of isolates was performed by the broth microdilution method on BHI according to EUCAST recommendations (https://www.eucast.org/clinical_breakpoints, accessed on 2 August 2022). Fresh bacterial cultures were used after overnight incubation at 37 °C in aerobic conditions. The range of dilution was 1:2 to 1:2048 for all antiseptic agents. Each antiseptic agent was tested in six independent assays in six replicates for each strain.

Antibiofilmogram® [44,60] is a diagnostic tool using the Biofilm Ring Test® to study antiseptic actions on biofilm formation [61]. Briefly, *P. aeruginosa* strains were subcultured on BHI agar at 37 °C for 24 h. Six colonies were inoculated into BHI broth and homogenized. The bacterial suspension was standardized to an optical density (OD) of 1.00 ± 0.05 at 600 nm and diluted 1:250 in BHI broth to obtain a final concentration of 4×10^6 CFU/mL using a defined calibration curve between OD and CFU/mL. Two hundred microliters of this bacterial suspension, 1% (vol/vol) magnetic beads (TON004) and antiseptics (20 µL of dilutions of each antiseptic) were then incubated, in triplicate, at 37 °C for 4 h (time needed to form *P. aeruginosa* biofilm in this model [17]) in a 96-well microplate (Falcon 96 Flat Bottom Transparent, Corning, United States). The microplate was placed onto a magnetic block for 1 min and analyzed. Due to the color of the antiseptic agent solutions, it was not possible to use a microplate scanner, and we compared the results visually. The MICb was therefore defined as the minimum concentration of the antiseptic agent that inhibited early biofilm formation. This MICb was visually detected as the minimum concentration that allowed brown spot formation at the bottom of the wells. Assays were replicated six times. Four wells without antiseptics filled with the bacterial suspension and magnetic beads were used as the positive control.

4.3. Antiseptic Effect on 72 h Biofilm Living Bacterial Load

The living bacterial load of 72 h-old biofilms before and after the antiseptic agent addition was quantified using an adapted method previously described [27]. Fresh overnight bacterial cultures were adjusted to an OD_{600 nm} of 1 ± 0.05 . The cultures were then diluted at 1:100 and incubated for 72 h at 37 °C without shaking in three flat-bottom 96-well plates for biofilm formation. Fresh culture medium was added every 24 h until 72 h. Wells with medium without bacterial culture were used as the negative control. At 72 h, one plate was used as the control for biofilm formation (C1). In the second plate, several dilutions (1:2 to 1:1028) of each antiseptic agent were added in three replicates. The plates were then incubated for an additional 24 h at 37 °C. The third plate served as a negative antiseptic

effect control (C2), containing only fresh culture medium incubated for an additional 24 h at 37 °C. After incubation, the microplates were washed three times with 200 µL of 1 × PBS. Finally, 200 µL of 1 × PBS was added to the well before biofilm disruption by sonication for 10 min at 40 kHz. Each well was then serially diluted, and the last dilution was plated on LB agar. The agar plates were then incubated overnight at 37 °C, and CFUs were counted to quantify the living bacterial load in each well. We determined the concentration of each antiseptic needed to obtain a 10-log reduction in the living bacterial load, since antiseptic agents are used to complement debridement offloading by 10 to 15%. The experiment was performed six times for each strain.

4.4. Dynamics of Antiseptic Agents on Biofilm

The previously described BioFlux™ 200 microfluidic system (Fluxion Biosciences Inc., Alameda, CA, USA) was adapted to assess the dynamics of biofilm formation and biofilm quantification, along with the dynamics of antiseptic agents in biofilm disruption [62]. This system is composed of microfluidic channels incorporated in 48-well plates. The bottom of the plate is made of a 180 µm glass coverslip, allowing microscopic examination [63]. Thus, the system was coupled with a Leica DM IRB inverted fluorescence microscope for biofilm visualization and a CoolSNAP FX black and white camera (Roper Scientific®, Trenton NJ, United States) for automated image acquisition and recording. Images were processed with MetaVue™ software (Molecular Devices, Sunnyside, CA, USA). Image analysis was processed using ImageJ® software. The analysis included 16-bit grayscale image adjustment with the threshold function, and the biofilm percentage was calculated with the “Analyze particles” function, as previously established [62,64]. Colonies of *P. aeruginosa* strains were resuspended in 3 mL of BHI and were incubated at 37 °C with shaking (220 rpm) overnight. A bacterial suspension was then prepared from this overnight culture standardized to an OD₆₀₀ of 0.1 ± 0.05 following a serial 1:200 dilution. The channel was first primed with 500 µL of medium without bacteria in the inflow well with a pressure setting of 1 dyne/cm² for 10 min. The remaining medium in the well was then withdrawn. The microfluidic channels were then inoculated by injecting the bacterial suspension from the output reservoir for 30 min at 1 dyne/cm². The setup was placed on the heating plate at 37 °C. Finally, the bacterial suspension was added to the inflow well for 72 h with pressure and temperature settings of 0.2 dyne/cm² and 37 °C. Fresh medium was added every 24 h to the input well, and the medium in the output well was discarded. Biofilms were recorded at 24, 48 and 72 h.

To mimic the first step of clinical management of a chronic wound, we developed an in vitro automatized debridement method to remove part of the pre-formed biofilm [26]. After 72 h of biofilm formation, a shear flow of 5 dynes/cm² was applied from the inflow well to the outflow well for 10 min. The flow was then reversed between the inflow and the outflow wells for 10 min. This process of flow reversion was performed twice. The aim of this flow inversion was to significantly reduce the biofilm constituted in the microfluidic channel to 10–15% of the remaining biofilm in the channel to mimic in vivo conditions [16]. After this in vitro automatized debridement, a dynamic flow (0.2 dyne/cm² at 37 °C for 24 h) of antiseptics was applied to evaluate their actions on the remaining pre-formed biofilm. The residual biofilm percentage after antiseptic contact was calculated by dividing the biofilm percentage after antiseptic contact by the biofilm percentage before antiseptic contact *100. The concentrations of antiseptics used corresponded to the commercialized compounds at their pure concentrations.

4.5. Statistical Analysis

All statistical analyses were carried out using the R® software version 4.0.2. A Wilcoxon Rank-Sum test was used to analyze the distribution of the living bacterial load results and assess the residual biofilm in the dynamic model.

5. Conclusions

In chronic wounds, pathological biofilms formed by *P. aeruginosa* are particularly prevalent and difficult to treat. Indeed, new therapeutic strategies are needed, and the determination of the in vitro efficiency against pre-formed biofilms is a great first step in the discovery of methods for the future management of these infections. Our study highlighted that sodium hypochlorite was the most effective antiseptic against biofilms formed by *P. aeruginosa*. This compound was active against these bacteria at concentrations lower than the commercialized one. It presented a MIC_b lower than MIC (1:8 vs. 1:3), demonstrating its activity against biofilm formation. Moreover, at this concentration, it reduced the living *P. aeruginosa* load formed in biofilms by 10-log, suggesting that there was no need to increase the antiseptic concentration to reduce mature biofilms. Finally, the use of sodium hypochlorite significantly reduced the biofilms after in vitro automatized debridement. Clinical studies are now necessary to confirm the results of this study, demonstrating that antiseptics are an interesting alternative solution for the management of DFUs.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms231911270/s1>.

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Publication scientifique N°3 :

**Antiseptic effectiveness in reducing biofilm
from *Staphylococcus aureus* strains isolated from
diabetic foot ulcer**

Koko Barrigah-Benissan, Jérôme Ory, Catherine Dunyach-Remy, Alex
Yiahiaoui Martinez, Jean-Philippe Lavigne, and Albert Sotto

Soumission en cours au journal « Pharmaceutics »

Publication scientifique N°3 : contexte et résumé introductif

Après l'évaluation des propriétés antibiofilm des 4 antiseptiques (HS, PHMB, PVPI et Octénidine) sur le biofilm de *Pseudomonas aeruginosa*, nous nous sommes ensuite intéressés à ces mêmes propriétés sur le biofilm de *Staphylococcus aureus*. Cette bactérie est en effet le microorganisme le plus fréquemment associé aux ulcères de pied chez les patients diabétiques[8,9].

Nous avons utilisé 4 souches de *S. aureus*. La souche de laboratoire Newmann, ainsi que 3 souches issues de prélèvements cliniques de débridement de plaies infectées à un grade 3 de pied chez les patients diabétiques : SAC1, SAC2 et SAC3. Nous avons utilisé la même stratégie de modèles multiples pour évaluer exhaustivement l'impact des antiseptiques à différentes étapes de la vie du biofilm : l'ATBFG® pour évaluer l'impact des antiseptiques sur la formation du biofilm, le modèle de dénombrement de la charge bactérienne vivante du biofilm pour évaluer la capacité des antiseptiques à réduire d'un log10 la charge bactérienne vivante d'un biofilm et enfin le modèle dynamique en flux, Bioflux® pour évaluer la capacité des antiseptiques à réduire significativement le biofilm après débridement. Nous avons également associé les modèles de biofilm au milieu plaie (CWM®) pour mimer le microenvironnement des plaies chroniques. Nous avons enfin après exposition du biofilm pendant 72h aux antiseptiques à l'intérieur du Bioflux®, effectué un nouveau débridement pour récupérer des souches sessiles libérées du biofilm. Nous avons estimé à nouveau les CMI des antiseptiques pour ces souches sessiles libérées pour évaluer une tolérance précoce aux antiseptiques.

Comme avec *P. aeruginosa*, les 4 antiseptiques ont démontré via le modèle du BRT® un effet inhibiteur sur la formation du biofilm de *S. aureus* à des concentrations très inférieures à celles des solutions commercialisées. En termes de dilution, ces concentrations étaient de 1 :20 pour l'hypochlorite de sodium, 1 :100 pour la PVPI et 1 :400 pour le PHMB et l'octénidine. Sur le modèle de dénombrement de la charge bactérienne, des dilutions de 1 :3 pour l'hypochlorite de sodium, 1 :8 pour la PVPI et 1 :100 pour le PHMB et l'octénidine ont été requises pour réduire d'un log10 la charge bactérienne vivante du biofilm monoespèce de *S. aureus*. Avec le modèle dynamique en flux, l'octénidine a été l'antiseptique avec le moins d'effet. L'octénidine a uniquement réduit significativement le biofilm de la souche de référence. L'effet du PHMB était limité à 2 souches cliniques. L'hypochlorite de sodium a été l'antiseptique le plus efficace pour réduire significativement le biofilm après le débridement *in vitro*, sur le biofilm des 4 souches utilisées. L'utilisation du milieu plaie a révélé une formation de biofilm facilitée ainsi qu'un biofilm plus dense. Cela a impacté tous les modèles par la nécessité de concentrations d'antiseptiques plus élevées pour atteindre le même effet antibiofilm par rapport au milieu contrôle, le BHI. Le seul antiseptique qui n'a pas été impacté par le milieu plaie était l'hypochlorite de sodium. A sa CMI, l'hypochlorite de sodium a eu un effet antibiofilm aussi bien sur la formation de biofilm, sur la réduction de la charge bactérienne d'un log10 mais également sur la réduction significative du biofilm post débridement *in vitro*. Après exposition de 72h, la CMI des antiseptiques pour inhiber la formation des souches sessiles libérées du biofilm était identique à celle des souches planctoniques initiales, supposant une absence de tolérance.

L'hypochlorite de sodium semble être le candidat idéal pour réduire la formation de biofilm et contenir la progression du biofilm post débridement. Ces résultats sont à confirmer par des évaluations sur du biofilm polymicrobien et bien évidemment sur des études cliniques randomisées de qualité.

Article

Antiseptics effectiveness in reducing *Staphylococcus aureus* biofilm

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Abstract: DFU is a major public health issue with severe complications. During DFU treatment, the main complication is biofilm formation. Biofilm is associated with wound chronicity and antibiotic resistance. Antiseptic agents are recommended in DFU treatment strategy to address biofilm and reduce antibiotic resistance. There is a lack of studies comparing antiseptic agents. We evaluated 4 antiseptics (Sodium Hypochlorite, Polyvinylpyrrolidone iodine (PVPI); PolyHexaMethyleneBiguanide (PHMB) and Octenidine) using multiple biofilm models. Biofilm was formed from one reference and 3 DFU isolated *S. aureus* strains. *Staphylococcus aureus* is the most prevalent microorganism in Diabetic Foot Ulcer (DFU). Antiseptic efficacy against biofilm formation and mature biofilm were evaluated using the Biofilm Ring Test (BRT) ® and a quantitative biofilm reduction test. The Bioflux ® model was adapted for evaluating antiseptics in dynamic conditions. Chronic wound media (CWM) was coupled with the models to mimic wound micro-environment. Low antiseptic concentrations inhibited biofilm formation. To reduce of 10 log the bacterial load of a 72h biofilm, 4 to 10 higher dilutions were required. PVPI could not be tested using the dynamic model. Our results in the dynamic model identified Sodium hypochlorite as a relevant antiseptic for DFU treatment.

Keywords: antiseptic; biofilm; DFU; *Staphylococcus aureus*; CWM; Antibiofilmogram®; chronic wound; PHMB; PVPI; Octenidine; Sodium Hypochlorite.

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1. Introduction

One adult in ten worldwide is estimated to suffer from Diabetes mellitus (DM)[1]. The number of DM patients is continuously increasing, from 151 million in 2000 to 463 million in 2019, now 536 million in 2021 and an estimation of 783 million for 2045[1,2]. Diabetic Foot Ulcer (DFU) is one of the main DM complications, affecting 15-25% of DM patients[3]with up to an estimated 42% 5 years mortality rate[4]. DFU is naturally colonized with microorganisms[5,6]. In 80% of chronic wounds as DFU; microorganisms aggregates in a community called biofilm [7,8]. Inside this biofilm, a self-produced extracellular polymeric substance (EPS) matrix protects microorganism[9,10]; making then unreachable to DFU treatments[9,11]. Consequently, Biofilm delays wound healing[12]. Biofilm also enhances antibiotic tolerance[13,14]and horizontal gene transfer, resulting in resistance diffusion and Multi drug resistant (MDR) strains selection [15,16]. For this reason, biofilm management is now a key point in DFU management[8,17]. Many antibiofilm strategies are recommended or under review for biofilm reduction (enzymes for EPS dispersal, nutraceuticals, phage therapy, next generation dressings, antimicrobi-

als...)[18]. The inappropriate use of antibiotics as antibiofilm agents can lead to MDR strains selection[9]; especially when up to 1000 times higher antibiotic concentrations are required for biofilm eradication, compared to planktonic bacteria[11]. In this context, Antibiotic use is limited to DFU infection[8,19]. On the other hand, despite the lack of international consensus for their use, antiseptics as Sodium hypochlorite (SH), Octenidine, Polyhexamethylene Biguanide (PHMB) and Polyvinylpyrrolidone iodine (PVPI) are increasingly given a pivotal role as recommended antimicrobials in DFU management[17,19–21]; especially due to the absence of reported resistance so far[19,22]. There is however a lack of evidence comparing antiseptic agents to recommend one antiseptic over others[23]. The major difficulty in comparing antibiofilm agents, is the lack of biofilm models representative of the chronic wound microenvironment[24,25]. The wound microenvironment modulates bacterial behavior and is crucial for bacterial survival in hostile environment[26]. *In vivo* biofilm study models fail in reproducing the wound microenvironment, are expensive and not reproducible depending on subject variation[24,25]. *In vitro* models fail to reproduce the wound micro-environment, and the interaction between microorganisms and host cells in the biofilm[24]. The extrapolation of the findings using the current models is limited [25,27]. To encompass this problem, a multiple model strategy is recommended in biofilm studies[25]. To mimic wound microenvironment, a relevant media called Chronic Wound Media (CWM) was recently developed for biofilm studies[28]. Another limitation of biofilm models is the microorganism used in biofilm formation models. Although *Staphylococcus aureus* is the most prevalent microorganism in DFU[29,30], biofilm studies mainly focus on *Pseudomonas aeruginosa* due to its strong biofilm forming ability[10,31].

Here in, we combined the CWM with 1 static (Antibiofilmogram ®), 1 quantitative microbial load reduction and 1 dynamic (Bioflux ®) models to study antibiofilm efficacy of 4 antiseptic agents (PHMB, PVPI, Octenidine and Sodium hypochlorite) on biofilms from 1 reference and 3 DFU isolated strains of *S. aureus*.

2. Materials and Methods

2.1 Media, strains, and antiseptic agents

Table 1: Media, strains, and antiseptic agents of the study

Media	Characteristics	Reference
BHI	Brain Heart Infusion	Sigma-Aldrich
LB	Luria Bertani broth	Sigma-Aldrich
CWM	Chronic Wound Medium, medium mimicking <i>in vivo</i> conditions encountered in chronic wounds	[28,32]
Strain		[28,32,33]
Newman	Reference strain	
SAC 1	<i>Staphylococcus aureus</i> clinical strain isolated on a Grade 3 DFI (patient n°1)	[28]
SAC 2	<i>Staphylococcus aureus</i> clinical strain isolated on a Grade 3 DFI (patient n°2)	[34]
SAC 3	<i>Staphylococcus aureus</i> clinical strain isolated on a Grade 3 DFI (patient n°3)	[34]
Antiseptics		
Sodium Hypochlorite (SH)	5g.L ⁻¹ (Dakin ®, Cooper, France)	
Polyvinylpyrrolidone iodine (PVPI)	100g.L ⁻¹ (Betadine ®, Mylan Medical, France)	
Polyhexamethylene Biguanide (PHMB)	1g.L ⁻¹ (Prontosan ® 0.1% undecylenamidopropylbetaine and 0.1% PHMB in aqueous solution; B. Braun Melsungen AG).	

Octenidine 0.5g.L⁻¹ (Octenelin® wound irrigation solution, Schuelke und Mayr, Norderstedt, Germany)

2.2 Susceptibility testing 76

The microdilution broth method was used for the determination of the MIC (Minimal Inhibitory Concentration), according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) recommendations (https://www.eucast.org/clinical_breakpoints). For adaptation to antiseptic agents, the BHI medium was used instead of the Mueller Hinton (MH) medium. A susceptibility test range of [1:2 to 1:2048] was tested for every antiseptic agent. Bacterial cultures were retrieved from a fresh aerobic 37°C overnight incubation for the experiments. Each antiseptic experiment was replicated 6 times. 77
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2.3 Impact of antiseptics and CWM on early biofilm formation kinetic 85

Biofilm ring test (BRT) ® (Biofilm Control®, Saint Beauzire, France) was used in studying the early biofilm formation kinetics, using the capacity of biofilm in blocking magnetic fields[35]. Succinctly, fresh *Staphylococcus aureus* bacterial cultures were standardized to a final 4.10⁶ (Colony Forming Unit per milliliter) CFU.mL⁻¹ concentration and a 200mL volume. A 1% (vol/vol) magnetic beads (TON004) solution was added to the bacterial suspension in a 96 well microtiter plate. The plate was set for 4 hours of incubation at 37°C without shaking. 100µL of liquid contrast were added to each well after incubation. Then a 5 min position on a magnetic block induced the submission to a magnetic field. The plate was scanned with the Biofilm Control Reader (BFC Elements ® 3) and analyzed with the Biofilm Control Software (BFC Elements ® 3). This software calculates Biofilm Formation Index (BFI) ranging from 0 to 20. Low BFI values (<2) indicate a strong biofilm formation profile while as high BFI values(>7) indicate low biofilm formation profile in microorganisms[36]. High BFI values are visually represented by a brown spot at the end of the well, when low BHI values show no spot and an homogenic suspension in the well. Human keratinocytes debris were removed from the CWM media used for BRT ® experiments , to avoid interaction with the magnetic beads. A line of 12 wells with magnetic beads and media was added to each plate to serve as control . 4 replicates were performed. 86
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The impact of antiseptic agents on early biofilm formation kinetics was investigated with the Antibiofilmogram(ATBFG)®. This test shares the same principle with the BRT® [37]. Antiseptic dilution (1:2 to 1: 1024) were added to the wells containing bacterial suspension and magnetic beads before incubation. The PVPI and Sodium hypochlorite solutions used in this study were colored and did not allow an exact MBIC value based of the BFI values[36] results as previously described[38]. We measured for the purpose of this study the partial MBIC (pMBIC) as the minimal concentration at which a brown spot formation is present at the bottom of the wells. The brown spots indicate no biofilm formation, suggesting an antibiofilm properties against biofilm formation early steps. The ATBFG® test was proceeded in BHI and CWM to evaluate the impact of antiseptics in a wound-like micro-environment. 104
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2.4 Antibiofilm properties against 72h static mature biofilm 115

Antiseptic efficacy against 72h mature biofilms was evaluated using the quantification of the bacterial load [39]. Briefly, an adjustment at 1 ± 0.05 optical density (OD₆₀₀) was proceeded on overnight bacterial cultures. To form a 72h static biofilm, the previous bacterial suspension went through a 1:100 dilution then 72h humid incubation at 37°C without shaking in 2 flat bottom 96 wells. Every 24h, fresh medium was added to the wells. Negative control included a well line with media only. Following the 72h of incubation, one plate was put in contact for 24h, with a 1:2 to 1:1028 range dilution of antiseptic agents. Negative control plate consisted of 24H contact with media solution. The 116
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other plate went through the quantification process as follows. (i) PBS solution washing 124
thrice; (ii) 200 μ L of PBS addition; (iii) Biofilm disruption with 10 min sonication at 40 125
kHz; (iv) 10-fold serial dilutions; (v) Last dilution plating on agar plates (vi) Plates incu- 126
bation for 24h at 37°C; (vii) CFU count after incubation. After 24h of incubation, the first 127
plate also went through quantification process. In practice, antiseptic agents are used to 128
complement clinical debridement by 10 to 15%[40]. We then determined the concentra- 129
tion of antiseptic required to reduce the bacterial living load by 10%.6 replicates of this 130
experiment were processed. 131

2.5 Antiseptic dynamics 132

Antiseptics dynamic on biofilm was evaluated using an adapted *in vitro* technol- 133
ogy[32], based on a microfluidic system[41], the Bioflux™. The system used in this study 134
consists of a 48 well plates with microfluidic channels connecting wells by pair (an inlet 135
well and an outlet well); a heat pad; an automated air compressor and an electromagnet- 136
ic regulator. These components, added to the laminar flow induced by the size of the 137
wells, produce a uniform biofilm formation and a precise pressure delivery in the whole 138
plate. A Leica DM IRB inverted fluorescence microscope and a CoolSNAP FX black and 139
white camera (Roper Scientific ®, Trenton NJ, United States) were coupled with the sys- 140
tem for visualizing biofilm formation and acquiring or recording automated images. The 141
plates of the system can be examined through microscope by their bottom, manufact- 142
ured in 180 μ m coverslip glass[42]. The temperature was set at 37°C throughout the exper- 143
iment. Before all else, the channels were primed with warm media, introduced in the 144
inlet wells for 10 min at a 1 dyne.cm⁻² flow. The channels were emptied after the 10 min 145
priming steps. Fresh bacterial suspension was added from the outlet well for 30 min at a 146
1dyne.cm⁻² flow. The suspension was prepared by an OD₆₀₀ adjustment at 1 ± 0.05 and a 147
1:200 dilution of a fresh overnight bacterial suspension. These 30 min are crucial for bac- 148
terial attachment. It was followed by a bacterial suspension transfer in the inlet well for 149
72h at a 0.2 dyne.cm⁻² flow. Every 24h, biofilm formation was visualized for the experi- 150
ment control microscope and recorded. Also, every 24h, fresh media was added in the 151
inlet well and the outlet well was emptied. After the 72h breakpoint, to mimic a clinical 152
management of a chronic wound, an automatized debridement was processed as previ- 153
ously described[32]. A 5 to 20 dyne.cm⁻² flow was applied from the inlet to the outlet 154
well, then reversed from the outlet to the inlet well. A visual control was performed eve- 155
ry 10 to 30 min depending on biofilm percentage until the residual biofilm was visually 156
estimated in a range of 10 to 20% of the biofilm formed at 72h. Biofilm images after au- 157
tomated debridement process were also recorded. The next step of the experiment con- 158
sisted in antiseptic application at commercial concentration and a 0.2 dyne.cm⁻² flow for 159
72h. Saline solution was applied as control. Every 24h, images of biofilm were recorded. 160
Imaging was processed with MetaVue™ software (Molecular Devices, Sunnyscale, CA, 161
USA) was used for. The ImageJ® software allowed images analysis including images 162
processing, coloring, scale bars inclusion and biofilm percentage calculation. The calcu- 163
lation was proceeded through the adjustment of 16-bit grayscale images using the 164
“threshold” function and the “Analyzes particles” function, as previously described 165
[41,43]. Biofilm percentage at each step was calculated as the surface of the well covered 166
in biofilm, divided by the entire surface of the well *100. These experiments were repli- 167
cated twice. 168

After automated debridement process and 72h antiseptic contact bacteria dispersed 169
from the biofilm was retrieved and went through another series of susceptibility tests to 170
evaluated adaptation to antiseptics after a long exposure. 171

2.6 Statistical analysis 172

R® software (4.0.2 version) was used for statistical analyses. Analysis of the living 173
bacterial load distribution results and the residual biofilm in the dynamic model were 174

performed with a Wilcoxon Rank Sum test. BFI values were subjected to a t-student test analysis. Significance levels were set a p value inferior to 0.05 .

