Staphylococcus aureus : molecular characterization of afghan isolates and study of conjugative transfer of linezolid resistance

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STAPHYLOCOCCUS AUREUS: MOLECULAR CHARACTERIZATION OF AFGHAN ISOLATES AND STUDY OF CONJUGATIVE TRANSFER OF LINEZOLID RESISTANCE

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Staphylococcus aureus : Caractérisation moléculaire des isolats Afghans et étude du transfert conjugatif de la résistance au linézolide

Résumé

1. Caractérisation moléculaire des isolats de *S. aureus* en provenance d'Afghanistan

Staphylococcus aureus (SA) est une cause majeure d'infections chez l'Homme et de nombreux animaux. L'un des principaux objectifs de cette thèse a été d'étudier les caractéristiques moléculaires et le profil de résistance aux antibiotiques des souches d'infection et de colonisation de SA/SA résistant à la méticilline (SARM) chez l'Homme et le bétail à Kaboul. Les souches ont été identifiées par des méthodes phénotypiques conventionnelles, et l'antibiogramme réalisé par diffusion. Les souches de SARM ont ensuite été caractérisées à l'aide de puces à ADN. Parmi les isolats de SA provenant d'infections humaines dans 2 hôpitaux de Kaboul entre Janvier et Juin 2017, la prévalence des SARM était élevée (66,3 %). Les 98 isolats de SASM et SARM appartenaient à 12 complexes clonaux et 27 clones distincts. La prévalence du portage nasal de SA et du SARM dans la communauté, plus précisément chez des étudiants (hors médecine) de l'Université de Kaboul, était respectivement de 33,3% et 12,7%. Les 19 isolats de SARM appartenaient à 4 clones. Enfin, les études réalisées chez l'animal ont montré qu'environ 11,8% des souches de SA responsables de mammite bovine à Kaboul étaient résistantes à la méticilline (2/17) et que la prévalence du portage nasal de SA chez les moutons était très faible (1,1 %). Globalement, la caractérisation moléculaire des isolats a mis en évidence une grande diversité génétique et la présence de multiples gènes de virulence et de résistance parmi les souches de SA/SARM circulant dans les établissements de soins, la communauté et le bétail à Kaboul.

2. Transfert conjugatif du gène de résistance au linézolide *cfr*

Le linézolide est un antibiotique de recours pour le traitement des infections à SARM. L'objectif de ce travail a éte d'étudier, i) le risque de transfert conjugatif de la résistance au linézolide médiée par le gène plasmidique *cfr,* de souches de *S. epidermidis* (SE) vers des souches de SA de divers fonds génétiques, et ii) le coût de ce transfert pour les souches receveuses et la stabilité des plasmides. Cinq souches de SE portant 4 plasmides différents (*pSA737, p12-02300, p-cfr-PBR-A, p-cfr-PBR-B*) portant le gène *cfr*, et des souches sensibles appartenant à 9 clones majeurs de SA circulant en France ont été respectivement utilisées comme souches donneuses et receveuses. Les réusltats obtenus montrent que certains clones, comme le clone ST8-MRSA-IV USA300,

présentaient un risque plus important d'acquisition des plamides proteur de gène *cfr*. La souche SE ST5 avait une plus grande capacité à transférer le plasmide *p-cfr-PBR-B* à différentes souches receveuses que la souche ST2 portant ce même plasmide. Certains plasmides, comme *pSA737* et *p12-02300,* étaient transférables à la plupart des souches receveuses testées. Les coûts de l'acqusition des plasmides portant le gène *cfr* étaient variables pour une même souche et entre les plasmides eux-mêmes. Enfin, il apparaît que les plasmides *cfr*+ étaient systématiquement plus stables dans les SE que dans les SA après des subcultures itératives sans pression de sélection. Au final, nos résultats ont montré que la résistance au linézolide, médiée par *cfr*, peut être transférée efficacement par conjugaison de SE aux clones pathogènes majeurs de SA circulant en France mais que le transfert est très variable en fonction des souches donneuses et receveusess ainsi que des plamides considérés.

Mots-clés

Staphylococcus aureus, SARM*,* caractérisation moléculaire, Kaboul, Afghanistan, linézolide, *cfr*, transfert conjugatif, fitness.

Staphylococcus aureus: Molecular characterization of Afghan isolates and study of conjugative transfer of linezolid resistance

Summary

1. Molecular characterization of *S. aureus* isolates from Afghanistan

Staphylococcus aureus (SA) is a major cause of infections in human and animals. One of the main objectives of this thesis was to investigate the molecular features and the antimicrobial resistance profile of SA and methicillin-resistant SA (MRSA) strains isolated from infection and colonization among humans and livestock in Kabul. All strains were identified by conventional phenotypic methods and antimicrobial susceptibility testing performed by disk diffusion. MRSA strains were then extensively characterized using DNA microarray. Among SA isolated from human infections in 2 hospitals of Kabul, from January to June 2017, MRSA prevalence was high at 66.3%. The 98 isolates (65 MRSA and 33 MSSA) were grouped into 12 clonal complexes and 27 distinct clones. The prevalence of nasal carriage of SA and MRSA among healthy population (*i.e.*, among non-medical university students) in Kabul was 33.3% and 12.7% respectively. Four CCs and 7 clones were identified in the 19 MRSA isolates. Regarding animal strains, 11.8% (2/17) of SA strains causing bovine mastitis were MRSA, belonging to 2 distinct clones, and a very low prevalence (1.1%) of SA nasal carriage was found in sheep in Kabul. Overall, molecular characterization of the isolates indicated a large genetic diversity and the presence of multiple virulence and antibiotic resistance genes among SA/MRSA strains circulating in the health-care facilities, community and livestock in Kabul.

2. Conjugative transfer of *cfr* linezolid resistance gene

Linezolid is one of the last resort antibiotics for the treatment of MRSA infections. The objective of this work was to investigate, i) the risk of conjugative transfer of linezolid resistance from *S. epidermidis* (SE) strains to various SA clones, and ii) to assess the fitness cost and stability of plasmids in recipient strains. Five SE strains harboring different *cfr*-carrying plasmids (*pSA737, p12-02300, p-cfr-PBR-A, p-cfr-PBR-B*), and 9 major clones of SA circulating in France were used as donors and recipients, respectively. The results showed that certain clones such as ST8-MRSA-IV USA300 was more at risk of acquisition of *cfr*-carrying plasmids. The SE ST5 strain was more able to transfer the plasmid *p-cfr-PBR-B* to different recipients, compared to SE ST2 harboring the same plasmid. The plasmids *pSA737* and *p12-02300* were transferred to most of the recipients tested. The fitness costs of *cfr*-positive plasmids were variable for the same strain and between

plasmids. Finally, the studied *cfr*-positive plasmids were systematically more stable in SE than in SA after consecutive sub-cultures without selection pressure. Finally, these results show that *cfr*-mediated linezolid resistance can be efficiently transferred to major pathogenic clones of SA circulating in France, but the transmission is variable according to donor and recipient strains as well as the relevant plasmids.

Keywords

Staphylococcus aureus, MRSA*,* molecular characterization, Kabul, Afghanistan, linezolid, *cfr*, conjugative transfer, fitness cost.

Affiliate laboratory

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ABBREVIATIONS

Agr	Accessory gene regulator
AIP	Autoinducing peptide
AST	Antibiotic susceptibility testing
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
CoNS	Coagulase-negative staphylococci
CRISPR	Clustered regularly interspaced short palindromic repeats
EUCAST	European committee on antimicrobial susceptibility testing
HA-MRSA	Healthcare-associated methicillin-resistant *Staphylococcus aureus*
HCWs	Healthcare workers
HGT	Horizontal gene transfer
ICU	Intensive care unit
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
LR	Linezolid-resistant
LR-CoNS	Linezolid-resistant coagulase negative staphylococci
LRSE	Linezolid-resistant *Staphylococcus epidermidis*
LSSE	Linezolid-susceptible *Staphylococcus epidermids*
LukF-PV	F component of Panton-Valentine leucocidin
LukS-PV	S component of Panton-Valentine leucocidin
MDR	Multi-drug resistant
MGEs	Mobile genetic elements
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
OD	Optical density
*ori*T	Origin of transfer
PBP	Penicillin binding protein
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PMNs	Polymorphonuclear cells
PSMs	Phenol-soluble modulins
PTC	Peptidyl transferase center
PVL	Panton-Valentine leucocidin
rRNA	Ribosomal RNA
SaPIs	*Staphylococcus aureus* pathogenicity islands
SCC*mec*	Staphylococcal chromosomal cassette *mec*
SEs	Staphylococcal enterotoxins
SSSS	Staphylococcal scalded skin syndrome
SSTIs	Skin and soft tissue infections
ST	Sequence type
TSA	Tryptic soya agar
TSB	Tryptic soy broth
TSST-1	Toxic shock syndrome toxin-1
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World health organization
WTA	Wall teichoic acid

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a major cause of hospital and community-acquired infections. It is most often responsible for skin and soft tissues infections (SSTIs), but can cause more severe diseases such as bacteremia, pneumonia, and serious deep-seated infections such as endocarditis and osteomyelitis. It can also cause infections related to medical instrumentation, such as central-line associated bloodstream infections. Since the 1960s, methicillin-resistant *S. aureus* (MRSA) was a truly global challenge. In the 2000s, in addition to the long-known healthcare-associated clones, novel strains have also emerged outside the hospital settings, in the community as well as in livestock. The emergence and spread of virulent clones expressing Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin-1 (TSST-1) and other superantigens, is an additional cause for concern. MRSA epidermiology is variable in the world with some strains predominating in geographically restricted settings while others have achieved pandemic spread.

Firstly, the severity and outcome of a *S. aureus* infection in an individual depend in part on the virulence (toxinic arsenal) of the bacterial clone responsible. Thus, epidemiological studies are interesting for public-health officials and infectious disease experts to analyze the geographic distribution of the virulent and multi-drug resistant (MDR) *S. aureus* clones that cause invasive infections, because this information should help them to understand how these pathogens spread and to better control them. Clones of *S. aureus* can be distinguished by "molecular typing" techniques based on the determination of clone-specific nucleotidic sequences in variable regions of the bacterial genome. Although molecular typing data for *S. aureus*/MRSA are abundantly available for Western Europe, North America or Australia only, few studies have been conducting to describe the epidemiology in the rest of the world, including Central Asia. Until now, no molecular typing study on *S. aureus*/MRSA clinical and community strains as well as animal strains, has been conducted in Afghanistan.

Secondly, in regard to the treatment of MDR gram-positive pathogens, glycopeptides have traditionally been the antibiotics of choice; but there may be difficulties with their use, including the emergence of glycopeptide-resistant or intermediate strains, a moderate tissue penetration, and the need to achieve and monitor adequate serum levels. Newer antibiotics such as linezolid, a synthetic oxazolidinone, are now available for the treatment of infections due to resistant gram-positive bacteria. Linezolid is effective in the treatment of infections caused by various gram-positive pathogens, including MRSA and enterococci. It has also been shown to be a cost-effective

treatment option in several studies, with its high oral bioavailability allowing an early change from intravenous to oral formulations with consequent of earlier patient discharge and lower inpatient costs.

Nevertheless, overuse of linezolid in the hospitals is a relevant factor for the emergence of linezolid resistant microorganisms. Mechanisms of resistance consist in i) ribosomal mutations which can not be transferred horizontally ii) horizontally transferable resistance genes which could be prevented by knowing their mechanism of spread and setting up preventive measures. According to some recent studies, the main reservoir for the transferable linezolid resistance genes (mainly *cfr*) are coagulase negative staphylococci (CoNS), especially *Staphylococcus epidermidis* (*S. epidermidis*), which may horizontally transfer the linezolid resistance genes to more pathogenic gram-positive cocci such as *S. aureus*, resulting severe infections difficult to treat. Understanding the mechanisms of transmission and the transfer capacity of the different *cfr*-carrying plasmids, as well as the propensity of *S. aureus* strains to acquire this kind of resistance or ability of *S. epidermidis* strains to transfer it, would help to take preventive measures in order to reduce the emergence and spread of new linezolid-resistant strains.

In this context, the first objective of this work was to investigate the molecular features and antibiotic resistance profile of *S. aureus*/MRSA clinical, community and livestock strains isolated in Kabul Afghanistan. Secondly, this work aimed to investigate the risk of conjugative transfer of *cfr*-carrying plasmids from linezolid-resistant *S. epidermidis* (LRSE) strains already isolated from the patients in various French hospitals, to varioius pathogenic clones of *S. aureus* currently circulating in France.

In the first part of this thesis, we present a literature review about *S. aureus* resistance genes, virulence determinants, and circulating clones in the regions around Afghanistan; then we explored the mechanisms of resistance to linezolid and horizontal gene transfer (HGT); finally we evaluated the fitness costs of the acquired plasmid on bacterial host. This part is concluded with relevant findings of literature review and working hypothesis. In second part, the experimental work is presented and results are discussed in light of previous literature. In the last chapter, the main findings are summarized and conclusions are followed by the perspectives for the continuation of this work.

PART 1: LITERATURE REVIEW

1. Characteristics of *S. aureus*

1.1. Generalities about *S. aureus*

The genus *Staphylococcus* was first identified by the Scottish surgeon Sir Alexander Ogston in 1880 in Aberdeen, United Kingdom. He discovered the microbe after noticing groups of bacteria in pus from a knee joint abscess during a procedure he was performing. Then, in 1884, the German scientist Friedrich Julius Rosenbach identified *S. aureus*, discriminating and separating it from *Staphylococcus albus*, a related bacterium (1). These designations remained until 1939, when, based on coagulase testing, Cowan differentiated *S. epidermidis* as a separate species (2). According to bacterial classification, *S. aureus* belongs to the kingdom of bacteria, phylum *Firmicutes*, class of *Bacilli*, order of *Bacillales* and family of *Staphylococcaceae* (Table 1).

S. aureus is a facultative anaerobic, gram-positive spherical bacterium of approximately 1µm in diameter forming grape-like clusters and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates (Fig. 1). It is nonmotile and does not form spores (1). *S. aureus* reproduces by binary fission. Complete separation of the daughter cells is mediated by *S. aureus* autolysin, and in its absence or targeted inhibition, the daughter cells remain attached to one another and appear as clusters (3). The catalase enzyme produced by *S. aureus* transforms hydrogen peroxide (H_2O_2) to oxygen and water. Catalase-activity is used to distinguish staphylococci from streptococci and enterococci. Among staphylococci, the coagulase test can be used to distinguish coagulase-positive staphylococci (mainly *S. aureus*, but also *S. hyicus*, *S. intermedius, S. pseudintermedius*) from coagulase-negative species such as *S. epidermidis* (4,5).

Table 1. Taxonomic classification of *S. aureus*. Retrieved on 18. Jan. 2021 from the Integrated Taxonomic Information System online-database (www.itis.gov)

Kingdom	***Bacteria*** Cavalier- Smith,2002
Subkingdom	***Posibacteria*** Cavalier- Smith,2002
Phylum	***Fermicutes*** Gibbons and Murray, 1976
Class	***Bacilli*** Ludwig et al., 2010
Order	***Bacilliales*** Prévot, 1953
Family	**Staphylococcaceae** Schleifer and Bell, 2010
Genus	***Staphylococcus*** Rosenbach, 1884
Species	***Staphylococcus aureus*** Rosenbach, 1884
	Direct Children:
Subspecies	***Staphylococcus aureus anaerobius*** De La Fuente et al., 1985
Subspecies	***Staphylococcus aureus aureus*** Rosenbach, 1884

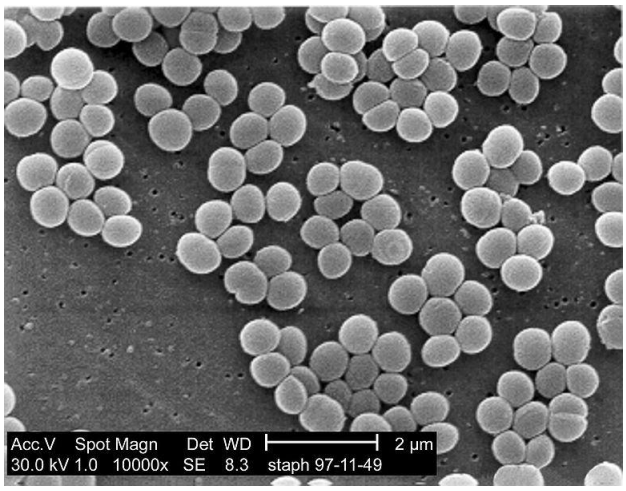

Fig 1. Morphology of *S. aureus*. The image is captured under a scanning electron microscope and 10,000x magnification (CDC public health image gallery).

S. aureus is part of human flora, and is primarily found in the nose and skin of approximately 25 to 30% of the general population without causing any harm (nasal carriage), but it is also a pathogenic bacterium that can cause various infections in humans (6). Higher carrier rates are seen in injection drug users, persons with insulin dependent diabetes, patients with dermatological conditions, and inpatients with long-term indwelling intravascular catheters. The carrier state is of clinical importance because any surgical intervention or exudative skin condition predispose the

carrier to a higher rate of infection compared with non-carriers, the infection usually being caused by the patient colonizing strain (7). The main reservoir sites are the nasal vestibule (35%) and the perineal region (20%), as well as the umbilical, axillary and interdigital regions (5-10%), from which dissemination can occur, causing disease and allowing transmission to other individuals (6). *S. aureus* is one of the first described pathogens in history of medicine. This is not surprising, however, as it was and is still one of the most common causes of infections in humans. It is of significant importance due to its ability to cause a plethora of infections as well as its capacity to adapt to diverse environmental conditions. *S. aureus* is one of the major causes of healthcare-associated (HA) and community-acquired (CA) infections. It is most often responsible for SSTIs, but can cause more severe diseases such as bacteremia, pneumonia, and serious deep-seated infections such as endocarditis and osteomyelitis. It can also cause infections related to medical instrumentation, such as central-line associated bloodstream infections (8). Infections are due to both methicillin-susceptible *S. aureus* (MSSA) and MRSA strains. Currently, there is little understanding of *S. aureus* mechanism of pathogenesis and the nature of protective immunity against infection. In recent decades, due to the evolution of bacteria and the abuse of antibiotics, the drug resistance of *S. aureus* and infection rate of MRSA has gradually increased in many countries (9–11).

S. aureus pathogenicity is mediated by numerous virulence factors and toxins, leading to either suppurative infections or toxin-mediated diseases, including toxic shock syndrome, staphylococcal food-borne diseases, and scalded skin syndrome (SSSS) (9). These virulence factors and toxins also allow *S. aureus* to address challenges presented by the human immune system. Because it has such elaborated tools, one might think that humankind would be highly vulnerable to severe infections by *S. aureus*. Interestingly, however, *S. aureus* maintains a fine control of its virulence factors and, in most cases, rarely causes severe life-threatening infections in healthy individuals.

S. aureus is naturally susceptible to most of the antibiotics that have been developed, however is able to acquire a whole range of resistance to antimicrobials. Resistance is acquired either by horizontal transfer of genes from outside sources, or by chromosomal mutations. The natural susceptibility of *S. aureus* led Alexander Fleming to discover the penicillin in 1928, and it was truly a miracle drug allowing to cure infections that were previously fatal. Yet, in the mid-1940s, only a few years after its introduction into clinical practice, penicillin resistance was encountered in hospitals and within a decade it had become a significant problem in the community (12). Emergence of antibiotic resistance by *S. aureus* can be visualized as a series of waves (Fig. 2).

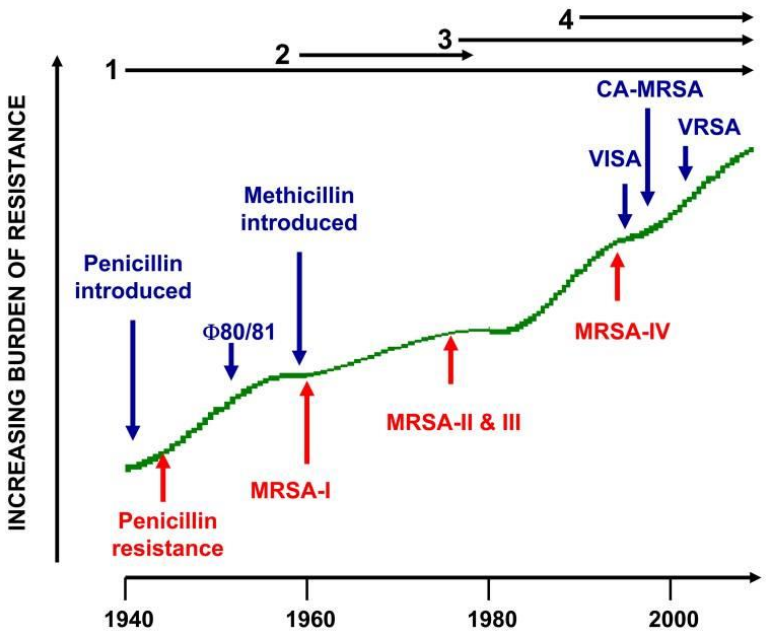

Fig 2. Emergence of antibiotic resistance by *S. aureus* over time illustrated by a timeline of the four waves of antibiotic resistance in *S. aureus*. Wave 1 began shortly after the introduction of penicillin into clinical practice in the 1940s. The first pandemic antibiotic resistant strains, from lineage named phage type 80/81 (Φ80/81), were penicillin resistant and produced PVL. Wave 2 began almost immediately after the introduction of methicillin into clinical practice with isolation of the first MRSA (Archaic clone), which contained type I Staphylococcal cassette chromosome mec (SCC*mec*) (MRSA-I) and continued into the 1970s as the Iberian clone. Wave 3 began in the mid-to-late 1970s with emergence of new MRSA strains, which contained novel SCC*mec* types, II and III (MRSA-II and III), marking the on-going worldwide pandemic of MRSA in hospitals and healthcare facilities. The increase in vancomycin usage for treatment of MRSA infections eventually led to emergence of vancomycin intermediate *S. aureus* (VISA) strains. Wave 4, which began in the mid-to-late 1990s, marks the emergence of MRSA strains in the community. CA-MRSA strains are generally susceptible to most antibiotics other than beta-lactams, and contain a smaller SCC*mec* element, type IV (MRSA-IV), and a variety of virulence factors, including PVL. Vancomycin-resistant *S. aureus* (VRSA) strains, of which approximately 10 have been isolated, exclusively in healthcare settings, were first identified in 2002 (10).

The first wave began in the mid-1940s with the emergence of penicillin-resistant *S. aureus* strains in hospitals.