3. Results

3.1 Susceptibility testing

Every antiseptic agent displayed the same MIC value in 6 replicates for each strain. We expressed values in concentration (10^{-3} g.L⁻¹) and in dilution ration from the commercial solution (V:V). SH MIC values were 83010^{-3} g.L⁻¹ and 1:6 for Newman and 166010^{-3} g.L⁻¹ (1:3) for the 3 clinical strains. PVPI MIC values were 1000010^{-3} g.L⁻¹ (1:10) for the 4 strains. PHMB values were 510^{-3} g.L⁻¹ (1:200) for all strains. Octenidine values were 2.5 (1:200) for all strains.

3.2 Antiseptics and CWM impact on biofilm formation kinetics

Biofilm Formation Index (BFI) values were low(0.03 [-0.03, 0.10] for the laboratory strain (Newman) and the clinical strains (SAC1, SAC2, and SAC3) [0.01-0.12].

The partial biofilm Minimum Inhibitory Concentrations (pbMIC) values were 0.125×10^{-3} g.L⁻¹ (1:40 dilution) for SH; 1000×10^{-3} g.L⁻¹ (1:100 dilution) for PVPI; 2.5×10^{-3} g.L⁻¹ (1:400) for PHMB and 1.25×10^{-3} g.L⁻¹ (1:400) for Octenidine. These values are lower than MIC values (7 to 14 folds for SH, 10 folds for PVPI and 2 folds for PHMB and Octenidine). Higher antiseptic concentrations were required to reach the pbMIC in CWM compared to BHI. pbMIC values are compared to MIC in Figure 1. The same values were obtained for the 6 replicates.

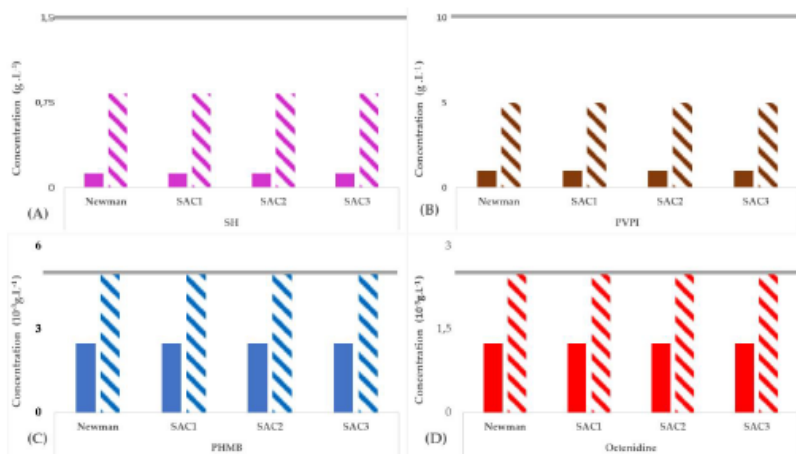


Figure 1: Comparison of Minimum Inhibitory Concentrations (MIC) and partial biofilm Minimum Inhibitory Concentration (pbMIC) values for (A) Sodium hypochlorite, (B) Povidone iodine, (C) PHMB and (D) Octenidine on 4 *Staphylococcus aureus* strains (a Newman reference strain and 3 clinical strains (SAC1, SAC2 and SAC3). pbMIC values are displayed in plain for BHI medium and striped for CWM medium. MIC values are represented as a plain greyline on top of pbMIC lines. Values are displayed in g.L⁻¹

3.3 Antibiofilm properties of antiseptics on a 72h mature biofilm

For all strains, the mean 72h old biofilm bacterial load values in BHI was inferior to the distribution in CWM. (p value= 0.0074, 0.0079, 0.0048 and 0.0022 respectively for Newman, SAC1, SAC2 and SAC3). No significant difference was found in the living bacterial load distributions of Newman reference strain compared to the clinical strains. Nor in BHI media(p value= 0.6095 for Newman vs SAC1; 0.5847 for Newman

vs SAC2 and 0.4576 for Newman vs SAC3); neither in CWM (p value= 0.1905 for Newman vs SAC1, 0.4555 for Newman vs SAC2 and 0.9 for Newman vs SAC3). The mean load values are represented in Figure 2.

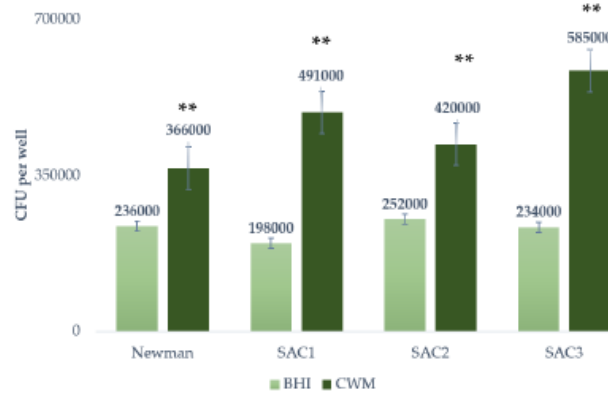


Figure 2: Mean living bacterial load of a 72h mature biofilm for (A) Newman reference strain and clinical strains (B) SAC1, (C) SAC2 and (D) SAC3 in BHI broth and Chronic Wound Media (CWM). Results are presented as the mean ± standard deviation. Average CFU per well was calculated from six independent experiments. Wilcoxon Rank Sum test using the R® software in its 4.0.2 version allowed comparison between BHI and CWM in terms of the mean bacterial load. **, $p < 0.01$.

Since bacterial load reduction is clinically achieved up to 90% with wound debridement[40], we determined the required concentrations for a 10log reduction in the living bacterial load of a 72h static biofilm (Table3). Higher PVPI, PHMB and Octenidine concentrations were required in CWM compared to BHI for that reduction. Sodium hypochlorite CMI concentration induced a 10log reduction in BHI as in CWM 72h static biofilm bacterial load.

Table 2: Antiseptic concentration (in 10^{-3}g.L^{-1}) and dilution ratios (in V:V) inducing a 10log living bacterial load reduction in a 72h static biofilm for reference strain Newman and 3 clinical strains (SAC1, SAC2 and SAC3) in BHI media and CWM.

	SH				PVPI				PHMB				Octenidine			
	BHI (V:V)		CWM (V:V)		BHI (V:V)		CWM V:V		BHI V:V		CWM V:V		BHI V:V		CWM V:V	
N	1666	1:3	1666	1:3	12.5	1:8	50	1:2	10	1:100	20	1:50	5	1:100	10	1:50
SAC1	1666	1:3	1666	1:3	12.5	1:8	50	1:2	10	1:100	20	1:50	5	1:100	10	1:50
SAC2	1666	1:3	1666	1:3	12.5	1:8	50	1:2	10	1:100	20	1:50	5	1:100	10	1:50
SAC3	1666	1:3	1666	1:3	12.5	1:8	50	1:2	10	1:100	25	1:40	5	1:100	10	1:50

3.4 Antiseptic dynamics

With the dynamic model, the biofilm formation was significantly faster in CWM with a higher biofilm formation percentage at 24 and 48h (p value < 0.05) for all strains. Visually, the 72h mature biofilm in CWM was denser and heavier compared to BHI.

Residual dynamic biofilm after debridement (control) and after 72h contact with antiseptic at commercial dilution is reported in Figure 3. The mean control of biofilm residual percentage at 72h post automated debridement process without antiseptic was

12% for all strains. This value was compared to the residual biofilm post automated debridement process and 72h antiseptic contact for all antiseptics on every strain. 234
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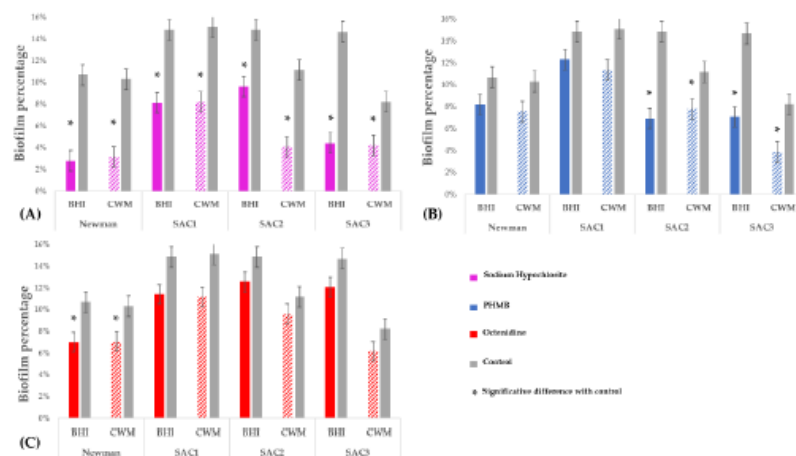


Figure 5. Residual biofilm percentage after in vitro debridement and after 72h antiseptic contact at commercial concentration for Sodium Hypochlorite(A, pink); PHMB (B, blue) and Octenidine (C, red) for Newman reference strain and 3 clinical strains (SAC1,SAC2 and SAC3), in dynamic flow model. Results are presented as the mean \pm standard deviation. A mean 12% biofilm was residual after in vitro debridement (grey control line). Residual percentage is represented in plain column for BHI medium and striped columns for CWM. *, $p < 0.1$; **, $p < 0.01$. 236
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3.5 Adaptation to antiseptic after a 72h exposure 243

After 72h exposure to antiseptic in the Bioflux™ microfluidic system, sessile bacteria freed from biofilm MIC values were evaluated. These MIC values were the same as MIC values of planktonic bacteria. 244
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4. Discussion 247

DFU is a raising public health challenge. Difficulties in making DFU infection diagnosis and the organization of DFU commensal microorganism in biofilm lead to impaired wound healing and MDR bacteria diffusion through inappropriate antibiotic use. Antiseptics may represent an alternative with a lack of resistance. Four antiseptic agents were evaluated in this study for their efficiency against *S. aureus* biofilm, the most prevalent bacteria in DFU. The study used a multiple model approach including a medium that mimics DFU microenvironment. 248
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While testing susceptibility, previous studies determined various MIC values for the tested antiseptic agents, as presented in Table 3. For SH, the $1666 \times 10^{-3} \text{g.L}^{-1}$ (1:6 dilution) found in this study is lower than those previously found [44–49]. The $10000 \times 10^{-3} \text{g.L}^{-1}$ (1:10 dilution) MIC value of PVPi found in this study higher than the 781.5 to $4094 \times 10^{-3} \text{g.L}^{-1}$ range found in previous studies [46,48,50–53]. The $5 \times 10^{-3} \text{g.L}^{-1}$ MIC value of PHMB found in the current study is consistent with the 1 to $10 \times 10^{-3} \text{g.L}^{-1}$ range of previous studies [51–58]. When testing MIC of Octenidine, previous studies found a MIC The $2.5 \times 10^{-3} \text{g.L}^{-1}$ (1:200 dilution) Octenidine MIC value found in the current study is at the top of the 0.5 to $2 \times 10^{-3} \text{g.L}^{-1}$ range previously found [51–53,59–62]. A direct comparison is nevertheless limited by differences in strains and media amongst studies, as previously documented [63,64]. Independently of the study and the methodology, MIC values are strong dilutions of the 4 antiseptics commercial solutions. These antiseptic solutions have antimicrobial properties against planktonic *S. aureus* at weak concentrations. 255
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Table 3: Reported MIC values of Sodium Hypochlorite, PVPI, PHMB and Octenidine

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Antiseptic	MIC	Dilution	<i>S. aureus</i> strain	Method	Study
Sodium Hypochlorite	1%	1:5	ATCC 6538	Personal	Estrela et al[44]
	0.025%	1:20	NCT6571	Personal	Locker et al[45]
	938 10 ⁻³ g.L ⁻¹	1:5	ATCC25923	Personal	Penna et al [46]
	512 10 ⁻³ g.L ⁻¹	1:10	60 clinical isolates	Microdilution broth	Kawamura et al [47]
	2000 10 ⁻³ g.L ⁻¹	1:2.5	1532 isolates	Microdilution broth	Morrissey et al [49]
	1250 10 ⁻³ g.L ⁻¹	1:4	ATCC 27853	Microdilution broth	Gupta et al[48]
	512-1024 10 ⁻³ g.L ⁻¹	1:10 -1:5	ATCC 6538	German Microdilution broth	Koburger et al[53]
	830-1666 10 ⁻³ g.L ⁻¹	1:6-1:3	Newman and 3 clinical isolates	Microdilution broth	Current study
PVPI	0.08%	1:125	ATCC 25923	Microdilution broth	Sa et al [50]
	781.5 10 ⁻³ g.L ⁻¹	1:128	ATCC 25923	Microdilution broth	Gupta et al[48]
	6250 10 ⁻³ g.L ⁻¹	1:16	ATCC 25923	Personal	Penna et al [46]
	2000 10 ⁻³ g.L ⁻¹	1:50	ATCC 29213	Microdilution broth	Hirsch et al [51]
	1028-4094 10 ⁻³ g.L ⁻¹	1:100-1:25	ATCC 6538 and 30 clinical isolates	Microdilution Broth	Dittman et al [52]
	512 10 ⁻³ g.L ⁻¹	1:200	ATCC 6538	Microdilution broth	Koburger et al[53]
	10000 10 ⁻³ g.L ⁻¹	1:10	Newman and 3 clinical isolates	Microdilution broth	Current study
PHMB	10 10 ⁻³ g.L ⁻¹	1:100	ATCC 29213	Microdilution broth	Hirsch et al [51]
	0.5-1 10 ⁻³ g.L ⁻¹	1:2000_-1:1000	ATCC 25923 and 3 clinical isolates	Microdilution broth	Lopez-Rojas et al[54]
	1 - 8 10 ⁻³ g.L ⁻¹	1:1000 to 1:125	40 clinical isolates	Microdilution broth	Zhou et al [55]
	1 - 2 10 ⁻³ g.L ⁻¹	1:1000 - 1:500	ATCC 6538 and 30 clinical isolates	Microdilution Broth	Dittman et al [52]
	1-2 10 ⁻³ g.L ⁻¹	1:1000: 1:500	100 clinical isolates	Microdilution broth	Fabry et al [56]
	0.5 - 8 10 ⁻³ g.L ⁻¹	1:1000	54 clinical isolates	Microdilution broth	Renzeni et al [57]
	1-2 10 ⁻³ g.L ⁻¹	1:1000: 1:500	ATCC 29213 and 15 clinical isolates	Microdilution broth	Assadian et al
	1 10 ⁻³ g.L ⁻¹ 5 10 ⁻³ g.L ⁻¹	1:1000 1:200	ATCC 15442 Newman and 3 clinical isolates	Microdilution broth Microdilution broth	Koburger et al[53] Current study
Octenidine	1 10 ⁻³ g.L ⁻¹	1:500	ATCC 29213	Microdilution broth	Hirsch et al [51]
	0.5 - 1 10 ⁻³ g.L ⁻¹	1:1000 -1:500	335 clinical isolates	Microdilution method	Zheng et al [59]
	0.5 -2 10 ⁻³ g.L ⁻¹	1:1000 -1:250	878 clinical isolates	Microdilution method	Htun et al [60]
	0.25 -2 10 ⁻³ g.L ⁻¹	1:2000 -1:500	ATCC 25923, ATCC 43300 and 2 clinical isolates	Microdilution method	Nicolae Dopcea et al [61]
	0.2-0.4 10 ⁻³ g.L ⁻¹	1:2500 -1:1250	ATCC 25923, ATCC 25 213 and 3 clinical isolates	Microdilution method	Grecka et al [62]
	2 10 ⁻³ g.L ⁻¹	1:250	ATCC 15442	Microdilution broth	Koburger et al[53]
	1 - 2 10 ⁻³ g.L ⁻¹	1:500 - 1:250	ATCC 6538 and 30	Microdilution Broth	Dittman et al [52]

		clinical isolates		
2.5 10 ⁸ g.L ⁻¹	1:200	Newman and 3 clinical isolates	Microdilution broth	Current study

The Antibiofilmogram test(ATBFG)®, based on the Biofilm Ring Test (BRT)® was adapted to assess the impact of antiseptic on *S. aureus* biofilm formation kinetics[38]. This test evaluated antiseptic impact on biofilm formation. The results were expressed as the partial biofilm Minimal Inhibitory Concentration (pbMIC). pbMIC results indicated that biofilm formation inhibition was obtained at 2 to 8 times lower values than the CMI for all antiseptic agents. To the best of our knowledge, this is the first study evaluating antiseptic agents on *S. aureus* biofilm using the ATBFG ® test. These results are significant for DFU management. They imply that the 4 antiseptic agents could be used in DFU management for preventing biofilm formation. ATBFG ® results also indicated that 2 to 6 folds higher concentrations were required to reach the pMBIC in CWM for all antiseptic agents, resulting in higher pMBIC values in CWM than in BHI. These values however are still 1:400 to 1:40 times lower than commercial solutions concentration. These results may be directly linked to the impact of CWM on biofilm formation. Prior works with CWM noted a significantly accelerated biofilm formation kinetic in CWM compared to BHI. [28,32]. As CWM boosts the biofilm forming capacity of *S. aureus*, higher antiseptic concentrations may be required to inhibit biofilm formation.

We evaluated the antibiofilm effect of the 4 selected antiseptics on a 72h mature static biofilm. 5:4 (PVPI) to 2 (Octenidine and PHMB) times higher concentrations than those required for biofilm formation inhibition were required for a 10log reduction in bacterial load. These results may be explained by the presence of the EPS that complicates access to microorganisms in the biofilm. Compared to BHI, 2 (Octenidine and PHMB) to 4 (PVPI) times higher antiseptic concentrations were required in the CWM for a 10log reduction of a 72h static biofilm bacterial load. Compared to CMI, 4(Octenidine and PHMB) to 10 (PVPI) higher concentrations were required in the CWM for a 10log reduction of a 72h static biofilm bacterial load. Only SH was effective in reducing bacterial load by a 10 log at MIC concentration independently of the medium. These results are likely to be related to the impact of the wound microenvironment on biofilm. The bacterial load of a 72H static biofilm in CWM was significantly higher than the bacterial load of a 72H static biofilm in BHI. The 72h static biofilm in CWM was denser and heavier compared to BHI, similar to what was previously reported in the wound bed[32]. The biofilm the heavier and denser biofilm in CWM may further limit antiseptic efficiency than the one in BHI. In addition, a decreased activity in presence of organic matter, as those present in the wound microenvironment is known and described for antiseptics[65]. PVPI, PHMB, and Octenidine, were previously reported to be inactivated by the presence of material load[66]. Sodium hypochlorite affected by organic matter as previously reported[67]. SH CMI value induced a 10log reduction in the bacterial load both in BHI and CWM. According to this model, SH seems to be the best antiseptic for reducing the bacterial load of a mature static biofilm. Nonetheless, a note of caution is due there since concentration values for the 10log reduction were lower than commercial values. At commercial value, all antiseptics induced a 10log reduction in a 72h static biofilm in both CWM and BHI. The 4 selected antiseptic are still effective in reducing by 10 log the bacterial load of a 72h static biofilm at commercial values.

In clinical practice, bacterial offloading is obtained with clinical debridement up to 85-90%. Antiseptic adjunction is complementary to that debridement. To mimic clinical conditions, we then assessed the antibiofilm properties of the 4 selected antiseptic agents using a dynamic fluidic model and an in vitro debridement. Using the dynamic model, and commercial solutions, PVPI could not be assessed due its dark color that prevented biofilm microscopic examination. Biofilm formation was significantly accelerated in the CWM compared to BHI in the dynamic model. Nutritionally balanced media like CWM close to the wound micro-environment are assumed to enhance biofilm formation[68].

These results endorse the previous ones that highlight the importance of wound micro-environment in biofilm studies and by extension, the importance of culture media in *in vitro* biofilm studies[24,25,28,31,68]. PHMB and Octenidine showed a strain dependent effect in the dynamic model. After *In vitro* debridement process, only Newman biofilm was significantly reduced by Octenidine. PHMB reduced SAC 2 and SAC3 biofilm percentages. On the other hand, SH significantly reduced biofilm percentage after *in vitro* debridement for all strains to a mean 6% biofilm percentage up to 72h after antiseptic contact. These results suggest SH prevented biofilm reformation after debridement in addition to biofilm reduction. The Bioflux ® dynamic model, coupled with the CWM was developed as a relevant tool for biofilm studies for mimicking wound micro-environment[32]. The findings using this model are estimated closer to clinical DFU treatment. We can then assume SH to be the best antiseptic for DFU treatment. It could be argued SH superiority is related to its mechanism of action. PHMB and Octenidine mode of action relies on their penetration through the bacterial membrane[69,70]. SH on the other way has a multiple mode of action that combines protein degradation, oxidizing action, direct interaction with the membrane and reactive oxygen species[67]. Interestingly, PHMB was efficient against 2 strains (SAC 2 and SAC3) while Octenidine was efficient only against Newman laboratory strain. A possible explanation may be the presence of betaine in the PHMB solution used in this study[71]. As surfactant agent, betaine was previously reported as biofilm disruptor agent that can induce a better tolerance and enhance membrane penetration[72]. This betaine biofilm disruptor effect may boost PHMB effect but even so ,PHMB action was limited to 2 strain biofilms over the 4 tested.

Following automated debridement process and a 72h contact with PVPI, Octenidine and Sodium Hypochlorite in the dynamic model, freed sessile bacteria from the 4 strains biofilm was retrieved. We performed a second susceptibility test to evaluate the impact of the 72h antiseptic contact on the strain's susceptibility. The 4 *S.aureus* strains did not show any adaptation to the antiseptic. The MIC values remained stable. The strains did not develop any adaptation to the antiseptic solutions as previously reported[26,73]. These findings could support the evidence for the absence of adaptation and resistance reported for PVPI, SH, PHMB and Octenidine[19,22,61,74,75]. A note of caution is due there as antiseptic contact was only for 72 hours.

One limitation of the study is the inability to assess PVPI with the dynamic flow model due to its dark color that blocked microscopical biofilm analysis. This problem is currently under reviewing. The major limitation of the study was the use of a single specie biofilm. In fact, DFU biofilm and more generally wound biofilm is predominantly multispecies [31,76]. In consequence, a further work will consist in adapting this study design on a multispecies biofilm. Notwithstanding its limitations, this was the first study combining new relevant tools as BRT ® Bioflux ® and quantitative load reduction models in the assessment of antiseptic agents.

5. Conclusions

Biofilm formed by *S.aureus* is one major challenge in DFU management. Alternative therapeutics . The present study was designed for assessing 4 antiseptic agents on *S. aureus* biofilms from one laboratory and 3 DFU isolated strains in conditions close to wound-like micro-environment. Sodium hypochlorite seems to be the most efficient antiseptic according to the findings of this study. At its MIC concentration, SH prevented biofilm formation, and induced a 10-log bacterial load reduction. At commercial concentration, its antibiofilm properties remained stable against the reference and the clinical strains under dynamic wound-live microenvironment conditions after debridement. These findings still need to be supported by another assessment on a multispecies biofilm. Clinical studies on the impact of antiseptic agents in biofilm reduction and chronic wound healing are required to support the results of this study which endorses antiseptic agents as a meaningful alternative treatment in DFU treatment.

Supplementary Materials: The following supporting information can be downloaded at: 373
www.mdpi.com/xxx/s1, Table S1: Susceptibility testing : MIC and pMBIC values; Table S2: Biofilm 374
formation percentage values in the Bioflux ® dynamic model; Table S3: Biofilm formation percent- 375
age values in the Bioflux ® dynamic model after debridement and after 72h antiseptic contact at 376
commercial concentration. 377

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CDR ; software, K.B.B.; validation, C.D.R., A.S. and J.P.L.; formal analysis, K.B.B., J.O. and A.Y.M.; 379
investigation K.B.B.; writing—original draft preparation, K.B.B; writing—review and editing, J.O., 380
C.D.R., A.S., and J.P.L.; supervision, A.S., and J.P.L.; project administration, J.O, C.D.R, J.P.L; fund- 381
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ware at the U1047 unit Chronic wound research section. 389

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III. PARTIE 3

EVALUATION DES ANTISEPTIQUES SUR DU BIOFILM BIESPECE

Article scientifique N°4

Sodium hypochlorite and PHMB antibiofilm properties on a diabetic foot ulcer isolated bispecies biofilm in a wound-like environment

Koko Barrigah-Benissan, Jérôme Ory, Catherine Dunyach-Remy, Alex Yiahiaoui Martinez, Jean-Philippe Lavigne, and Albert Sotto

Écriture en cours

Version à la date du 15 octobre 2022

Publication scientifique N°4 : contexte et résumé introductif

Après une évaluation des antiseptiques sur du biofilm monoespèce, nous nous sommes intéressés au biofilm bi-espèces. Dans le microenvironnement clinique des plaies chroniques, les microorganismes se développent majoritairement sous la forme d'un biofilm polymicrobien[211]. Les deux espèces prédominantes de ce biofilm polymicrobien sont *S. aureus* et *P. aeruginosa*[10,123,209].

Nous avons utilisé pour ces expérimentations, 2 couples de souches cliniques : le couple SAC1/PAC1 et le couple SAC2/PAC2. Ces souches ont été isolées de prélèvements de débridement de plaies d'ulcères de pied chez les patients diabétiques infectées à un grade 3. Nous avons tout d'abord utilisé le modèle de quantification de la charge bactérienne vivante du biofilm pour évaluer l'efficacité du biofilm à éradiquer la charge bactérienne vivante des biofilm monoespèce de ces souches. Nous avons ensuite utilisé le modèle dynamique du Bioflux ® pour étudier la formation d'un biofilm bi espèces avec les 2 couples de souches. Nous avons utilisé les 2 antiseptiques les plus prometteurs dans les évaluations sur le biofilm monoespèce : l'hypochlorite de sodium et le PHMB.

La formation de biofilm monoespèce a été significativement accélérée dans le biofilm monoespèce comme lors des travaux précédents. L'hypochlorite de sodium a été efficace dans l'éradication significative du biofilm monoespèce de 72h de toutes les souches à la moitié de sa concentration commerciale. A la moitié de sa concentration commerciale, l'efficacité du PHMB dans l'éradication d'un biofilm mature de 72h est limité pour toutes les souches. Comparément au biofilm monoespèce, la formation de biofilm bi espèces a été plus rapide, particulièrement dans le milieu plaie (CWM®). Le sodium hypochlorite a significativement réduit le biofilm bi-espèces post débridement

automatisé indépendamment du milieu utilisé. Cet effet a persisté 72h après application. Cela suppose que comme trouvé lors de nos évaluations précédentes, l'effet antibiofilm de l'hypochlorite de sodium persiste jusqu'à 72h mais que cet effet antibiofilm passe aussi par l'inhibition de la formation d'un nouveau biofilm. L'effet du PHMB était moins évident. Il s'est limité au milieu contrôle le BHI pour les 2 biofilm bi espèces. Le PHMB était inefficace dans le milieu CWM ®.

Ces résultats permettent de positionner l'hypochlorite de sodium en avant dans la prise en charge des ulcères de pied chez les patients diabétiques par rapport au PHMB.

Article

Sodium Hypochlorite and PHMB antibiofilm properties on a DFU isolated mixed species biofilm in a wound-like microenvironment

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Abstract: In vitro biofilm models are a mandatory step in antimicrobial evaluation for DFU treatment. In a previous work, Sodium hypochlorite (SH) and polyhexamethylenebiguanide (PHMB) were found good candidates for wound antiseptics for a 10 log reduction of single species mature biofilms of *S.aureus* and *P. aeruginosa*. This work aims to confirm their antibiofilm properties. We first evaluated their ability to eradicate a 72h single specie mature biofilm of *S.aureus* and *P. aeruginosa* grew in static conditions. We also evaluated their efficiency in reducing mixed species biofilm of *S.aureus* and *P. aeruginosa* in a microfluidic dynamic model. Both evaluations were proceeded using the CWM®, a media model that mimics chronic wound microenvironment. With the single specie static biofilm model, both antiseptic agents performed total biofilm eradication. In the microfluidic system, PHMB showed a media dependent effect and was unable to significantly reduce biofilm after in vitro debridement. SH on the other hand, significantly reduced biofilm percentage up to 72h after its application. This work positioned sodium hypochlorite as a pertinent wound antiseptics agent in DFU treatment.

Keywords: biofilm; DFU; wound microenvironment; CWM®; *S.aureus*; *P. aeruginosa*; antiseptics; Sodium hypochlorite; PHMB

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1. Introduction

Chronic wounds are a serious burden for patients, professional health workers and the public health systems and economics worldwide[1]. Their prevalence is estimated at 1 to 2% of the population[1]. Diabetic foot ulcers (DFU) particularly are constantly increasing with diabetes mellitus prevalence. Around 10% of the world global population is now estimated living with diabetes mellitus[2]. 10 to 34% of them is estimated to suffer from DFU at least once in their life[1]. DFU treatment is challenging due to its multiple origin factors but also its numerous complications. Infection is one of the main and the hardest to treat DFU complications. Wounds are naturally colonized with microorganisms. The distinction between colonization and infections is quite tricky, leading to an excessive use of antibiotics which is a significant contributory factor to multidrug resistance[3]. Furthermore, DFU chronicity is linked to the development of the wound microorganisms in a biofilm mode[4]. Biofilm is believed ubiquitous in chronic wounds, with the ability to reform quickly after removal[1]. It is a development mode as a community of microorganism with a slow growth, embedded in a protective extracellular

matrix[5] This matrix, protects microorganism inside the biofilm from antimicrobial activity[5]. Biofilm conformation facilitates resistance gene transfers[6,7], contributing also to multidrug resistance progression[8]. As for now, biofilm management in DFU treatment consists in adequate wound bed preparation and wound debridement[1,9]. That said, debridement is described to eradicate biofilm by 80 to 90%[10]. In addition, in the 24h following debridement biofilm reformation begins[11]. As a consequence, alternative approaches other than antibiotics and wound debridement for DFU management are crucial[3]. Antiseptic agents are one promising alternative approach for DFU treatment[3]. Despite the absence of an international consensus, there are more and more recommendations supporting their use in DFU treatment[12,13]. The regain of interest in wound antiseptics is motivated by the lack of described resistance so far in the main antiseptic agents recommended in chronic wound treatment[13]. Yet, their clinical efficiency is yet to be proven and there is not enough data supporting the recommendation of one agent over others[14]. Until then, *in vitro* biofilm studies are a precious tool for evaluating antiseptic agents and orientate clinical studies[15]. For a relevant extrapolation to real-life situations, *in vitro* antibiofilm agents evaluation should incorporate mature biofilm, wound microenvironment and interactions between host component and microorganisms[15,16]. We recently developed an *in vitro* medium named Chronic Wound Medium (CWM®) that imitates chronic wound microenvironment[17]. In previous works, we showed that PHMB and sodium hypochlorite were effective antibiofilm agents for 10 log reduction in the living microbial load of 72h mature single species biofilms of *S. aureus* and *P. aeruginosa* in this wound-like microenvironment[18]; making them a good complement for wound debridement. We studied inside a biofilm, the bacterial cooperation of *S.aureus* and *P. aeruginosa*[19]. In this work, we evaluated their efficiency for total eradication of the single species biofilms. In chronic wounds like DFU, biofilm is mostly polymicrobial[20]. The evaluation models of antimicrobials should therefore comprise a polymicrobial biofilm in addition to the wound microenvironment[15,16,21]. Also, recent reviews recommend the use of visualization technologies and opened models during biofilm studies[15,16,21–23]. *Staphylococcus aureus* and *P. aeruginosa* are the most prevalent species encountered in DFU[24]. Therefore, in this work, we analyzed the formation of a mixed species biofilm of *S.aureus* and *P. aeruginosa* in a microfluidic dynamic model. We evaluated Sodium hypochlorite (SH) and Polyhexamethylenebiguanide (PHMB) antibiofilm properties on this mixed species biofilm.

2. Materials and Methods

2.1. Strains and media

2 media and 4 strains were used for this work, as presented in Table 1. All strains were all isolated from Grade 3 DFU infections. 2 strains were *S. aureus* and the 2 others *P. aeruginosa*. Strains were transferred from a Brain heart infusion (BHI) agar. Colonies were resuspended overnight in 3mL liquid media (BHI and CWM®) at 37°C on a rotation shaker at 200 rpm.

CWM® is a media developed in our institute that reproduces wound microenvironment[17]. It comprises heat-inactivated human serum, debris of human keratinocytes and hemolyzed human blood at a pH of 8, as encountered in clinical wounds. We used this CWM® and BHI broth as control.

Table 1: Strains and media in the current study

Strains	Characteristics	Reference
SAC 1	<i>Staphylococcus aureus</i> clinical strain isolated on a Grade 3 DFI*, along with PAC1 (patient n°1)	[17,25]
SAC 2	<i>Staphylococcus aureus</i> clinical strain isolated on a Grade 3 DFI*, along with PAC2 (patient n°2)	[19,25]
PAC 1	<i>Pseudomonas aeruginosa</i> clinical strain isolated from a	[17,25]

PAC 2	Grade 3 DFI*, along with SAC1 (patient n°1) <i>Pseudomonas aeruginosa</i> clinical strain isolated from a Grade 3 DFI*, along with SAC2 (patient n°2)	[19,25]
Media		
BHI	Brain heart infusion	Sigma-Aldrich
CWM®	Chronic wound medium ®. A medium that imitates the microenvironment encountered <i>in vivo</i> in chronic wounds	[17,19,25]

*DFI= diabetic foot ulcer infection

2 antiseptic agents were evaluated. Sodium hypochlorite (SH), (5g.L-1 (Dakin ®, Cooper, France); and Polyhexamethylene Biguanide (PHMB), (1g.L-1 (Prontosan ® 0.1% undecylenamidopropyl-betaine and 0.1% PHMB in aqueous solution; B. Braun Mel-sungen AG).

2.1. Single specie biofilm formation and quantification

Quantification of bacterial survival before and after antiseptic exposure was evaluated as follows with a counting system previously described[26]. Fresh overnight bacterial cultures were transferred to 3 flat bottoms 96wells plates for 72h humid incubation at 37°C; after a 1 ± 0.05 OD₆₀₀ nm adjustment and a 1:100 dilution. Every 24h, fresh medium either BHI or CWM was added. At 72h, the plates were supplemented with an equal volume of an antiseptic agent at commercial concentrations or saline solution. These plates were incubated for 24h more at 37°C. Afterwards, every well went through 3 washing steps with 200 µL of 1× PBS. A final 200 µL of 1× PBS was added to the wells. They were additionally sonicated for 10 min at 40 kHz for biofilm disruption. Then, every well was serially diluted, and the last dilution was plated on BHI agar. This BHI agar was incubated overnight at 37°C. After incubation, CFU (Colony Forming Unities) were counted. The living bacterial load before and after antiseptic or saline application were compared.

These experiments were replicated 4 times per strain and per medium.

2.2. Mixed species Biofilm formation in a wound like environment in a microfluidic system

Mixed species biofilm formation was performed with a microfluidic system, named BioFlux™ 200 (Fluxion Biosciences Inc, Alameda, CA, USA). The protocol for biofilm growth in this system was adapted from a previously described one[27]. This system is an automated continuous flow analyzer with standard size well plates. It comprises microfluidic channels that connect 2 wells in each plate, an inlet one and an outlet one. An automated air compressor, an electromagnetic regulator and the laminar flow induced by the size of the channels, allow precise pressure delivery and uniform biofilm growth in each channel and well. A heat pad allows temperature control. These experiments were replicated 4 times per strain and per medium.

Fresh overnight cultures were serially diluted at 1:200 and standardized to a 0.1 ± 0.05 OD₆₀₀. A mixed bacterial suspension of the co-isolated couples SAC1/PAC1 or SAC2/PAC2 was prepared with equivalent ratio of each specie. 500 µL of medium without bacteria was inserted in the inflow well with a pressure setting of 1 dyne/cm² for 10 min for microfluidic channels priming purposes. The remaining medium in the well was discarded after 10min. The mixed bacterial suspension was injected from the outlet well to the inlet well for 30 min at 1 dyne/cm². The plate was placed on the heating pad, set at 37 °C. Finally, the mixed bacterial suspension was injected in the inlet well for 72 h with a 0.2 dyne/cm² pressure and at 37 °C. Every 24h, newly prepared medium was added in the inlet well, and the medium in the outlet well was discarded.

2.3. In vitro automated debridement and antiseptic application

An *in vitro* automated debridement step was developed as previously described, for imitation of the clinical debridement step in DFU treatment[28]. This debridement aims

to remove parts of the pre-formed biofilm and to keep a 10 to 15 % remaining biofilm similar to what was described in real life settings[29]. After 72h of biofilm formation, a 5 to 20 dyne.cm⁻² flow was applied for 10 min first from input to output well, then reversed from output to input well. The remaining biofilm was visually assessed each 10 min on the microscope. This step was repeated until the visual residual biofilm was estimated from a range of 10 to 20 %. Following the *in vitro* debridement, antiseptic solutions were applied with a 0.2 dyne/cm² from inlet to outlet well for 72h, with a visual control every 24h.

2.4. Mixed biofilm visualization and analyzis before and after antiseptic contact

Bottoms of BioFluxTM 200 plates are manufactured in 180 µm coverslip glass, easing microscopic examination[27]. The BioFluxTM 200 system was coupled with a CoolSNAP FX black and white camera (Roper Scientific®, Trenton NJ, United States) for automated image acquisition and recording. It was also associated with a Leica DM IRB (Leica Biosystems, Nanterre, France) inverted fluorescence microscope for biofilm visualization. MetaVueTM software (Molecular Devices, Sunnyscale, CA, USA) allowed image processing. Every 24h of biofilm formation, pictures of the microfluidic charnels were recorded. Pictures were recorded again after debridement and every 24h after antiseptic solutions insertion in the channels.

Images analysis was achieved using the ImageJ® software. ImageJ® “threshold” function operated the 16-bit grayscale images adjustment to the bacterial structure. “Analyzes particles” function applied the biofilm percentage calculation as previously established[30,31]. Biofilm percentage was calculated as the surface of the well covered with biofilm divided by the entire well surface multiplied by 100.

These experiments were replicated twice per strain couple and per medium.

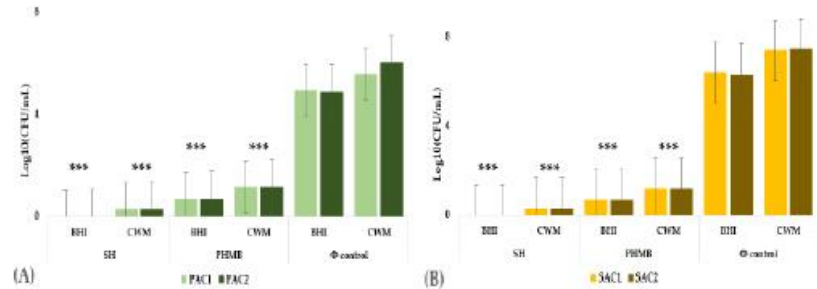
2.5. Statistical analyzes

Analyzes were carried using a Wilcoxon rank sum test and R studio® software in its 4.0.2 version.

3. Results

3.1. Sodium hypochlorite and PHMB can significantly reduce the bacterial load of a static 72h biofilm in a close model

PHMB and Sodium hypochlorite significantly reduced the bacterial living load of a 72h single specie mature biofilm grew in closed static conditions. For SAC1, the log₁₀ CFU per mL went down in BHI media from 6.39 to 0 for SH and to 0.699 for PHMB. In CWM® the decrease was from 7.39 to 0.301 for SH and to 1.175 for PHMB. Regarding SAC2, this decrease was in BHI from 6.32 to 0 for SH and to 0.301 for PHMB; and from 7.4 to 0.7 for SH and to 1.176 for PHMB in CWM®. For PAC1, the decrease was 4.93 to 0 for SH and to 1.176 for PHMB in BHI and in CWM® from 5.59 to 0.3 for SH and to 1.18 for PHMB. Finally, PAC2 CFU per mL log₁₀ decrease with antiseptic agents’ application was in BHI: from 4.88 to 0 for SH and to 0.699 for PHMB; and in CWM®: from 6.059 to 0.3 for SH and too 1.176 for PHMB (Figure1).



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Figure 1: Impact of antiseptic agents on the living bacterial load of a 72h single specie biofilms in static conditions for *P. aeruginosa* strains PAC 1 and PAC2 (A) and *S.aureus* strains SAC1 and SAC2 (B) in BHI broth and CWM® (Chronic wound media). ***p<0.001.

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3.2. Biofilm formation is enhanced in the CWM®

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Mixed species biofilm formation was significantly enhanced in CWM® compared to BHI media at 24h, 48h and 72h. This was recorded as a biofilm visually heavier and denser as represented in Figure 2 and biofilm percentages significantly increased in CWM® as reported in Figure 3. For SAC1/PAC1, mean biofilm percentages were 26.52% in BHI and 60.27% at 24h, 50.27% in BHI and 71.23% in CWM® at 48h; 74.08 and 86.36% in CWM® at 72h. For SAC2/PAC2; these percentages were 27.95% in BHI and 48.63% at 24h; 60.77% in BHI and 76.31% in CWM® at 48h; 71.33% in BHI and 91.83% in CWM® at 72h. See supplementary data S1 for further details.