The introduction of methicillin marked the beginning of the second wave of resistance. The first reports of MRSA was published in 1961 (13). However, the specific gene, *mecA*, which encodes the low affinity penicillin binding protein 2a (PBP2a) responsible for the phenotypic resistance to methicillin, was not identified until over 20 years later. Unlike penicillinase-mediated resistance, which is narrow in its spectrum, methicillin resistance confers resistance to almost all of the beta-

lactam antibiotic class (penicillins, cephalosporins, and carbapenems). Among the earliest MRSA clinical isolates, is the archetypal strain COL, a member of the "archaic" clone of MRSA and perhaps the most studied MRSA strain, which was isolated from a patient in Colindale, United Kingdom in 1960 (12). COL is a member of the most successful of all MRSA lineages (*e.g*., the Iberian and Rome clones), which includes both HA- and CA-strains. This archaic clone of MRSA strains circulated in hospitals throughout Europe until the 1970s (14). Its isolates also reported from hospitals in the United States (15,16), but the rest of the world was largely spared and these early MRSA clones never gained a foothold in the community. By the 1980s, for unclear reasons, archaic MRSA strains had largely disappeared from European hospitals, marking the end of the second and the beginning of the third wave of antibiotic resistance. Descendants of the archaic MRSA clone (*e.g*., the Iberian and Rome clones) and other highly successful MRSA lineages emerged (15). These strains disseminated worldwide, leading to the global MRSA pandemic in hospitals that continues to the present.

Although the global spread, MRSA were still confined mainly to hospitals and other healthcare facilities. Due to increased burden of MRSA infections in hospitals the use of vancomycin as part of anti-MRSA treatment intensified, and under this selective pressure, vancomycin intermediate *S. aureus* (VISA, which are not inhibited *in vitro* at vancomycin concentrations below 4 to 8 μg/mL) and vancomycin-resistant *S. aureus* (VRSA, showing high-level resistance to vancomycin and harbouring the *vanA* gene from vancomycin-resistant enterococci, VRE) strains emerged (17). In the 2000s, linezolid was commercialized and appeared as one of the last resort antibiotic for the treatment of severe infections due to MDR-MRSA strains (18), which will be explained in a separate chapter of this thesis.

The invasion of the community by MRSA constitutes the fourth and latest wave of antibiotic resistance described for *S. aureus*. Some of the first cases of CA-MRSA infections occurred in indigenous populations in Western Australia, United States and Canada in the early 1990s, suggesting that they were either descendants of hospital strains or community strains that had acquired *mecA* from hospital strains by HGT (12). Outbreaks and epidemics of CA-MRSA now occur worldwide, although the specific clones that have emerged vary with geographical location. CA-MRSA strains are generally susceptible to most antibiotics other than beta-lactams, and contain a smaller SCC*mec* element, type IV, and a variety of virulence factors, including PVL. Their genotypes indicate that they are not related to endemic hospital clones and these community strains are susceptible to numerous antibiotics to which hospital strains are routinely resistant.

1.2. Virulence determinants of *S. aureus*

S. aureus is able to produce many virulence factors, such as hemolysins, leukocidins, proteases, enterotoxins, exfoliative toxins, and immune-modulatory or immune-evasion factors. The expression of these factors is tightly regulated during growth. The accessory gene regulator (*agr*) system, known as the quorum-sensing system of *S. aureus*, is a crucial regulatory component involved in the control of bacterial virulence factor expression. A number of *in vivo* experiments showed that many factors, including cellular immune factors and nutrient conditions, affect the expression of virulence factors, suggesting that the mechanisms of regulation of virulence factors *in vivo* are complex and not only dependent on *agr* (19).

For the most of diseases caused by *S. aureus*, pathogenesis is multifactorial. However, there are correlations between some clinical features and expression of specific virulence determinants, which suggests their role in a particular disease *e.g*., PVL and tissue necrosis. The application of molecular biology has led to advances in unraveling the pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced, and many protein toxins have been purified. With some staphylococcal toxins (*e.g.,* Enterotoxins A-G, TSST-1), symptoms of human disease can be reproduced in animals with the purified protein toxins, lending an understanding of their mechanism of action (20).

The broad range of infections caused by *S. aureus* is related to a number of virulence factors that allow it to adhere to surface, invade and/or avoid the immune system, and cause harmful toxic effects to the host (Fig. 3).

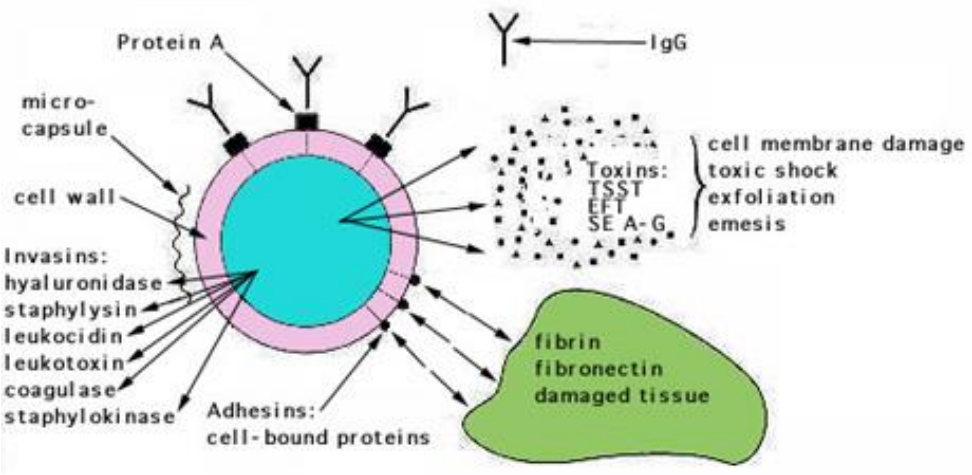

Fig 3. Virulence determinants of *S. aureus* (18).

The important virulence factors of *S. aureus* which exist in all strains, because encoded in the core genome, are: (1) surface proteins that promote colonization of host tissues that are called adhesins; typical members of the family of adhesins called microbial surface components recognizing adhesive matrix molecules (MSCRAMM) are staphylococcal protein A (*Spa*), fibronectin-binding proteins A and B (*FnbpA* and *FnbpB*), collagen-binding protein, and clumping factor (Clf) A and B; (2) invasins that promote bacterial spread in tissues (kinases, hyaluronidase); (3) surface factors that inhibit phagocytosis (capsule, protein A); (4) biochemical components that enhance bacterial survival in phagocytes (carotenoids, catalase); (5) immune evasion (protein A, coagulase); (6) membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukocidins).

The most important virulence factors encoded by mobile genetic elements (MGEs) and horizontally transmissible to other *S. aureus* strains are: PVL, staphylococcal enterotoxins A-G, TSST-1, which are described later in more detail.

The *agr* quorum-sensing system

S. aureus has evolved a complex regulatory network to control its virulence. One of the main functions of this interconnected network is to sense various environmental cues and respond by altering the production of virulence factors necessary for survival in the host, including cell surface adhesins and extracellular enzymes and toxins. Of these *S. aureus* regulatory systems, one of the best studied is the *agr* system (21). This system allows *S. aureus* to sense its own population density and translate this information into a specific gene expression pattern. Besides *agr*, this pathogen uses other two-component systems to sense specific cues, and coordinates responses with cytoplasmic regulators of the *SarA* protein family and alternative sigma factors. These divergent regulatory systems integrate the various environmental and host-derived signals into a network that assures optimal pathogen response to the changing conditions.

The *agr* system is a crucial regulatory component in *S. aureus*, conserved throughout the species. It is a 3 kb locus showing highly conserved and hypervariable regions (22) (Fig. 4). The sequence of this hypervariable segment is the target of PCR amplification for defining *agr* types 1 to 4 (23). *agr* is the gene cluster that encodes the peptide quorum-sensing system that senses the local concentration of a cyclic peptide signalling molecule (Fig. 5). The signal sensed by the *agr* system is an autoinducing peptide (AIP), which can be 7–9 amino acids in length and contains a 5-membered thiolactone ring between the C-terminal end and a conserved cysteine residue. The AIP signal accumulates in the extracellular environment, and once it reaches a critical concentration,

usually at a "quorum" cells in the population, the system is activated. *S. aureus* uses the *agr* system to adapt to changing environmental conditions during growth and to regulate virulence. The *agr* system consists of two adjacent transcripts, called RNAII and RNAIII, whose expression are driven by the P2 and P3 promoter, respectively. The RNAII transcript is an operon of four genes, *agrBDCA*, that encode the machinery of the quorum-sensing system, and the RNAIII transcript is the major effector and regulates the expression of most *agr*-dependent target genes. As shown in Fig. 5, AgrD is the ribosomal peptide precursor of AIP and is proteolytically processed by AgrB, an integral membrane-bound peptidase. AgrB mediated cleavage of AgrD results in the formation of an enzyme-bound thiolactone intermediate, and through an unclear mechanism this structure is transported across the membrane. The type I signal peptidase SspB performs the final processing step to release the mature AIP into the extracellular environment. Once outside the cell, AIP is then sensed by AgrC, the membrane-bound histidine sensor kinase of the AgrCA two-component system. The binding of AIP to the AgrC receptor leads to histidine autophosphorylation, and this signal is relayed to the aspartate receiver on the response regulator AgrA. The phosphorylated AgrA can then bind to the P2 and P3 promoters to drive expression of RNAII and RNAIII, respectively. Expression of RNAII, which encodes for all components of the *agr* system, effectively leads to a positive feedback loop. This autocatalytic regulation is a hallmark of quorum-sensing systems and enables *S. aureus* to readily produce exoproteins, even though growth is slowed down (21).

One-third of the N-terminal region of the AgrB product and nearly half of the C-terminal region of the AgrC product are highly conserved. The intervening sequences, which include two-thirds of the C-terminal region of the AgrB product, the whole of AgrD, and about half of the N-terminal region of the AgrC product, are highly divergent and constitute the hypervariable region. It is the variations among these hypervariable regions that divide *S. aureus* into 4 *agr* specificity groups (I to IV) (22). There is evidence that specific *agr* biotypes are associated with specific clinical features. For example, most menstrual toxic shock syndrome (TSST-1) strains belong to *agr* group III (24), whereas those belonging to *agr* group IV have been found to be associated with the production of exfoliatins and to be involved in bullous impetigo (23).

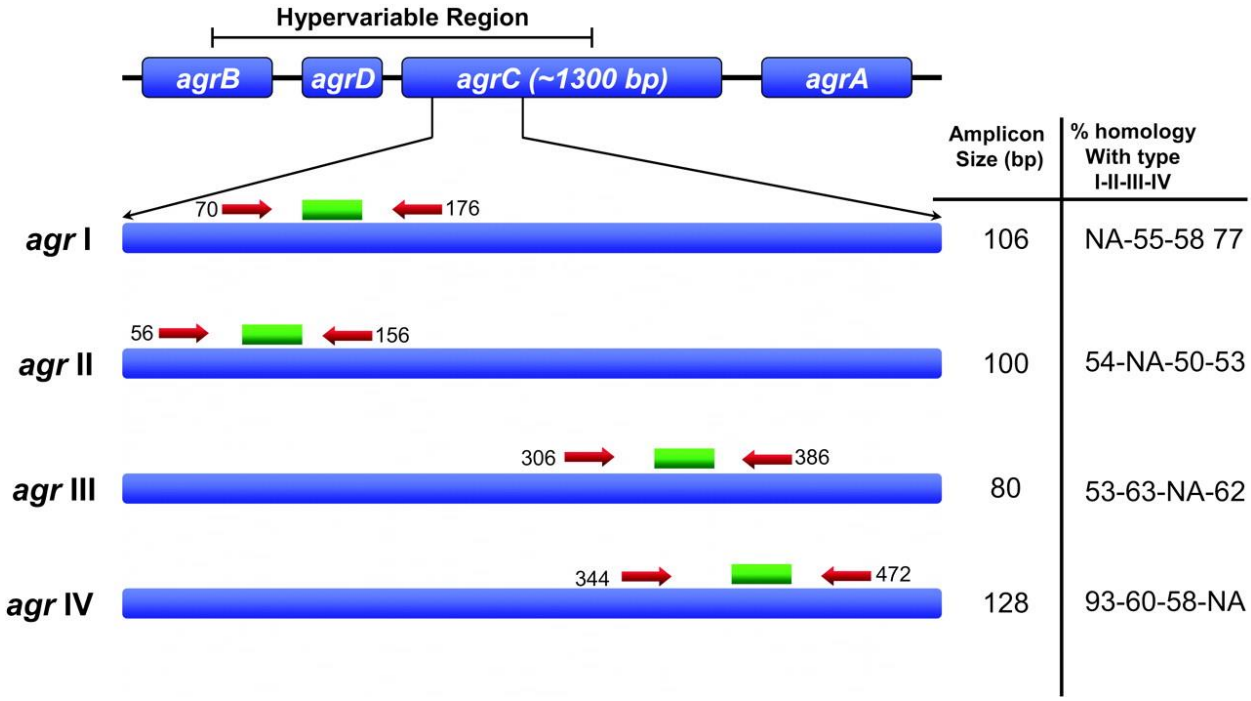

Fig 4. Schematic representation of the *S. aureus agr* locus that contains variable and conserved regions. Numbers indicate oligonucleotide positions based on the 5′ to 3′ sequence. All primers and probes were selected in the first half of the AgrC gene, in a region containing enough sequence divergence between the four *agr* groups to allow specificity of the PCR. The exact length of amplicon obtained for each *agr* type and the percentage of homology observed in the PCR-targeted region are also indicated. NA, not applicable (23).

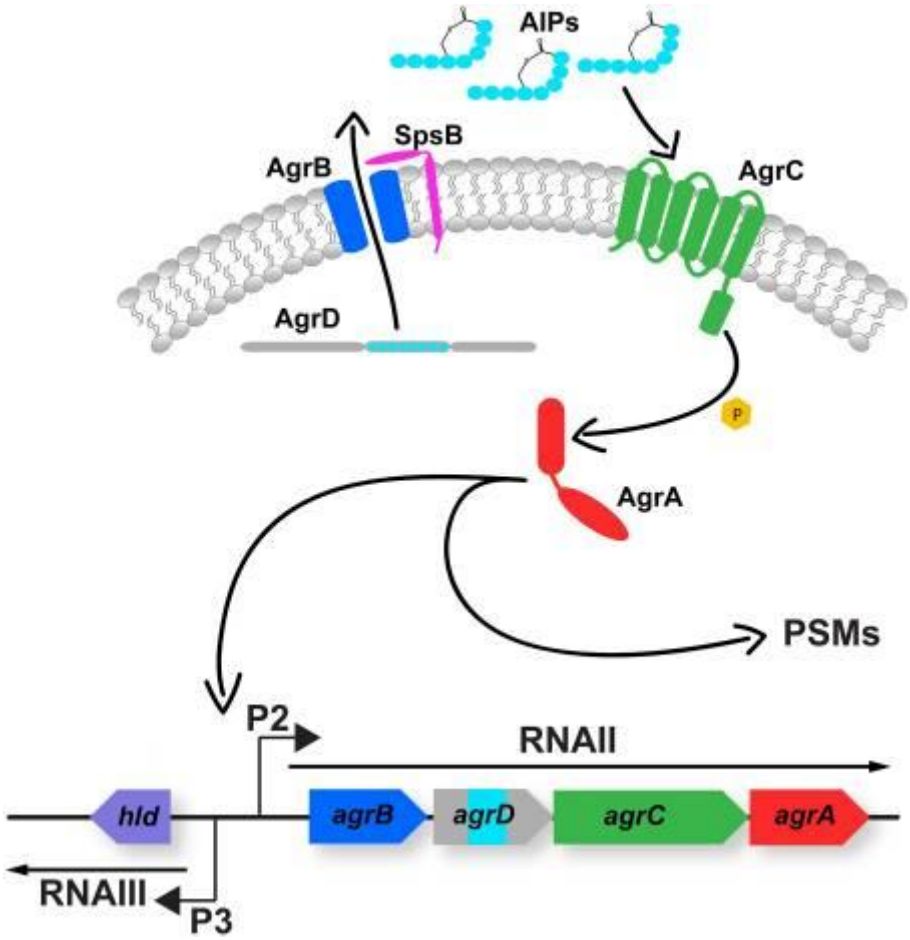

Fig 5. A schematic representation of the molecular organization, signal biosynthesis and transduction cascade of the *agr* quorum-sensing system. The autoinducing peptide (AIP) signal is encoded within the AgrD peptide. AgrD is processed and transported into the environment by AgrB, with the aid of signal peptidase SpsB. When the extracellular AIP concentration reaches a critical level, the signal is sensed by the histidine kinase AgrC, which undergoes auto-phosphorylation. Then the phosphate is relayed to AgrA, which in turn can bind the P2 and P3 promoters, driving expression of the RNAII and RNAIII transcripts, respectively. The RNAII transcript harbors the *agrBDCA* operon, encoding the primary machinery for AIP biosynthesis and detection. RNAIII is the main effector molecule of the *agr* system and drives expression of downstream target genes. Phosphorylated AgrA also binds the promoters for the phenol-soluble modulins (PSMs) genes, leading to their expression (21).

1.2.2. Clinically important horizontally transmissible virulence factors

S. aureus strains can acquire several virulence factors directly associated with specific clinical presentations, which are described below in more detail:

1.2.2.1. Panton Valentine Leukocidin (PVL)

PVL is a toxin composed of two components, LukS-PV and LukF-PV strongly associated with skin and soft-tissue infections and are comparatively rare in invasive diseases (25). These two components encoded with a single operon, are secreted before they assemble into a pore-forming heptamer on neutrophil membranes, leading to neutrophil lysis (25) (Fig. 6). PVL, encoded by two contiguous and cotranscribed genes lukF-PV and lukS-PV, is involved in chronic furunculosis and necrotizing pneumonia (26,27). The toxin has been linked to community-onset MRSA infections worldwide (28), but some CA-MRSA strains do not carry the PVL genes (29,30). Sequencing data suggest that circulating CA-MRSA strains might be directly derived from a historical PVL-producing penicillin-resistant clone phage type 80/81 that circulated in the 1950s and 1960s and was highly virulent. Additionally, PVL has well established leukocidal properties, and causes dermonecrosis when injected into rabbits (25).

The PVL genes are found on several different phages, *i.e.,* ΦPVL, Φ108PVL, ΦSLT, ΦSa2958, ΦSa2MW and ΦSa2USA (31). There is 20% to 27% sequence homology between the two PVL protein compounds LukS-PV and LukF-PV. *In vitro*, PVL induces the lysis of several cell types involved in host defense such as neutrophils, monocytes, and macrophages. However, PVL is not hemolytic (32,33). High concentration (200 nM) of PVL induces polymorphonuclear cell (PMN) necrosis by activation of calcium channels and osmotic leakage from the cytosol. Conversely low concentration (10 nM) of PVL induces the formation of pores in the mitochondrial membrane, and thereby cell apoptosis (34). Expression of LukS-PV and LukF-PV could be positively controlled by *SarA*, *agr* and *SaeRS* systems, while negatively regulated by *Rot. SarA* and *Rot* are essential regulators for the inducing effect of beta-lactams on PVL production (35).

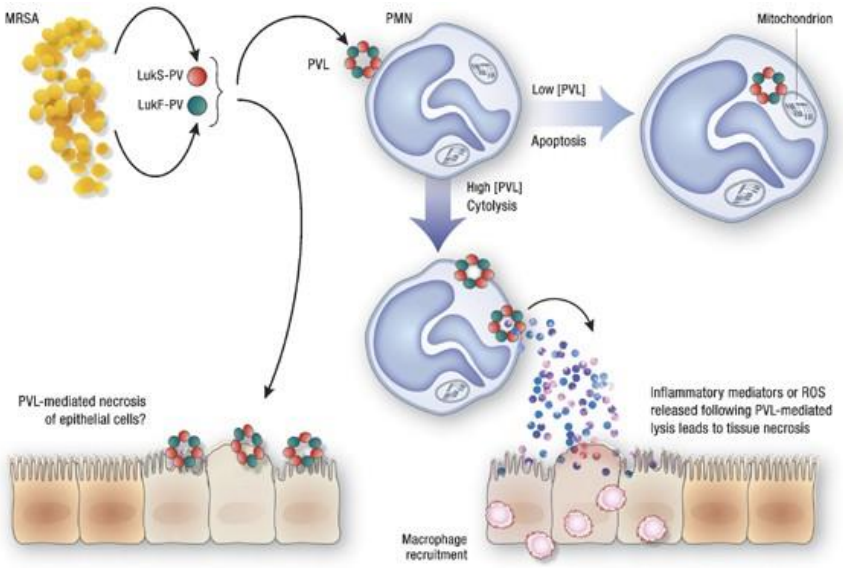

Fig 6. Model of the contribution of PVL to tissue necrosis. The two PVL components, LukS-PV and LukF-PV, are secreted from *S. aureus* before they assemble into a pore-forming heptamer on PMN membranes. High PVL concentrations cause PMN lysis whereas low concentrations mediate PMN apoptosis by directly binding to mitochondrial membranes. Tissue necrosis could result from release of reactive oxygen species (ROS) from lysed PMNs. Alternately, release of granule contents from lysed PMNs could set in motion an inflammatory response, eventually resulting in tissue necrosis. It is unlikely that PVL has a direct necrotic effect on epithelial cells (33).

1.2.2.2 TSST-1 and enterotoxins

Staphylococcal toxic shock syndrome (TSS) is a toxin-mediated acute life-threatening illness, mediated by a complex interaction of superantigens with the host resulting in extensive immune dysregulation, characterized by high fever, rash, hypotension, multi organ failure (involving at least 3 or more organ systems), and shock (9,36). This syndrome can also include severe myalgia, vomiting, diarrhea, headache, and nonfocal neurologic abnormalities (37). The syndrome is attributed in particular to TSST-1. TSST-1 has been associated with almost all menstrual TSS (mTSS) and half of nonmenstrual TSS (nmTSS) cases, while staphylococcal enterotoxins A, B, and C (*sea*, *seb*, and *sec*) are involved in the remaining nmTSS cases (38). TSST-1 and staphylococcal enterotoxins (SEs) are the paradigm of a large family of pyrogenic exotoxins called superantigens. Superantigens are proteins that generate a powerful immune response by binding to Major Histocompatibility Complex (MHC) class II molecules on antigen-presenting cells and T cell receptors on T cells, and can cause food poisoning or systemic shock (39). *S. aureus* can produce a large number of superantigens. Aside from TSST-1, it can produce at least 17 different enterotoxins (*sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, seq, ser, seu*) (40).

Most genes coding for enterotoxins are located on MGEs such as plasmids, bacteriophages or pathogenicity islands (41). Thus, horizontal transfer between strains is not rare. These bacterial proteins are known to be pyrogenic and are connected to significant human diseases that include food poisoning characterized by nausea, vomiting, abdominal cramping, diarrhea; and toxic shock syndrome (42). These toxins induce mononuclear cells to produce the cytokines tumor necrosis factor-alpha, interleukin-1beta and interleukin-6, which mediate fever, shock, and tissue injury (40).

2. The clonal distribution and diversity of *S. aureus* isolates in the regions around Afghanistan

Given its epidemiology changing with time and place, timely updated information on *S. aureus* strains carrying clinically important virulence factors and circulating in the geographical area is essential for the prevention and control of this pathogen. This information is also important for clinicians dealing with staphylococcal infections.

In recent years, MRSA have become a truly global challenge (43). In addition to the long-known healthcare-associated clones, novel strains have also emerged outside of the hospital settings, in the community as well as in livestock. The emergence and spread of virulent clones expressing PVL, TSST-1 and other superantigens is an additional cause for concern (44).