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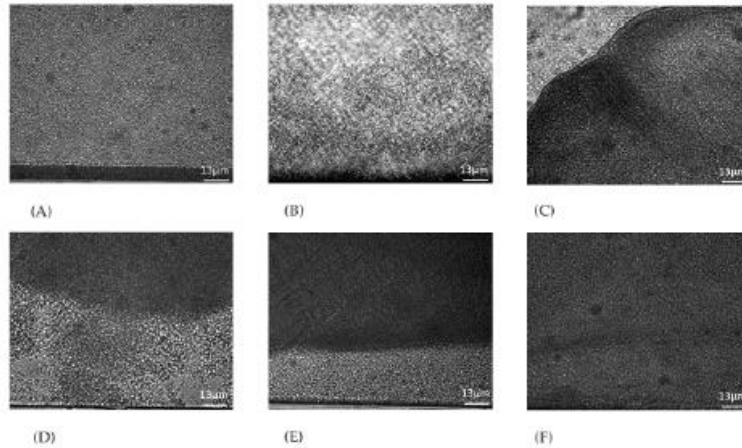
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Figure 2: Images of SAC1/PAC1 mixed species biofilm formation in BHI media at 24h (A), 48h(B) and 72h (C) ; and CWM® at 24h (D), 48h (E) and 72h (F). Biofilm is represented as black areas on the glass white area.

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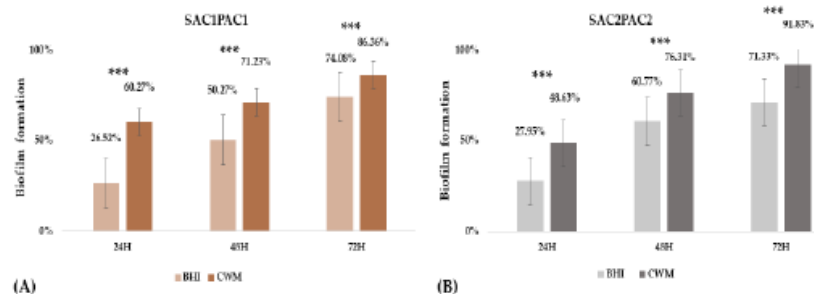


Figure 3: Mean percentages of mixed species biofilm formation (*S. aureus* and *P. aeruginosa*) in the Bioflux™200 system in BHI media compared to CWM® at 24h, 48h and 72H for SAC1/PAC1 (A) and SAC2/PAC2 (B). Statistics for percentage per media comparison were performed with a Wilcoxon rank sum test and R Studio® software in its 4.0.2 version. ***p<0.001

3.3. Sodium hypochlorite is more effective than PHMB for biofilm reduction

A mean 10% biofilm remained after *in vitro* debridement. Sodium hypochlorite significantly reduced mixed species biofilm after *in vitro* automatized debridement independently of the media. SAC1PAC1 biofilm was reduced from a mean 10% to 4.18% in BHI (p<0.001) and 3.83% in CWM® (p<0.01). SAC2PAC2 biofilm was reduced from 10% to 2.89% in BHI (p<0.001) and to 3.49% in CWM® (p<0.001). PHMB on the other hand reduced the mixed species biofilms after *in vitro* debridement only in BI media. SAC1PAC1 biofilm was reduced from 10% to 6.22% in BHI (p<0.001) and 8.25% in CWM® (p=0.15). SAC2PAC2 biofilm was reduced from 10% to 6.60% in BHI (p<0.01) and 9.39% in CWM® (p=0.22). Biofilm reduction with antiseptic agents after debridement is reported in Figure 4.

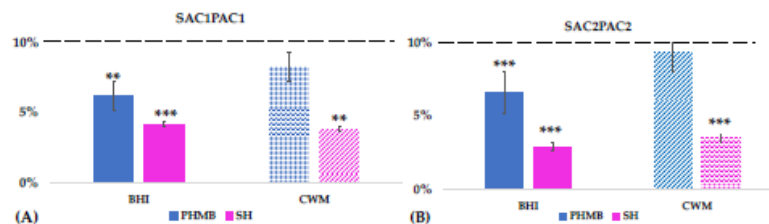


Figure 4: Percentages of biofilm reduction in the preformed mixed species biofilm for SAC1/PAC1 (A) and SAC2/PAC2 (2) using PHMB solution (Blue) and sodium hypochlorite (pink). A mean 10% of biofilm remained after *in vitro* automatized debridement before antiseptic application as represented by the black line. Results are presented as the mean percentage. ***p<0.001, **p<0.01

4. Discussion

Biofilm formation is problematic in DFU treatment as it leads to wound impairment and DFU healing delay[32]. DFU microbiota development in biofilm mode complicates further DFU treatment by making infection diagnosis inaccurate[3]. To avoid multidrug resistance spread, DFU alternative treatment options to antibiotics are required. The evaluation of these alternative options for now is based on biofilm models due to the lack of relevant clinical studies[14]. PHMB and Sodium hypochlorite were previously described as relevant antibiofilm agents for single specie biofilm reduction in complement of wound debridement[18]. This study aims to investigate further their antibiofilm properties on mature biofilm eradication and on mixed species biofilm reduction. One principal limit of biofilm *in vitro* models is their lack of wound microenvironment imita-

tion. To bypass this issue, we performed our analysis in a wound-like media, the CWM® that mimics wound microenvironment.

Applying sodium hypochlorite and PHMB on equal volumes of bacterial suspensions containing a 72h mature static biofilm of *S. aureus* or *P. aeruginosa* strains resulted in complete eradication of these biofilms. These 2 antiseptic solutions previously significantly reduced the bacterial load of 72h mature static biofilm by 10 log with high dilutions. These results indicate PHMB, and SH could be interesting candidates for wound debridement. Ever so in biofilm, microorganisms have a slower growth rate and cannot be always cultivated[33,34]. Therefore, pertinent antibiofilm properties evaluation can not only rely on culture methods[16,22].

Using a dynamic microfluidic system, we were able to induce a mixed species biofilm formation under shear flow in a wound-like microenvironment. Biofilm formation was significantly enhanced in the wound-like media, supporting this media as a pertinent model for biofilm studies[17,18,25]. With an automated debridement procedure, we reduced the biofilm percentage to 10%, like clinical conditions. Applied to this residual biofilm, SH reduced it to a mean 3% and PHMB to a mean 8% In BHI media. When using CWM® which enhanced biofilm thickness in addition to the mixed species, while SH reduced the biofilm to still a mean 3%, PHMB was ineffective in biofilm reduction, showing a media dependent efficiency. Many antimicrobials penetration is limited by the biofilm extracellular matrix and the high protein concentration in the wound microenvironment[35]. Limited penetration due to the extra polymeric substance is a common survival strategy that disable many antimicrobial efficiency[36]. SH seems to evade this strategy. Plus, SH antibiofilm effects were still effective 72h after first application. These results may seem contradictory to some previous study which found sodium hypochlorite ineffective in reducing mature biofilm[23,35]. However, a quick comparison shows that commercial solutions investigated in these studies have 2.5 times lower concentrations than the one used in this work. These results implicate SH can also inhibit biofilm formation as previously demonstrated on single species biofilm[18]. The properties of SH as mature biofilm reduction and biofilm formation inhibition extend to mixed species biofilm. This work reinforces the potential of SH as a good antiseptic candidate for wound antiseptics.

5. Conclusions

The association of CWM® and the microfluidic Bioflux™ 200 system is a relevant tool for biofilm studies and antimicrobial agents' evaluation. It allowed the identification of Sodium hypochlorite as a relevant agent for DFU treatment in complement of wound debridement for reducing biofilm formation. Clinical studies assessing antibiofilm agents should comprise Sodium hypochlorite evaluation.

Supplementary Materials: The following supporting information can be downloaded at www.mdpi.com/xxx/s1 Table S1: Biofilm formation percentages in the Bioflux™ 200 system

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Data Availability Statement: Data supporting the reported results can be found in the Bioflux software at the U1047 unit in the file: Chronic wound research section. 269
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IV. Discussion

Les plaies chroniques sont un problème de santé majeur qui affectent environ 2% de la population mondiale[212,213]. Les ulcères de pied chez les patients diabétiques en particulier, sont en constante augmentation avec le vieillissement de la population et l'accroissement de l'obésité. De 151 millions de personnes en 2000, la proportion de patients diabétiques au niveau mondial est passé à 536 millions en 2021 avec une projection de 783 millions pour 2045 [62,214]. Il y a donc actuellement en 2022, 10% de la population mondiale qui est estimée souffrir de diabète sucré[202]. 10 à 15% environ de cette population de patients diabétiques aura des problèmes d'ulcères de pied [215]. Une majorité de ces ulcères se compliquera d'infections. La causalité de ces infections persistantes est associée au biofilm, retrouvé dans 60 à 90%[5] des infections chroniques et qui augmente le risque d'amputation[5,88,206]. Ce biofilm complique la prise en charge des ulcères de pied chez les patients diabétiques. Il induit une tolérance accrue des microorganismes, favorisant ainsi l'antibiorésistance. Il apparaît donc plus que nécessaire de valider des thérapeutiques alternatives comme les antiseptiques dans la prise en charge des ulcères de pied chez les patients diabétiques[157]. Afin d'évaluer l'effet antibiofilm des antiseptiques dans cette indication, nous avons axé ce travail autour de 3 principales questions :

- L'élaboration d'une stratégie d'évaluation des antiseptiques *in vitro* qui permet de prendre en compte le biofilm et le microenvironnement des ulcères de pied chez les patients diabétiques.
- L'utilisation de la stratégie élaborée pour évaluer les différents antiseptiques sur du biofilm monoespèce et du biofilm bi-espèce avec des isolats cliniques des deux espèces les plus fréquemment isolées d'ulcères de pied chez les patients diabétiques.

- L'établissement d'une comparaison pertinente des différents antiseptiques utilisables dans la prise en charge des ulcères de pied chez les patients diabétiques.

4.1 Stratégie d'évaluation *in vitro* des antiseptiques dans la prise en charge des ulcères de pied de patient diabétiques

Une évaluation pertinente d'antimicrobiens dans la prise en charge de plaies chroniques devrait prendre en compte le biofilm et de préférence un biofilm mature et polymicrobien[194,207]. La première étape de formation du biofilm est l'adhésion des microorganismes sous forme planctonique entre elles ou à une surface[208]. La formation du biofilm est un processus dynamique, avec des étapes qui peuvent se chevaucher et coexister, depuis cette étape d'adhésion à la dispersion en passant par la maturation. La plupart des modèles d'étude des plaies chroniques et du biofilm présentent de nombreuses limites comme vu plus haut. Nous avons établi une stratégie d'évaluation basée sur la combinaison de multiples modèles pour évaluer les antiseptiques aux différentes étapes clés du processus de formation de biofilm.

La première étape à évaluer était l'état planctonique des bactéries en amont de la formation du biofilm. Un rapide tour de la littérature nous a montré que les méthodes habituelles d'évaluation des antiseptiques sont très disparates. Nous utilisons donc à cet effet les recommandations de l'EUCAST pour évaluer la CMI des antiseptiques sur le même modèle de microdilution utilisé pour l'évaluation des antibiotiques.

Ensuite notre évaluation portera sur l'étape d'adhésion. Nous avons identifié le Biofilm Ring Test® comme modèle d'étude pertinent de cette étape. Ce modèle est bien connu et utilisé dans notre unité de recherche en routine. Mais également au laboratoire de microbiologie du Centre Hospitalier Universitaire (CHU) de Nîmes.

L'unité de recherche INSERM U1047, axée sur l'étude de la virulence bactérienne et des infections chroniques comporte une sous unité spécialisée dans les plaies chroniques. Composée de cliniciens-chercheurs (infectiologues, hygiénistes et microbiologistes cliniciens) et de microbiologistes chercheurs, cette sous-unité tente depuis des années à travers l'étude de données fondamentales à répondre aux problématiques rencontrées en pratique clinique au quotidien sur la prise en charge et la prévention des infections d'ulcères de pied chez les patients diabétiques et la recherche d'alternatives thérapeutiques. Cette sous-unité s'est spécialisée dans l'étude des coopérations et de la virulence bactérienne des différents microorganismes isolés dans les plaies chroniques. Des travaux précédents ont porté sur l'étude de l'adaptation de *S. aureus* dans des conditions de stress similaires à ceux rencontrés dans les ulcères de pied chez les patients diabétiques : l'hyperglycémie, l'ischémie (anaérobiose), la présence de concentrations sub-inhibitrices d'antibiotiques [216]. Ces études préliminaires ont abouti au développement du milieu plaie (CWM®) qui fait l'objet d'un brevet européen[200]. Ce milieu est un modèle d'étude qui permet de mimer le micro-environnement des plaies. Il est composé de bouillon de Bolton, constitué de peptone à 79,5% et de débris de kératinocytes qui mime les tissus endommagés, de sérum humain décomplémenté à 20%, qui mime le sérum humain avec les protéines matricielles et effectrices de l'immunité, de sang humain hémolysé à 0,5% qui mime les globules rouges, de sels minéraux. Il a un pH tamponné à 8 pour mimer l'alcalinité du microenvironnement des plaies chroniques[200]. Ce milieu permet la culture des microorganismes les plus souvent isolés des plaies chroniques mais aussi des cultures polymicrobiennes[88].

Nous avons couplé ce milieu à un modèle quantitatif pour l'évaluation de l'étape de formation du biofilm. Ce modèle de quantification de la charge bactérienne vivante à l'intérieur du biofilm permet le dénombrement des cellules bactériennes vivantes après

destruction de la capside matricielle extracellulaire du biofilm. L'évaluation de la charge bactérienne d'un biofilm mature de 72h avant puis après contact avec un antiseptique pendant une durée déterminée permet de déterminer l'impact des antiseptiques sur cette charge bactérienne d'un biofilm mature de 72h.

L'unité de recherche a également fait l'acquisition du dispositif Bioflux® 200 au cours de ces travaux de recherche. Cela a permis la mise au point d'un modèle d'étude dynamique ouvert, qui permet un apport continu de nutriments, un système contrôlable de flux proche de celui des flux sanguins, et un système réglable de température[201]. L'association CWM® et Bioflux® a permis d'élaborer un milieu innovant et qui compense les limites de la majorité des modèles expérimentaux d'étude du biofilm[201]. Pour mimer au mieux les conditions cliniques de prise en charge des ulcères de pied chez les patients diabétiques, nous avons établi un protocole de débridement automatisé. Ce protocole de débridement consiste en une réversion du flux à débit élevé au travers des canaux microfluidiques du système pour éliminer le biofilm mature formé les 72h précédentes. 10 à 15% de biofilm résiduel est maintenu comme retrouvé en pratique clinique[35,160,194]. En pratique, les antiseptiques sont utilisés pour compléter le débridement à l'étape de nettoyage ou de rinçage des plaies[172]. Nous avons donc utilisé l'association du Bioflux® et du milieu plaie (CWM®) pour l'évaluation de la capacité des antiseptiques à réduire le biofilm résiduel post débridement et pour les comparer entre eux.

Toujours dans la perspective de mimer au mieux les conditions réelles des ulcères de pied chez les patients diabétiques, nous avons utilisé des souches cliniques isolées de débridement d'ulcères de pied de patients diabétiques à la « clinique du pied diabétique » du CHU de Nîmes.

4.2 Évaluation *in vitro* des antiseptiques

La première étape d'évaluation des antiseptiques portait sur leur capacité d'inhibition des souches planctoniques de *P. aeruginosa* et *S. aureus*. Malgré l'absence de référentiel, les majorités des études publiées sur les CMI des antiseptiques évalués se sont basées sur des techniques de microdilution comme celles recommandées par l'EUCAST qu'elles ont ensuite adapté. Une comparaison des valeurs de CMI retrouvées au cours de ces différentes études est exposée dans le **tableau 2**.

Pour faciliter les comparaisons, les résultats seront exprimés en fraction de dilution par rapport à la solution commerciale d'antiseptique utilisée pour les expérimentations.

Concernant *P. aeruginosa*, des dilutions de 1:20 à 1:5 ont été retrouvées pour la CMI de l'hypochlorite de sodium sur diverses souches. Le travail actuel a trouvé une dilution de 1:3 au-dessus de cet intervalle. Pour la PVPI, la valeur de 1:8 trouvée lors de nos expérimentations est comprise dans l'intervalle de 1:100 à 1:2 trouvé dans la littérature. La dilution de 1 :64 trouvée pour le PHMB est également dans l'intervalle de 1:512 à 1:32 de la littérature. Il en est de même pour l'Octénidine avec une valeur de 1 :64 comparé avec un intervalle de 1:500 à 1:32 dans la littérature.

Pour *S. aureus*, les valeurs trouvées pour l'hypochlorite de sodium sont de 1:6 à 1:3 selon la souche utilisée. Elles sont également au-dessus de l'intervalle de dilution trouvé dans la littérature de de 1:20 à 1:4. Il en est de même pour la PVPI, avec une valeur de 1:10, au-dessus de l'intervalle de la littérature de 1:200 à 1:16. Pour le PHMB, nous avons trouvé une valeur de 1:200, qui est incluse dans l'intervalle de la littérature de 1:2000 à 1:100. Enfin, la valeur de 1:200 que nous avons trouvée pour la CMI de l'octénidine sur les souches de *S. aureus* font partie de l'intervalle de la littérature de 1:2500 à 1:250 de dilution de la solution commerciale utilisée dans ces travaux.

Tableau 2 : Résumé des différentes publications sur la concentration minimale inhibitrice de l'hypochlorite de sodium, de la polyvidonepyrrolidone iodine (PVPI), du polyhexaméthylènebiguanide (PHMB) et de l'octénidine sur des souches de *Pseudomonas aeruginosa*

Antiseptiques	CMI	Dilution	Souches <i>Pseudomonas</i>	Méthode	References
Sodium Hypochlorite	1%	1:5	ATCC 27853	Personnelle	[217]
	0.025%	1:20	NCIMB 12469	Personnelle	[218]
	800 mg.L ⁻¹	1:6	ATCC27853	Microdilution	[219]
	512 mg.L ⁻¹	1:10	60 souches cliniques	Microdilution	[220]
	400 mg.L ⁻¹	1:12	Souche clinique	Microdilution	[219]
	312.5 mg.L ⁻¹	1:16	ATCC 27853	Microdilution	[221]
	312.5 mg.L ⁻¹	1:16	10 souches cliniques	Microdilution	[221]
	1,666 mg.L ⁻¹	1:3	PAO1 and 3 souches cliniques	Microdilution	Travaux actuels
PVPI	1,024 mg.L ⁻¹	1:100	ATCC 15442	Microdilution	[222]
	5%	1:2	UCBPP-PA14	Personnelle	[223]
	0.04%	1:250	ATCC 15442	Microdilution	[224]
	7,500 mg.L ⁻¹	1:16	ATCC 27853	Microdilution	[225]
	12,500 mg.L ⁻¹	1:8	PAO1 and 3 souches cliniques	Microdilution	Travaux actuels
PHMB	2 mg.L ⁻¹	1:512	ATCC 15442	Microdilution	[222]
	4 mg.L ⁻¹	1:256	ATCC 27853	Microdilution	[45,226]
	8 mg.L ⁻¹	1:258	3 souches cliniques	Microdilution	[45,226]
	16-32 mg.L ⁻¹	1:64 - 1:32	25 souches cliniques	Microdilution	[227]
	7.8 mg.L ⁻¹	1:128	ATCC 27853	Microdilution	[45,226]
	15.6 mg.L ⁻¹	1:64	Souches cliniques	Microdilution	[219]
	10 mg.L ⁻¹	1:100	ATCC 27853	Microdilution	[225]
	15.6 mg.L ⁻¹	1:64	PAO1 and 3 souches cliniques	Microdilution	Travaux actuels
Octénidine	2 mg.L ⁻¹	1:256	ATCC 15442	Microdilution	[222]
	4-16 mg.L ⁻¹	1:128-1:32	2 souches cliniques	Microdilution	[228]
	3.9 mg.L ⁻¹	1:128	ATCC 27853	Microdilution	[219]
	3.9 mg.L ⁻¹	1:32	Souche clinique	Microdilution	[219]
	1 mg.L ⁻¹	1:500	ATCC 27853	Microdilution	[225]
	7.8 mg.L ⁻¹	1:64	PAO1 and 3 souches cliniques	Microdilution	Travaux actuels

Les comparaisons directes des différentes valeurs trouvées sont néanmoins délicates au vu des différences de souche, de milieu et de méthodologie qui peuvent influencer les résultats [229,230]. Les différentes valeurs de CMI retrouvées dans la littérature sont résumées dans le **tableau 3**.

Tableau 3 : Résumé des différentes publications sur la concentration minimale inhibitrice de l'hypochlorite de sodium, de la polyvidone pyrrolidone iodine (PVPI), du polyhexaméthylènebiguanide (PHMB) et de l'octénidine sur des souches de *S. aureus*

Antiseptic	MIC	Dilution	<i>S. aureus</i> strain	Method	Study
Sodium Hypochlorite	1%	1:5	ATCC 6538	Personnelle	Estrela et al[217]
	0.025%	1:20	NCT6571	Personnelle	Locker et al[218]
	938 10 ⁻³ g.L ⁻¹	1:5	ATCC25923	Personnelle	Penna et al [231]
	512 10 ⁻³ g.L ⁻¹	1:10	60 souches cliniques	Microdilution	Kawamura et al [220]
	2000 10 ⁻³ g.L ⁻¹	1:2.5	1532 souches cliniques	Microdilution	Morrissey et al [232]
	1250 10 ⁻³ g.L ⁻¹	1:4	ATCC 27853	Microdilution	Gupta et al[221]
	512-1024 10 ⁻³ g.L ⁻¹	1:10 -1:5	ATCC 6538	German Microdilution	Koburger et al[222]
	830-1666 10 ⁻³ g.L ⁻¹	1:6-1:3	Newman and 3 souches cliniques	Microdilution	Travaux actuels
PVPI	0.08%	1:125	ATCC 25923	Microdilution	Sa et al [224]
	781.5 10 ⁻³ g.L ⁻¹	1:128	ATCC 25923	Microdilution	Gupta et al[221]
	6250 10 ⁻³ g.L ⁻¹	1:16	ATCC 25923	Personnelle	Penna et al [231]
	2000 10 ⁻³ g.L ⁻¹	1:50	ATCC 29213	Microdilution	Hirsch et al [225]
	1028-4094 10 ⁻³ g.L ⁻¹	1:100-1:25	ATCC 6538 and 30 souches cliniques	Microdilution	Dittman et al [233]
	512 10 ⁻³ g.L ⁻¹	1:200	ATCC 6538	German Microdilution	Koburger et al[222]
	10000 10 ⁻³ g.L ⁻¹	1:10	Newman and 3 souches cliniques	Microdilution	Travaux actuels
PHMB	10 10 ⁻³ g.L ⁻¹	1:100	ATCC 29213	Microdilution	Hirsch et al [225]
	0.5-1 10 ⁻³ g.L ⁻¹	1:2000_ - 1:1000	ATCC 25923 and 3 souches cliniques	Microdilution	Lopez-Rojas et al[234]
	1 - 8 10 ⁻³ g.L ⁻¹	1:1000 to 1:125	40 souches cliniques	Microdilution	Zhou et al [227]
	1 - 2 10 ⁻³ g.L ⁻¹	1:1000 - 1:500	ATCC 6538 and 30 souches cliniques	Microdilution	Dittman et al [233]
	1-2 10 ⁻³ g.L ⁻¹	1:1000: 1:500	100 souches cliniques	Microdilution	Fabry et al [235]
	0.5 - 8 10 ⁻³ g.L ⁻¹	1:1000	54 souches cliniques	Microdilution	Renzeni et al [236]
	1-2 10 ⁻³ g.L ⁻¹	1:1000: 1:500	ATCC 29213 and 15 souches cliniques	Microdilution	Assadian et al
	1 10 ⁻³ g.L ⁻¹	1:1000	ATCC 15442	German Microdilution	Koburger et al[222]
	5 10 ⁻³ g.L ⁻¹	1:200	Newman and 3 souches cliniques	Microdilution	Travaux actuels
Octénidine	1 10 ⁻³ g.L ⁻¹	1:500	ATCC 29213	Microdilution	Hirsch et al [225]
	0.5 - 1 10 ⁻³ g.L ⁻¹	1:1000 -1:500	335 souches cliniques	Microdilution	Zheng et al [237]
	0.5 -2 10 ⁻³ g.L ⁻¹	1:1000 -1:250	878 souches cliniques	Microdilution	Htun et al [238]
	0.25 -2 10 ⁻³ g.L ⁻¹	1:2000 -1:500	ATCC 25923, ATCC 43300 and 2 souches cliniques	Microdilution	Nicolae Dopcea et al [239]
	0.2-0.4 10 ⁻³ g.L ⁻¹	1:2500 -1:1250	ATCC 25923, ATCC	Microdilution	Grecka et al [240]

		25 213 and 3 souches cliniques		
2 10 ⁻³ g.L ⁻¹	1:250	ATCC 15442	German Microdilution	Koburger et al[222]
1 - 2 10 ⁻³ g.L ⁻¹	1:500 - 1:250	ATCC 6538 and 30 souches cliniques	Microdilution	Dittman et al [233]
2.5 10 ⁻³ g.L ⁻¹	1:200	Newman et 3 souches cliniques	Microdilution	Travaux actuels

L'étape suivante de notre évaluation consistait en l'évaluation de l'effet des antiseptiques sur l'étape d'adhésion des bactéries lors de la formation du biofilm avec le modèle de l'Antibiofilmogram®. Les souches utilisées ont été évaluées en amont avec le Biofilm Ring Test® qui avait confirmé leur capacité à adhérer à la plaque. Les 4 antiseptiques évalués ont inhibé précocement l'adhésion de toutes les souches à la plaque. Aussi bien les souches de laboratoire que les souches cliniques et ce, pour les 2 espèces étudiées *S. aureus* et *P. aeruginosa*. Cela suppose par extrapolation que les antiseptiques évalués ont tous la capacité d'empêcher la formation de biofilm. A notre connaissance, ces résultats sont la première évaluation des antiseptiques avec ce modèle. Classiquement, en utilisant des modèles *in vitro*, les antiseptiques sont évalués quant à leur capacité d'éradication du biofilm, indépendamment de l'étape du biofilm et de leur impact sur la stratégie de traitement du patient. Notre approche permet de rajouter une autre dimension à l'évaluation de ces composés

L'étape d'après consistait en l'évaluation de l'effet des antiseptiques sur du biofilm mature dans des conditions statiques avec le modèle de dénombrement de la charge bactérienne vivante après rupture de la capsid matricielle extracellulaire. Nous avons évalué la capacité des antiseptiques à réduire la charge bactérienne vivante d'un biofilm mature. Avec ce modèle, tous les antiseptiques ont réduit efficacement d'un log10 la charge bactérienne des biofilms matures monomicrobiens de *S. aureus* et *P. aeruginosa* à leurs concentrations commerciales et ce indépendamment du milieu utilisé.

Au sein du biofilm, certains microorganismes sont dits « persistants ». Ces microorganismes ont un métabolisme réduit, qui se traduit par des cellules viables mais non cultivables. Bien que non cultivables, ces cellules sont infectantes et sont estimées très résistantes aux antimicrobiens[241]. Du fait de ces propriétés, ces cellules ne sont pas identifiables par des modèles de quantification de la charge bactérienne du biofilm. Il est par conséquent également impossible d'évaluer l'efficacité des antiseptiques sur ces cellules à l'intérieur du biofilm. Aussi, la dernière étape de nos expérimentations a porté sur une évaluation des antiseptiques au plus proche possible de la réalité des pratiques cliniques d'utilisation. Cette évaluation a été effectuée avec un modèle dynamique de biofilm/plaie chronique, le Bioflux®. Elle a consisté en l'application des antiseptiques sur un biofilm de 72H après une étape de débridement automatisé, comme dans la pratique clinique. A ce niveau, nous avons observé un effet souche dépendant et milieu dépendant de l'octénidine. Le PHMB a également présenté un effet souche dépendant mais uniquement sur les souches de *S. aureus*. L'hypochlorite de sodium a été efficace sur tous les biofilms indépendamment du milieu utilisé. L'effet de réduction significative de biofilm a persisté jusqu'à 72h après contact de l'antiseptique. Cela suppose une inhibition de la formation du biofilm comme suggéré avec le modèle de l'Antibiofilmogram®.

Après 72h de biofilm suivi d'un débridement et de 72H de contact avec les antiseptiques dans le milieu CWM®, les souches évaluées de *S. aureus* ont présenté la même sensibilité à l'octénidine, au PHMB et à l'hypochlorite de sodium. Les CMI équivalentes au CMI en amont de la formation de biofilm sous-entendent que la tolérance aux antiseptiques ne s'est pas mise en place. A ce jour, aucun mécanisme de résistance n'a été décrit pour ces antiseptiques[171,239,242-244].

4.3 Comparaison des antiseptiques & Impact du milieu CWM® sur le biofilm et l'activité des antiseptiques

L'impact du milieu plaie sur le biofilm a été observé dès les étapes de formation précoce du biofilm évaluées avec le BRT®. Des travaux précédents ont confirmé une adhésion accélérée des microorganismes à la surface de la plaque de culture avec le CWM®. Cette adhésion accélérée suppose par extension une formation de biofilm accélérée[200]. Lors de nos expérimentations, nous avons également observé une adhésion accélérée avec le CWM®. Cela s'est matérialisé au cours de l'évaluation des antiseptiques par la nécessité d'utilisation de concentrations 2 à 6 fois plus élevées d'antiseptiques pour inhiber la formation du biofilm de *S. aureus* avec le modèle de l'Antibiofilmogram® dans du milieu CWM®, en comparaison au milieu contrôle. Toutefois, les concentrations requises pour inhiber la formation de biofilm en milieu CWM® étaient bien en dessous des concentrations commerciales. Aux concentrations commerciales utilisées en pratique, tous les antiseptiques ont la capacité d'inhiber la formation de biofilm, indépendamment du milieu utilisé.

Avec le modèle de dénombrement de la charge bactérienne vivante, la charge bactérienne dénombrée avec le milieu CWM® était significativement plus importante que la charge bactérienne avec le milieu contrôle. Cette supériorité a été observée aussi bien pour le biofilm monomicrobien de *S. aureus* que pour celui de *P. aeruginosa*. Le milieu CWM® a encore une fois impacté l'activité des antiseptiques. L'hypochlorite de sodium était le seul antiseptique à réduire significativement le biofilm d'un log10 à la valeur de sa CMI indépendamment du milieu utilisé. La PVPI, le PHMB et l'octénidine ont nécessité une augmentation de 10 fois les concentrations utilisées pour réduire la charge bactérienne dans le milieu contrôle comparé au CWM®. Cet effet peut s'expliquer par la formation d'un biofilm plus dense avec le milieu CWM® qui

limiterait l'accès des antiseptiques aux microorganismes à l'intérieur du biofilm. De plus l'effet des antiseptiques est réputé être limité en présence de matières organiques comme le sang, les débris cellulaires, et les protéines plasmatiques qui sont présentes dans le milieu CWM®[245–247]. L'hypochlorite de sodium ne semble pas être affecté par la matière organique comme précédemment suggéré[170]. Néanmoins, encore une fois les concentrations requises pour la diminution de la charge bactérienne d'un log de 10 étaient bien en deçà des concentrations commerciales utilisées. Aux concentrations maximales utilisables dans ce modèle qui correspondent à la moitié de la concentration de la solution commerciale, tous les antiseptiques ont été capables d'éradiquer significativement la charge bactérienne vivante d'un biofilm mature statique de 72H de toutes les souches dans le milieu contrôle. Dans le CWM®, la capacité d'éradication du biofilm était moins significative pour l'octénidine. Les propriétés antibiofilm des autres antiseptiques étaient moins impactées par le milieu CWM®.

Une formation significativement accélérée du biofilm a également été notée lors de l'utilisation du modèle dynamique du Bioflux®. Avec ce modèle également, le biofilm formé était plus dense et visuellement plus opaque. Ces remarques se sont appliquées aussi bien pour les biofilms monoespèce de *S. aureus* et *P. aeruginosa* que pour le biofilm bi-espèces des deux espèces. Les antiseptiques ont cette fois ci été évalués dans des conditions proches de la clinique. A savoir utilisation des concentrations commerciales après une étape de débridement. En ce qui concerne les antiseptiques, l'impact du milieu CWM® sur leur efficacité s'est limité cette fois ci à l'octénidine avec la souche PAO1 puisque son efficacité s'est limitée au milieu contrôle et non au CWM®. L'efficacité de l'octénidine était limitée à la souche de laboratoire de *S. aureus* Newman dans les 2 milieux. Pour les souches de *P. aeruginosa*, l'octénidine a significativement réduit le biofilm post débridement des souches cliniques PAC4 et PAC1 indépendamment du milieu. Son effet sur la souche de laboratoire PAO1 s'est

limité au milieu de contrôle. Le PHMB malgré un effet significatif sur toutes les souches de *P. aeruginosa*, n'était efficace que sur 2 souches cliniques de *S. aureus*. La supériorité de l'efficacité de l'hypochlorite de sodium s'est confirmée lors de son évaluation conjointe avec le PHMB sur du biofilm bi-espèces de *S. aureus* et *P. aeruginosa*. L'hypochlorite de sodium était le seul antiseptique efficace sur tous les biofilms, mono comme bi-espèces, indépendamment du milieu. Le PHMB a présenté un effet milieu dépendant et biofilm dépendant. Il a été efficace uniquement sur le S2P2 et le S1P1 dans milieu contrôle et non dans le milieu CWM®.

Ces résultats sont en contradiction avec des études précédentes qui ont noté un manque d'efficacité de l'hypochlorite de sodium sur du biofilm mono ou polymicrobien[248–251]. Toutefois, en analysant de plus près ces études, on note que les solutions évaluées contiennent des concentrations très faibles d'hypochlorite de sodium et de chlore actif, en moyenne 80 parties par million (ppm). La solution utilisée lors de nos travaux contenait 5000 ppm de chlore actif.

V. Conclusion & Perspectives

En combinant plusieurs modèles innovants, ces travaux ont permis d'évaluer les antiseptiques à différents niveaux du processus de formation du biofilm dans un microenvironnement similaire à celui des plaies. Cette évaluation par extrapolation, donne une idée de l'efficacité des antiseptiques dans la prise en charge des plaies chroniques. Nous avons établi les premières évaluations de l'efficacité des antiseptiques sur l'inhibition de la formation du biofilm de *S. aureus* et *P. aeruginosa* en nous basant sur le modèle de l'Antibiofilmogram[®]. En utilisant des modèles innovants, nous avons pu démontrer la supériorité de l'hypochlorite de sodium sur le PHMB et l'octénidine dans la réduction significative du biofilm post débridement automatisé dans un microenvironnement similaire à celui d'un ulcère de pied chez les patients diabétiques. Cela suggère que l'hypochlorite de sodium serait une meilleure option thérapeutique pour la prise en charge des ulcères de pied chez les patients diabétiques.

Nos travaux se rajoutent à la liste de ceux qui mettent en avant l'importance du microenvironnement des plaies chroniques et la nécessité d'utiliser des milieux d'étude mimant le microenvironnement des plaies chroniques[32,131,199,200,207,226]. Ils ont également confirmé précédents résultats et validé le modèle d'étude du CWM[®] dans l'étude des plaies chroniques comme les ulcères de pied chez les patients diabétiques.

La PVPI a été identifiée dans notre revue de la littérature comme un antiseptique avec un fort potentiel. Elle n'a pas pu être évaluée dans nos travaux à cause de sa coloration foncée qui a empêché les visualisations au microscope. Une alternative serait l'utilisation de souches fluorescentes pour pallier ce problème. Une autre alternative pourrait être l'utilisation de kits d'identification de viabilité cellulaire par fluorescence comme le kit « The Filmtracer[™] LIVE/DEAD[™] Biofilm Viability Kit » (propidium iodide et Syto9[™], Thermo Fisher Scientific, Waltham, MA, USA). Ce kit contient 2

solutions fluorescentes qui associent leurs propriétés de fluorescence et de fixation à la membrane cellulaire des microorganismes pour identifier la viabilité cellulaire à l'intérieur du biofilm. Les cellules vivantes sont colorées en vert et celles mortes en rouge. Une estimation de la viabilité cellulaire avant et après leur application pourrait donner une idée de l'efficacité de l'effet antibiofilm des antiseptiques.

Actuellement, l'association Bioflux/microscope permet une identification visuelle du biofilm. Toutefois, l'évaluation de la viabilité est limitée à la mobilité des cellules lors de l'identification visuelle microscopique. Cette évaluation est donc sujet dépendant. De plus, la visualisation des cellules persistantes au sein du biofilm avec un mode de développement latent est impossible. Une fluorescence corrélée à la viabilité cellulaire comme celle proposée par le kit « The Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit » pourrait remédier à cette limite.

Plusieurs améliorations pourraient être apportées au modèle afin de simuler au mieux une plaie chronique et son microenvironnement. Une première ébauche a été suggérée dans des travaux précédents par l'ajout de glucose dans le milieu plaie pour mimer l'hyperglycémie constante retrouvée chez les patients diabétiques[88].

Un autre versant abordé dans l'unité de recherche est la distribution spatiale des microorganismes dans le biofilm[252]. Grâce à la microscopie fluorescente et les souches fluorescentes utilisées dans cette optique, l'impact des antiseptiques sur la distribution spatiale au sein d'un biofilm polymicrobien pourrait permettre l'identification des mécanismes d'action des antiseptiques sur le biofilm. Les mécanismes d'action décrits actuellement sont basés sur l'effet des antiseptiques sur les souches planctoniques.

Même si le modèle bi-espèces, utilisé dans ces travaux est déjà une grande avancée, en pratique, le biofilm des plaies chroniques est décrit comme polymicrobien avec de multiples espèces qui coopèrent entre elles. Une représentation plus proche de

la réalité serait donc d'incorporer en plus de *S. aureus* et de *P. aeruginosa*, plusieurs espèces bactériennes et fongiques, notamment les plus retrouvées dans les plaies chroniques comme les corynébactéries et les entérocoques et les espèces du genre *Candida*.

Aussi, ces travaux ont effleuré le versant de la tolérance aux antiseptiques en évaluant la CMI des antiseptiques sur les bactéries sessiles libérées du biofilm monomicrobien de *S. aureus*. L'exposition pendant 72h du biofilm aux différents antiseptiques n'a pas induit de tolérance à l'hypochlorite de sodium, au PHMB et à l'octénidine. En pratique, une tolérance est induite avec des durées d'exposition plus longues. Il serait dans cette idée pertinent d'évaluer la tolérance avec des durées d'exposition plus longues aux antiseptiques. Un séquençage des souches utilisées permettrait aussi de compléter l'information génétique de ces souches sur les gènes de virulence et de résistance aux antiseptiques. Cela permettrait aussi d'étudier l'existence éventuelle des résistances croisées avec les antibiotiques.

Une des innovations du modèle dynamique utilisé durant ces travaux était l'ajout des débris cellulaires fibroblastiques dans le milieu CWM® qui permettent de mimer au mieux la plaie. Une amélioration pourrait consister en l'incorporation de cellules vivantes du tissu cutané en plus des débris. Cette incorporation pourrait s'établir par le tapissage des canaux microfluidiques de l'automate des cellules fibroblastiques et/ou de kératinocytes. Un modèle similaire est en effet décrit pour l'étude des maladies cardiovasculaires [253,254].

Enfin, les résultats de ces travaux gagneraient à être validés sur des modèles animaux. La validation la plus pertinente resterait néanmoins des études cliniques multicentriques de grande ampleur, pour confirmer l'efficacité des antiseptiques et les comparer entre eux.

VI. Références

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RESEARCH

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Clinical factors associated with peripherally inserted central catheters (PICC) related bloodstream infections: a single centre retrospective cohort



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Abstract

Background Despite their spread in daily practice, few data is available on clinical factors associated with peripherally inserted central catheter (PICC)-related bloodstream infections (PR-BSI). We aimed to assess the PR-BSI incidence, microbiology, and factors associated with PR-BSI with a focus on clinical symptoms.

Methods We conducted a retrospective cohort study in a French university hospital. We screened all PICC insertions performed from April 1st, 2018, to April 1st, 2019, and included PICC insertions in adult patients. We assessed the PR-BSI incidence, the factors associated with PR-BSI using a Cox model, and negative and positive predictive values (NPVs and PPVs) of each clinical sign for PR-BSI.

Results Of the 901 PICCs inserted in 783 patients (38,320 catheters days), 214 PICCs (24%) presented with a complication. The most prevalent complication was PR-BSI (1.9 per 1000 catheter days; 8.1% of inserted PICCs). Enterobacteriales ($N=27$, 37%) and coagulase negative Staphylococci ($N=24$, 33%), were the main microorganisms responsible for PR-BSI. Factors independently associated with occurrence of PR-BSI were fever (hazard ratio 13.21, 95% confidence interval 6.00–29.11, $p < 0.001$) and chills (HR 3.66, 95%CI 1.92–6.99, $p < 0.001$). All clinical signs and a duration of PICC maintenance ≥ 28 days, had a low PPVs ($\leq 67.1\%$) but high NPVs ($\geq 92.5\%$) for PR-BSI.

Conclusions Monitoring of clinical signs, especially fever and chills, with caution and limitation of device maintenance duration, could improve PICC management.

Keywords PICC, CR-BSI, Fever, Chills, PICC maintenance duration

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Background

Since their introduction in late 1970s [1], peripherally inserted central catheters (PICC, also known as PICC-line) widely spread in daily practice. They are indicated for intermediate-term venous access (7 days to 6 months) for some concrete indications as irritant or vesicant infusions (total parenteral nutrition or chemotherapy), difficult venous access and prolonged antimicrobial therapies [2]. PICC insertion occurs through a peripheral upper arm vein, avoiding iatrogenic complications and ensuring a safe and easy removal compared to other central venous catheters (CVCs) which they partially replaced [3]. Despite some advantages, mechanical complications as occlusion, accidental withdrawal [4], deep venous thrombosis [5], and catheter-related bloodstream infections (CR-BSIs) [6, 7] were reported during PICC use. Rates of PICC-related bloodstream infections (PR-BSIs) compared to other CVCs infections has been a controversial and disputed subject. Now PR-BSI rates are recognized similar to those of conventional CVCs [8, 9]. Nonetheless, in a recent national public health survey in France, one quarter of the 30 million of catheters implanted every year presented with a CR-BSI. Among these infections, 16.8% were related to a PICC, making PR-BSI the most important and preventable PICC-related complication [10–12].

Whilst abundant literature is available on PICC related complications, PR-BSI risk factors are still poorly assessed [13]. Published data remains scarce [9, 13–18], particularly for medical ward inpatients and outpatients, since most of the data focuses on intensive care unit (ICU) [19].

A study aiming to assess PICC-related complication incidence, with a highlight on PR-BSI, to describe the microorganisms involved and to assess risk factors associated with PR-BSI with a focus on clinical signs, is lacking.

Methods

Study aim, design and settings

This study aims to assess PICC-related complication incidence, particularly the PR-BSI incidence and to describe the microorganisms involved and risk factors associated with PR-BSI with a focus on clinical signs in patients from a tertiary hospital.

We conducted a retrospective, single centre, observational cohort study in Nimes University Hospital, from April 1st, 2018, to April 1st, 2019. In the medical imaging department of this 2094-beds University Hospital, single or double lumen PICC (Bard Access Systems, Salt Lake City, UT, USA) ultrasonography guided insertions are performed on inpatients and outpatients 5 days a week.

Each PICC insertion request is forwarded to, registered, and validated or not by the Pharmacy Department. PICC insertion is performed in aseptic conditions according to the French Society of Infection Control (SF2H) guidelines [20]. After insertion, PICC position is verified with a chest X-ray and adjusted if required. Normal saline is used for preventing lumen occlusion and maintaining PICC patency, as recommended by national and international guidelines [2, 20].

Patients

We screened all consecutive patients with at least one PICC placement between April 1st, 2018, and April 1st, 2019, using the pharmacy registry of PICC insertion requests. All adult patients were included in the study. When a patient had more than one PICC placement, all PICC placements were considered. Patients under 18-year-old and those lost to follow-up between PICC insertion and PICC removal were excluded.