For decades after the identification of the first MRSA isolate in the 1960s, MRSA was regarded as a typical HA pathogen. The epidemiology of HA-MRSA is commonly described by geographical location and temporal dynamics in the distribution of particular clones; however, it is established that CC1, CC5, CC8, CC22, CC30, and CC45 are predominant around the world (45). The main changes in the global epidemiology of MRSA during the last decades are associated with the emergence of distinct genetic lineages of CA-MRSA in the 1990s (46), and of livestock associated MRSA (LA-MRSA) in the 2000s (43).

The epidemiology of MRSA varies considerably worldwide, and the spread of several CA-MRSA clones and their dissemination into hospitals have made it more difficult to understand the epidemiology (47). CA-MRSA lineages are genotypically and phenotypically unrelated to MDR HA-MRSA, and have recently started to replace the formerly pandemic HA-MRSA clones (CC5, CC8, CC22, CC36, and CC45) in healthcare facilities (48). CA-MRSA infections are dominated by five lineages: sequence type (ST) ST1-MRSA-IV (USA400), ST8-MRSA-IV (USA300), ST30-MRSA-IV (Pacific/Oceania; South West Pacific clone), ST59-MRSA-IV/V (USA1000, Taiwan), and ST80-MRSA-IV (European CA-MRSA), each being geographically restricted (49). However, these originally continent-specific clones have spread to other parts of the world; ST1-MRSA-IV (USA400) clone, for example, has been detected in Europe and Asia. Some of PVL-positive clones, such as ST1-MRSA-IV and ST30-MRSA-IV, are pandemic, having been detected in America, Europe, and Asia (50). ST80 –MRSA-IV, known as the European clone but originated in Africa, has also been reported in Libya, Jordan, and Lebanon (51,52).

In general, there are less data regarding the circulating clones of *S. aureus*/MRSA in livestock in comparison to human. Based on literature review, the most detected clones are ST398-MRSA,

ST130-MRSA causing bovine infections (53). Reports also show that various *S. aureus* clonal complexes including*:* CC1, CC9, CC22, CC30, CC97, and CC705 isolated from livestock infections worldwide (43,54–56). There are also various reports of LA-MRSA transmission to human worldwide including Asia, especially the pandemic ST398-MRSA strain (43,53,55,57).
Indeed, Asia the most densely populated continent, has one of the highest prevalence rates of HA-MRSA and CA-MRSA in the world (58–60). In central Asia, including Afghanistan, studies have mainly focused on the prevalence of MRSA, but few data are available about their molecular characteristics and the circulating clones. Below, we present the updated information obtained from literature review about the circulating *S. aureus*/MRSA clones in Afghanistan's neighboring countries and the surrounding region.

2.1. Pakistan

In Pakistan, a multicentric study in four hospitals showed a MRSA rate of 41.9% among infected strains in 2006–2008 (61). ST239-MRSA-III was the dominant genotype among HA-MRSA isolates in Pakistan in this period (102). Another study focused on molecular characterization of *S. aureus* isolates causing community- and hospital-acquired SSTIs among patients in a tertiary care hospital in the Malakand district of the Khyber Pakhtunkhwa Province of Pakistan: forty-five *S. aureus* isolates were characterized by microarray hybridization. Twenty isolates (44 %) were MRSA, whereas 22 (49 %) were PVL-positive (62). Fourteen MRSA isolates harboured PVL genes. The dominant clones were CC121-MSSA (n = 15, 33 %) and the PVL-positive "Bengal Bay Clone" (ST772-MRSA-V; n = 13, 29 %). The PVL-positive CC8-MRSA-IV strain "USA300" was found once (62). In a joint study related to epidemiological typing of MRSA isolates from patients in Pakistan and India, a total of 60 non-duplicate MRSA isolates were collected from three tertiary care hospitals in Pakistan and one in India. CC8 was the dominant clonal complex (57/60) and was present in both Pakistan and India. Thirty-four of the 57 isolates carried SCC*mec* type III/IIIa and the remainder carried SCC*mec* type IV (63). In another study, 44 MRSA isolates were collected from two tertiary care hospitals of the Rawalpindi district of Pakistan (64). Six CCs and 19 clones were identified. The most frequently identified strains belonged to the PVL-positive CC772-MRSA-V Bengal Bay Clone (10 isolates; 22.3%), ST239-MRSA [III + ccrC] (5 isolates); and CC8-MRSA-IV strain, as well as CC6-MRSA-IV (both with 4 isolates; 9.1% each). As expected, several of the strains detected indicated epidemiological links to the Middle Eastern/Arabian Gulf region.
In a research in Agha Khan University Hospital in Karachi between June 2006 and July 2007, 126

clinically significant MRSA isolates were studied (65). Among the HA-MRSA isolates, variants of SCC*mec* type III were prevalent, whilst SCC*mec* type IV or variants were predominant in the CA-MRSA isolates. The prevalent genotypes circulating in Pakistan at the time of study were ST8-MRSA-IV and ST239-MRSA-III in the community and hospital settings, respectively. A set of hospital acquired isolates collected in 1997 were characterized for comparison and the results suggest that an increase in genetic diversity occurred over the period 1997–2007 as a result of either microevolution or likely the importation of strains from surrounding areas.

Concerning epidemiology of MRSA among livestock in Pakistan, a recent study conducted in Rawalpindi-Islamabad in slaughterhouses and meat shops showed a high prevalence of MRSA in chicken (77%), beef (63%) and mutton (50%) (66). In another study in public and private farms of Faisalabad, the prevalence of MRSA in bovine milk was 34%, and cattle 38% (67), but there is no information about their molecular characteristics.

2.2. India

In India, two multicentric studies described comparable MRSA rates of 41% and 45% among *S. aureus* isolates in 2008–2009 and 2011, respectively (68,69). A single-institute study, including 50 MRSA isolates causing SSTIs in southern India in 2011, showed that ST239-MRSA-III strains accounted for 32% of all isolates, and were multiresistant to mupirocin, amikacin, cotrimoxazole, erythromycin, rifampin and tetracycline with high frequencies (58). In 2002 and 2005, four isolates with vancomycin MICs of 16 to 64 mg/L were identified in northern India by screening 783 clinical *S. aureus* isolates with the agar dilution method (70). None of these vancomycin-resistant isolates carried *vanA* or *vanB*. Another clinical VRSA isolate (vancomycin MIC 64 mg/L), identified in Kolkata in 2005, harboured *vanA* (71). As most of the reported VISA and VRSA isolates were clinical isolates, it was noteworthy that two nasal VRSA isolates carrying *vanA* were identified during a routine nasal carriage survey of VISA/VRSA strains in an intensive-care unit in northern India (72). Between 2006 and 2009, 412 MRSA isolates from a mixed hospital- and community-associated patient population from Mumbai were evaluated, and it was found that 34% of the isolates carried SCC*mec* IV and 41% carried SCC*mec* V. Multilocus sequence typing (MLST) of 29 SCC*mec* type IV isolates and 13 SCC*mec* type V isolates identified ST22-MRSA-IV and ST772-MRSA-V clones (73). Furthermore, a study of MRSA in healthy and diseased individuals in India in 2010 showed the predominance of ST22-MRSA-IV harbouring PVL and TSST-1 genes (74). Variable resistance markers were detected in ST22-MRSA-IV PVL-positive isolates: *blaZ*,

ermC, *aacA-aphD*, *aadD*, *dfrA* and Q6GD50 (*fusC*, SCC-borne fusidic acid resistance). Another study aimed to determine the antibiotic susceptibility pattern of *S. aureus* and the circulating clones in Bangalore, India in a tertiary-care hospital. Out of the 92 collected strains, 52.2% were MRSA, isolated from community-acquired and hospital-acquired infections in 60.4% and 39.6% of cases, respectively. *S. aureus* isolates were also highly resistant to erythromycin (54.3%) and ciprofloxacin (70.6%). MRSA were found to be significantly more resistant to gentamicin, cotrimoxazole and ciprofloxacin, than MSSA, but no significant difference was observed between CA- and HA- *S. aureus*. ST217-MRSA-IV PVL-positive appeared as a new emerging and prevalent clone, but ST772-MRSA-V PVL-positive Bengal Bay clone remained the predominant clone (75). In a study, *S. aureus* strains were collected from in and around Bangalore and three other cities of India from HA- and CA-infections or nasal carriage in order to determine their lineage. The isolates belonged to 15 STs. The dominant MRSA clones were ST22-MRSA-IV and ST772-MRSA-V among healthy carriers and patients. In this study authors reported three novel clones, two MSSA isolates belonging to ST291-MSSA (related to ST398-MSSA which is livestock-associated), and two MRSA clones, ST1208-MRSA-V, and ST672-MRSA-IV as emerging clones for the first time. Sixty-nine percents of isolates carried PVL genes along with many other toxins. There was more diversity of STs among MSSA than MRSA (76).

In regard to the LA-MRSA, a study was carried out to evaluate the prevalence of *S. aureus* CC-398 in chicken and mutton marketed in retail outlets of Chennai, India (77). Of the 40 chicken isolates and 40 mutton isolates, 28 and 21 isolates belonged to CC398 (70% and 52.5%), respectively. In a study in west Bengal of India, the prevalence of MRSA in bovine mastitis was 9.6%, and *spa*-typing showed that isolates belonged to t304 and t6297 (78).

2.3. Iran

In Iran, a systematic review reported a MRSA rate of 43.0% in human infections between 2000 and 2016 (79). In another study at the Arak University of Medical Sciences campus, nasal swabs were collected from students without infection over a 3-month period in 2012 (80). One hundred fifty-four (22%) *S. aureus* strains were isolated from the anterior nares of 700 healthy students. Seven (4.5%) of isolates were confirmed as MRSA. MRSA isolates belonged to SCC*mec* types IV (n = 6) and V (n = 1). The predominant ST was ST22-MRSA-IV in 3 isolates, and the 4 other STs were ST25-MRSA-IV, ST859-MRSA-IV, ST14-MRSA-V, and ST15-MRSA-IV. Another study conducted in southwest of Iran (Kerman) among clinical isolates of *S. aureus* (81) reported 56.8%

of MRSA. All MRSA isolates were susceptible to vancomycin and linezolid. The most frequent SCC*mec* types were type III (48.31%) followed by type V (19.1%), type I (16.85%), and type IV (3.37%). The PVL genes were detected in 3.1% of isolates (2 MRSA and 3 MSSA isolates). REP-PCR typing divided the 92 MRSA isolates into 10 distinct clusters. In a latest study conducted in Iran in burn patients, 15.1% of MRSA isolates harbored PVL genes and the majority of MRSA strains carried SCC*mec* type III (71.7%). ST239-MRSA-III (34%) was the most common genotype followed by ST239-MRSA-III (24.5%), ST15-MRSA-IV (15.1%), ST22-MRSA-IV (13.2%), and ST239-MRSA-III (13.2%). Resistance to mupirocin was confirmed in 19.8% of MRSA strains belonged to ST15-MRSA-IV (40%), ST22-MRSA-IV (23.3%), ST239-MRSA-III (20%), and ST239-MRSA-III (16.7%) clones. (82). In a systemic review and meta-analysis concerning epidemiology of PVL-harboring *S. aureus* in cutaneous infections in Iran, the pooled prevalence of PVL in cutaneous infections was estimated at 27.9%, with a range from 7.4% to 55.6% (83). In another study among hospital staff nasal carriers in Babol, the rate of *S. aureus* nasal carriage was found to be 33.3% and 70% of those strains were identified as MRSA with a frequency of PVL genes of 5%. Based on multiplex PCR assays, four different SCC*mec* types were detected as 35.7% of the MRSA isolates belonged to SCC*mec* type I, 14.2% to SCC*mec* type III, 7.1% to SCC*mec* type II and 3.5% to SCC*mec* type IV (84). In a study about clonal dissemination pattern of PVL-producing *S. aureus* strains isolated from hospitalized patients in Tehran, the prevalence of PVL in *S. aureus* strains from clinical specimens was 29.2%. All the PVL-positive *S. aureus* strains were confirmed as MRSA. Five CCs and nine different clones were detected in this study. The most frequent CC was ST22-MRSA-IV (42.8%) followed by ST30-MRSA-IV (21.5%), ST8-MRSA-IV (17.2%), ST772-MRSA-V (11.4%), and ST80-MRSA-IV) (7.1%) (85). In a cross-sectional study in Motahari teaching hospital in Tehran, pus/wound swabs from SSTIs were collected in burn patients between January-August 2013 showed that MRSA isolates belonged to two major CCs; CC8 (ST239, ST585, ST2732, ST1294) and CC30 (ST30, ST36, ST1163) and four singletons. Subsequent analysis of MRSA isolates revealed that the most prevalent SCC*mec* type was type III (55.8%) followed by type IV (34.9%) and type II (2.3%). The prevalence of PVL-positive MRSA strains was high (20%). In a study among healthy students residing in dormitories of universities in Urmia, nasal screening identified 19.6% *S. aureus* and 2.6% MRSA carriers respectively (86). SCC*mec* typing showed that most MRSA strains belonged to SCC*mec* type IV (n = 14; 77.8%). Only 1 (5.6%) MRSA isolate carried the PVL genes. In a recent study, *S. aureus* clinical isolates were collected from two Tehran hospitals between February 2014 and March 2015.

Of 116 isolates, 13 (11.2%) harboured the PVL genes, 5 isolates (38.4%) were MRSA with *agr* group I and SCC*mec* type III. High diversity was found among PVL-positive strains by pulsed field gel electrophoresis (PFGE). The TSST-1 gene was found only in MSSA (87). In a cross-sectional study in four hospitals affiliated to Shahid Beheshti University of Medical Sciences in Tehran, a total of 1,161 non-duplicated clinical *S. aureus* isolates were obtained from different clinical specimens from August 2013 to July 2018 (88). A total of 14 STs, 26 *spa*-types, 3 *agr* types (no *agr* IV), and 9 CCs were identified. Nine (9.1%) of the isolates were MRSA and distributed in nine CCs, whereas the MSSA isolates were less diverse, which mainly belonged to CC22 (7.95%) and CC30 (7.95%).

In regard to livestock strains, a study on referred clinical and subclinical bovine mastitis milk samples in Ahvaz province of Iran, showed a low prevalence of MRSA (1.3) (89); while in another study on referred subclinical mastitis milk in Kurdistan province, the prevalence of MRSA was high (11.6%) (90). In a nasal carriage study, the proportion of *S. aureus* -positive nasal swabs from cattle, sheep and goats were 5.06%, 4.1% and 25%, respectively, only one (1/11; 9.1%) strain of MRSA was detected in sheep nasal swabs (91).

2.4. China

A nationwide study, including 18 hospitals in 14 cities in 2005–2006 in China, showed that 77.1% of the MRSA clinical isolates belonged to the ST239-MRSA-III lineage, and that 15.5% belonged to the ST5-MRSA-II lineage (92). Most of the ST239 strains were resistant to tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, and ciprofloxacin (92). Between January 2005 and January 2006, 18 (14.9%) of 121 MRSA isolates were positive for PVL genes in a teaching hospital in Wenzhou, China (93). Six STs (ST88-MRSA-III, ST239-MRSA-III, ST398-MRSA-III, ST25-MRSA-III, ST30-MRSA-IV and ST59-MRSA-I) were found among these 18 PVL-positive MRSA isolates. In a study from four maternity and children's' hospitals in Guangzhou (94), a total of 131 *S. aureus* clinical isolates were obtained between 2015 and 2018. The prevalence of MRSA was 48.9%. In total 12 STs and 5 CCs were detected in MRSA isolates, while 22 STs and 10 CCs were detected in MSSA isolates. Among MRSA isolates the top three STs were ST59-MRSA-IV, ST338-MRSA-III, and ST45-MRSA-IV represented 78.1%, and among MSSA isolates, ST188-MSSA, ST1-MSSA, and ST398-MSSA, representing 35.8% of isolates. In another epidemiologic investigation, a total of 3695 *S. aureus* isolates was recovered from 2008 to 2017 in a teaching hospital in Shanghai, China; the prevalence of predominant HA-

MRSA clone, ST239-MRSA-III significantly decreased over the study period (from 20.3% to 1%) and replaced by the continually growing ST5-MRSA-II clone (from 0% to 17.3%) (95). In a major study in order to better understand the national molecular epidemiology and resistance profiles of HA-MRSA in China, a laboratory-based multicenter surveillance study was conducted (96). ST239-MRSA-III, and ST5-MRSA-II weree the predominant HA-MRSA.

In a study exploring the molecular evolution of MRSA at Peking Union Medical College Hospital, a total of 466 nonduplicate *S. aureus* isolates, including 302 MRSA and 164 MSSA isolates recovered from 1994 to 2008 were characterized (97). The 302 MRSA isolates were classified into 9 STs. From 1994 to 2000, the most predominant MRSA clone was ST239-MRSA-III. Another clone, ST5-MRSA-II emerged in 2002 and persisted at a low prevalence rate. The 164 MSSA isolates were classified into 40 STs. ST398-MSSA was the most common MLST type for MSSA, followed by ST59-MSSA, ST7-MSSA, ST15-MSSA, and ST1-MSSA.

In regard to livestock MRSA strains, a study reported a MRSA prevalence of 2.7% in bovine mastitis with isolates belonging to distinct clones: ST97-MRSA-IV, ST965-MRSA-IV, ST6-MRSA-IV and ST9-MRSA-SCC*mec*-not typeable (98). In another study, two LA-MRSA isolates (ST398-MRSA-V) recovered from bulk tanks of cow milk samples in two geographically distant farms in China. Whole-genome analysis strongly suggested that these strains were closely related to the HA- ST398-MRSA-V and CA- ST398-MRSA-V strains in China (99).

2.5. Russia

In the literature review, information about *S. aureus* and MRSA circulating clones in Russia is very scarce. In a prospective study, *S. aureus* isolates were collected from paediatric and adult in-patients and out-patients in Vladivostok from August 2006 to April 2007 (100). Among the 63 *S. aureus* isolates, 48% were MRSA. HA- strains accounted for 93% of all MRSA isolates. The major MRSA clone was the ST-239-MRSA-III PVL-negative, representing 90% of MRSA. This clone was MDR, including 41% of isolates resistant to rifampicin. CA-MRSA isolates ($n = 2$) were assigned to ST30-MRSA-IV PVL-positive, and ST8-MRSA-IV PVL-negative. In another study in Saint Petersburg of Russia, *S. aureus* isolates recovered from hospital patients (n=716), and healthy carriers (n=2053) between 2011-2014. The detection rates of MRSA among hospital strains and healthy carrires were 67% and 0.6%, respectively. HA-MRSA demonstrated high resistance to ciprofloxacin, gentamicin, and chloramphenicol, and elevated MIC (2 μg/mL) for vancomycin in 26% of isolates. The predominant clones among HA-MRSA were ST8-MRSA-IV and ST239-

MRSA-III, constituted 83.1% of the isolates; other HA-MRSA isolates belonged to CC5, CC22, CC97 and CC398 (101). Among healthy carriers the ST22-MRSA-IV clone accounted for 84.6% of the MRSA isolates; other isolates belonged to CC5.

In regard to *S. aureus* animal strains, no information about MRSA is available. The only study about *S. aureus* among animals was conducted in West of Russia; it did not detect any MRSA on milk samples obtained from cow farms. The whole genome sequencing identified ST97-MSSA as the most common type in this region (102).

2.6. Middle East

MRSA has emerged as an important pathogen in HA- and CA- infections in many countries in the Middle East, over the last decade (103). With the global transmission of MRSA, the local epidemiology within countries in the Middle East is changing, owing to the introduction of new strains, with the intercontinental exchange of several clones. ST80-MRSA-IV is one common clone detected in different countries within the region (103). A study in the Eastern Province of Saudi Arabia revealed high clonal diversity among the isolates of nasal colonization among healthy carriers, HCWs and medical students, with 19 different *spa* types, 12 CCs, and 7 STs detected (104). *S. aureus* isolates were present in the nares of 37% of the healthy carriers and 26% of the medical students and HCWs. Sixteen percent of the all isolates were MRSA. The most common strains were CC15-MSSA, ST80-MRSA-IV, ST22-MRSA-IV, and ST5-MRSA-IV. In a review article related to the genotype distribution of MRSA infections in the hospitals in the Kingdom of Saudi Arabia, showed that the pandemic Vienna/Hungarian/Brazilian clone (CC8/ST239-MRSA-III) was the most frequent in Saudi regions (Riyadh and Damamm) (105). Several other clones such as Barnim/UK-EMRSA-15 (CC22-MRSAIV), Southwest Pacific clone (ST30-MRSA-IV) and European community-associated-MRSA clone (CC80-MRSA-IV) have been detected in Riyadh region. In another study including 1,327 MRSA isolates obtained from clinical samples in 13 Kuwait hospitals from 1 January to 31 December 2016, 261 *spa* types were identified with *spa* types t688, t304, t860, t127, t044, t311, t002, t223, t267, t019, t3841, t005, t084, t852, and t657 constituting 51.0% of the isolates. A total of 102 MRSA strains indentified as novel variants in this study were investigated further. They belonged to 14 clonal complexes with CC361 (32; 32.3%), CC30 (15; 14.7%), CC22 (13; 12.7%) and CC1 (11, 10.7%) as the dominant CCs. Forty-six (45.1%) of these isolates were positive for PVL and 89 (87.2%) were resistant to fusidic acid mediated by *fusC* (106). A study conducted throughout the Gaza strip by Biber *et al*. (107) found

that around 13% of healthy children and their parents were MRSA carriers with the predominance of ST22-MRSA-IV (75%). Interestingly, of all MRSA isolates, 8.5% were PVL-positive and belonged to ST80-MRSA-IV. Khalil *et al.* (51) performed molecular characterization of 103 *S. aureus* (41 MRSA and 62 MSSA) recovered from stool and nose specimens collected from children admitted to the Jordan university hospital between August and October 2008. Genotyping revealed 17 different MRSA *spa* types, and 31 different MSSA *spa* types. Fourty-one percent of the isolates were MRSA. The clustering revealed that ST80-MRSA-IV was the dominant type. Among the MRSA isolates, 34% were PVL-positive, while it was 1.6% for MSSA. A more recent study was conducted on 132 *S. aureus* clinical isolates recovered in a period of 6 months in Beirut (52). The proportion of MRSA collected in this study was 30%, with PVL being detected in 54% of MRSA and 12% of MSSA isolates. Clustering of SCC*mec* types with MLST identified seven MRSA and 20 MSSA clones, and confirmed that the ST80-MRSA-IV PVL-positive was the dominant clone in Lebanon, followed by ST30-MSSA PVL-positive.

In regard to the livestock *S. aureus* strains, a study was conducted in Saudi Arabia to determine the presence and genetic characteristics of *S. aureus* isolated from raw retail meat sold in Riyadh, Saudi Arabia from March-December 2014. The Camel meat had 20.8% rate of MRSA contamination, lamb 16.7%, beef 12%, while poultry meat had 3.5%. The MRSA isolates were grouped into 4 CCs namely CC1-MRSA-IV, CC15-MRSA-V, CC80-MRSA-IV PVL-positive, and CC88-MRSA-IV PVL-positive (108). A recent study conducted on 1010 goats (235 milk samples and 775 nasal samples) from 25 flocks in eastern province of Saudi Arabia to study the prevalenc of MRSA in goat farms. The prevalence of MRSA in mastitic milk was 9.2%, in normal milk 0.6% , and in nasal swabs 2.6% (109).