Data collection

Patient's demographic, clinical and biological data were collected from the hospital electronic medical record. Age, sex, weight, height, body mass index (BMI), medical history, reason for PICC placement, insertion date, removal date, type of PICC, site of insertion, reason for PICC removal, ongoing treatment (especially corticosteroids or other immunosuppressive treatments) and vital status at PICC removal were collected. When a bloodstream sample was diagnosed positive by the microbiology laboratory, more information was collected on the laboratory software (number of samples collected, number of positive samples, central and peripheral blood culture results, insertion site culture and catheter culture results, lag time between central and peripheral positive blood cultures, microorganism identification and antimicrobial resistance). Patients' Charlson comorbidity index was calculated [21]. Microorganisms resistance diagnosis were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [22]. An adjudication committee, made up of an infection control specialist (J.O.), an infectious disease physician (A.S) and an intensivist (R.L.), analysed the medical records to ensure the diagnosis met definition criteria of PICC-related infections (PRI) [23]. For each case of BSI, alternative sources of infection were carefully checked by the adjudication committee by reviewing the patient chart and all microbiological culture results. In case of discrepancy, diagnosis was discussed between the committee members until a consensus was reached.

Definitions

We defined PRI according to the French Intensive Care Society (SRLF) guidelines [23], which are in line with the Centers for Disease Control and Prevention (CDC) and the European Centre for Disease Control and Prevention (ECDC) guidelines [24–26].

We defined PICC colonization as a quantitative catheter culture $\geq 10^3$ CFU/mL (according to Brun-Buisson) without bacteraemia or clinical signs [23].

We defined non-bacteraemia PRI (NB-PRI), in the absence of bacteraemia, as a combination of: (i) a quantitative catheter culture $\geq 10^3$ CFU/mL and (ii) (a) signs of local infection (purulent discharge from the PICC insertion site or tunnel infection); and/or (b) systemic signs, with complete or partial resolution of systemic signs of infection within 48 h after PICC removal [23].

We defined PR-BSI as an association of: (i) the occurrence of either bacteraemia or fungaemia during the 48-h period surrounding catheter removal (or a suspected diagnosis of PRI when the PICC is not removed immediately); (ii) and either a positive culture with the same microorganism on one of the following samples: insertion site culture, or catheter culture $\geq 10^3$ CFU/mL or positive central and peripheral blood cultures with the same microorganism, with a central/peripheral positive blood culture lag time > 2 h, with central blood cultures being positive earlier than the peripheral ones [23].

Statistical analysis

PICC insertion was the unit for statistical analyses. Data are described as median and interquartile range (IQR) or number and percentage as appropriate. We assessed factors associated with PR-BSI using a Cox model. Factors with a p -value ≤ 0.1 in the univariate analysis were included in the multivariable analysis. Results of the Cox model were reported as hazard ratio (HR) with 95% confidence interval (95% CI). We plotted receiver operating characteristic (ROC) curves for clinical signs associated with PR-BSI and for PICC maintenance duration. We calculated the negative and positive predictive values (NPVs, PPVs) for each clinical sign, and identified the optimal cut-off value of catheter duration for PR-BSI occurrence by maximizing the Youden index. All tests were two-sided and p -values less than 0.05 were considered statistically significant. We performed all analyses using *R* software, version 4.2.0 (The *R* Foundation for Statistical Computing, Vienna, Austria).

Results

From April 1st, 2018 to April 1st, 2019, amongst 1091 PICCs inserted in 952 patients, 901 PICCs inserted in 783 patients met inclusion criteria and were included in the analysis (Fig. 1). The median follow-up was 21 days (IQR, 9–43). More than a half of PICCs were removed at the end of intravenous therapy ($N=529$; 59%), 38% ($N=346$) because of a complication and 3% ($N=26$) after port implantation. The median time for PR-BSI occurrence was 30 (IQR, 16–76).

Characteristics of the study population are presented in Table 1. The median age for patients with a PICC insertion was 70.9 years (IQR, 59.4–79.8). The median BMI was 24.5 kg/m² (IQR, 21.2–28.9). More than a half of patients had a peripheral vascular disease, a quarter had a chronic heart failure and 22% a metastatic cancer. The median Charlson comorbidity index was 6 (IQR, 3–9).

Antimicrobial therapy ($N=408$; 45%); chemotherapy ($N=240$; 27%) and difficult venous access ($N=154$; 17%) were the main reasons for PICC insertion. Inserted PICCs had mostly one lumen ($N=712$; 79%) and were inserted in the left basilic or brachial veins ($N=470$; 52%, or $N=209$; 24%). The median PICC maintenance duration was 21 days (IQR, 9–43) accounting for 38,320 catheter days, and PICCs were essentially removed at the end of IV therapy ($N=529$; 59%). However, an accidental withdrawal before the end of therapy occurred in 69 cases (7%), and PICCs were also removed immediately in case of CR-BSI suspicion ($N=124$; 14%) or death ($N=117$; 13%). Characteristics of PICCs are summarized in Table 1.

Around one quarter of PICCs ($N=214$, 24%) presented with a complication (Table 2). The first complication encountered was PR-BSI ($N=73$, 8.1%, 1.9 per 1000 catheter days). Accidental withdrawal ($N=61$, 6.8%, 1.6 per 1000 catheter days), vein thrombosis ($N=14$, 1.6% and 0.4 per 1000 catheter days), catheter occlusion ($N=12$, 1.3%, 0.3 per 1000 catheter days), local signs of inflammation ($N=4$, 0.4%, 0.1 per 1000 catheter days) and NB-PRI ($N=3$, 0.4%, < 0.1 per 1000 catheter days) were less frequent.

Among the microorganisms involved in PR-BSI, Enterobacterales were the main species ($N=27$, 37%), followed by Coagulase Negative Staphylococci (CoNS) ($N=24$; 33%), *Staphylococcus aureus* ($N=7$; 10%), *Candida* species ($N=7$; 10%) and non-fermenting Gram-Negative Bacilli ($N=5$; 7%). Polymicrobial CR-BSI with two species accounted for 19 cases (26%) and 5 cases (7%) were documented with more than 2 species. Moreover, 47 PICCs presented with a colonization (6.8%, 1.2 per 1000 catheter days), predominantly due to CoNS ($N=33$, 70%) and Enterobacterales ($N=6$, 13%).

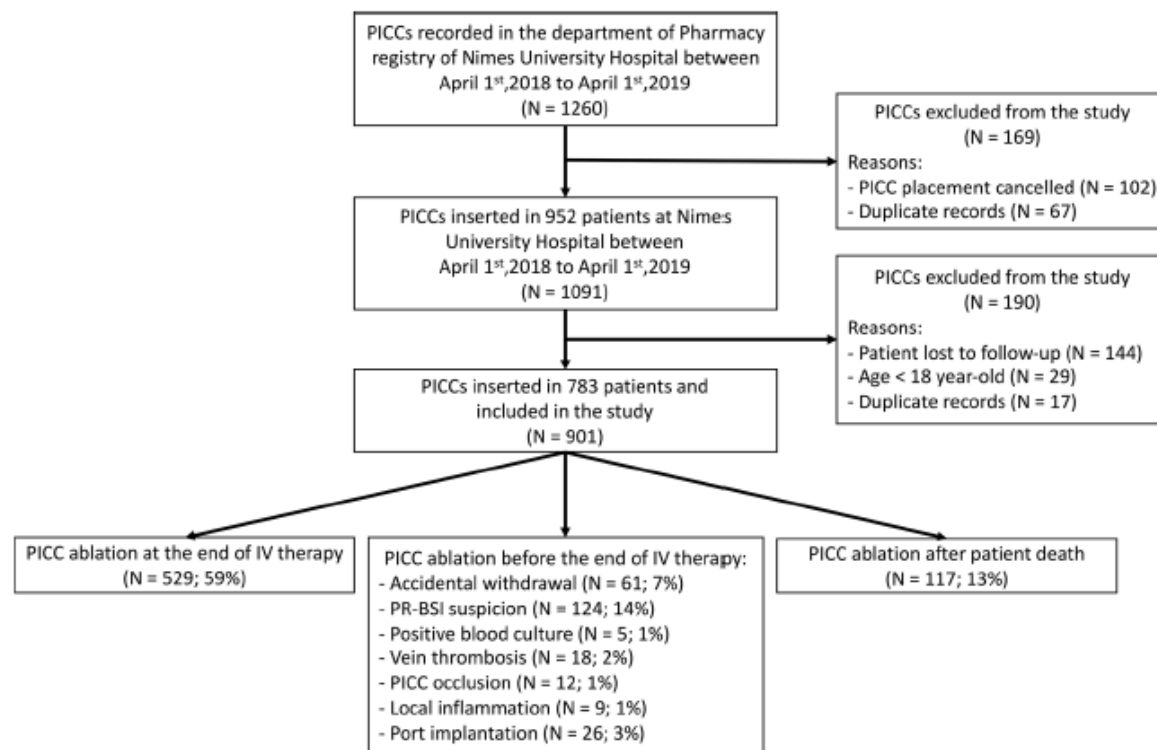


Fig. 1 Flow chart of peripherally inserted central catheters (PICC) placement

Characteristics of the species involved in infectious complications are listed in Table 3.

Fever and chills were the most common clinical signs in 84% and 64% of patients with PR-BSI, respectively. These two clinical signs showed the best prediction capacity for PR-BSI: area under the ROC curve (AUC) 0.8828, 95%CI 0.8391–0.9265 for fever and 0.808, 95%CI 0.7524–0.8636 for chills (Fig. 2). Importantly, all clinical signs, namely pain or rash at PICC insertion site, fever, chills, or dyspnoea had high specificity but low sensitivity for PR-BSI detection (Table 4). Accordingly, the NPVs of clinical signs were satisfactory, especially for fever (98.5%) and chills (96.9%), whereas the PPV were low. The higher PPV were 66.7% for the occurrence of a rash at PICC insertion site and 67.1% for the occurrence of chills (Table 4).

As illustrated in the Fig. 3, the risk of PR-BSI increased with the duration of PICC maintenance. The risk increased mainly during the first 6 months with a probability of PR-BSI at 32.2% (95%CI 22.5–40.7) at day-180. The AUC of PICC maintenance duration for PR-BSI development was 0.6349, 95%CI 0.5715–0.6984 and the optimal cut-off value of catheter day associated with PR-BSI development was 28 days. Similar to clinical signs, the PPV for PR-BSI development of a PICC maintenance

duration ≥ 28 days was very low at 11.2% whereas the NPV was at 93.9%.

In the univariate analysis the variables associated with occurrence of PR-BSI were: an history of immunosuppressive treatments (either corticosteroids or immunosuppressors, or both; HR 1.79, 95%CI 1.38–2.32, $p < 0.001$), an history of malignancy (solid tumour or haematological malignancy; HR 1.68, 95%CI 1.2–2.36, $p = 0.003$), dementia (HR 8.25, 95%CI 1.12–61, $p = 0.04$), insertion of a double-lumen PICC (HR 2.24, 95%CI 1.41–3.58, $p < 0.001$) and occurrence of clinical signs: pain at insertion site (HR 6.65, 95%CI 3.01–14.7, $p < 0.001$), rash at insertion site (HR 10.2, 95%CI 4.36–23.8, $p < 0.001$), fever (HR 30.9, 95%CI 16.5–57.7, $p < 0.001$), chills (HR 25.6, 95%CI 15.2–43, $p < 0.001$).

In the multivariable analysis only fever (HR 13.21, 95%CI 6.00–29.11, $p < 0.001$) and chills (HR 3.66, 95%CI 1.92–6.99, $p < 0.001$) were independently associated with the occurrence of PR-BSI (Table 4).

Discussion

This retrospective cohort study reports the results of 901 PICC insertions in 783 patients accounting for 38,320 catheters days. 214 PICCs (24%) presented with

Table 1 Characteristics of the study population and peripherally inserted central catheters (PICC)

	Total (N = 901) N (%) or median (IQR)	No PR-BSI (N = 828) N (%) or median (IQR)	PR-BSI (N = 73) N (%) or median (IQR)
Patients, N	783	713	70
Sex ratio M/F	500/401	461/367	37/33
Age (year)	70.9 (IQR, 59.4–79.8)	71.0 (IQR, 59.8–80.5)	69.8 (IQR, 58.8–74.1)
Body Mass Index (kg/m ²)	24.5 (IQR, 21.2–28.9)	23.8 (IQR, 20.7–28.1)	24.4 (IQR, 21.1–28.7)
Charlson comorbidity index	6 [3–9]	6 [3–8]	7 [4–10]
<i>Main comorbidities</i>			
Corticosteroids	141 (18%)	112 (16%)	29 (41%)
Immunosuppressors	101 (13%)	78 (11%)	23 (33%)
Solid tumour	142 (18%)	129 (18%)	13 (19%)
Metastatic solid tumour	178 (23%)	151 (21%)	27 (38%)
Haematological malignancy	69 (9%)	51 (7%)	18 (26%)
Leukaemia	46 (6%)	36 (5%)	10 (14%)
Lymphoma	23 (3%)	15 (2%)	8 (11%)
<i>PICC lumen</i>			
1 lumen	712 (79%)	669 (81%)	43 (59%)
2 lumens	189 (21%)	159 (19%)	30 (41%)
<i>Reason for PICC insertion</i>			
Antimicrobial therapy	408 (45%)	397 (48%)	11 (15%)
Chemotherapy	240 (27%)	201 (24%)	39 (53%)
Difficult venous access	154 (17%)	143 (17%)	11 (15%)
Total parenteral nutrition	52 (6%)	44 (5%)	8 (11%)
Iterative venous access	47 (5%)	43 (5%)	4 (5%)
<i>Insertion site</i>			
Left basilic vein	470 (52%)	436 (53%)	34 (47%)
Right basilic vein	135 (15%)	124 (15%)	11 (15%)
Left brachial vein	209 (24%)	189 (22%)	21 (29%)
Right brachial vein	72 (8%)	67 (8%)	5 (7%)
Left cephalic vein	12 (1%)	10 (1%)	2 (3%)
Right cephalic	1 (<1%)	1 (<1%)	0 (0%)
Femoral	1 (<1%)	1 (<1%)	0 (0%)
PICC maintenance (days)	21 (IQR, 9–43]	20 (IQR, 9–41]	30 (IQR, 16–76)
<i>Reason for PICC removal</i>			
End of IV therapy	529 (59%)	529 (64%)	0 (0%)
Accidental withdrawal	61 (7%)	60 (7%)	1 (1%)
PR-BSI suspicion	124 (14%)	62 (7%)	62 (85%)
Positive blood culture	5 (1%)	2 (<1%)	3 (4%)
Vein thrombosis	18 (2%)	17 (2%)	1 (<1%)
PICC occlusion	12 (1%)	11 (1%)	1 (<1%)
Local inflammation	9 (1%)	8 (1%)	1 (<1%)
Port implantation	26 (3%)	25 (3%)	1 (<1%)
Death	117 (13%)	114 (14%)	3 (4%)

BSI Bloodstream infection, COPD Chronic obstructive pulmonary disease, IQR Interquartile range, PR-BSI PICC related bloodstream infection, IV Intravenous

a complication, mainly a PR-BSI (N = 73, 8.1%) with a 1.9 per 1000 catheter days incidence rate. Enterobacteriales (N = 27, 37%) and CoNS (N = 24, 33%) were the main microorganisms involved in PR-BSI. Moreover, we

highlighted the importance of clinical signs for PR-BSI monitoring, reporting fever and chills as factors independently associated with PR-BSI occurrence.

Over the last decade, PICC use increased in hospitals [27], particularly in university hospitals [10]. PR-BSIs

Table 2 Type, rate and incidence of peripherally inserted central catheters (PICC) complications

Complications	Number of PICC	Rates (%)	Incidence (per 1000 catheter days)
PR-BSI	73	8.1	1.9
Accidental withdrawal	61	6.8	1.6
PICC colonization	47	5.2	1.2
Vein thrombosis	14	1.6	0.4
Catheter occlusion	12	1.3	0.3
Local inflammation	4	0.4	0.1
NB-PRI	3	0.3	<0.1

PR-BSI PICC related bloodstream infection, NB-PRI Non bacteraemia PICC related infection

also increased at the same time [10, 28, 29]. One important finding of this study was the 1.9 per 1000 catheter days incidence rate of PR-BSI, which was in line with the median 2.1 per 1000 catheter days incidence rate reported in meta-analyses of international studies [9, 30, 31]. Previous studies found various incidence rates ranging from 0.6 to 3.3 per 1000 catheter days [4, 16–18, 32]. Importantly, differences in PR-BSI incidence may be related to the time period of the studies and differences in catheter-related infection definitions across these studies [16]. Moreover, several definitions used in numerous

studies were reported unsuitable for research [33]. Nonetheless, we must acknowledge the PR-BSI incidence rate found in this study was above the 1 per 1000 catheter days threshold target for CR-BSI prevention intervention suggested by infection control experts [12]. In this global context, CVCs and particularly PICC were pointed as priorities for infection prevention measures [28]. Multimodal process for better CR-BSI prevention and control including practice change based on knowledge, education, and behavioural interventions in our hospital are encouraged [12]. Recent guidelines [34] suggested, practical healthcare workers team education and training, promoted by a multidisciplinary team with checklists, continuous improvement programs and bundles implementation [35].

Another significant finding of this work was the importance of clinical monitoring of PR-BSI. Indeed, this study identified a strong correlation between clinical signs such as fever and chills, and PR-BSI. Most importantly, clinical signs showed high specificity and NPV but unsatisfactory sensibility and PPV. The absence of clinical signs is therefore significantly indicative of the absence of PR-BSI as suggested by others in ICU settings [36]. Amongst clinical signs, fever and chills have the best NPVs. Fever was previously reported as indicative of CR-BSI along with other infection signs [37, 38]. In line with previous studies [39–41], we reported that prolonged duration of

Table 3 Species involved in infectious complications of peripherally inserted central catheter (PICC)

Species	PR-BSI (N, %)	PICC colonization (N, %)	Total (N, %)
<i>Gram positive cocci</i>			
Coagulase Negative Staphylococci	24 (33%)	33 (70%)	57 (48%)
Linezolid resistant	2 (3%)	0 (0%)	2 (2%)
<i>Staphylococcus aureus</i>	7 (10%)	1 (2%)	8 (7%)
MRSA	5 (7%)	0 (0%)	5 (4%)
<i>Enterococcus spp</i>	2 (3%)	1 (2%)	3 (2.5%)
<i>Streptococcus spp</i>	1 (1%)	0 (0%)	1 (1%)
<i>Gram negative bacilli</i>			
Enterobacterales	27 (37%)	6 (13%)	33 (28%)
3GCR	28 (38%)	0 (0%)	28 (23%)
ESBL	10 (14%)	0 (0%)	10 (8%)
AMPC	16 (23%)	0 (0%)	16 (13%)
CRE	7 (10%)	0 (0%)	7 (6%)
Non-fermenters	5 (7%)	3 (6%)	8 (7%)
Pip-Taz resistant <i>P. aeruginosa</i>	2 (3%)	0 (0%)	2 (2%)
Ceftazidime resistant <i>P. aeruginosa</i>	1 (1%)	0 (0%)	1 (1%)
CR <i>P. aeruginosa</i>	1 (1%)	0 (0%)	1 (1%)
<i>Candida spp.</i>	7 (10%)	3 (6%)	11 (9%)
Total	73	47	120

CR Carbapenem resistant, CRE Carbapenem resistant Enterobacterales, ESBL Extended spectrum beta-lactamase, MRSA Methicillin resistant *Staphylococcus aureus*, Pip-Taz Piperacillin-tazobactam, PR-BSI PICC related bloodstream infection, 3GCR 3rd generation cephalosporin resistance

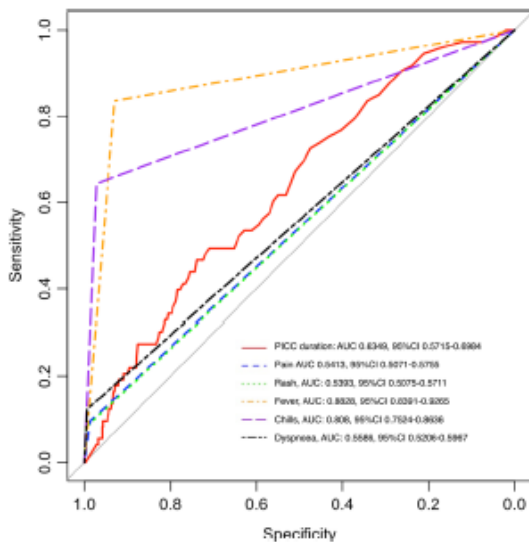


Fig. 2 Receiver operating characteristics (ROC) curves for peripherally inserted central catheter (PICC)-associated bloodstream infection on the basis of the number of catheter days (red line) and clinical signs such as pain (blue dashed line), rash at the PICC insertion site (green pointed line), fever (orange dashed line), chills (purple dashed line), dyspnoea (black dashed line). Area under the ROC curves (AUCs) are given with the 95% confidence interval (95% CI)

catheterization increased the risk for PR-BSI (see supplementary materials). However, our work underlined the poor ability of PICC maintenance duration to predict PR-BSI. Indeed, the threshold value of 28 catheter days

was associated with very low PPV but satisfactory NPV for PR-BSI, as previously reported [42]. These results suggest a close clinical signs monitoring could rapidly help diagnose and treat PR-BSI particularly in patient with a prolonged PICC dwelling time. These results also supports the recent recommendation of catheter duration limitation to the shortest requested to limit PR-BSI [34]. Available data also suggests that clinicians should limit the number of catheter lumen [9, 14, 43] and concurrent catheter [34].

Immunocompromised patients, particularly those treated with immunosuppressive treatments and/or with metastatic cancer have been identified at risk for PR-BSI. Immunosuppression, particularly neutropenia, was previously reported as risk factor for PR-BSI [40, 44–47], prompting clinicians to monitor PICC in immunocompromised patients with caution. Surprisingly, those patients did not seem at higher risk for PR-BSI in our study. During the study, a high nurse-to-patient ratio in haematology and oncology wards, local guidelines, and specific courses on PICC use for nurses were available in our institution. This may have impacted the quality of PICC care which has been widely reported to decrease PR-BSI incidence rate [34].

Previous studies on PR-BSI microbiology reported CoNS as the predominant microorganisms [11, 13, 39, 48]. Nonetheless, recent evidences [48–51] suggested a change in this trend with the rising of Gram negative bacilli as main microorganisms associated with PR-BSI. With a majority of PR-BSI related to Enterobacterales (37%), the results of our study tend to support the later.