2.7. Afghanistan

In Afghanistan, the prevalence of MDR bacteria including MRSA has been reported high and it can be linked to the over prescription of antibiotics in healthcare facilities (110,111). Unfortunately, no molecular studies have been conducted until now on *S. aureus* strains that infect or colonize humans and animals in Afghanistan, to characterize the circulating clones including their virulence and antibiotic resistance determinants, as well as their epidemiological links. A recent study concerning *S. aureus* infections diagnosed in Kabul hospitals highlighted a high rate of methicillin resistance (56.2%), and MDR-MRSA (MRSA resistant to ≥ 3 different classes of antibiotics) (91.4%) compared to the neighboring countries (112). In parallel, an overuse of antimicrobials was

described during the last decade in primary healthcare clinics of five major provinces of Afghanistan, including Kabul (113).

A recent study conducted in 3 public hospitals of Kabul to analyze the rate of prescription of antibiotics from July to August 2017, showed that 66% of the prescriptions contained at least 1-3 antibiotics, with an average of 1.5 antibiotics per prescription (114). Such widespread use of antimicrobial therapies is a major public health concern and have likely contributed to the increase of MDR microorganisms including MRSA in Afghanistan.

In regard to the prevalence and molecular characteristics of *S. aureus* strains in livestock, no study has been conducted in Afghanistan yet.

3. Genetics of antimicrobial resistance in *S. aureus*

The species *S. aureus* and in particular MRSA is among the "high priority" list of World health organization (WHO), for which there is an urgent need to control the emergence of resistance (115). One of the challenges confronting the treatment of *S. aureus* infection is resistance to many commonly used antimicrobial drugs. *S. aureus* strains that are resistant to multiple antimicrobial compounds, including most available classes of antibiotics and some antiseptics, are a major threat to patient care. It is now evident that the evolution of multi-resistance is driven, either by the acquisition of antimicrobial resistance genes via HGT transfer, or chromosomal mutation (116). However, the successive discoveries of HGT and extra-chromosomal DNA elements quickly confined gene mutation to a secondary role in the evolution of antibiotic-resistant bacteria. It is now clear that in most instances, resistance to multiple antimicrobial agents in the staphylococci is driven by the acquisition of MGEs such as plasmids, transposable genetic elements (insertion sequences and transposons) and genomic islands (117).

Here we describe the main mechanisms of *S. aureus* resistance to different classes of antibiotics.

3.1. Resistance to beta-lactams

Penicillins and other antibiotics in the beta-lactam family kills bacteria by inhibition of the last step in peptidoglycan synthesis through binding of the beta-lactam ring to bacterial transpeptidase involved in cross-linking peptides and preventing new cell wall formation. The target of the action of beta-lactam antibiotics are known as penicillin-binding proteins (PBPs) that are involved in the synthesis of peptidoglycan (118).

Two mechanisms confer penicillin resistance in staphylococci. The most important is production of beta-lactamase, which inactivates penicillin by hydrolysis of its beta-lactam ring. The second mechanism confers resistance to methicillin due to a penicillin-binding protein, PBP2a, encoded by *mecA* or its homologue the *mecC* gene (119). Methicillin resistance is associated with the acquisition of a particular resistance island called staphylococcal cassette chromosome *mec* (SCC*mec*), an exogenous piece of DNA, which is discussed later.

blaZ-encoded penicillin resistance has been thoroughly investigated. The resistance is coded by an operon clustered together the structural gene *blaZ*, its repressor gene *blaI*, and a signal transducer-sensor protein gene *blaR1* (120). Four types of *blaZ* product (A, B, C, D) have been distinguished by serotyping and differences in hydrolysis of selected β-lactam substrates. Types A, C and D are

usually located on plasmids, whereas type B typically resides in the chromosome (121). *blaZ* has also been identified as the cause of penicillin resistance among CoNS (122,123). Transfer of *blaZ* and several other resistance genes between CoNS and *S. aureus* has been reported indicating that CoNS may act as a resistance gene reservoir for *S. aureus* (120,124–127).

3.1.1. Methicillin-resistant *Staphylococcus aureus* (MRSA)

MRSA was first described in the 1960s in Europe, about two years after the methicillin began to be used to treat *S. aureus* infections. MRSA can be responsible for difficult-to-treat nosocomial infections in humans. MRSA are also called MDR-*S. aureus* or oxacillin-resistant *S. aureus* (ORSA) (128). MRSA are resistant to most of beta-lactams including oxacillin, penicillin, amoxicillin, cephalosporins and carbapenems (129). The widespread use of antibiotics has also accelerated the emergence of HA-MRSA strains by acquiring multiple resistance genes, to become resistant to aminoglycosides, macrolides, sulfonamides, tetracyclines and fluoroquinolones (130). MRSA were initially associated with healthcare settings, including hospitals and other healthcare environments. However, it has now also emerged as a major cause of community-associated infections and has created reservoirs in both settings. Hence, MRSA is no longer only a nosocomial pathogen. The frequency of CA-MRSA infections have increased since they were first described in the 1980s (8).

The resistance to methicillin is due to a PBP2a encoded by *mecA* or *mecC* gene found in SCC*mec* which is inserted inside chromosomal DNA of *S. aureus*. The rapid emergence of MRSA raised the hypothesis that *mecA* was already present in the staphylococcal gene pool prior to the introduction of methicillin. In fact, a ubiquitous homologue named *mecA1*, with 80% nucleotide identity to *mecA* has been identified in the primitive coagulase-negative *Staphylococcus sciuri* (131). Several lines of evidence suggest that *mecA1* is the precursor of *mecA*. While *mecA1* does not confer resistance to β-lactams in *S. sciuri*, there are reports of β-lactam-resistant strains that have alterations in the promoter region of this gene (132). When introduced experimentally into a *S. aureus* strain, *mecA1* was able to confer β-lactam resistance and produce a protein with properties similar to PBP2a (133,134). Additional *mecA* homologues have been identified in related species, including a *mecA* homologue (*mecA2*) with 90% nucleotide identity with *mecA* in *Staphylococcus vitulinus* (135). Furthermore, *mecA* along with its regulators, *mecI* and *mecRI*, has been identified in a small number of *Staphylococcus fleurettii* isolates (136). Despite the

importance of *mecA* in the epidemiology of antibiotic resistant staphylococci, the evolutionary history of this gene has remained unclear (137).

mecC was first identified in May 2007, in southwest England: an epidemiological survey of bovine mastitis led to the isolation of *S. aureus* LGA251 strain from a bulk milk sample in a farm tank (138). In this study, the authors found the isolate resistant to methicillin but *mecA*-negative. By sequencing its whole-genome, they found a homologue of *mecA* on SCC*mec* element of the isolate, which was called *mecC*. Further studies identified 65 isolates positive for *mecC* not only from dairy cattle but also from humans. This included an isolate obtained in 1975 from Danish human blood, suggesting that although *mecC* gene-has been recently identified, it has probably caused infections for over 40 years (138). A third group of *mec* gene homologues (*mecB* and *mecD*) has been reported to occur both chromosomally and on a plasmid of *Macrococcus caseolyticus*, a close genetic relative of *Staphylococcus* (8). In a recent study at the University Hospital of Münster, Germany, during routine MRSA screening, they recovered a *S. aureus* isolate UKM4229 from a combined nasal-throat swab of an inpatient to carry a *mecB* gene previously described for *Macrococcus caseolyticus* but not for staphylococcal species (139).

3.1.2. Staphylococcal Cassette Chromosome *mec*

Methicillin resistance is associated with the acquisition of particular resistance island called staphylococcal cassette chromosome *mec* (SCC*mec*), an exogenous piece of DNA, variable in size, that is absent from the methicillin-susceptible strains. It was discovered that the emergence of methicillin-resistant staphylococcal lineages was due to the acquisition and insertion of the SCC*mec* element into the chromosome of susceptible strains. This mobile 21- to 60 kb genetic element is the defining feature of MRSA strains and is responsible for conferring the broad-spectrum beta-lactam resistance (8). SCC*mec* may also contain other genetic structures, such as Tn554, *pT181*, and *pUB110*, which are responsible for conferring resistance to other non-beta-lactam drugs (117). The high diversity in the structural organization and the genetic content of these elements has resulted in their classification into types and subtypes. To summarize, there are three basic structural/genetic elements in SCC*mec*: the *mec* gene complex, containing the *mec* gene (*mecA, mecB, mecC*, and/or *mecD*) and its regulatory elements that control its expression (*mecR1*, encoding a signal transducer protein, and *mecI*, encoding a repressor protein); the *ccr* gene complex, encoding the site-specific recombinases, *i.e.,* cassette chromosome recombinase (*ccr*) genes (*ccrAB* and/or *ccrC*); and regions other than *mec* and *ccr* within the SCC*mec* element that

are designated as "joining regions" (J-regions) and are classified into three subgroups, J1-3 (Fig. 7). These cassette components are nonessential and may contain determinants for additional antimicrobial resistance (140). The J1 region is the region located between the right chromosomal junction and the *ccr* gene, upstream of the *ccr* gene. The J2 region is the region between the *ccr* gene complex and the *mec* gene complex. Similarly, the J3 region is located between the *mec* gene complex and the left chromosomal junction, *i.e.,* downstream of the *mec* gene complex.

Based on the location of the regulatory genes upstream or downstream of *mec* gene and/or disparities in the insertion sequences, the *mec* gene complex has been categorized into various classes. In addition, various combinations of *ccr* gene allotypes have given rise to different types of *ccr* gene complex. The chromosomal excision and integration of types I to IV SCC*mec* are catalyzed by the site-specific recombinases *ccrA* and *ccrB*, and *ccrC* for type V (141). It is the combination of these classes of the *mec* gene complex and the type of *ccr* gene complex that results in the classification of SCC*mec* elements into types. These elements are further divided into subtypes centered on the variations in the J regions within the same combination of *mec*-*ccr* complex. To date there are a total of 13 types of SCC*mec* identified in MRSA strains (SCC*mec* type I to SCC*mec* type XIII) (8).

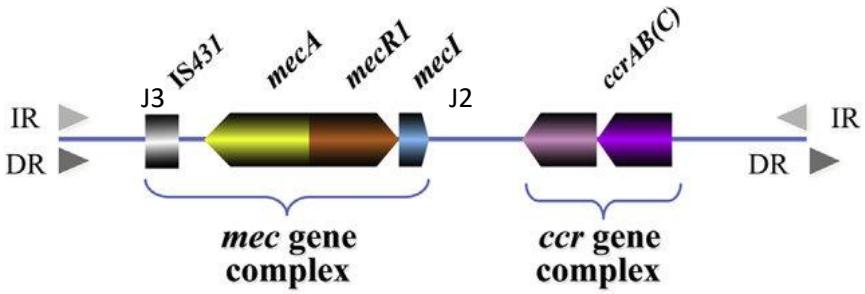

Fig 7. The structure of SCC*mec* (140). SCC*mec* is composed of two essential gene complexes. One is *mec*-gene complex, encoding methicillin resistance (*mecA* gene) and its regulators (*mecI* and *mecR1*), and the other is *ccr*-gene complex that encodes the movement (integration to and precise excision form the chromosome) of the entire SCC element. IR, inverted repeat; DR, direct repeat.

3.2. Resistance to macrolides, lincosamides & streptogramins (MLS)

Macrolide antibiotics such as erythromycin and azithromycin, lincosamides such as lincomycin and clindamycin, and streptogramins such as virginiamycin and pristinamycin, arrest protein synthesis by binding the 50S ribosomal subunit and causing dissociation of the peptidyl-tRNA during elongation (142). Three mechanisms are mainly responsible for acquiring resistance to MLS antibiotics in staphylococci: (1) target site modifications by methylation or mutation; (2) active efflux of antibiotics; or (3) inactivation of antibiotics. The first mechanism includes target site modifications by a methylase encoded by one or more of the *erm* genes (*ermA, ermB, ermC, ermT*), methylating 23S rRNA and thereby altering binding sites for MLS antibiotics (143). Phenotypically, this resistance appears either inducible (resistant to 14- and 15-membered macrolides and susceptible to 16-membered macrolides, lincosamides and streptogramin B) or constitutive (resistant to all forms of these antibiotics) (144). The second mechanism involves a macrolide efflux pump encoded by *msrA* and/or *msrB* genes. This pump protein belongs to the ABC transporter family and exports 14-membered macrolides and streptogramin B antibiotics from bacterial cells, while lincosamide and streptogramin A antibiotics remain unaffected (145). The third mechanism encompasses several enzymes. A lincosamide nucleotidyltransferase encoded by the *lnuA* gene confers resistance only to lincosamides. *vgaA/B* genes have been characterized as a determinant of streptogramin A resistance. Finally, the macrolide phosphotransferase C encoded by the *mphC* gene inactivates some macrolide antibiotics (146).

3.3. Resistance to aminoglycosides

Aminoglycosides inhibit protein synthesis by binding to the A site on the 16S ribosomal RNA of the 30S ribosomal subunit and promoting mistranslation by inducing codon misreading on delivery of aminoacyl transfer RNA. This results in error prone protein synthesis, allowing for incorrect amino acids to assemble into a polypeptide that is subsequently released to cause damage to the cell membrane and elsewhere (147). Mechanisms of bacterial resistance to aminoglycosides are diverse. The most common mechanism is inactivation of aminoglycosides, by a family of enzymes named aminoglycoside-modifying enzymes that are encoded by MGEs (148). In staphylococci resistance to gentamicin, kanamycin, and tobramycin is mediated by a bifunctional acetyltransferase-phosphotransferase gene (*aacA-aphD)* (149). This gene is carried by the IS256-

flanked composite transposon Tn4001, found on large staphylococcal multiresistance plasmids such as *pSK1* and *pSK41*, or on the chromosome (150). A number of plasmids have been reported that mediate resistance to neomycin/kanamycin, and tobramycin via adenyltransferase activity encoded by *aadD* specified by plasmid *pUB110*. Resistance to neomycin/kanamycin is mediated by aminoglycoside 3'-phosphotransferase activity encoded by *aphA3*, and resistance to streptomycin is mediated by aminoglycoside 6′ adenyltransferase activity encoded by *aadE*, carried by Tn5404.The plasmid is integrated within the SCC*mec*II cassette that occurs in some MRSA strains (151–153).

3.4. Resistance to tetracyclines

Tetracyclines inhibit protein synthesis by binding the 30S ribosomal subunit and preventing association of aminoacyl-tRNA with its acceptor site (154). Most tetracycline-resistant bacteria including *S. aureus* have acquired tetracycline resistance genes (*tet*). Two main mechanisms of resistance to tetracycline have been described in *S. aureus*: active efflux, resulting from the acquisition of the plasmid-located *tetK* and *tetL* genes and ribosomal protection by elongation factor-like proteins that are encoded by chromosomal or transposonal *tetM* or *tetO* determinants (155). Resistance to tetracycline can also be mediated by mutations that cause increased expression of various chromosomally encoded efflux pumps, such as Tet38 (156). Studies have shown that tetracycline-resistant MRSA isolates commonly contain either *tetM*, *tetK* or both determinants (155,157).

3.5. Resistance to phenicols

Phenicols, mainly chloramphenicol, cause a bacteriostatic effect by binding the 50S ribosomal subunit and inhibiting the transpeptidation step during protein synthesis (158). Resistance to chloramphenicol in *S. aureus* is most frequently due to the activity of an inducible detoxification enzyme, chloramphenicol acetyltransferase (CAT) (159). Genes for CAT in *S. aureus* are exclusively carried by plasmids within the size range of 2.9 to 5.1 kb. All *S. aureus* CAT enzymes are of the classical A type, with subtypes A-7, -8 and -9 carried on the plasmids *pC221*, *pC223* and *pC194*, respectively (160).The four characterized chloramphenicol resistance plasmids *pC221*, *pUB112*, *pC223* and *pC194* have distinct replication regions, suggesting diverse evolutionary

histories for each plasmid (161). The binding site for linezolid closely overlaps that of chloramphenicol and presumably compounds with related structures (162). Chloramphenicol is only used topically to treat conjunctivitis, but a fluorinated derivative of thiamphenicol called florfenicol is used in veterinary medicine (163). Presumably, florfenicol has the same mechanism of action as chloramphenicol by interfering with the aminoacyl end of aa-tRNA and inhibiting peptidyltransferase. Another florfenicol-chloramphenicol resistance gene, designated *fexA*, encoding a transmembrane efflux protein in staphylococci (164). The *fexA* protein differs from all previously known proteins involved in the efflux of chloramphenicol and florfenicol.

3.6. Resistance to glycopeptides

The glycopeptides are a group antimicrobial agents that show antibacterial activity against gram-positive organisms through inhibition of cell-wall synthesis, through binding to the d-alanyl-d-alanine terminus of the lipid II bacterial cell-wall precursor, preventing cross-linking of the peptidoglycan layer (165).

Vancomycin is a glycopeptide antibiotic that is widely used to treat serious infections caused by MRSA strains. It binds to the dipeptide D-Ala4-D-Ala5 of lipid II and prevents transglycosylation and transpeptidation catalysed by PBP2 and PBP2a, and antagonises peptidoglycan remodeling (165). The main mechanism of resistance to vancomycin is owing to the presence of enzymes that produce lower-affinity binding precursors in which the carboxy-terminal d-alanine residue is replaced by either d-lactate or d-serin (165). Following the emergence of vancomycin resistance in *Enterococcus*, rapid resistance developed in *S. aureus* (VRSA) as a result of HGT of *vanA* operon from resistant enterococci (166,167).The resistance mediated by *vanA* gene located on MGEs, is the most common mechanism of resistance in enterococci and is the only one detected in VRSA (MIC $\geq$ *16* µg/mL) to date (168,169). VISA strains (MIC= 2-16 µg/mL) do not harbor foreign MGEs; rather, the reistance happens due to mutations during treatment of the invading pathogen (165).

3.7. Resistance to antibiotics by miscellaneous genes

The trimethoprim resistance gene from plasmids *pSK 1*, designated *dfrA*; and plasmid *pKKS2187* designated *dfrk*, encodes the production of a trimethoprim-resistant dihydrofolate reductase (170,171).

Resistance to fusidic acid (a bacteriostatic antibiotic that blocks bacterial protein synthesis by locking elongation factor G (EF-G) to the ribosome) is encoded by *fusB* and *fusC* genes which could be carried by plasmids such as by the 21 kb plasmid *pUB101*; however, it can also be chromosomal. The *fusB* protein has been shown to bind EF-G and protect the staphylococcal translation apparatus from the inhibitory effects of fusidic acid (172).

Mupirocin, a protein synthesis inhibitor, is widely used as a topical agent to reduce nasal carriage of MRSA in hospital patients and staff and also indicated as a topical agent to treat skin infections. Resistance to mupirocin exists in two forms: low-level resistance (MIC 8–256 mg/mL) due to mutations in the native isoleucyl-tRNA synthetase gene and high-level resistance (MIC 256 mg/mL) mediated by the plasmid-associated *mupR* gene which encodes an extra isoleucyl-tRNA synthetase (173).

The *cfr* gene act for combined resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin (174), which is discussed in detail in the next chapter.

Fosfomycin is a broad-spectrum bactericidal antibiotic that interferes with cell wall synthesis in both gram-positive and gram-negative bacteria by inhibiting the synthesis of peptidoglycan by blocking the formation of N-acetylmuramic acid. *fosB*, a Mn^{2+}-dependent fosfomycin-inactivating enzyme found in *S. aureus*, is one of three related enzymes (encoded by *fosA, fosB*, and *fosX*) that confer resistance to fosfomycin resulting in a modified compound with no bactericidal properties (175).

A specific focus is made on linezolid in the next chapter of this literature review, since resistance to linezolid in staphylococci is one of the two main objectives of this work.

4. Linezolid and mechanisms of resistance of staphylococci

Linezolid is an oxazolidinone drug that was approved in 2000 for difficult-to-treat nosocomial infections caused by MRSA (176). The chemical structure of linezolid is shown in Fig. 8. The activity of the compound is increased by the morpholino group in the first ring (from the left) and the fluoride atom in the second ring (177). Linezolid is available in intravenous and oral formulations, which has provided this agent as an attractive alternative for treating numerous infection types, including hospital-acquired pneumonia caused by *S. aureus,* infections caused by vancomycin-resistant *Enterococcus faecium*, complicated skin and skin structure infections (SSSIs), uncomplicated SSSIs caused by MSSA or *Streptococcus pyogenes*, and community-acquired pneumonia caused by *Streptococcus pneumoniae* and infected intensive care unit (ICU) patients (18,178).

The compound is a synthetic antibiotic that acts as a protein synthesis inhibitor by binding to the ribosomal peptidyl transferase center (PTC) on the bacterial 23S ribosomal RNA (Fig. 9A), thereby inhibiting transition of the aminoacyl-tRNA to the A site and stopping the growth of bacteria by preventing formation of the fMet-tRNA-30S ribosome-mRNA initiation complex (179).

Unfortunately linezolid does not escape the rule of bacterial resistance and its increasing use has been accompanied by the emergence of resistance in human, the first linezolid-resistant *Staphylococcus* (LRS) was reported in a patient with peritonitis undergoing oral linezolid treatment during peritoneal dialysis in 2001 (180). Since then, the occurrence of linezolid-resistant strains has been reported worldwide (181,182). Resistance to linezolid in clinical isolates is still rare (178), and this antibiotic remains active against >98% of staphylococci, with resistance identified in 0.05% of *S. aureus* and 1.4% of CoNS (183). Among CoNS, *S. epidermidis* have been largely considered as a genetic reservoir of linezolid resistance genes (especially *cfr*) for other pathogenic bacteria including *S. aureus* (184).

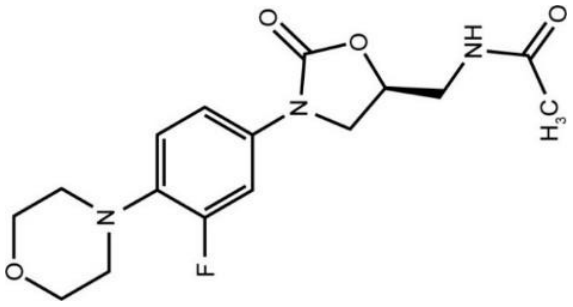

Fig 8. Chemical structure of linezolid. The empirical formula of the compound is C16H20FN3O4 (molecular weight: 337.35 g/mol) (18).

4.1. Chromosomal resistance to linezolid

Analysis of high-resolution structures of linezolid showed that it binds to a deep cleft of 50S ribosomal subunit that is surrounded by 23S rRNA nucleotides (185). Linezolid-resistant CoNS and *S. aureus* most commonly have chromosomal mutations in 23S rRNA binding site and / or mutations in genes encoding the 50S ribosomal proteins L3, L4 and L22 of the peptide translocation centre of the ribosome (186,187). Mutations in the *rrl* gene encoding 23S rRNA result in a ribosome conformational change and a loss of affinity for linezolid for its target (179) (Fig. 9A). The most common mutation in clinical isolates is the G2576T mutation (the substitution of thymine for guanine at position 2576) (188). The accumulation of this mutation in the different copies of the *rrl* gene that staphylococci possess (5 to 6 copies) leads to an increase of linezolid MIC but also confers cross resistance to lincomycin (189). The mutations of the genes encoding the L3, L4, and L22 ribosomal proteins (*rplC*, *rplD,* and *rplV* genes) modify the accessibility of the binding site of linezolid on 23S rRNA (Fig. 9B). These two proteins interact closely with the PTC site of the 23S rRNA (190). These types of resistances are chromosomal, and are not horizontally transferable.

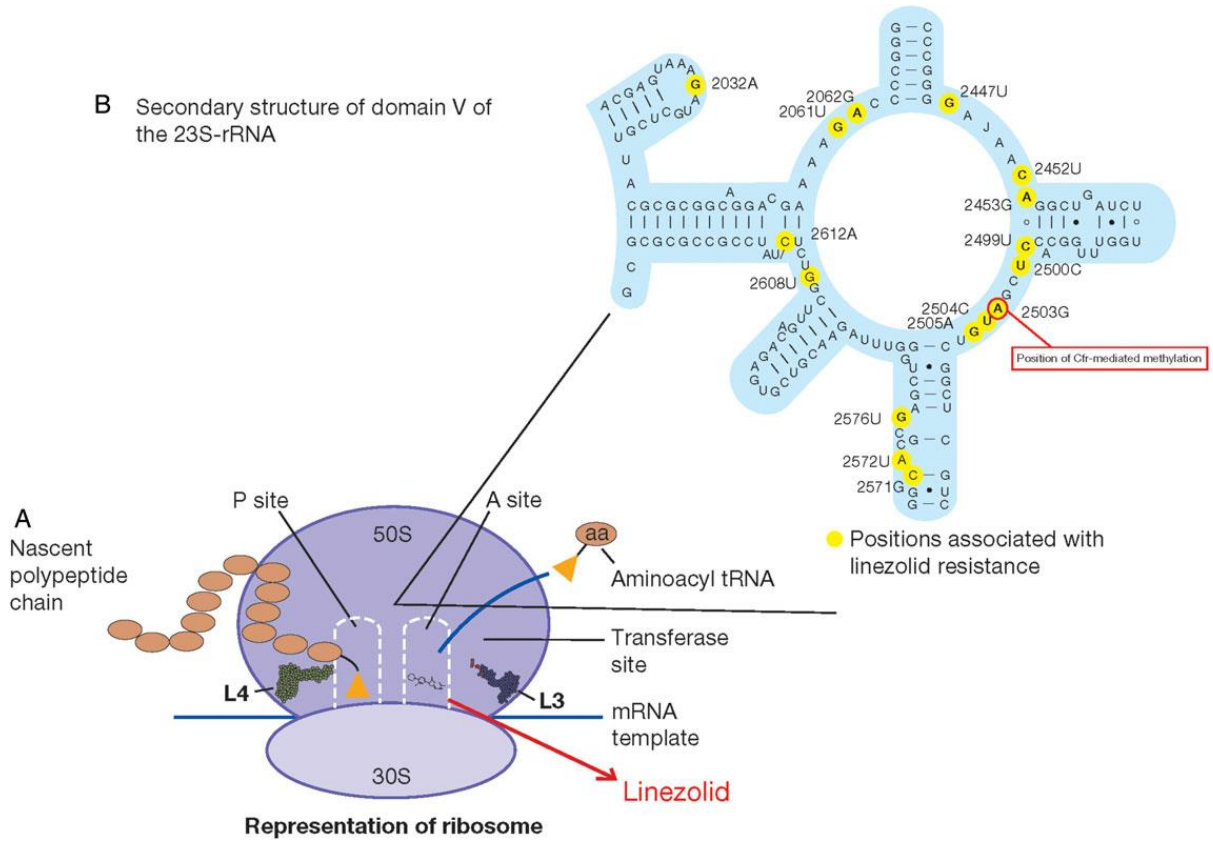

Fig 9. Schematic representation of the mechanism of action of linezolid, and linezolid-resistance mechanisms. **A**, Linezolid interferes with the positioning of aminoacyl transfer RNA (tRNA) by interactions with the peptidyl transferase center. Ribosomal proteins L3 and L4, associated with resistance, are shown. **B,** Representation of domain V of 23S ribosomal RNA (rRNA) showing mutations associated with linezolid resistance. Position A2503, the target of *cfr* (chloramphenicol-florfenicol resistance) methylation, is highlighted. mRNA, messenger RNA (187).

4.2. Plasmid-encoded resistance to linezolid

Plasmids conferring multi-drug resistant are usually conjugative, capable of initiating not only their own transfer but also that of other plasmids, and possess mechanisms to control their copy-number in the cell and/or replication ability (191). Unfortunately, the transferable modification of 23S rRNA can cause resistance to linezolid. Until now, three transferable resistance genes to linizeolid have been identified. These are the *cfr* (chloramphenicol-florfenicol resistance), *optrA* (oxazolidinone-phenicol transferable resistance), and *poxtA* (phenicol-oxazolidinone-tetracycline resistance) genes (192).

The *cfr* gene was identified in 2000 in a strain of *S. sciuri* obtained from nasal swab of a calf suffering from a respiratory tract infection in Germany (193). Originally, *cfr* was restricted to

staphylococci of animal origin where it was probably selected for, by usage of florfenicol in veterinary medicine (152). The *cfr* gene encodes a methyltransferase (*cfr* protein) that methylates adenine nucleotide of the 23S rRNA at position 2503 (A2503) (Fig. 9B). This base is in close proximity to the overlapping binding sites causing reduced or abolished binding of many antibiotics that bind to the PTC of the bacterial ribosome (194). Methylation of A2503 results in a phenotype commonly referred to as PhLOPSA, and confers resistance to at least five antimicrobial classes (phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics) (195). The *cfr* gene doesn't confer cross-resistance to tedizolid, which differs from other oxazolidinones by possessing a modified side chain at the C-5 position of the oxazolidinone nucleus and has an optimized C- and D-ring system that improves potency through additional binding site interactions (196). Importantly, tedizolid can be used against certain linezolid-resistant bacterial strains carrying the *cfr* gene, in the absence of certain ribosomal mutations conferring reduced oxazolidinone susceptibility (196).

Dissemination of the *cfr* gene among staphylococci is of great concern, since evidence of transferability of the gene has been provided both *in vitro* and *in vivo* (197,198). The *cfr*-positive CoNS are increasingly isolated in hospitals probably due to high consumption of linezolid. This emerging resistance to linezolid in CoNS is of concern because their ubiquity in commensal mucocutaneous flora makes them potential reservoirs of resistance, some of which are transferable to the more virulent *S. aureus* strains and *Enterococcus spp* (184). Until now, the *cfr* gene has mainly been identified in strains belonging to *Staphylococcus* but it has also been found in *Enterococcus*, *Bacillus*, *Proteus vulgaris*, *Escherichia coli*, *Macrococcus caseolyticus*, *Jeotgalicoccus pinnipedialis*, and *Streptococcus suis* (194). The *cfr* gene has been associated with different plasmid vehicles detected in different countries, and the ability of *cfr*-carrying plasmids to be transmitted between strains and species is of global concern.

Resistance to linezolid is also mediated by the *optrA* determinant. This is widely encountered in enterococci from human and animal sources in China (199). It is also found in CoNS (*S. sciuri*) (200), but has not yet been reported in *S. aureus;* although this is likely to only be a matter of time. It codes for a protein belonging to the ABC-F subfamily of ATP-binding cassette proteins Vga, Lsa, and Mcr (ABC transporters). *optrA* confers resistance to phenicols, linezolid but also to tedizolid, unlike the *cfr* gene which does not cause cross-resistance to tedizolid. The resistance mechanism of *optrA* relies almost certainly on ribosomal protection (201). Like *cfr*, *optrA* was likely selected due to extensive usage of florfenicol in intensive animal farming in China.

Recently a new linezolid resistance gene has been described in a clinical MRSA strain in Italy (202). This is the *poxtA* gene which encodes a protein that is 32% identical to *optrA* and exhibits structural features typical of the F lineage of the ATP-binding cassette (ABC transporters) protein superfamily that cause antibiotic resistance by ribosomal protection. It confers resistance to phenicols, all oxazolidinones and tetracycline.

As one of the axis of our work focus on conjugative transfer of *cfr*-carrying plasmids from *S. epidermidis* strains to *S. aureus* major clones, we described below the characteristics of *cfr*-carrying plasmids known until now.

4.3. *cfr*-carrying plasmids

The *cfr* gene is often found on plasmids, and if chromosomal it seems always associated with insertion elements. By conducting a literature review to date, more than 40 different *cfr*-carrying plasmids have been reported, differing in size ranging from 7.1 to 97 kb. Three plasmids also harbor another resistance gene to oxazolidinones (*optrA*). Bases on sequence analysis, a number of these *cfr*-harbouring plasmids show similarity or identity in size and/or in immediate *cfr* downstream and uppstream regions (Table 2). In addition, most of studied plasmids also carryied other antibiotic resistance genes in *cfr* upstream or downstream region such as: florfenicol-chloramphenicol exporter gene (*fexA*), resistance to aminoglycosides (*aacA-aphD, aadD, aadE, aphA3*), resistance to tetracylines (*tetK, tetL*), resistance to MLS antibiotics (*ermA, ermB, ermC*, *ermT, msrA*, *lnuA, IsaB*), resistance to trimethoprim (*dfrk*), and resistance to penicillin (*blaZ*) (Table 2). Moreover, *cfr*-carrying plasmids are not limited to staphylococci, but are rather distributed across staphylococci and enterococci. Additionaly, plasmids of the same subtypes, *e.g., pSCFS3* and derivatives, have been isolated from different animal hosts and staphylococcal species (Table 2). Some of the plasmids have been fully or partially sequenced, and in most of them, the immediate genetic environment of *cfr* has been analysed and shown to encode transposases and mobilization proteins. Only a small number of these plasmids have been characterized as conjugative plasmids (127,203,204), however co-mobilization of certain plasmids facilitated by helper plasmids cannot be excluded (186). The identification of vectors such as *pSCFS3*-like plasmids in the United States (205), the *pSP01*, and *pSCFS7*-like plasmids in European countries (127,206–208), or *pLRSA417* and *pSS-01*-like plasmids in China (209,210) may indicate a geographically distribution of the *cfr*-vectors. The *cfr* gene has been identified in close proximity to different insertion sequences, which most probably also play an important role in its dissemination (209).

Table 2. Summary of the characteristics of the various *cfr*-carrying plasmids described in literature.

#	Name of *cfr*-carrying plasmids	Plasmid size	LRG	Coexistence of other resistance genes	Origin of plasmids	Authors
1	*pLRSA417*	39.5 kb	*cfr*	*aacA-aphD*	MRSA and CoNS	Jia Chang Cai *et al*. 2015
2	*pERGB*	50 kb	*cfr*	*tetL*, *ant(4')Ia, dfrk*	MRSA	Enrique Ruiz de Gopegui *et al*. 2012
3	*pSCFS6*	43 kb	*cfr*	*fexA, IsaB*	*S. warneri* and *S. simulans*	Corinna Kehrenberg *et al.* 2006
4	*pSCFS7*	45 kb	*cfr*	*fexA*	*S. aureus,* MRSA-USA300	Anna C Shore *et al*. 2010
5	*pSCSF1*	16.5 kb	*cfr*	*ermC*	*S. sciuri*	Stefan Schwarz *et al.* 2000
6	*pSCFS3; pSCFS4; pSCFS5; pSCFS2*	41 kb	*cfr*	*fexA*	Staphylococci of animal and human origin	Corinna Kehrenberg, Stefan Schwarz. 2006
7	*p426-3147L*	75 kb	*cfr*	-	*S. epidermidis*	Rodrigo E Mendes *et al.* 2008
8	*p004-737X*	55 kb	*cfr*	-	*S. aureus*	
9	*pSE1243*	39.3 kb	*cfr*	*fexA*	*S. epidermidis*	Rodrigo E Mendes. 2013
10	*pSA737*	39.3 kb	*cfr*	*fexA*	*S. aureus*	
11	*pSA1900*	7.9 kb	*cfr*		*S. aureus*	
12	*p12-00322, p12-02178, p12-02179*	36.7 kb	*cfr*	*IsaB*	*S. epidermidis*	Jennifer Bender *et al.* 2015
13	*p12-02300, p12-01787, p12-01631*	38.9 kb	*cfr*	*fexA*	*S. epidermidis*	
14	*pSR01*	39.5 kb	*cfr*	*aacA-aphD*	MRSA	Dandan Wu *et al*. 2020
15	*pSR02*	28 kb	*cfr*	*aacA-aphD, aadD, tetK*	MRSA	
16	*pSR03*	24.6 kb	*cfr*	*blaZ*	MRSA	
17	*p603-50427X*	97 kb	*cfr*	-	*Enterococcus faecalis*	Lorena Diaz *et al*. 2012
18	*pFSIS1608820*	28 kb	*optrA, cfr*	*fexA, ermA, ermB, aphA3,*	*Enterococcus faecium*	Gregory H Tyson *et al.* 2018
19	*pWo28-3*	60.5 kb	*optrA, cfr*	*fexA, aadD, aacA-aphD, ble*	*S. sciuri*	Dexi LI *et al*. 2016
20	*p-cfr-PBR-A*	38.7 kb	*cfr*	*fexA*	CoNS	Laurent Dortet *et al*. 2018

21	*p-cfr-PBR-B*	40.2 kb	*cfr*	*blaZ, msrA, aadD*	*S. epidermidis*	
22	*pSP01*	76.9 kb	*cfr*	*blaZ, msrA, aadD*	*S. epidermidis*	Andrea Brenciani *et al*. 2015
23	*pSP01.1*	49 kb	*cfr*	*blaZ, msrA, aadD*	*S. epidermidis*	
24	*pLRSA417 like vector*	49 kb	*cfr*	*aacA-aphD*	CoNS	Le Thuy Thi Nguyena *et al.* 2020
25	*pSEM13-0451*	85.6 kb	*cfr*	*IsaB, ermT*	MRSE	Alexandros Lazaris *et al.* 2017
26	*pEF12-0805*	72.9 kb	*cfr, optrA*	*lnuB, lnuE, ermA, ermB, aphA3, aadE*	VRE	
27	*pSAM12-0145*	41.6 kb	*cfr*	*fexA*	MRSA	Anna C. Shore *et al*. 2016
28	*pSAM13-0401*	27.5 kb	*cfr*	*IsaB*	MRSA	
29	*pSS-01*	40 kb	*cfr*	*accA-aphD, fexA*	*S. cohnii* and *S. saprophyticus*	Yang Wang *et al*. 2017
30	*pSS-02*	35.4 kb	*cfr*	-	*S. saprophyticus* and *S. sciuri*	
31	*pSS-03*	7.1 kb	*cfr*	*ermC*	*S. arlettae, S. cohnii, S. saprophyticus,* and *S. sciuri*	
32	*pBS-01*	16.4 kb	*cfr*	*ermB*	*S. cohnii, S. saprophyticus,* and *S. sciuri*	
33	*pSS-02 like plasmid*	35.4 kb	*cfr*	*fexA*	*S. haemolyticus* and *S. cohnii*	Lanqing Cui *et al.* 2013
34	*p2823634, p2823586, and p2823605*	*pSA737* like plasmids	*cfr*	-	MRSA	Jeffrey B. Locke *et al.* 2014
35	*pMSA16*	7.5 kb	*cfr*	*fexA, ermA*	bovine MRSA	Xiu-Mei Wang *et al*. 2012
36	*pHK01, pRM01, pRA01*	*pSCFS3* and *pSS-01* like plasmids	*cfr*	*fexA*	*S. cohnii*	Hongbin Chen *et al*. 2013

LRG: linezolid resistance gene

Following is a schematic representative of a *cfr*-carrying, conjugative multi-resistance plasmid (*pSP01*, 76.9 kb) (Fig. 10), isolated from a clinical isolate of *S. epidermidis* in Italy (211).

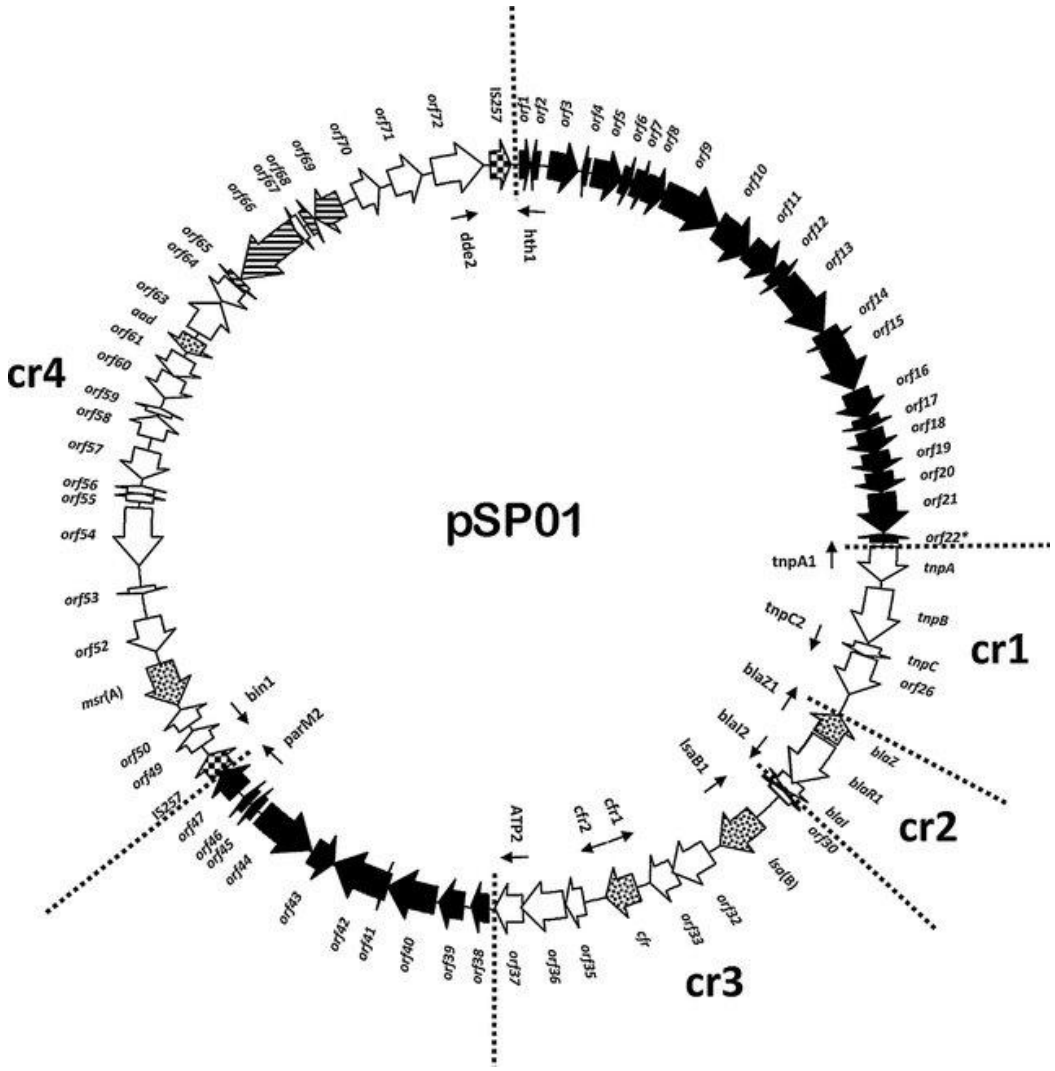

Fig 10. Schematic representation of the *cfr*-carrying plasmid *pSP01* from *S. epidermidis* ST23. The 73 ORFs of the plasmid, represented as arrows pointing in the direction of transcription, are indicated with their number (*orf1* to *orf73*) or with a more explanatory designation where appropriate. The ORFs of the two backbone regions are represented as black arrows. Those of the four cargo regions (*cr1* to *cr4*) are represented as white arrows, spotted in case of antibiotic resistance genes, striped in case of genes involved in heavy metal resistance, and chequered in case of ISs. Thin arrows inside the circular ORF map indicate the primer pairs used for the stability tests of each cargo region (211).

The above-mentioned plasmid is transferable to *S. aureus* by conjugation and transformation, and displays a distinctive mosaic structure, with four cargo regions (here designated *cr1* to *cr4*) interpolated into a backbone which shows high-level identity (95%) and complete synteny to that of *S. aureus* plasmid *pPR9* (Pérez-Roth *et al*. 2010). Whereas *cr1* lacks drug resistance genes, *cr2*, *cr3*, and *cr4* carry a variety of such genes; *cr2* is formed by the *bla* complex genes *blaZ*, *blaR1*, and *blaI*; *cr3* is the genetic context of the *cfr* gene and also contains the clindamycin exporter gene *IsaB*; and *cr4* contains *msrA* (macrolide efflux), *aadD* (aminoglycoside N6′-acetyltransferase), and a gene cluster for heavy metal resistance. The number and importance of these additional resistance genes make *pSP01* unique among the *cfr* plasmids reported to date. Remarkably, of the four cargo regions of *pSP01*, two contain insertion sequences (ISs): *cr3* shows a single IS element (*IS21-558*) formed by two partially overlapping ORFs, *istAS* and *istBS* (Kehrenberg *et al.* 2007) (*orf32* and *orf33* in *pSP01*); and *cr4* is bracketed between two identical IS257 elements. Transpositionally active forms of the IS21-558 element are involved in the mobility of the multiresistance gene *cfr* (174).

5. Horizontal gene transfer mechanisms in Staphylococci

5.1. Conjugation and mobilization

Conjugation is a highly evolved and efficient mechanism facilitating DNA transfer in bacteria. A bacterial cell that has received genetic material (such as plasmid) from another bacterium by conjugation is called transconjugant. A distinction is made between conjugative plasmids (which have the genes allowing their transfer from one bacterium to another by conjugation), mobilizable plasmids (which require the presence of a conjugative plasmid in order to be able to be transferred), and non-mobilizable plasmids (which can only be transferred by transformation).

In staphylococci, it is estimated that a small number of plasmids carry conjugation-gene clusters required for autonomous conjugative transfer, however new mechanisms of conjugative mobilization have recently been described, which may resolve the paradoxical underestimation of conjugative plasmid transfer in staphylococci (212). Plasmids code for genes involved in many aspects of microbial biology, including detoxication, virulence, ecological interactions, and antibiotic resistance. Autonomously-transferring conjugative plasmids carry both mating-pore genes and genes for DNA processing, single-stranded DNA (ssDNA) replication and recruitment of ssDNA to the mating pore. DNA is recruited to the mating pore by the relaxase protein, which binds, cleaves and covalently attaches to a recognition sequence called the origin-of-transfer (oriT), forming (often with accessory proteins) a nucleoprotein complex referred to as the relaxasome (212). The relaxasome is recruited to the mating-pore through interactions with a mating-pore component called the VirD4 coupling protein, after which it is transferred to recipient cells through a type-IV secretion system (Fig.11). Relaxases can additionally be involved in rolling-circle-like plasmid replication in the donor bacterium and recircularization and replication of plasmid DNA in the recipient. Plasmid conjugation systems can therefore be considered an evolutionary amalgam of type IV protein secretion systems that have evolved to transfer protein-tied DNA, with rolling circle replicases (or recombinases) that have evolved an association with the type IV secretion system through relaxase-coupling-protein interactions (212).