Table 4 Factors independently associated with peripherally inserted central catheters (PICC) related bloodstream infection (PR-BSI), and predictive values of clinical signs for PR-BSI

Variables	Multivariate analysis			Diagnostic ability of clinical signs			
	Hazard ratio	95% CI	p-value	Sensitivity	Specificity	PPV	NPV
<i>Patient conditions</i>							
Immunosuppr. therapy ¹	1.07	0.80–1.43	0.63	–	–	–	–
Malignancy ²	1.08	0.67–1.75	0.75	–	–	–	–
Dementia	1.62	0.18–14.58	0.67	–	–	–	–
<i>Clinical signs</i>							
<i>Local signs</i>							
Pain	0.55	0.17–1.81	0.33	9.6%	98.7%	38.8%	92.5%
Rash	2.82	0.82–9.69	0.10	8.2%	99.6%	66.7%	92.5%
<i>Systemic signs</i>							
Fever	13.21	6.00–29.11	<0.001	83.6%	93.0%	51.3%	98.5%
Chills	3.66	1.92–6.99	<0.001	64.4%	97.2%	67.1%	96.9%
Dyspnoea	1.24	0.53–2.94	0.62	12.0%	99.4%	64.3%	93.0%
PICC duration ≥ 28 days	–	–	–	53.4%	62.8%	11.2%	93.9%

¹ at least one of the following: corticosteroids, azathioprine, mycophenolate mophetil, tacrolimus, ciclosporin. ²solid tumour or haematological malignancy. PPV Positive predictive Value, NPV Negative Predictive Value, 95% CI 95%confidence interval

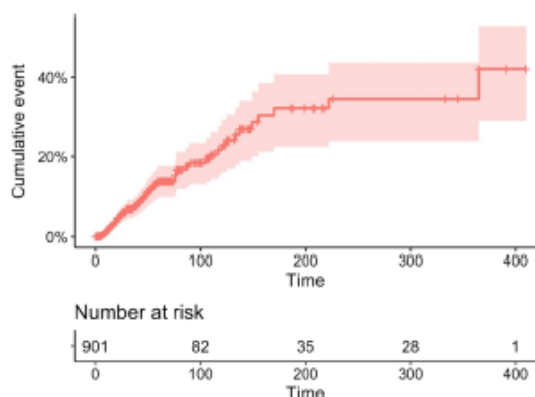


Fig. 3 Incidence of peripherally inserted central catheters (PICC) related bloodstream infection during PICC maintenance (in days) with 95% confidence interval

Yet, CoNS are still significantly associated with PR-BSI (33%) and responsible for most of PICC colonisations. The large proportion of immunocompromised patients in our cohort (almost a half of patients had a haematological malignancy or a cancer, and more than two thirds of patients with a PR-BSI), may explain the high prevalence of Enterobacterales associated PR-BSIs since these patients are most at risk to be infected with their own Enterobacterales [52]. Another possible explanation for these results was the inclusion of patients in home-hospitalization. This finding has important implications for the implementation of infection control bundle in PR-BSI prevention, especially regarding hand disinfection and skin antiseptics during PICC care and dressing management [12, 35].

This study also reported low rates of PICC related complications other than PR-BSI. We found an accidental withdrawal incidence rate of 1.6 per 1000 catheter days ($N=61$, 6.8%), lower than those reported by Valbousquet et al. (2.8 per 1000 catheter days) [17] and Grau et al. 8.0% [16]. However, others found lower rates at 2.4% [4] and 5% [18], respectively, but reported rates instead of incidence which limited comparison with our results. Importantly, accidental withdrawal was identified as a common complication in PICC use especially in patients older than 70 year-old, which is the median age in our study population [16, 53]. We reported a vein thrombosis incidence rate at 0.4 per 1000 catheter days, also lower than those previously reported [19, 53, 54]. PICCs are described as more thrombogenic than CVCs [19, 55], particularly in cephalic vein position [56]. Consequently, PICC were mainly inserted in basilic, or brachial vein as recommended in our local protocol. In addition, more than a third (27%) of the patients received

an anticoagulant therapy. This may have limited the incidence rate of thrombosis in our cohort.

This study has several limitations. First, the single centre design of the study could limit extrapolation of the results. Second, the relatively small size of our cohort limited the weight of some factors such as BMI > 40 kg/m², number of lumen or total parenteral nutrition which are recognized at risk factors for CR-BSI [40, 57]. However, to the best of our knowledge, this study is the first to analyse the association between clinical signs and PR-BSI. Third, the retrospective design of the study limits our analyses to available data in medical records and may induce bias in data collection and results interpretation. Some risk factors such as the microbial colonization at the catheter hub and at insertion site, or the outpatient/inpatient status, could not be assessed. However, combining medical, microbiological, and administrative data, added to the adjudication committee for PR-BSI diagnosis in accordance with international expert consensus tends to limit this bias.

Conclusions

Complications occurred in 24% of PICC, and PR-BSI was the most prevalent one with a 1.9 per 1000 catheter days incidence rate. PR-BSIs were mainly caused by Enterobacterales and CoNS. Clinical signs and PICC maintenance duration ≥ 28 catheter days, had better NPVs than PPVs for PR-BSI diagnosis. Fever and chills had the best NPV and were independently associated with PR-BSI occurrence.

These results suggest that health workers should cautiously monitor PICC insertion site, and especially fever and chills, and limit the duration of PICC maintenance to the minimum required. They also prompt patients to self-monitoring. Further studies are mandatory to assess whether improving patients and healthcare workers education on PICC management through the development of an infection prevention bundle and continuous evaluation could reduce PR-BSI under 1 per 1000 catheter days.

Abbreviations

AUC	Area under the ROC curve
BMI	Body mass index
CDC	Centers for disease control and prevention
CoNS	Coagulase negative staphylococci
CR	Carbapenem resistant
CRE	Carbapenem resistant enterobacterales
CR-BSI	Catheter-related bloodstream infections
CVC	Central venous catheters
ECDC	European centre for disease control and prevention
ESBL	Extended spectrum beta-lactamase
EUCAST	European committee on antimicrobial susceptibility testing
HR	Hazard ratio
IQR	Interquartile range
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
NB-PRI	Non-bacteraemia PRI

NPV	Negative predictive value
PICC	Peripherally inserted central catheter
Pip-Taz	Piperacillin-tazobactam
PPV	Positive predictive value
PR-BSI	PICC-related bloodstream infection
ROC	Receiver operating characteristic
SF2H	French society of infection control
SRLF	French intensive care society
3GCR	3rd generation cephalosporin resistance
95%CI	95% confidence interval

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Author contributions

Conceptualization, A.S. and R.L.; methodology, A.S. and R.L.; software, K.B.-B. and R.L.; validation, J.O., A.S., R.L. and J.-P.L.; formal analysis, K.B.-B., J.O., A.S. and R.L.; investigation K.B.-B.; writing—original draft preparation, K.B.-B.; writing—review and editing, J.O., R.L., and A.S.; supervision, R.L.; project administration, C.S., A.M., J.-P.B., A.S. and R.L. All authors reviewed the final version of the manuscript.

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Availability of data and materials

The authors consent to share the collected data with others. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. Data will be available immediately after the main publication and indefinitely.

Declarations

Ethics approval and consent to participate

The Institutional Review Board of Nîmes University Hospital approved the study protocol (No. 22.03.06) and waived the need for signed patient consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Environmental detection of SARS-CoV-2 in hospital rooms in different wards of a university hospital

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SUMMARY

Background: Transmission of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) can occur through direct, indirect, or close contact with infected people. However, the extent of environmental contamination is unknown. The nature of the relation between patients' symptoms and SARS-CoV-2 environmental shedding remains unclear. The aim of this study was to assess the relationship between patient coronavirus disease 2019 (COVID-19) status and environmental contamination.

Methods: Between May and November 2020, environmental swabs were taken before and after room disinfection at day 7 after symptom onset in a cohort of patients clinically or biologically diagnosed with COVID-19. Twelve surfaces per room were collected in 13 rooms. Sample analysis was performed by reverse transcription polymerase chain reaction (RT-PCR) for SARS-CoV-2 detection [SARS-CoV-2 R-Gene (biométrieux, Marcy l'Etoile, France)]. Clinical data (day of illness, symptoms, RT-PCR results) was collected from the clinical software.

Results: Five medical units were included in the study. Of 156 samples collected in 13 rooms, five rooms (38.5%) presented 11 SARS-CoV-2-positive samples. These positive samples were detected on eight different surfaces. There was no association between detection of SARS-CoV-2 and patient age ($P=1$) or patient symptoms ($P=0.3$).

Conclusion: Viral shedding during COVID-19 appears to be unrelated to the presence of symptoms, patient age, and low-value cycle threshold of patient's test. This study supports the evidence for the environmental shedding of SARS-CoV-2 until at least 7 days after symptom onset. It emphasizes the need for strict compliance with contact precautions, hand hygiene, the correct use of personal protective equipment and room disinfection for the routine care of patients with COVID-19.

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Introduction

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Since late 2019, the emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has led to a severe respiratory infection named coronavirus disease

2019 (COVID-19) that spread rapidly into a global outbreak and pandemic. The underlying mechanisms of viral transmission in healthcare environments remain unclear. Transmission of SARS-CoV-2 can occur through direct, indirect, or close contact (within 1 m) with infected people [1]. However, direct or close human-to-human contact with infected secretions (e.g. saliva, respiratory droplets) are the main transmission routes [1]. Therefore, droplet and contact isolation precautions are the key recommended precautions for COVID-19 patient care [1]. Other routes of transmission are suspected, such as ocular route [2], faecal oral route [3], airborne, or fomite transmission [4–6]. However, for fomite transmission, the extent of environmental contamination remains unclear. Several studies have described the persistence of SARS-CoV-2 and its viability on surfaces [4–10]. Infectious SARS-CoV-2 has been found on different environmental contaminated surfaces for periods ranging from an hour to days [1]. Numerous studies have described the analysis of surfaces and hospital rooms for the detection of SARS-CoV-2 virus [11]. Yet most failed to address the nature of the relation between patient COVID-19 status and the SARS-CoV-2 environmental shedding [11]. This information is critical for understanding SARS-CoV-2 transmission and developing effective infection control procedures.

The aim of this study was to assess the relationship between patient COVID-19 status and environmental contamination.

Methods

This retrospective, monocentric study was approved by the local institutional review board (IRB number: 22.03.05). From May to November 2020, environmental samplings were performed in different units at Nîmes University Hospital (France) in rooms according to two inclusion criteria: (1) The presence of a patient with clinically or biologically diagnosed COVID-19. The COVID-19 diagnosis occurred between symptom onset (Day 1) and environmental sampling (Day 7). The clinical diagnosis of COVID-19 was based on French Health Society guidelines [12,13] and established by medical doctors. The diagnosis was orientated by the presence of at least one of the following symptoms (fever, persistent cough, fatigue, rhinitis, dyspnoea, headache, nausea, disorientation, oxygen dependence, myalgia, shortness of breath, diarrhoea, abdominal pain, chest pain, sore throat, anosmia or ageusia) and confirmed with chest scan results. The biological diagnosis of COVID-19 was established by reverse transcription polymerase chain reaction

(RT-PCR) performed on nasopharyngeal swabs. (2) The patient in the sampled room was at Day 7 after COVID-19 symptom onset (Day 1).

The methodology timeline from symptoms onset (Day 1) to environmental sampling (Day 7) is represented in Figure 1.

Clinical information was collected from the hospital clinical data software: date of onset of symptoms, clinical presentation, patient clinical status at the sampling day, hospitalization date and, when available, patient's RT-PCR date and cycle threshold (Ct) values. The delay between diagnosis and environmental sampling was estimated in days.

Environmental sampling was performed before room disinfection. Each sampled room was occupied by patient at least 24 h prior to disinfection. Following the local health institute recommendations during the pandemic, room disinfection was performed each day by detergent disinfectant, with virucide activity (European Norm 14 476+A2) according to the French infection control society guidelines [14]. To confirm the efficiency of the local disinfectant, a set of rooms were sampled after room disinfection.

Twelve surfaces were sampled in each room in the presence of patients. The close surfaces sampled were: nurse call controller, bedside rail or bed lifting bar, telephone or television remote, overbed table; and the remote surfaces sampled were: bathroom doorknob, bathroom sink, toilet seat, doorknob, air vent, bathroom sink tap and toilet button. These surfaces are among the most touched surfaces by both patients and health workers. Sampling was performed by dry sterile swabs (GongDong, bioMérieux, Marcy l'Etoile, France) covering the whole surface of the different selected points of sampling. After sampling, swabs were dipped in viral transport medium (MicroTest MART, Thermo Scientific, Waltham, MA, USA) and vigorously agitated. The medium was immediately transported to the Department of Virology within a 30-min timelapse and stored at 4 °C.

SARS-CoV-2 was quantified from RNA extracted from each clinical and environmental sample using an automated nucleic acid extraction system (Chemagic, PerkinElmer, Waltham, MA, USA). Samples were analysed using RT-PCR (ARGENE SARS-CoV-2 R-Gene, bioMérieux) with a maximum Ct of 40 targeting three viral genes: the N gene of the nucleocapsid, the RNA dependent polymerase gene (RDRP) and the E gene, which is common to all the SARS viruses. Testing and reading were carried out following manufacturers' instructions. At the time of the study, samples were considered negative after 40 cycles of PCR without any

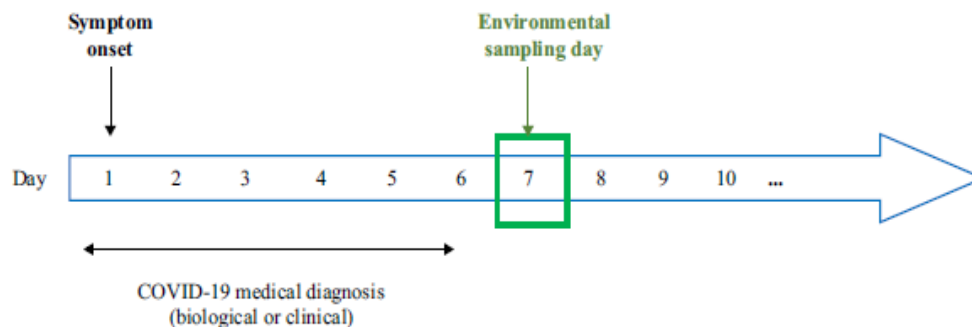


Figure 1. Timeline of sampling environmental in room with patients clinically or biologically diagnosed with coronavirus disease 2019 (COVID-19).

Table 1
Sampled rooms, units, and patient's information [symptoms, age and cycle threshold (Ct) values]

Room	Units	Patient information		Positive surface samples			Delay between diagnosis and environmental sampling (days)	Ct (N) values of positive surface samples
		COVID-19 symptoms	Age (years)	Ct (N) values of patient's test	Close environment	Remote environment		
1	IDU	Asymptomatic	97	25.39	2/5	2/7	4/12	38.7; 35.57; 33.20; 35.46
2	NH	Fever, rhinitis, oxygen dependence	84	23.6	0/5	0/7	0/12	-
3	NH	Cough, dyspnoea, fever	92	33.88	0/5	0/7	0/12	-
4	IDU	Cough, fever, dyspnoea, fatigue	69	37.35	0/5	0/7	0/12	-
5	IDU	Cough, fever, dyspnoea, headache	82	29.31	0/5	0/7	0/12	-
6	IDU	Cough, fever, dyspnoea, rhinitis	69	34.24	0/5	0/7	0/12	-
7	IDU	Cough, rhinitis, headache	83	20.13	1/5	0/7	1/12	35
8	ICU	Bilateral pneumonia, oxygen dependence, dyspnoea	46	CD	0/5	0/7	0/12	-
9	ICU	Bilateral pneumonia, cough, dyspnoea, fatigue	78	CD	0/5	0/7	0/12	-
10	LTCU	Oxygen dependence, dyspnoea, cough, nausea	74	CD	1/5	1/7	2/12	37.7; 39.3
11	LTCU	Fever, dyspnoea, oxygen dependence, diarrhoea	76	CD	0/5	0/7	0/12	-
12	GU	Fever, cough, disorientation	89	28	1/5	0/7	1/12	38.7
13	GU	Fever, cough, disorientation	91	CD	3/5	0/7	3/12	31.71; 34.05; 29.75

COVID-19, coronavirus disease 2019; CD, clinical diagnosis; GU, geriatric unit; ICU, intensive care unit; IDU, infectious disease unit; LTCU, long-term care unit; NH, nursing home.

SARS-CoV-2 RNA detection. The Ct value of each gene was recorded. The Ct value of the N gene was used as reference. The delay between swabbing and processing (extraction and RT-PCR) of samples was around 24 h.

Statistical association with environmental contamination was analysed using Fisher's exact test. The distribution of the Ct values of the RT-PCR tests were analysed with a Wilcoxon rank sum test. A P-value inferior or equal to 0.05 was considered statistically significant. Statistical analyses were performed using the statistical software R version 4.0.2.

Results

Thirteen rooms were sampled before room disinfection in five medicals units: infectious diseases unit (IDU, N = 5), geriatric unit (GU, N = 2), intensive care unit (ICU, N = 2), long-term care unit (LTCU, N = 2) and a nursing home (NH, N = 2). Three rooms were sampled in May 2020 during the first wave of the pandemic, four rooms in August 2020 and six other rooms during the second wave in November 2020. At inclusion, five patients were diagnosed on clinical criteria and eight on both clinical and biological criteria. A total of 156 and 52 samples were analysed, respectively, before and after room disinfection. Four rooms were sampled in IDU after room disinfection with a total of 52 analysed samples. Environmental samples were performed an average of 5 days (2–7 days) after nasopharyngeal test (Table 1).

Before disinfection, five rooms (38%, 5/13) presented positive samples (Table 1). Interestingly, no rooms in the ICU or NH showed environmental SARS-CoV-2 contamination. Positive environmental samples had a Ct range between 29.75 and 39.3 (Table 1). Eight samples were positive in the immediate patient environment [nurse call controller (N = 2), bedside rail (N = 2), bed lifting bar (N = 1), telephone (N = 2), overbed table (N = 1)] and three in patient's remote environment [bathroom doorknob (N = 1), bathroom sink tap (N = 1), toilet seat (N = 1)] (Table 1, Figure 2). During room sampling, 12 of 13 patients were symptomatic. The eight patients tested by RT-PCR presented a Ct range between 20.13 and 37.35. However, patients in the contaminated rooms presented low Ct values between 20.13 and 28. Interestingly, the room (Room 1) with the highest number of positive samples (4/12 corresponding to bedside rail, doorknob, bed lifting bar and toilet seat) corresponded to the only asymptomatic patient at the moment of the inclusion (Table 1). The mean delay between the patient's nasopharyngeal test and environmental sampling was 4.4 days for positive rooms, and 5.5 days for negative rooms (Table 1), without significance (P=0.2897). Environmental contamination showed no significant association with patient age (P=1) or patient symptoms (P=0.3). However, Ct values of nasal RT-PCR were significantly lower than the Ct values of environmental RT-PCR (28.7 vs 38.5, respectively; P=0.0074). Moreover, the patients were significantly older in contaminated rooms than in non-contaminated rooms (mean age = 89 years vs 74; P=0.0046). After disinfection, no room presented positive samples.

Discussion

This study evaluated the relationship between SARS-CoV-2 positivity of patients and the environmental contamination of their room. We also reported the environmental persistence or

Table II
Positive environmental samples and their locations in patients' rooms

Surfaces	Number of positive samples	Close or remote environment (C or R)
Nurse call controller	2	C
Bedside rail	2	C
Bed lifting bar	1	C
Room telephone	2	C
Overbed table	1	C
Bathroom doorknob	1	R
Bathroom sink	1	R
Toilet seat	1	R
Doorknob	0	R
Air vent	0	R
Bathroom tap	0	R
Toilet button	0	R
Total	11	8 C and 3 R

not of SARS-CoV-2 at day 7 after clinical onset of symptoms in 13 patients diagnosed with COVID-19. Despite daily room disinfection, 7 days after the first COVID-19 symptoms, SARS-CoV-2 was found in five patients' close and remote environment before disinfection. After disinfection, no virus was found. This finding reinforces the need for daily disinfection during the care of a COVID-19 patient and also the prevention of pathogens cross-transmission, as recommended by the French national infection control society [14].

A review found an environmental contamination rate ranging from 0 to 75% in ICU and from 0 to 61% in medical units [11]. Our study showed contamination rates in the lower part of these ranges, with 7% in medical units and 0% in ICU. Among the five investigated units, three (GU, IDU and LTCU) had room

contamination whereas two (ICU and NH) did not. The disinfection protocol and room layout were the same in all units. Moreover, the studied population was similar in terms of age, symptoms and Ct values.

Our study selected patients admitted for COVID-19 at hospital, and surface samples were performed 7 days after symptom onset. Other studies have focused on different populations (healthy asymptomatic workers, patients at a long-term care facility or a nurse facility independently of the time of symptom's onset, passengers, and crewmembers of a ship) and various viral sources (respiratory, rectal, urinary, gastrointestinal tract/stool, blood, breast milk, semen samples, surfaces inside and outside patients' rooms) [11]. These other studies on environmental contamination also assessed different surfaces and used different sampling procedures. In some samples, sampling was made with sterile premoistened swabs [4–8], while we used dry sterile swabs. Moreover, some variations in the laboratory analyses procedures (e.g. extraction kits and targets of RT-PCR) could be observed. For example, only the envelope (E) gene was detected in Zhou *et al.*'s study [6], whereas we detected three genes, increasing the sensitivity of the assay. These differences in methodology hamper direct comparison between studies. However, all these studies corroborated the presence of SARS-CoV-2 on room surfaces, representing a potential source of contamination and cross-transmission.

Interestingly, in our study, positive environmental samples were recovered in one asymptomatic patient room at inclusion, and negative environmental samples were recovered in eight rooms of symptomatic patients. Moreover, no significant association between patient symptoms and environmental contamination was noted. These conclusions have been previously reported [5,6,9]. Thus, environmental contamination appeared unrelated to patient symptomatology, although firm conclusions are not possible as only one asymptomatic patient



Figure 2. Surfaces sampled in patient's room. A, nurse call controller; B, bedside rail; C, bed lifting bar; D, telephone or television remote; E, overbed table; F, bathroom doorknob; G, bathroom sink; H, toilet seat; I, air exhaust grill; J, bathroom sink tap; K, toilet button. One other point (doorknob) was not pictured. In red, the positive severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) points. In white, the negative SARS-CoV-2 points.

was included. Moreover, for symptomatic patients with environmental contamination, the positive surfaces were mainly located in the patient's close environment (nurse call controller, telephone, bedside rail, bed lifting bar, overbed table), whereas no association was observed between close environment and symptoms. The infectious respiratory secretions produced through coughing or sneezing are the main factor of close contamination [1]. Few studies distinguished close from remote environment when studying environmental contamination. Our results support frequent room disinfection during the hospitalization of a COVID-19 patient, particularly of the immediate close environment or high touch-surfaces, as suggested by the World Health Organization [15]. Notably, despite the significantly greater age of patients in contaminated rooms (mean age = 89 years vs 74 in non-contaminated rooms; $P=0.0046$), no significant association between patient age and environmental contamination ($P=1$) was observed, suggesting that age had no role in environmental contamination, as previously reported [4,10].

All the included patients in this protocol were bedridden and could not use the toilet. In contaminated rooms, SARS-CoV-2 virus was detected in both remote and close patient's environment (Figure 2). Thus, we could suggest that this contamination resulted from: (1) hand contamination either by the patient or the healthcare workers [16] or from the healthcare workers' personal protective equipment (PPE) such as gloves [17]; or (2) respiratory droplets or respiratory aerosols from the patient [4,18]. Another interesting finding is that bedside rail, nurse call controller and room telephone were the most contaminated surfaces. These objects are among the most touched surfaces by both patients and health workers [15]. This reinforces the need for hand hygiene and the correct use of PPE by healthcare workers [15]. This study contributes to the recent debate regarding the duration of the infection control precautions during the hospital care of patients with COVID-19.

Our study has several limitations. Firstly, we did not evaluate the viability of the SARS-CoV-2 collected in the environmental swab samples. Only viable virus can cause an infection. Despite the detection of SARS-CoV-2 virus in the environment, virus viability and infectivity cannot be determined by RT-PCR. Casia et al. observed that coronaviruses' infectivity declines in environment with decline rates from 99% at 5 min to 83% at 3 days depending on dried or wet mucine state, while still remaining infectious [19]. In addition, a review has highlighted that given the difficulties in culturing infectious virus from clinical specimens during a pandemic, using viral RNA load as a surrogate remains plausible for generating careful clinical hypotheses [11].

Secondly, our population size is relatively small. Nevertheless, we investigated a representative panel of units and COVID-19-positive patients hospitalized at the time of the study.

In conclusion, our study supports the evidence for the environmental shedding of SARS-CoV-2 until at least 7 days after symptoms onset. Age and patient's RT-PCR Ct values were not significantly associated with room contamination, but the results of this study give a hint that absence of symptoms did not influence environmental contamination. Further studies exploring the link between patient symptoms and environmental contamination would be worthwhile. Our findings emphasize the need for strict compliance with contact

precautions, the reinforcement of hand hygiene, the correct use of PPE by health workers and daily disinfection even after 7 days for the routine care of COVID-19 patients, as recommended by health authorities.

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Author contributions

Conceptualization, J.O., A.S. and J.P.L.; methodology, K.B.B., J.O., A.S. and J.P.L.; software, K.B.B.; validation, J.O., A.S. and J.P.L.; formal analysis, K.B.B., J.O., A.B. and R.S.; investigation, K.B.B. and J.O.; writing—original draft preparation, K.B.B., J.O. and J.P.L.; writing—review and editing, A.B., R.S. and A.S.; supervision, A.S. and J.P.L.; project administration, A.S.; funding acquisition, A.S. and J.P.L. All authors read and agreed to the published version of the manuscript.

Conflict of interest statement

None declared.

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Évaluation des appareils de protection respiratoire au centre hospitalier universitaire de Nîmes lors de l'épidémie de Covid-19

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RÉSUMÉ

Introduction. Suite à l'apparition du Sars-CoV-2, des mesures relatives aux risques épidémiques émergents ont été mises en place par l'ensemble des établissements de santé, entraînant des tensions d'approvisionnement en masques FFP2. L'objectif de cette étude réalisée pendant la crise sanitaire était d'évaluer la qualité des masques FFP2 reçus par le CHU de Nîmes avant leur utilisation. **Matériel et méthode.** Ces évaluations concernent la présence du marquage CE, la conformité aux normes relatives aux appareils de protection respiratoire (EN 149:2001+A1:2009) et la date limite d'utilisation. En complément, sont réalisés des contrôles visuels de l'intégrité du conditionnement, de la solidité des attaches et de l'aspect du masque. Son étanchéité est évaluée par le contrôle d'ajustement (*Fit check*) sur au moins trois testeurs ayant des morphologies faciales hétérogènes, complété par un test d'ajustement qualitatif (*Fit test*). **Résultats.** Les 50 masques testés respectent tous les normes attendues ou des normes équivalentes. Seuls 15 masques ont satisfait au test d'étanchéité. Aucun masque avec une plicature verticale conforme à la norme chinoise KN95 n'a réussi le test d'étanchéité. **Conclusion.** L'étanchéité des masques FFP2 doit systématiquement être contrôlée pour assurer une protection optimale des soignants. Les normes qualifient les capacités de filtration des masques mais pas leur étanchéité. La prévention du risque de transmission du Sars-CoV-2 ne se limite pas au port du masque, elle passe aussi par une protection oculaire, une hygiène des mains adaptée, une désinfection régulière des surfaces et un renouvellement fréquent de l'air.

MOTS-CLÉS

Alerte sanitaire – Gestion de crise – Masque – Équipement de protection individuel – Contrôle qualité.

ABSTRACT

Evaluation of respiratory protection devices during the Covid-19 outbreak

Introduction. The Sars-CoV-2 outbreak led to the implementation of measures to fight emerging epidemic risks in all health-care facilities. This gave rise to supply pressures concerning FFP2 masks. This study aims to assess the quality of FFP2 masks before their use during the sanitary crisis. **Materials and methods.** This evaluation concerned the presence of CE labelling, the compliance with respiratory protection device (RPD) standards (EN 149:2001 +A1 2009) and use-by dates. In addition, the integrity of packaging, the resistance of the ties and the appearance of the masks were all checked visually. Airtightness was Fit-Checked with at least three testers with different facial morphologies. This was completed by a qualitative Fit test. **Results.** All the 50 masks tested were in agreement with expected or equivalent standards. Only 15 masks passed the airtightness tests. All the masks with a vertical fold recognised by the Chinese KN95 standard failed the test. **Conclusion.** Healthcare professionals should know the exact indications of FFP2 masks and master Fit-checking. These masks offer optimal protection but should be reserved for specific indications. The prevention of Sars-CoV-2 transmission is not restricted to the sole wearing of a respiratory protection device. It also includes eye protection, adequate hand hygiene and the regular disinfection of hard surfaces.

KEYWORDS

Health warning – Crisis management – Mask – Protective equipment – Quality control.

Contexte crise sanitaire

Suite à l'apparition du Sars-CoV-2¹, des mesures de prévention contre les risques épidémiques émergents ont été mises en place dans les établissements de santé pour maîtriser la diffusion de ce nouveau virus [1]. La prise en charge des patients atteints ou suspects de la Covid-19² nécessite de disposer d'équipements de protection individuelle (EPI) pour protéger les soignants. Ces EPI sont les masques chirurgicaux, les masques FFP2³, les tabliers en plastique, les visières ou lunettes de protection, les surblouses et les gants. L'application de ces mesures par l'ensemble des établissements de santé a entraîné des tensions d'approvisionnement auprès des fournisseurs, notamment pour les masques FFP2. Pour pallier ce manque, des appels aux dons (particuliers et commerces) et des achats auprès d'autres fournisseurs que ceux engagés par appel d'offres ont été réalisés. Par la suite,

les approvisionnements ont été coordonnés par l'État. La diversité des masques FFP2 reçus à l'hôpital nous a imposé de vérifier leur conformité aux normes attendues, ainsi que leur étanchéité selon l'avis de la Société française d'hygiène hospitalière (SF2H) [2]. Cette étanchéité a été vérifiée par le contrôle de l'ajustement (*Fit check*) et un test qualitatif (*Fit test*) [3]. L'objectif de cette étude est d'évaluer la qualité des appareils de protection respiratoire (APR) avant leur utilisation lors de la crise sanitaire.

Matériel et méthode

Cette étude a été réalisée au centre hospitalier universitaire de Nîmes de mai 2020 à février 2021. Elle a consisté à tester l'ensemble des masques FFP2 obtenus par divers moyens d'approvisionnement. Ces masques étaient issus de donations des entreprises locales, de la dotation de l'État et du marché public (Figure 1).

Vérification de la conformité des masques

Dès réception à la plateforme logistique et avant la délivrance aux services de soin, des vérifications sont réalisées sur la présence du marquage CE, la conformité à

1- Severe acute respiratory syndrome coronavirus 2, coronavirus 2 du syndrome respiratoire aigu sévère.
2- Coronavirus disease 2019, maladie à coronavirus 2019.
3- Filtering facepiece, « pièce faciale filtrante » de classe 2.

Figure 1 – Exemples de masques testés, issus des donations des entreprises locales, de la dotation de l'État et du marché public : à plicature verticale, à plis et en forme de coquille.



Masque à plicature verticale

Masque forme coquille

Masque pliable

la norme relative aux appareils de protection respiratoire (EN 149:2001+A1:2009) et la date limite d'utilisation [4]. Selon l'avis du ministère du Travail, les masques peuvent être utilisés 24 mois après la date de péremption. Pour chaque référence reçue, des contrôles visuels de l'intégrité du conditionnement et de l'aspect du masque sont réalisés, et la solidité des élastiques et de la barrette nasale de maintien est vérifiée.

Vérification de l'étanchéité des masques

L'étanchéité des APR est évaluée par le test d'ajustement *Fit check* [3] sur au minimum trois personnes. L'objectif est de sélectionner le modèle et la taille qui permettent d'obtenir une étanchéité optimale quelle que soit la morphologie du visage. En raison de la pénurie de masques, une grande réactivité est nécessaire et les testeurs sont choisis de manière aléatoire parmi les personnes présentes dans le service le jour de l'essai. Le périmètre crânien de chaque testeur est mesuré et classé selon trois tailles : petite (inférieur à 55 cm), moyenne (entre 55 cm et 58 cm) et grande (supérieur à 58 cm). Les porteurs de barbe de trois jours et plus sont exclus de l'étude. Le contrôle d'étanchéité (*Fit check*) se déroule en six étapes (Figure 2) : placer le masque sur le visage en intégrant nez, bouche et menton ; placer correctement les élastiques selon le type de masque (un au-dessus et un en dessous des oreilles, ou autour des oreilles) ; ajuster le masque sur le nez en pinçant la barrette nasale ; ajuster le masque sous le menton jusqu'à la base du cou ; obturer la surface filtrante avec les mains ; inspirer et bloquer sa respiration quelques secondes et expirer. Si l'étanchéité est bonne, le masque tend à se plaquer légèrement sur le visage et se maintient plaqué jusqu'à l'expiration ; dans le cas contraire, le masque doit être réajusté et l'essai renouvelé. Si l'étanchéité du masque est confirmée, un test complémentaire d'ajustement qualitatif *Fit test* est réalisé [3], dont le principe repose sur l'absence de détection de l'odeur et de la saveur sucrée (saccharine FT-10) dans une chambre d'inhalation. Avant chaque essai, le testeur place sa tête, sans masque, dans une chambre

d'isolation jusqu'aux épaules. La nébulisation d'une solution de saccharine permet de contrôler sa sensibilité au goût sucré. Un rinçage de la bouche est ensuite réalisé, suivi d'un temps d'attente de quelques minutes. L'expérience est renouvelée avec le testeur équipé du masque dans les six situations suivantes : respiration normale, respiration profonde, lecture à voix haute ou chant, inclinaison de la tête de gauche à droite avec une pause intermédiaire pour inhalation, inclinaison de la tête de haut en bas et inversement avec inhalation en position haute. Si le testeur détecte un goût ou une odeur sucrée, le test est stoppé, un repositionnement du masque est demandé, puis un second test est réalisé. Le masque est considéré conforme s'il y a absence de détection d'odeur et de saveur sucrée dans les six positions.

Résultats

La totalité des masques possédait la norme CE et la norme EN 149 ou une norme équivalente [5]. Les non-conformités de plusieurs références étaient liées à une fragilité des élastiques et à une date limite d'utilisation dépassée depuis plus de 24 mois [4]. Au total, 50 références de masques ont été évaluées par 208 tests sur des personnes représentant les trois tailles de périmètre crânien (Tableau I). Le périmètre crânien moyen était de 55,9 cm (écart type : 1,76 [53-57,5]). Seules 15 références de masques (30%) ont satisfait au test d'étanchéité complet. La conformité de ces masques était respectivement de 28,57% (n=10/35), 37,10% (n= 46/124) et 36,7% (n=18/49) pour les périmètres crâniens de petite, moyenne et grande taille. Aucun masque avec une plicature verticale conforme à la norme chinoise KN95 (Figure 3) n'a été validé par le premier contrôle d'étanchéité (*Fit check*).

Discussion

L'émergence rapide de la Covid-19 à l'échelle mondiale, début 2020, a eu un impact considérable sur les approvisionnements en EPI indispensables à la maîtrise du risque infectieux. Sans connaissance précise du mode

Figure 2 – Les six étapes du contrôle d'étanchéité *Fit check*



Source : Réseau de prévention des infections associées aux soins. Information pour les professionnels de santé en médico-social & soins de ville. Bordeaux, 2020. 2 p. Accessible à : https://www.preventioninfection.fr/?jet_download=10809 (Consulté le 15-06-2021).

Tableau I – Résultats des tests d'étanchéité de 50 références de masques.

Masques testés	Norme		Résultat de l'étanchéité selon l'évaluateur						Étanchéité
	Évaluateur :		E1	E2	E3	E4	E5	E6	
	Taille :		S	M	M	M	M	L	
1	KN95		non	non				non	non
2	KN95		non	non				non	non
3	KN95		non	non				non	non
4	KN95		non	non				non	non
5	KN95		non	non				non	non
6	EN149:2001		non	non				non	non
7	EN149:2001		oui	oui				oui	oui
8	EN149:2001		oui		oui			oui	oui
9	EN 149:2001+A1:2009		non		non			non	non
10	KN95		non		non			non	non
11	KN95		non		non			non	non
12	KN95		non		non			non	non
13	KN95			non		non		non	non
14	KN95			non		non		non	non
15	KN95			non		oui		oui	non
16	KN95			non		oui		oui	non
17	KN95			non		non		non	non
18	EN 149:2001+A1:2009		oui	oui	oui	oui	oui	oui	oui
19	KN95		non	non	non	non	non	oui	non
20	EN 149:2001+A1:2009		non		oui	oui	non	oui	oui
21	EN 149:2001		non		oui	oui	non	non	oui
22	KN95		non	non	non	non	non	non	non
23	KN95		non	non	non	oui	non	non	non
24	EN 149:2001		oui	oui	oui	oui	oui	oui	oui
25	EN 149:2009		non	non	non	non	non	non	non
26	KN95		non	non	non	oui	non	non	non
27	KN95				non	non		non	non
28	EN 149:2001+A1:2009			oui	oui	oui	non	oui	oui
29	EN 149:2009		non	oui	oui	non	non	oui	non
30	KN95			non	non	non	non	non	non
31	EN 149:2001+A1:2009			oui	oui	oui	non	oui	oui
32	EN 149:2001+A1:2009		oui	non	oui	oui		oui	oui
33	KN95			non	non	non		non	non
34	KN95			non	non	non		non	non
35	NR			non	non	oui		non	non
36	KN95		non	non	non		non	non	non
37	EN 149:2009		non	non	oui		non	oui	non
38	KN95		non	non	non		non	non	non
39	KN95		non	non	non		non	non	non
40	EN 149:2001+A1:2009				oui			oui	oui
41	KN95		non		non			non	non
42	EN 149:2001		oui	oui		oui	oui	non	oui
43	EN 149:2009		oui	oui		oui	oui	oui	oui
44	EN 149+A1 2009		oui	oui	oui	oui	oui	oui	oui
45	EN 149:2001+A1:2009		oui		oui	oui	oui	oui	oui
46	EN 149+A1:2009		oui		oui	non	oui	oui	oui
47	EN 149:2001+A1:2009		non		non	non	non	non	non
48	EN 149:2001+A1:2009				non	non		non	non
49	EN 149:2001+A1:2009			oui				oui	oui
50	KN95		non		non	non	non	non	non

Figure 3 – Masque à plicature verticale conforme à la norme chinoise KN95.



de transmission du virus, des précautions complémentaires « air » et « contact » ont été recommandées par la SF2H et la Société de pathologie infectieuse de langue française [1]. L'application de ces recommandations a entraîné une surconsommation de masques FFP2. Ces derniers sont utilisés pour la prise en charge de patients auprès desquels sont appliquées les précautions complémentaires « air », comme ceux suspects ou atteints de tuberculose pulmonaire, ou « gouttelettes » pour la réalisation de gestes invasifs au niveau des voies respiratoires à risque d'aérosolisation (p. ex. en cas de grippe). De nouveaux modes d'approvisionnement en masques FFP2 ont été déployés pour pallier les difficultés rencontrées, sous forme de dons sollicités par un appel via les réseaux sociaux et les radios locales, puis de dotations par l'État pour chaque établissement. Ces nouveaux circuits d'approvisionnement ont entraîné une nouvelle organisation hospitalière dans le cadre du contrôle des normes et de l'utilisation de ces masques. L'équipe opérationnelle d'hygiène a testé l'ensemble des masques reçus. Malgré le marquage CE et les équivalences revendiquées avec la norme européenne [5], les masques conformes à la norme chinoise KN95 ne sont pas adaptés à toutes les morphologies de visages. D'où un défaut d'étanchéité qui est une potentielle source de contamination pour les professionnels de santé. L'équipe de Lucas Kleinjohann et Christophe Lange [8] a également montré ce défaut d'étanchéité de 9 masques FFP2 respectant des normes européennes ou la norme chinoise KN95 par des tests sur plus de 40 personnes. Ils concluent que les masques KN95 ne sont pas étanches pour l'ensemble des testeurs. Par ailleurs, l'agence fédérale américaine chargée de formuler des recommandations pour la prévention des accidents du travail et des maladies professionnelles⁴ souligne après de multiples

tests qu'au moins 60% des masques KN95 ne satisfont pas aux exigences. Ses recommandations sont déterminées, d'après des essais sur un panel de testeurs, par la longueur et la largeur du visage. À compter de juin 2021, ces tests d'étanchéité intégreront aussi le périmètre crânien, en plus de la largeur et de la longueur du visage [7]. Suite aux résultats de notre étude, une déclaration a été effectuée sur le portail national du signalement des événements sanitaires indésirables du ministère de la Santé⁵ dans la rubrique des événements liés aux infections associées aux soins, catégorie « Défaut de qualité d'un équipement de protection individuelle Covid-19 » pour l'ensemble des masques présentant un défaut de conformité. La forme de ces masques semble plus adaptée aux morphologies de type asiatique. Une étude a en effet montré des variations significatives de forme entre les visages de types caucasien et asiatique en mesurant de multiples paramètres dont le périmètre crânien : les crânes chinois sont plus ronds que les crânes caucasiens, avec l'arrière de la tête et le front plus plats. Les auteurs de l'étude soulignent que ces mesures anthropométriques doivent être prises en compte lors de la conception de produits, notamment pour déterminer la largeur et le sens de plicature des masques FFP2 [8]. Parmi les masques conformes, plusieurs références ont été mises à disposition dans les services de soins afin que chaque professionnel puisse disposer d'un masque adapté à la morphologie de son visage [2,9]. Cependant, à ce jour, il n'y a pas de corrélation établie entre la contamination d'un soignant et le type de masque FFP2 porté. L'inconfort généré par le port d'un masque FFP2 peut conduire à des manipulations plus fréquentes voire à un positionnement inadéquat [10,11]. L'évolution des connaissances sur l'infection par la Covid-19 a depuis poussé les experts à recommander le port du masque FFP2 uniquement pour la réalisation de gestes invasifs au niveau des voies respiratoires à risque d'aérosolisation [12-15]. Ces nouvelles recommandations ont contribué à la diminution de la consommation des masques FFP2 et à une fluidification des approvisionnements des établissements de santé.

Conclusion

Malgré les difficultés d'approvisionnement en masques FFP2 lors de la crise sanitaire, leur étanchéité a été systématiquement contrôlée pour assurer une protection optimale des soignants. À l'occasion de prochains achats, nos tests d'étanchéité prendront en compte le périmètre crânien, en plus de la largeur et de la longueur du visage. Les normes qualifient les capacités de filtration des masques mais pas leur étanchéité. Avant la distribution, une évaluation de l'étanchéité des masques FFP2 par un *Fit check* est à réaliser systématiquement,

4- National Institute for Occupational Safety & Health, Institut national pour la sécurité et la santé au travail.

5- Accessible à : https://signalement.social-sante.gouv.fr/psig_ihm_utilisateurs/index.html#/accueil (Consulté le 09-06-2021).

ce test facile et rapide ne nécessitant pas de matériel particulier. Par ailleurs, le professionnel doit connaître les indications précises du port du masque FFP2, et maîtriser le contrôle d'étanchéité (*Fit check*). Ce dernier garantit une protection et une étanchéité optimales et est à réserver à des indications ciblées [9]. La prévention du risque de transmission du Sars-CoV-2 ne se limite pas

au port d'un masque [16]. La transmission par contact ne doit pas être sous-estimée lors de la prise en charge de patients, et il doit être prévu une protection oculaire [17], une hygiène des mains [18] et une désinfection régulière des surfaces par du détergent désinfectant répondant notamment à la norme virucide EN 14476, sans oublier une aération adaptée. ■

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Annexe 4 : Liste des autres productions scientifiques

1. RICAI 2020 : Réunion Interdisciplinaire de Chimiothérapie Infectieuses
Communication orale sur **Persistence environnementale du SARS-Cov-2 dans des chambres de patients hospitalisés**
2. Congrès de la Société Française d'Hygiène Hospitalière (SF2H) 2021: Poster sur **Evaluation des appareils de protection respiratoire au CHU de Nîmes pendant l'épidémie de Covid-19**
3. Microbes 2022 : Congrès national de la Société Française de microbiologie : Poster sur **Antibiofilm properties of antiseptic agents on Diabetic Foot Ulcer isolated *Pseudomonas aeruginosa* biofilm**