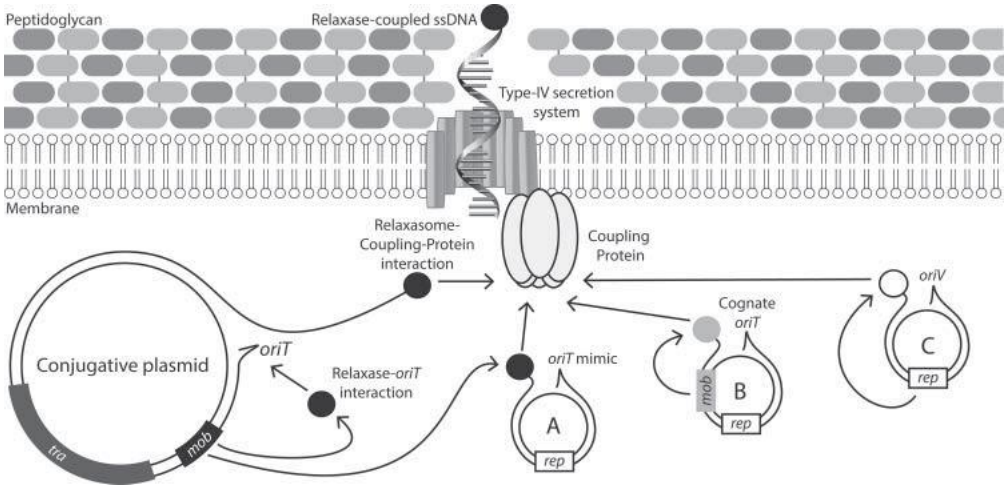

Fig 11. Mechanisms of conjugative mobilization in staphylococci. The conjugative plasmid encodes all genes required for formation of the mating pore, as well as the coupling protein, DNA relaxase and an *oriT*. Mobilizable plasmids can exploit the conjugative-plasmid mating pore by either: (A) encoding a mimic sequence of the conjugative-plasmid *oriT*, (B) encoding a distinct relaxase (Mob) compatible with the conjugative plasmids coupling protein and its own *ori*T, or (C) carrying a replicative relaxase (Rep) compatible with the conjugative-plasmid coupling protein (212).

Staphylococcal isolates are frequent hosts for diverse antimicrobial-resistance plasmids. Closely related members of the *pSK41/pGO1* plasmid family were first documented by several groups in the early 1980s as the basis of emergent gentamicin resistance (213–216). In addition to aminoglycoside resistance, these plasmids have been found to variously confer resistance to penicillins, trimethoprim, bleomycin, tetracycline, antiseptics and disinfectants, mupirocin, and macrolides, lincosamides and streptogramin B (212). The resistance genes responsible for these phenotypes are usually encoded by small plasmids co-integrated between copies of IS257/IS431 within the *pSK41/pGO1* plasmid. Notably, plasmids of this type have subsequently been associated with linezolid and high-level vancomycin resistance (186,217).

More recently a second distinct family of staphylococcal conjugative plasmids was characterized. *pWBG749* was found in a strain from a remote indigenous Australian community in 1995 (218,219). *pWBG749*-family conjugative plasmids carrying penicillin, aminoglycoside and vancomycin-resistance genes have since been identified (220,221).

A third distinct family of conjugative plasmids, which was designated the *pWBG4* family, were first identified as a third unique type of conjugative plasmid in 1985 by Townsend *et al.* (222). The first of these, *pWBG14*, confers aminoglycoside, macrolide, lincosamide, and spectinomycin resistance and was identified in a strain originally isolated from Royal Perth Hospital in 1968.

Conjugative transfer of related plasmids *pWBG4* and *pWBG25* was subsequently demonstrated (223,224). A *pWBG4*-family plasmid conferring trimethoprim resistance, *pWBG707*, was identified in a Malaysian isolate in 1992 (225). The putative *pWBG4* conjugation gene cluster (*detA-detV*) is clearly distinct from that of *pSK41* and *pWBG749*. The *detA-detV* region was additionally identified on several contemporary staphylococcal plasmids associated with the dissemination of *cfr*-gene-encoded linezolid resistance in both human and animal-isolated staphylococci (212). The *pWBG4*-family plasmid *pSA737* was found in the first American example of *cfr*-mediated linezolid resistance in a human *S. aureus* isolate (205), and was subsequently identified in 19 isolates from 2 Ohio hospitals (226). The *cfr*-carrying plasmid *pSA737* was isolated in France from patients in the Nantes University Hospital between 2015 and 2017 from 13 MRSE and 3 *S. aureus* strains (data not published). *pWBG4*-family *cfr*-carrying plasmids have now been identified in China (*pHK01*) (227), Germany (*p12- 02300*) (186), and Ireland (*pSAM12-0145*) (205). Despite the wide distribution of *pWBG4*-like plasmids, the clinical importance of linezolid and the prominence of the *detA-detV* conjugation gene cluster, only one of these studies reported laboratory conjugation experiments confirming that the *cfr*-carrying *pWBG4*-like plasmid was conjugative (205).

Mobilizable plasmids are those which carry DNA-transfer genes required for formation of all or part of the relaxasome, but lack genes required for mating pore formation. Mobilizable plasmids have an ability to exploit conjugative plasmids for horizontal dissemination, but are non-mobile in cells that lack mobile elements carrying compatible mating-pore genes. The vast majority of documented mobilizable plasmids exploit conjugative element mating-pores by encoding their own relaxase (Mob) that acts on the plasmid's cognate oriT (228).

In summary, to date no accurate data exist to estimate the proportion of conjugative, mobilizable, or nontransmissible plasmids in staphylococci (228). On the other hand, it is clear that there are at least 3 distinct families of conjugative plasmids (based on their distinct conjugation-gene clusters of relaxase and *OriT* sequences) in staphylococci namely: *pSK41*, *pWBG749* and *pWBG4.* Data suggest that these 3 families of conjugative plasmids, alongside the mobilizable plasmids, currently contributing to the horizontal spread of resistance mechanisms against last-resort antimicrobials such as vancomycin and linezolid (212).

5.2. Transduction

Transduction is a process by which DNA is transferred from one bacterium to another via bacterial viruses known as bacteriophages (Fig. 12). The life cycle of bacteriophage uses the bacterial cell replicational, transcriptional, and translation machinery to make new virions. This process is a relatively low fidelity event during which pieces of bacterial chromosome (or a plasmid) can accidentally be packaged into the bacteriophage capsid. The bacteriophage then undergoes a lytic cycle, which creates a bloom of new particles upon lysis of the host cell (229). Following a second round of lytic infection, the bacteriophages infect recipient bacteria and inject the foreign DNA (viral and bacterial) into the cells. In this transduction event, the transferred bacterial DNA can integrate into the recipient bacterium's genome through homologous recombination or recircularize into a replicating plasmid (229). The final result is the movement of bacterial genetic information from one strain to another. In nature, transduction is a part of HGT mechanis through which antibiotic-resistance genes are exchanged between bacteria (230). In addition, bacteriophages are also responsible for the mobilization of *S. aureus* pathogenicity islands (SaPIs), which encode major toxin genes, such as those of TSST-1 and other superantigens (230). The vast majority of *S. aureus* phages known so far are double-stranded DNA phages belonging to the *Siphoviridae* family of the Caudovirales order (231).

In *S. aureus*, phage transduction is thought to play a major role in HGT, since most of the *S. aureus* isolates are lysogenized. The DNA size that can be packed in this transducing phage (siphoviruses) is up to 39–43 kb (232). Horizontal gene transfer between *S. aureus* is due to generalized transduction via bacteriophage, and all clinical isolates have prophages in their genomes (233). Generalized transduction occurs when induced phage particles package host chromosomal or plasmid DNA instead of replicating phage DNA, and on cell lysis these particles deliver host DNA to new recipient *S. aureus* (234). Transfer of phage DNA (transduction of phage followed by lysogeny) between colonizing *S. aureus* populations has been reported in patients with cystic fibrosis (235).

In a recent study, the potential spreading of the *cfr* gene by transduction was tested by using staphylococcal transducing phage MR83a (184). Phage was amplified by infecting the strain N315-45 (containing pSCFS7-like plasmids), and its ability to transduce the *cfr* gene was tested in the recipient strains N315, COL and Mu50 (both strains were able of acquiring *cfr* by filter-mating methodology from N315-45). All recipient strains were able to obtain *cfr* by transduction, generating the *cfr*-positive T-N315-45, T-COL-45 and T-Mu50-45 strains. These results showed

that bacteriophage-mediated transmission of the *cfr* gene could occur between MRSA strains. In the case of *Enterococcus spp*., no *cfr in vitro* transmission was observed by transduction for any strain tested (184).

Recently, phage-mediated HGT of SaPIs between major staphylococci pathogens has been reported (236). Surprisingly, the *S. aureus* ST395 lineage-specific phage Φ187 was capable of transferring SaPIs between ST395 isolates and many CoNS because of shared properties in the cell wall teichoic acid (WTA) as surface receptors for the above phage (236). Genomic and biochemical analyses of the ST395 WTA biosynthesis pathway have further suggested the occurrence of previous HGT events between ST395 isolates and CoNS, most likely via Φ187-related phages (237). Because most pathogenic CoNS, such as *S. epidermidis*, have a glycerol-phosphate WTA backbone resembling that of ST395 isolates, Φ187 might be a suitable tool to transfer plasmid DNA to CoNS strains that are otherwise difficult to transform.

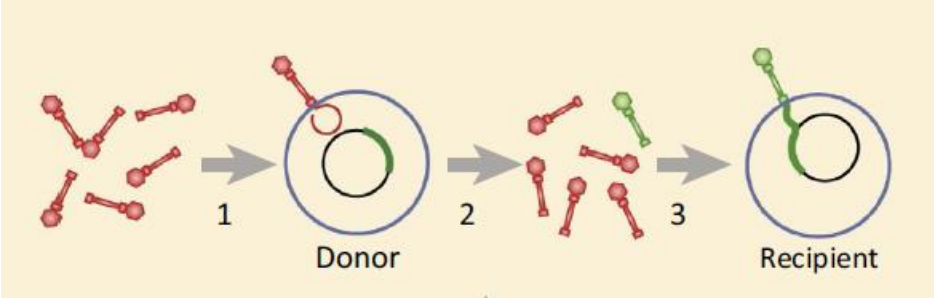

Fig 12. A schematic representation of phage-mediated DNA transfer in *S. aureus* by generalized transduction. (1) transducing phages (red) infect susceptible donor bacteria, (2) and upon lysis new phages are produced as well as rare transducing particles (green) containing bacterial DNA, (3) upon a new round of infection, the DNA of the transducing particle is delivered and established in a recipient bacterium (238).

5.3. Transformation

Transformation allows naturally competent bacteria to take up DNA from the environment and integrate the DNA into the chromosome by recombination. In *S. aureus,* expression of competence gene is regulated by an alternative sigma factor, SigH located in its genome; however despite these discoveries there is still very few evidence that transformation is a commonly occurring event in staphylococci (238).

For bacterial species that are not known to be naturally competent, such as *S. aureus* and *S. epidermidis*, electroporation is an efficient method in the laboratory for introducing genetic material into the cell. The technique uses electrical pulses to transiently permeabilize bacterial cell membranes, which allows the passage of plasmid DNA across the membranes (239). In this technique, an applied electric pulse produces an electric field across the cell membrane that alters the transmembrane potential of the cell in a side-specific manner. Due to the directional flow of current, the membrane becomes hyperpolarized on the side facing the anode of the electroporator, and depolarized on the side facing the cathode (Fig. 13). When the field strength is high enough, the areas of the membrane directly facing the electrodes become electropermeabilized due to the transmembrane potential difference reaching a critical value (240). Although many models attribute electropermeabilization to pore formation, the alternative theory is that the membrane simply becomes destabilized by the increase in potential difference, leading to formation of transient permeable domains but not necessarily defined pores (240). Either way, it is clear that the electric field increases membrane permeability in localized areas to allow for the transport of small molecules across the membrane. Negatively charged DNA is directed to the part of the cell membrane facing the cathode by electrophoresis from the applied electric field (241). There, the DNA interacts with the electropermeabilized membrane and forms aggregates (242). The subsequent steps, which include membrane recovery and DNA migration into the cytoplasm, are still not well understood and require further characterization (240). Most available techniques used for genetic manipulation (*e.g*., electroporation of shuttle plasmids) often fail, most likely because of strong genetic barrier mechanisms, such as restriction-modification (R-M) systems or clustered regularly interspaced short palindromic repeats (CRISPR), previously shown to impede HGT events between bacteria (243,244).

Until now, there are very few studies about the transfer of *cfr* gene by electroporation. In a study in China, two different *cfr*-carrying plasmids (50 kb and 7.1 kb) from MRSA isolates were successfully transferred to a recipient strain (*S. aureus* RN4220) by electroporation (245).

In another study by Dexi Li *et al.*, electrotransformation of the multiresistant plasmid *pWo28-3* (60.5 kb), carrying *cfr* and *optrA* genes isolated from *S. sciuri*, was successful from *S. sciuri* into *S. aureus* RN4220 (200).

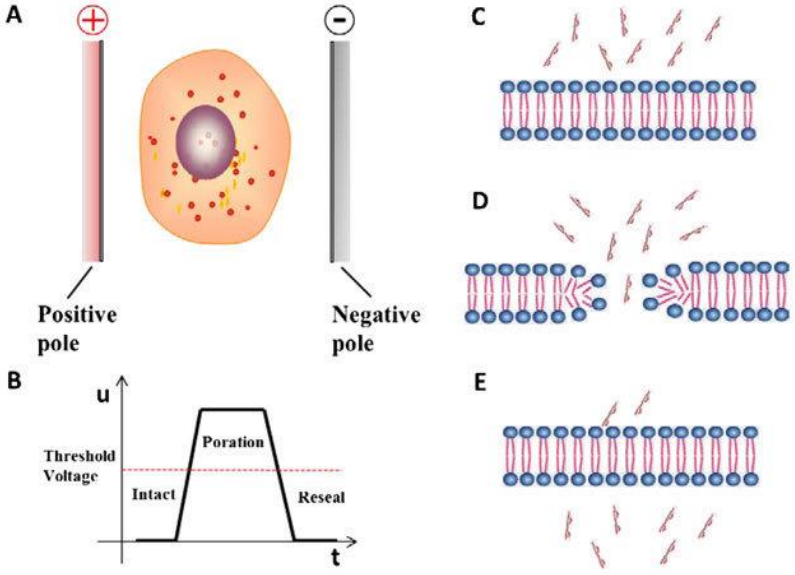

Fig 13. The principle of electroporation. **A**, a lively cell is exposed in the external pulse electric field. **BC**, when the strength of external electric field exceeds the threshold voltage. **D**, the transient pore is formed in the cell membrane and the exogenous nucleic acids are delivered into the cell. **E**, then, the cell membrane resealing happens over a range of minutes after the strength of external field dropping down to the threshold voltage (246).

6. Fitness and metabolic effects of the acquired plasmid on the bacterial host

HGT mediated by the spread of plasmids fuels evolution in bacteria. Plasmids carry genes necessary for plasmid replication and transmission, and the so-called accessory genes that under specific environmental conditions provide beneficial traits, such as antibiotic resistance, tolerance to heavy metals, or virulence (247). An important example of the ability of plasmids to catalyze bacterial adaptation and evolution is their instrumental role in the global spread of antibiotic resistance, which constitutes a major threat to public health (248). Although plasmids provide bacteria with new adaptive genes, they also produce physiological alterations that often translate into a reduction in bacterial fitness, manifesting as a reduced growth rate and weakened competitiveness of plasmid-bearing strains under conditions that do not select for plasmid-encoded genes (249). However this fitness reduction can be alleviated over time through compensatory mutations in the plasmid and/or the host chromosome (250). In-depth studies carried out in bacteria have shown that adaptive evolution in conditions selecting for plasmid carriage can increase plasmid stability by reducing the fitness cost of an initially costly plasmid-host association. Thus, periods of selection may provide sufficient time for plasmid-host adaptations to occur, enhancing plasmid persistence once selection is removed (251). However, the parameters governing plasmid fitness cost remain largely unexplored, and thus, it is not possible to predict the evolution of the same plasmid in different bacterial clones and the fitness effects of different plasmids in the same clone (250). For example, the lab of J. Lindsay showed that differences in fitness between two prevalent MRSA clones were unrelated to the presence of large antibiotic resistance plasmids (252). On the other hand, a recent study investigating the coevolution over the last 32 years of the *pSK1* plasmid family in the Australian *S. aureus* ST239-MRSA-III lineage revealed that *pSK1* plasmid maintenance is linked to multiple structural variations caused by the insertion sequences IS256 and IS257 (253).

The fitness effects of plasmids therefore have a crucial influence on their ability to associate with new bacterial hosts and consequently on the evolution of plasmid-mediated antibiotic resistance. However, the molecular mechanisms underlying plasmid fitness cost remain poorly understood (228). The fitness cost imposed by plasmids, coupled with the potential plasmid loss during bacterial cell division, can hinder the survival of plasmids in bacterial communities (248). Generally chromosomal resistance mutations carry a larger cost than acquiring resistance via a plasmid; this may explain why resistance often evolves by plasmid acquisition. Furthermore the

cost of plasmid acquisition increases with the range of encoded resistance. This suggests a potential limit on the evolution of extensive multi-drug resistance via plasmids (250). There are multiple sources of fitness costs related to HGT, such as the inhibition of bacterial replication or expression machinery, the biosynthetic cost associated with the new plasmid DNA and proteins, and deleterious interactions between the newly acquired genes and bacterial regulatory networks (249). To better understand the fitness cost mechanims, we dissect below the cost produced by plasmids during the different phases of their biology in the host bacterium (Fig. 14).

6.1. Plasmid reception

The first step of plasmid acquisition by a new host is the physical arrival of the plasmid in the cell by HGT mechanisms. Conjugation is considered the most important mechanism of plasmid transmission among bacteria (248). During conjugation, the plasmid enters the new cell as single-stranded DNA, leading to the induction of a transient activation of the SOS response (254–257). The SOS response is a bacterial stress response triggered by an increase in single-stranded DNA in the cell, which leads to a rise in mutation and recombination rates. The activation of SOS also leads to inhibition of cell division, which may be translated into a reduction into bacterial fitness in the short term (248).

6.2. Plasmid integration

Following successful transfer to a recipient cell, certain plasmids can integrate into the chromosome of the host (258). Although the integration of a plasmid carrying adaptive genes in the chromosome may be potentially advantageous for the host, this nonspecific integration can disrupt protein coding or regulatory regions or can interfere in the expression of genes flanking the integration site, entailing fitness costs in the recipient bacterium (259).

6.3. Plasmid replication

There is evidence that plasmid replication carries a fitness cost for the bacterial host. A particularly interesting observation regarding replication-related fitness costs is that plasmid DNA is significantly richer in AT than the host chromosome (260). Rocha and Danchin proposed that this difference might reflect the higher energy cost of G and C and the lower availability of these nucleotides in the host cell compared with A and T/U (261).

Plasmids usually encode their own replication-initiation (Rep) proteins, enabling auto control of plasmid copy number (262). These proteins subsequently recruit several other DNA polymerases and helicases from the bacterial host to proceed with plasmid replication (248). Multicopy plasmids probably require a high level of Rep protein expression to maintain their high copy number, which could increase the fitness cost (248). M. Alex Smith and Michael J. Bidochka, reported significant impacts of plasmid size and copy number of *pBluescript*-derived plasmids encoding ampicillin resistance, on bacterial host fitness in starvation media with and without the presence of antibiotics (263). Bacterial growth rate was reduced when maintaining larger plasmids. They hypothesized that metablic stress of maintaining plasmids in nonselective environments may be alleviated through appearance of instability in plasmid segregation and/or reduction in the plasmid copy number. In a recent study, T. LaBreck, and D. Scott Merrell demonstrated a fitness cost associated with carriage of the large *pC02* conjugative plasmid found in *S. aureus*. This MDR plasmid also carries genes known to be associated with antiseptic resistance (*i.e*., chlorhexidine and triclosan). However, subinhibitory concentrations of either chlorhexidine or triclosan suppressed this fitness cost (264).

6.4. Conjugation

The fitness cost associated with conjugation comes primarily from the high ATP demand for mating-channel formation and plasmid DNA translocation (248). To minimize this cost, plasmids tightly control the expression of conjugative systems. The result is a general repression of conjugation genes, with only a few cells in the population expressing the conjugative machinery (248). Conjugation can, however, be depressed, either through chemical signaling or through transcriptional overshooting of conjugative genes in new recipient cells (265). Experimental analysis showed that transcriptional overshooting allows a transient activation of plasmid functions immediately after plasmids invade a new host, suggesting an adaptive strategy for plasmids to be highly infective without damaging their hosts (266).

6.5. Expression of plasmid-encoded genes

The main biosynthetic burden associated with plasmid carriage is likely the expression of plasmid-encoded genes (248). The costs associated with gene expression can arise from gene transcription, translation, or subsequent interactions between plasmid-encoded proteins and cellular networks or

cytotoxic effects. Transcription is not considered a major cost (267), and the cost of plasmid gene expression appears to be predominantly linked to the translation of protein encoding plasmid genes (268). The cost of translation is determined by the difference between plasmid mRNA abundance and the balance of cellular tRNAs, amino acids, and ribosome (269), leading to reduced translation efficiency in the cell (254). The effect of this cost is the reduction of vertical plasmid transmission; however, the transient overexpression of conjugative genes helps the plasmid to rapidly spread horizontally in a bacterial population with available recipient cells. Another possible deleterious effect of plasmid gene translation is the depletion of the host cell amino acid pool (270). Amino acid starvation reduces bacterial growth rate and impacts bacterial physiology and fitness.

6.6. Effects of plasmid-encoded proteins on bacterial physiology

Plasmids bring new proteins to the host bacteria, and the potential effects of these proteins on bacterial physiology are impossible to predict. Plasmid-encoded proteins can cause fitness costs due to unwanted interactions with cellular networks or cytotoxic effects (248). In most cases, the deleterious interactions involve the plasmid Rep protein. Rep proteins connect extensively with host protein networks because they need to recruit many cellular enzymes, such as DNA polymerases and helicases, in order for plasmid replication to proceed (271). These interactions can result in the sequestration of the cellular replication machinery, altering the replication network and activating stress responses (256).

6.7. Mechanisms for minimizing plasmid cost

Once the plasmids are successfully established inside the host bacterium, it needs to adopt strategies to cope with the presence of such plasmids. One of the key factors for minimizing plasmids costs is transcriptional regulation by the host proteins such as transcriptional repressors that silence the expression of acquired genes (264). Plasmids also use specific regulators to control the transcription of their own genes, especially those related to plasmid main functions (248). In contrast, the transcription of plasmid-encoded accessory genes (generally related to antibiotic resistace) is not always beneficial. These accessory genes are frequently encoded in MGEs genetic such as integrons with high expression levels imposing a potential fitness cost to bacterial host (272).

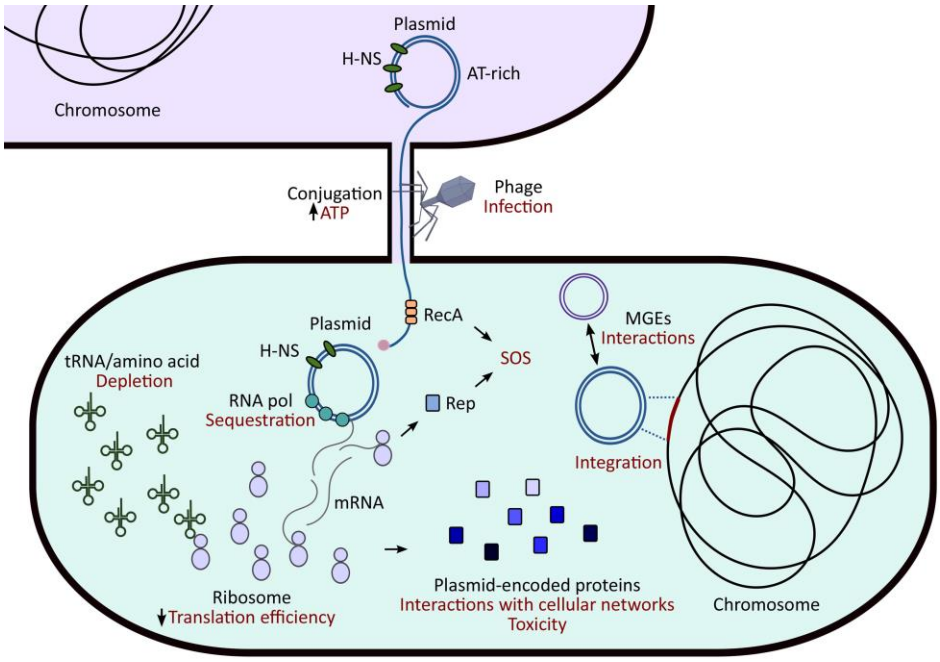

Fig 14. Fitness costs produced by plasmids. Potential fitness effects produced by the plasmids during their life cycle in the bacterial host (248).

7. Conclusion of the literature review section and working hypothesis

7.1. Epidemiology and molecular characteristics of *S. aureus*/MRSA in the regions surrounding Afghanistan

This literature review shows that the epidemiology of MRSA varies considerably on a global basis. Studies conducted in Afghanistan's three neighboring countries with large migratory movements, especially Pakistan, Iran and India show a high prevalence of MDR and virulent MRSA strains (46%-56%) and very diverse molecular characterstics, with predominance of ST772-MRSA-V (Bengal Bay clone), ST239-MRSA-III, ST22-MRSA-IV, CC8-MRSA-IV carrying PVL, TSST-1 and various resistance genes. Other predominant circulating clones of MRSA in the regions surrounding Afghanistan are various MDR-MRSA strains including ST25, ST859, ST14, ST398, ST59, ST30, ST8, USA900, CC15, ST30, CC1, ST80, ST88, ST25, CC30, CC239, CC9, CC5, CC1920, and CC59.

Existing data shows that prevalence of MRSA in the livestock in the region varies from 2.7% to 63% which is alarming. Various clones of MRSA circulating in livestock including, ST398, ST98, ST965, ST6, CC80, CC88 harboring various virulence genes. The predominant LA-MRSA in the region was ST398-MRSA-V with reports of transmission to human or causing zoonotic infections. Based on the available data, we can conclude that Afghanistan and its surrounding countries in west Asia, central Asia, south Asia, and east Asia is among the regions with the highest prevalence of MDR-MRSA strains harboring various virulence genes (PVL, TSST-1, enterotoxins) both in humans and animals. Given the changing epidemiology, timely updated information on epidemic of *S. aureus* strains in local and neighboring countries is essential for the prevention and control of this pathogen.Until now, in Afghanistan no information exist about molecular characteristics and circulating clones of *S. aureus*, especially MRSA strains which infect or colonize human and livestock.

7.2. Conjugative transfer of linezolid resistance

Based on the literature review, since 2000 linezolid has been widely used in the treatment of clinically-important gram-positive bacteria, including MDR-MRSA. Acquired resistance to linezolid emerged shortly after introduction of the drug in clinical practice. Initially, the reported resistance mechanisms were confined to ribosomal alterations, by mutations involving either the 23S rRNA or ribosomal proteins L3, L4 and L22. Subsequently, transferable resistance determinants to linezolid have also emerged, including the *cfr* gene and, most recently, the *optrA* and *poxtA* genes.

Numerous studies in several countries reported that *cfr* is the most frequently detected resistance gene to linezolid in staphylococci and the *cfr*-positive CoNS are increasingly isolated in hospitals worldwide due to high consumption of linezolid (186,208,273–277). Until now, more than 40 different *cfr*-carrying plasmids have been reported. The resistance to linezolid in clinical isolates is still rare and it remains active against >98% of staphylococci. Resistance to linezolid identified in 0.05% of *S. aureus* and 1.4% of CoNS. Among CoNS, *S. epidermidis* has been largely considered to act as a genetic reservoir of *cfr* gene for other pathogenic bacteria. The potential for dissemination is underscored by the frequent location of *cfr* on MGEs, typically plasmids, which are important vehicles for its spread to the more virulent and MDR *S. aureus*/MRSA strains that has become a global concern. In the recent years, there have been some outbreaks of *cfr*-carrying *S. aureus*/MRSA infections in the hospitals, including the pandemic ST22-MRSA-IV clone (180,205,278) that may have acquired the resistance gene from CoNS through HGT. Knowing the mechanism of linezolid resistance transfer to *S. aureus* clinical isolates, the genetic backgrounds of recipients that are more at risk of acquisition, or donors more capable to transfer the *cfr*-carrying plasmids, persistence of these plasmids within bacterial population in non-selective conditions, as well as conjugative transfer of each *cfr*-carrying plasmids to *S. aureus* strains, will help health care professionals to establish effective preventive measures against the spread of linezolid resistance to more pathogens.

To date, the information about conjugative transfer of *cfr*-carrying plasmids from CoNS to *S. aureus* strains is very scarce. No data exist about the role of genetic background of *S. epidermidis* and *S. aureus* strains in the plasmid transfer or acquisition, respectively. It is also important to know which plasmids are more transmissible to *S. aureus* clinical isolates via conjugation. Finally, information are lacking about the persistence of these plasmids within staphylococci population in

non-selective conditions, and the fitness cost imposed by these plasmids to *S. aureus* clinical isolates.

PART 2: EXPERIMENTAL STUDIES

The experimental studies are divided into two axes:

A. **Molecular characterization of *S. aureus* isolates from Afghanistan:** The main objective of this study was to determine the epidemiology and molecular characteristics of *S. aureus* strains isolated from human and livestock infection and colonization in Kabul.

   The main sub-objectives were:

   - To determine the molecular features and antibiotic susceptibility patterns of *S. aureus* strains isolated from clinical samples in the health facilities of Kabul.
   - To determine the molecular features and antibiotic susceptibility patterns of *S. aureus* strains isolated from human nasal carriers in Kabul.
   - To determine the molecular features and antibiotic resistance profile of *S. aureus* strains isolated from livestock (bovine mastitis/and nasal carriage in sheep) in Kabul.

B. **Conjugative transfer of linezolid resistance:** The main objective of the study was to study the conjugative transfer of linezolid resistance from *cfr*-postive LRSE to various clones of *S. aureus* circulating in France.

The main sub-objectives were:

- To study the role of genetic background of donors or recipients in the transfer or acquisition of various *cfr*-carrying plasmids, respectively.
- To study the transmission capability of different *cfr*-carrying plasmids.
- To study the plasmid fitness cost in transconjugants.
- To study the plasmid stability in transconjugants, under non-selective conditions.

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1. First publication: "Antibiotic resistance profile and molecular characterization of *Staphylococcus aureus strains* isolated in hospitals in Kabul, Afghanistan"

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Overuse of antibiotics and taking antibiotics without prescription is very common in Afghanistan (113). This widespread use of antibiotics could have contributed to selection and spread of MDR organisms including MRSA. Recent studies in Afghanistan and neighboring countries have shown a high prevalence of MRSA in healthcare settings and in the community (58,61,112,279). Knowledge of epidemic *S. aureus*/MRSA clones can help in the development of effective strategies to aid in controlling spread, and optimizing treatment.

In Afghanistan, no data are available about circulating clones of *S. aureus*. Molecular characterization and determination of virulence and resistance genes of the major *S. aureus* circulating clones in Afghanistan is an important step to treat and establish preventive measures to contain the spread of these pathogens in the community and healthcare facilities. Thus, this study was conducted to determine the molecular features and antibiotic resistance patterns of *S. aureus* strains isolated from clinical samples in two main hospitals of Kabul (Maiwand and Ibn-Sina). These two health facilities were selected because they have standard microbiology labs and perform most of the bacteriological analyses in Kabul. Standard microbiological procedures were conducted on clinical samples with minimum delay for culture; confirmatory tests and antibiotic susceptibility testing (AST) were performed in Kabul. The conserved strains were then brought to France for molecular analysis including detection of *mecA*/*mec*C genes, *agr* typing and DNA microarray for identification of clones.

The result showed various resistance genes to different classes of antibiotics among isolated strains, of which 66.3% were MRSA and all harboured *mec*A gene. After molecular typing of the isolates, a wide clonal diversity was observed with 12 different clonal complexes (CCs), 13 distinct clones of MRSA and 14 distinct clones of MSSA. Three MRSA clones predominated: the Southwest Pacific Clone (CC30-MRSA-IV PVL-positive), the Bengal Bay Clone (ST772-MRSA-V PVL-positive), and the CC22-MRSA-IV TSST1+ clone.

Our findings indicate that prevalence of MRSA isolates obtained from patients in Kabul hospitals was quite high when compared to the neighboring countries. Most of *S. aureus* isolates, especially MRSA, were MDR and harbored different virulence genes. This diversity and the clones identified reflect epidemiological links, including travels, migrations, commerce, and international military actions, with South West Pacific, Indian and Middle Eastern/Arabian Gulf regions, as well as Western Europe, USA and Africa. Major efforts have to be made to monitor and reduce the spread of these MDR pathogens in Kabul healthcare facilities and establish surveillance and infection control departments in major hospitals of Kabul and other provinces of Afghanistan.

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ORIGINAL ARTICLE

Antibiotic resistance profile and molecular characterization of *Staphylococcus aureus* strains isolated in hospitals in Kabul, Afghanistan

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Abstract

The aim of this study was to investigate the molecular features and the antibiotic resistance profile of 98 clinical *Staphylococcus aureus* isolates collected during 6 months in two hospitals of Kabul, Afghanistan. For all isolates, antimicrobial resistance patterns were determined by the disc diffusion method (including methicillin resistance which was detected using cefoxitin). The presence of the *mec*A/*mec*C genes was detected by PCR. Strains were then extensively characterized using microarray analysis. Of the 98 *S. aureus* isolates, methicillin-resistant *S. aureus* (MRSA) prevalence was high at 66.3%. Antibiotic susceptibility testing also revealed a high resistance rate to penicillin (100%), erythromycin (66.3%), ciprofloxacin (55.1%), and cotrimoxazole (40.8%). Resistance to tobramycin was detected in 25.5%, to gentamicin in 16.3%, to chloramphenicol in 34.7%, and to doxycycline in 23.5% of the isolates. All the MRSA isolates were *mec*A-positive and none of them harbored *mec*C. Isolates were grouped into twelve clonal complexes and twenty-seven distinct clones. The most frequently detected clones were the Southwest Pacific clone (CC30-MRSA-IV PVL+) (21/65 MRSA, 32.3%), the CC22-MRSA-IV TSST-1+ clone (11/65 MRSA, 16.9%), and the Bengal Bay clone (ST772-MRSA-V PVL+) (11/65 MRSA, 16.9%). The PVL genes were found in 59.2% (46/65 MRSA and 12/33 methicillin-susceptible *S. aureus*, MSSA) and *tst1* gene in 16.3% of isolates. This molecular study highlights the high prevalence of MRSA and the large genetic diversity of the *S. aureus* isolates circulating and detected in two hospitals of Kabul, with the presence of multiple virulence and antibiotic resistance genes.

Keywords *Staphylococus aureus* · MRSA · Antibiotic resistance · Molecular typing · Kabul · Afghanistan

Introduction

Staphylococcus aureus is a major human pathogen responsible for serious infections in both community and hospital settings [1]. *S. aureus* can cause a wide range of infections, from minor skin infections, such as carbuncles, folliculitis, and impetigo, to deep-seated (abscesses or cellulitis) or life-threatening infections such as pneumonia, osteomyelitis, bacteremia, toxic shock syndrome, or endocarditis. The global emergence and spread of methicillin-resistant *S. aureus* (MRSA) in hospitals since the 1960s are a public health concern and limit the therapeutic arsenal for severe staphylococcal infections. Indeed, healthcare-associated (HA-) MRSA are usually resistant to most antimicrobial agents. In the case of severe infections due to these multi-resistant strains, glycopeptides are often the drugs of last resort; however, isolates with reduced susceptibility to vancomycin are frequently reported [2], and some vancomycin-resistant *S. aureus* (VRSA)

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strains harboring the *van*A gene from enterococci have also been described but are very rare [3]. On the other hand, the emergence and spread of community-acquired (CA-) MRSA clones expressing the Panton-Valentine leukocidin (PVL) in the last two decades are an additional cause for concern and have changed the epidemiology of MRSA [4, 5]. These strains are generally associated with recurrent skin and soft tissue infections (SSTIs) but can also cause necrotizing pneumonia or destructive bone and joint infection [6]. Interestingly, some CA-MRSA clones predominate in geographically restricted areas while others have achieved a pandemic level.

In central Asia, including Afghanistan, studies about MRSA have mainly focused on the prevalence of MRSA, but few data are available about the circulating clones. In this part of the world, *S. aureus* infections in healthcare settings show a high prevalence of MRSA [7]. For example, in Pakistan, a multicentric study in four hospitals showed a MRSA rate of 41.9% in 2006–2008 [8]. In India, two multicentric studies described comparable MRSA rates of 41% and 45% in 2008–2009 and 2011, respectively [9, 10]. In Iran, a systematic review reported a mean MRSA rate of 43.0% between 2000 and 2016 [11] and several VRSA isolates were described [12]. More specifically, in Afghanistan, a recent study concerning *S. aureus* infections diagnosed in Kabul hospitals highlighted a high rate of methicillin resistance (56.2%) and an increase in the prevalence of multidrug-resistant (MDR)-MRSA compared to the neighboring countries [13]. In parallel, an overuse of antimicrobials was described during the last decade in primary healthcare clinics of five major provinces of Afghanistan, including Kabul [14] and, in 2014, some authors raised the question of over prescription of antibiotics in a district hospital in Kabul where more than 50% of out-patients were prescribed at least one antibiotic [15]. This widespread use of antimicrobial therapies could have contributed to the increase of MRSA prevalence in Afghanistan and the selection of MDR clones.

Nevertheless, although molecular typing data for *S. aureus* and MRSA are abundantly available for Western Europe, North America, or Australia, few studies have been conducting to describe *S. aureus* epidemiology in the rest of the world, including Central Asia and more specifically, Afghanistan. In this context, the aim of this work was to investigate the molecular features of *S. aureus* strains isolated from clinical samples in two hospitals of Kabul, as well as their antibiotic resistance profile.

Materials and methods

Strains collection

From January to June 2017, all *S. aureus* isolates cultured from in- and out-patient samples in two hospitals located in the center of Kabul (Maiwand and Ibn-Sina hospitals) were collected. During the 6-month study period, a total of 98 isolates of *S. aureus* obtained from 98 individual patients were characterized. The strains were isolated from various types of clinical samples: pus (skin and soft tissue infections; 65.3%), ear pus (18.4%), blood (7.1%), urine (3.1%), vaginal swab (3.1%), tracheal aspirations (2%), and semen (1%). Out of the 98 patients, 33 (33.7%) were women and 65 (66.3%) were men. The mean age was 19 years ([1–75] years, median 12 years).

Phenotypic assays

Standard microbiological procedures were conducted on clinical samples with minimum delay for culture, confirmatory tests, and antibiotic susceptibility testing (AST). Presumptive *S. aureus* isolates were subcultured for 18–24 h at 37 °C onto blood agar base medium (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood. Confirmatory tests were carried out for the identification of *S. aureus* isolates, by performing Pastorex Staph Plus (Bio-Rad, Marnes-la-Coquette, France) and coagulase assay. AST was performed on Mueller-Hinton agar (Oxoid) by Kirby Bauer disc diffusion method according to the 2017 guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [16]. The following panel of antibiotics was tested: penicillin G, cefoxitin (used to detect methicillin/oxacillin resistance), tobramycin, gentamicin, erythromycin, clindamycin, ciprofloxacin, chloramphenicol, doxycycline, rifampicin, and cotrimoxazole.

Detection of *mec*A/*mec*C genes and *agr* typing by multiplex PCR

Cellular DNA was obtained from *S. aureus* colonies grown overnight on blood agar plates using DNA Extraction Kit (Promega, USA) in accordance with the manufacturer's instructions. Detection of the *mec*A and *mec*C genes and *agr* typing were performed by multiplex PCR using primers already published [17–19].

Molecular characterization of strains by DNA microarray

DNA was extracted and purified using commercial extraction kits (DNeasy kit and Promega instrument; Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA microarray Identibac *S. aureus* genotyping® (Alere Technologies, Jena, Germany) was used as previously described [20]. This microarray allows the detection of 336 different target sequences corresponding to 185 genes and their allelic variants. The assignation of isolates to clonal complexes (CCs) was determined by comparison of the hybridization profiles to previously typed multi-locus sequence typing reference strains [20].

Statistical analysis

Statistical analysis was done using SPSS 21 (IBM Inc., Chicago). Binary logistic regression was used to determine the association between MRSA or MSSA status, gender, and age. Chi-square test was used to compare the resistance profiles and the presence of virulence genes between MRSA and MSSA (methicillin-susceptible *S. aureus*). A p value less than 0.05 was considered statistically significant.

Results

Antibiotic susceptibility testing

Out of the 98 *S. aureus* isolates, all were resistant to penicillin and 65 (66.3%) were methicillin-resistant (Table 1). Twenty-five (25.5%) were resistant to tobramycin, 16 (16.3%) to gentamicin, 65 (66.3%) to erythromycin, 11 (11.2%) to clindamycin, 54 (55.1%) to ciprofloxacin, 34 (34.7%) to chloramphenicol, 23 (23.5%) to doxycycline, 3 (3.1%) to rifampicin, and 40 (40.8%) to cotrimoxazole. MRSA isolates were statistically more frequently resistant to gentamicin ($p = 0.002$), erythromycin ($p < 0.001$), ciprofloxacin ($p < 0.001$), and cotrimoxazole ($p = 0.017$) than MSSA (Table 1). The difference of MRSA distribution was not statistically significant according to gender ($p = 0.39$) and age ($p = 0.25$).

Molecular characterization of *S. aureus* isolates

Various resistance genes were detected in the 98 collected isolates: *blaZ* in almost all isolates (97/98, 99%), *msr*A and *mph*C in 43/98 (43.9%), *erm*C in 16/98 (16.3%), *aph*A3 in 44/98 (44.9%), *aad*D in 22/98 (22.4%), *aac*A-*aph*D in 16/98 (16.3%), and *tet*K in 39/98 isolates (39.8%) (Table 2). No strain harbored the resistance genes *van*A, *qac*A, *qac*C, *fus*B, *mup*R, and *cfr*. The presence of *msr*A, *mph*C, *aac*A-*aph*D, *aph*A3, *sat*, and *dfr*A was higher in MRSA than in MSSA whereas the presence of *lnu*A, *tet*K, and *tet*Efflux was higher in MSSA. Out of the 65 isolates phenotypically resistant to methicillin, all harbored the *mec*A gene and no isolate was positive for *mec*C. Concerning the staphylococcal chromosome cassette *mec* (SCC*mec*) element of these MRSA isolates, 47 (72.3%) harbored SCC*mec* type IV, 17 (26.2%) harbored SCC*mec* type V, and one (1.5%) harbored SCC*mec* type III.

Regarding the molecular typing of the isolates by microarray, a wide clonal diversity was observed. The 98 isolates belonged to twelve different clonal complexes (CCs) and were assigned to 27 distinct clones (Tables 3 and 4). Three CCs (CC1, CC22, and CC30) dominated and accounted for 65.3% of the isolates; four other CCs (CC8, CC15, CC121, and CC398) accounted for 24.5%.

The most frequent detected clone was the Southwest Pacific clone (CC30-MRSA-IV PVL+), which accounted for 21.4% (21/98) of all isolates and 32.3% (21/65) of MRSA. It was mostly isolated from skin and soft tissue infections. Isolates of this clone were resistant to multiple antibiotics, particularly oxacillin (100%), erythromycin (95.2%), ciprofloxacin (95.2%), and cotrimoxazole (57.1%). Various resistance genes were detected in these isolates: *blaZ* operon, *msr*A, *mph*C, *fos*B and *tet*Efflux in all isolates; *aph*A3 and *sat* in 19/21 (90.5%) isolates; *aad*D and *tet*K in 1/21 (4.8%). The enteroxin gene cluster *egc* (*seg*, *sei*, *sem*, *sen*, *seo*, and *seu*) was detected in all these isolates.

The second most frequent detected clone was the Bengal Bay clone (ST772-MRSA-V PVL+), which accounted for 11.2% (11/98) of all isolates and 16.9% (11/65) of MRSA. Isolates of this clone were also resistant to multiple antibiotics, particularly oxacillin (100%), tobramycin (63.6%), gentamicin (54.6%), erythromycin (100%), ciprofloxacin (100%), chloramphenicol (63.6%), and doxycycline (63.6%). Various resistance genes were detected in these isolates: *blaZ* operon, *msr*A, *mph*C, *fos*B, and *tet*Efflux in all isolates; *tet*K in 6/11 (54.6%); and *aac*A-*aph*D in 5/11 (45.5%). Nine enterotoxin genes (*sea*, *sec*, *sel*, and the *egc*) were detected in almost all of these isolates (Table 3). Another clone, the CC22-MRSA-IV TSST1+ (toxic shock syndrome toxin-1) clone, accounted for 11/98 (11.2%) of all isolates and 11/65 (16.9%) of MRSA isolates. Isolates of this clone were less resistant to antibiotics: oxacillin (100%), erythromycin (27.3%), doxycycline (27.3%), rifampicin (18.2%), and cotrimoxazole (36.4%). Various resistance genes were detected in these isolates: *blaZ* operon in 10/11 (90.9%), *erm*C in 2/11 (18.2%), *dfr*A in 11/11 (100%), and *tet*K in 3/11 (27.3%). The *egc* cluster was detected in all of these isolates. All other clones of MRSA were detected in 6 or less isolates (< 7%) and presented a high diversity (Table 3).

Table 1 Antibiotic resistance of the 98 *S. aureus* isolates collected during 6 months, from January to June 2017, in two hospitals in Kabul (MSSA, $n = 33$, and MRSA, $n = 65$)

Antibiotic	All isolates *n* (%)	MRSA *n* (%)	MSSA *n* (%)	*p* value
Penicillin G	98 (100)	65 (100)	33 (100)	-
Tobramycin	25 (25.5)	19 (29.2)	6 (18.2)	0.236
Gentamicin	16 (16.3)	16 (24.6)	0 (0)	*0.002*
Erythromycin	65 (66.3)	51 (78.5)	14 (42.4)	*< 0.001*
Clindamycin	11 (11.2)	9 (13.8)	2 (6.1)	0.249
Ciprofloxacin	54 (55.1)	45 (69.2)	9 (27.3)	*< 0.001*
Chloramphenicol	34 (34.7)	24 (36.9)	10 (30.3)	0.515
Doxycycline	23 (23.5)	16 (24.6)	7 (21.2)	0.707
Rifampicin	3 (3.1)	2 (3.1)	1 (3.0)	0.99
Cotrimoxazole	40 (40.8)	32 (49.2)	8 (24.2)	*0.017*

Significant *p* values are shown in italic

Table 2 Presence of resistance genes in 98 *S. aureus* isolates collected during 6 months, from January to June 2017, in two hospitals in Kabul (MSSA, $n = 33$, and MRSA, $n = 65$).

	Gene	All isolates *n* (%)	MRSA *n* (%)	MSSA *n* (%)	*p* value
Beta-lactam resistance	*blaZ*	97 (99.0)	64 (98.5)	33 (100)	0.47
	*mec*A	65 (66.3)	65 (100)	-	-
	*mec*C	0 (0)	0 (0)	-	-
Macrolide/lincosamide resistance	*erm*A	1 (1.0)	1 (1.5)	0 (0)	0.47
	*erm*B	1 (1.0)	0 (0)	1 (3.0)	0.16
	*erm*C	16 (16.3)	16 (24.6)	5 (15.2)	0.82
	*lnu*A	7 (7.1)	0 (0)	7 (21.2)	*< 0.001*
	*msr*A	43 (43.9)	38 (58.5)	5 (15.2)	*< 0.001*
	*mph*C	43 (43.9)	38 (58.5)	5 (15.2)	*< 0.001*
Aminoglycoside resistance	*aac*A-*aph*D	16 (16.3)	16 (24.6)	0 (0)	*0.002*
	*aad*D	22 (22.4)	13 (20.0)	9 (27.3)	0.42
	*aph*A3	44 (44.9)	41 (63.1)	3 (9.1)	*< 0.001*
	sat	44 (44.9)	41 (63.1)	3 (9.1)	*< 0.001*
Cotrimoxazole resistance	*dfr*A	16 (16.3)	16 (24.6)	0 (0)	*0.002*
Fusidic acid resistance	*fus*B	0 (0)	0 (0)	0 (0)	-
Tetracycline resistance	*tet*K	39 (39.8)	21 (32.3)	18 (54.8)	*0.03*
	*tet*M	2 (2.0)	1 (1.5)	1 (3.0)	0.62
	*tet*Efflux	80 (81.6)	48 (73.8)	32 (97.0)	*0.005*
Choramphenicol resistance	*cat*	1 (1.0)	0 (0.0)	1 (3.0)	0.16
	cfr	0 (0)	0 (0)	0 (0)	-
	*fex*A	0 (0)	0 (0)	0 (0)	-
Mupirocin resistance	*mup*R	0 (0)	0 (0)	0 (0)	-
Fosfomycin resistance	*fos*B	65 (66.3)	46 (70.8)	19 (57.6)	0.19
Vancomycin resistance	*van*A	0 (0)	0 (0)	0 (0)	-

Significant *p* values are shown in italic

Out of the 33 MSSA isolates, 6 (18.2%) belonged to the CC15 and did not harbor genes for PVL, TSST-1, and enterotoxins. Five MSSA isolates (15.2%) belonged to the CC121-MSSA PVL+ clone and harbored the resistance genes *blaZ*, *fos*B, *tet*K, and *tet*Efflux, and virulence genes encoding PVL and enterotoxins (*seb*, and *egc*). Five MSSA isolates (15.2%) belonged to the ST291/813 and four (12.1%) belonged to the CC30-MSSA PVL+ clone. All other clones of MSSA were detected in three or less isolates (4% of all strains) but showed high diversity (Table 4).

The overall rate of PVL-positive isolates was 59.2% (58/98), these isolates belonging to 8 MRSA and 5 MSSA clones. The prevalence of PVL genes was higher in MRSA than in MSSA (70.8% vs 36.4%, $p = 0.001$). The arginine catabolic mobile element (ACME) locus was present in only one strain of MRSA belonging to the USA300 clone (ST8-MRSA-IV PVL+), isolated from an ear pus. Contrary to PVL, few isolates harbored the TSST-1 (16/98, 16.3%). Table 5 shows the prevalence data for virulence genes in the *S. aureus* isolates collected.

Discussion

This epidemiological study investigated the antibiotic resistance profile and performed the molecular characterization of MRSA and MSSA clinical isolates at two main health facilities in Kabul. The overall prevalence of MRSA was high at 66.3% (65/98). A very high genetic diversity was detected using microarray analysis, with 27 distinct clones among the 98 isolates collected during a 6-month period.

This study is the first molecular study of *S. aureus* clinical isolates in Afghanistan and reports the various clones circulating in its capital. The prevalence of MRSA in this study was higher compared to findings of similar studies conducted in neighboring countries such as Pakistan [21], Iran [22], Saudi Arabia [23], and Turkey [24]. In this study, a wide clonal diversity was detected with twelve different clonal complexes, along with 13 distinct MRSA and 14 MSSA clones. The typing results revealed a predominance of three MRSA clones, representing 44% of the isolates: Southwest Pacific (CC30-MRSA-IV PVL+), Bengal

Table 3 Distribution and characteristics of the 65 MRSA isolates collected from January to June 2017 in two main hospitals in Kabul

CC (number of isolates)	Clone assignment (Alere)[a]	*n*	% of isolates	*agr* type	SCC*mec* type	PVL	TSST	Enterotoxin genes	Antibiotic resistance genes (other than *mec*A)	Antibiotic resistance associated with methicillin resistance[b]
CC1 (12)	CC1-MRSA-IV PVL+, USA400	1	1.5	3	IV	Pos	Pos	*sea*, *seh*, *sek*, *seq*,	*blaZ*, *erm*C, *aad*D, *tet*K, *tet*Efflux	Tob, Ery, Dox, Sxt
	ST772-MRSA-V PVL+, Bengal Bay Clone	11	16.9	2	V	Pos	Neg	*sea*, *sec*, *seg* (10/11), *sei*, *sel*, *sem*, *sen* (10/11), *seo*, *seu*	*blaZ*, *msr*A, *mph*C, *aac*A-*aph*D (5/11), *fos*B, *tet*K (6/11), *tet*Efflux	Tob (7/11), Gen (6/11), Ery, Cli (1/11), Cip, Chl (7/11), Dox (7/11), Sxt (3/11)
CC8 (7)	CC8-MRSA-IV, USA500	4	6.2	1	IV	Neg	Neg	*seb*, *sek*, *seq*	*blaZ*, *erm*C (3/4), *aac*A-*aph*D, *aad*D, *aph*A3, *sat*, *fos*B, *tet*Efflux	Tob, Gen, Ery, Cli (3/4), Cip, Chl (3/4), Sxt
	CC8-MRSA-IV, UK-EMRSA-14/WA MRSA-5	1	1.5	1	IV	Neg	Neg		*blaZ*, *aac*A-*aph*D, *aad*D, *fos*B, *tet*Efflux	Tob, Gen, Cip, Sxt
	ST8-MRSA-[IV + ACME] PVL+, USA300	1	1.5	1	IV	Pos	Neg	*sek*, *seq*	*blaZ*, *msr*A, *mph*C, *aph*A3, *sat*, *fos*B, *tet*Efflux	Ery, Cip
	ST239-MRSA-[III + SCC-mer+ccrC], Vienna/Hungarian/Brazilian Clone	1	1.5	1	III	Neg	Neg	*sea*, *sek*, *seq*	*blaZ*, *erm*A, *aac*A-*aph*D, *aph*A3, *tet*K, *tet*M, *fos*B, *tet*Efflux	Tob, Gen, Cip, Chl, Sxt
CC22 (16)	CC22-MRSA-IV TSST-1+	11	16.9	1	IV	Neg	Pos	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*	*blaZ* (9/11), *erm*C (2/11), *dfr*A, *tet*K (3/11)	Tob (1/11), Ery (3/11), Cip (1/11), Chl (2/11), Dox (3/11), Rif (2/11), Sxt (4/11)
	CC22-MRSA-IV PVL+	5	7.7	1	IV	Pos	Neg	*seg*, *sei*, *sem*, *sen*, *seo* (4/5), *seu*	*blaZ*, *erm*C (4/5), *aac*A-*aph*D, *aad*D, *dfr*A	Tob, Gen, Ery (4/5), Cli (1/5), Cip (2/5), Chl (3/5), Sxt (4/5)
CC30 (28)	CC30-MRSA-IV PVL+, Southwest Pacific clone	21	32.3	3	IV	Pos	Neg	*sea* (1/21), *seg*, *sei*, *sem*, *sen*, *seo*, *seu*	*blaZ*, *msr*A, *mph*C, *aad*D (1/21), *aph*A3 (19/21), *sat* (19/21), *tet*K (1/21), *fos*B, *tet*Efflux	Tob (1/21), Ery (20/21), Cli (3/21), Cip (20/21), Chl (6/21), Dox (1/21), Sxt (12/21)
	CC30-MRSA-V/VT PVL+, WA MRSA-124	6	9.2	3	V	Pos	Neg	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*	*blaZ*, *erm*C (1/6), *msr*A (5/6), *mph*C, *aph*A3 (5/6), *sat*, *tet*K, *fos*B, *tet*Efflux	Tob (1/6), Ery (5/6), Cip (4/6), Chl (1/6), Dox (3/6), Sxt (1/6)
	CC30-MRSA-IV PVL+/TSST-1+	1	1.5	3	IV	Pos	Pos	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*	*blaZ*, *msr*A, *mph*C, *aad*D, *tet*K, *fos*B, *tet*Efflux	Tob, Ery, Sxt
CC88 (1)	CC88-MRSA-IV PVL+	1	1.5	3	IV	Pos	Neg	*sep*	*blaZ*, *tet*K, *tet*Efflux	None
CC509 (1)	CC509-MRSA-IV	1	1.5	3	IV	Neg	Neg	*sem*, *seo*	*blaZ*, *tet*K, *tet*Efflux	None

[a] MLST and clone assignments correspond to the comparison of the hybridization profiles on the DNA microarray to previously typed MLST reference strains [17]

[b] Tob, tobramycin; Gen, gentamicin; Ery, erythromycin; Cli, clindamycin; Cip, ciprofloxacin; Chl, chloramphenicol; Dox, doxycycline; Rif, rifampicin; Sxt, cotrimoxazole

Table 4 Distribution and characteristics of the 33 MSSA isolates collected from January to June 2017 in two main hospitals in Kabul

CC (number of isolates)	Clone assignment (Alere)[a]	*n*	% of isolates	*agr* type	PVL	Exfoliative toxin	TSST	Enterotoxin genes	Antibiotic resistance genes	Antibiotic resistance[b]
CC1 (2)	CC1-MSSA PVL+	1	3.0	3	Pos	Neg	Pos	*sea*, *seh*, *sek*, *seq*	*bla*Z, *tet*Efflux	Pen
	ST848-MSSA-[ccrAB1]	1	3.0	2	Neg	Neg	Neg	*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*	*bla*Z, *fos*B, *tet*Efflux	Pen
CC5 (1)	CC5-MSSA	1	3.0	2	Neg	Neg	Pos	*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*	*bla*Z, *msr*A, *mph*C, *aph*A3, *sat*, *tet*K, *fos*B, *tet*Efflux	Pen, Ery, Sxt
CC15 (6)	CC15-MSSA	6	18.2	2	Neg	Neg	Neg		*bla*Z, *erm*C (3/6), *lnu*A (4/6), *aad*D (4/6), *tet*K (4/6), *fos*B, *tet*Efflux	Pen, Tob (4/6), Ery (3/6), Clin (1/6), Cip (2/6), Chl (1/6), Dox (2/6), Sxt (2/6)
CC22 (1)	CC22-MSSA	1	3.0	1	Neg	Neg	Neg	*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*	*bla*Z, *erm*C, *tet*K	Pen, Ery
CC30 (5)	CC30-MSSA	1	3.0	3	Neg	Neg	Neg	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*	*bla*Z, *msr*A, *mph*C, *aph*A3, *sat*, *fos*B, *tet*Efflux	Pen, Ery, Cip
	CC30-MSSA PVL+	4	12.1	3	Pos	Neg	Pos (1/4)	*sea* (1/4), *sec* (1/4), *seg*, *sei*, *sel* (1/4), *sem*, *sen*, *seo*, *seu*	*bla*Z, *lnu*A (1/4), *aad*D (3/4), *tet*K (3/4), *fos*B, *tet*Efflux,	Pen, Tob (2/4), Ery (2/4), Clin (1/4), Chl (1/4), Sxt (1/4)
CC45 (2)	CC45-MSSA	2	6.1	1	Neg	Neg	Neg	*sec* (1/2), *seg*, *sei*, *sel* (1/2), *sem*, *sen*, *seo*, *seu*	*bla*Z, *msr*A (1/2), *mph*C (1/2), *tet*Efflux	Pen, Ery (1/2), Cip (1/2), Chl (1/2)
CC88 (1)	CC88-MSSA PVL+	1	3.0	3	Pos	Neg	Neg	*sep*	*bla*Z, *tet*Efflux	Pen
CC121 (6)	CC121-MSSA	1	3.0	4	Neg	Neg	Neg	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*	*bla*Z, *fos*B, *tet*Efflux	Pen
	CC121-MSSA PVL+	5	15.2	4	Pos	Neg	neg	*seb* (4/5), *seg*, *sei*, *sem*, *sen*, *seo*, *seu*	*bla*Z, *tet*K (3/5), *fos*B, *tet*Efflux	Pen, Ery (1/5), Cip (1/5), Chl (2/5), Dox (1/5), Sxt (2/5)
CC398 (5)	ST291/813-MSSA	5	15.2	1	Neg	Pos	Neg		*bla*Z, *erm*B (1/5), *erm*C (1/5), *aph*A3 (1/5), *sat* (1/5), *tet*K (3/5), *tet*M (1/5), *tet*Efflux	Pen, Ery (1/5), Cip (3/5), Chl (3/5), Dox (1/5)
CC1021 (1)	CC1021-MSSA-[ccrAB2+] PVL+	1	3.0	3	Pos	Neg	Neg	*sea*, *seb*, *sei*, *sek*, *sem*, *sen*, *seo*, *seq*, *seu*	*bla*Z, *tet*K, *tet*Efflux	Pen, Ery, Rif, Sxt
ST1278 (3)	ST1278-MSSA-[ccrA1]	3	9.1	3	Neg	Neg	Neg	*seh*	*bla*Z, *lnu*A (2/3), *msr*A (2/3), *mph*C (2/3), *aad*D (2/3), *tet*K (2/3), *tet*Efflux	Pen, Ery (2/3), Cip (1/3), Chl (2/3), Dox (2/3)

[a] MLST and clone assignments correspond to the comparison of the hybridization profiles on the microarray to previously typed MLST reference strains [17]

[b] Pen, penicillin G; Tob, tobramycin; Gen, gentamicin; Ery, erythromycin; Cli, clindamycin; Cip, ciprofloxacin; Chl, chloramphenicol; Dox, doxycycline; Rif, rifampicin; Sxt, cotrimoxazole

Table 5 Presence of virulence genes in 98 *S. aureus* isolates collected during 6 months (January–June 2017) in two hospitals in Kabul (MSSA, $n = 33$, and MRSA, $n = 65$)

Group	Gene	All isolates *n* (%)	MRSA *n* (%)	MSSA *n* (%)	*p* value
Toxic shock syndrome toxin gene	*tst1*	16 (16.3)	13 (20.0)	3 (9.1)	0.167
Enterotoxin genes	*sea*	17 (17.3)	14 (21.5)	3 (9.1)	0.124
	sep	2 (2.0)	1 (1.5)	1 (3.0)	0.622
	seb	9 (9.2)	4 (6.2)	5 (15.2)	0.145
	sec	16 (16.3)	11 (16.9)	5 (15.2)	0.823
	seg	70 (71.4)	54 (83.1)	16 (48.5)	*0.001*
	seh	5 (5.1)	1 (1.5)	4 (12.1)	*0.024*
	sei	72 (73.5)	55 (84.6)	17 (51.5)	*< 0.001*
	sek	9 (9.2)	7 (10.8)	2 (6.1)	0.446
	sel	16 (16.3)	11 (16.9)	5 (15.2)	0.823
	sem	73 (74.5)	56 (86.2)	17 (51.5)	*< 0.001*
	sen	71 (72.4)	54 (83.1)	17 (51.5)	*0.001*
	seo	72 (73.5)	55 (84.6)	17 (51.5)	*< 0.001*
	seq	9 (9.2)	7 (10.8)	2 (6.1)	0.446
	seu	72 (73.5)	55 (84.6)	17 (51.5)	*< 0.001*
Panton-Valentine leukocidin	*lukFPVL-lukSPVL*	58 (59.2)	46 (70.8)	12 (36.4)	*0.001*
Exfoliative toxins	*etd*	5 (5.1)	0 (0.0)	5 (15.2)	*0.001*
Miscellaneous virulence genes or loci	ACME	1 (1.0)	1 (1.5)	0 (0.0)	0.474
	sak	79 (80.6)	53 (81.5)	26 (78.8)	0.745
	chp	73 (74.5)	46 (70.8)	27 (81.8)	0.236
	scn	97 (99.0)	64 (98.5)	33 (100)	0.474

Significant *p* values are shown in italic

Bay (ST772-MRSA-V PVL+), and CC22-MRSA-IV TSST-1+ clones. The 24 other clones were present in one to 6 isolates.

The main limitation of this study is that only *S. aureus* isolates collected in two hospitals of Kabul were included. Therefore, even if these hospitals perform most of the bacteriological analyses in Kabul, the results do not reflect the global epidemiology in Afghanistan and are only representative of *S. aureus* epidemiology for infections diagnosed in hospitals. Nevertheless, the huge diversity of strains highlighted by the typing results illustrates the position of Kabul as a center of intense international exchanges.

Three different MRSA and two different MSSA clones were identified within CC30. The Southwest Pacific clone (CC30-MRSA-IV PVL+, USA1100) was the most prevalent clone, detected in 32.3% of MRSA isolates. This MDR and PVL+ clone is widely distributed in the Pacific islands and was first identified in New Zealand among the Samoan immigrant population [25]. It is a widespread CA-MRSA clone in Australia and has been found in several European countries as well as in Hong Kong, Taiwan, the USA, Saudi Arabia, the United Arab Emirates, and Iran [25–27]. The major occurrence of this clone in Afghanistan could possibly be linked to the large migratory movements of Afghans to Australia, Saudi Arabia, Iran, European countries, and some of the southwest Pacific countries, especially Indonesia, and Malaysia. The WA MRSA-124 clone (CC30-MRSA-V/VT PVL+), detected in 9.2% of MRSA isolates, is another virulent and MDR clone and was isolated mainly from SSTIs. The same lineage has already been described in Egypt [28] and in a patient living in the German/Polish border region and presenting a SSTI [4].

Two MSSA and two MRSA clones were identified within the CC1. Within this clonal complex, the Bengal Bay clone (ST772-MRSA-V PVL+), detected in 19.6% of MRSA isolates, is nowadays the dominant clone in India [29, 30]. As the Southwest Pacific clone, this one is also PVL+ and MDR. Studies from Pakistan, Nepal, and Australia also reported the predominance of this clone [31–33] which has become endemic in these regions. The presence of this clone in Kabul may illustrate an epidemiological link between Afghanistan and the neighboring countries, especially India and Pakistan where many Afghan families travel for medical treatment, and many young students go to pursue their higher education. This clone appears to be an increasing public health threat in several countries in the region, as it can be encountered in hospital as well as in community settings [31]. One PVL+ isolate, collected in Kabul from a SSTI, belonged to the USA400 pulsotype, one of the first-described PVL+ CA-MRSA. This one was reported to cause fatal infections in healthy children in the late 1990s in the USA [34] and then sporadically described in Australia, Germany, and

the UK. Prior to this study, USA400 had not been reported in the neighboring countries such as Pakistan and Iran.

Two MRSA and one MSSA clones were identified within the CC22. This is a common and widespread clonal complex, and different MRSA lineages have emerged from this genetic background. The CC22-MRSA-IV TSST-1+ clone, detected in 19.6% of all MRSA isolates, has been mostly reported in Saudi Arabia and the Middle East region [25]. Its presence in Afghanistan could be related to the large movements of Afghans to Saudi Arabia for pilgrimage each year. Seven MRSA isolates were assigned to the CC22-MRSA-IV PVL+ . This clone has been described in Germany, Australia, England, Ireland, Hong Kong, and the United Arab Emirates [4, 35]. Both clones were reported in hospital-acquired infections from Pakistan as well [31].

Four different MRSA clones were identified within the CC8. Among them, the USA500 clone (CC8-MRSA-IV PVL-) was predominant. This clone has been identified in the USA, Australia, the UK, and Ireland, and sporadically in Germany [4, 36]. Interestingly, only one isolate was identified as the well-known USA300 (CC8-MRSA-IV PVL+) clone. This clone of CA-MRSA, mainly reported in the USA, was also reported in numerous countries in Europe or Asia such as Saudi Arabia, Qatar, and Pakistan [4, 25, 32, 37]. One isolate was also identified as belonging to the Vienna/Hungarian/Brazilian clone (ST239-MRSA-III). This HA-MRSA lineage is probably the oldest pandemic MRSA strain and has been reported in many European countries [4, 38], as well as in Asia including Saudi Arabia [25], Iran [27], and Pakistan [32].

The overall prevalence of PVL among *S. aureus* isolates collected in Kabul was 59.2%, these isolates belonging to 8 MRSA and 5 MSSA clones, with a higher prevalence in MRSA. This high prevalence and the diversity of PVL+ isolates must be highlighted and could be linked to the import of different strains via international exchanges. Contrary to other countries, we did not observe the predominance of one single CA-MRSA clone such as the USA300 in North America. In studies in neighboring countries, the prevalence of PVL was generally lower: from 0 to 12.7% in Turkey [39], from 7.4 to 55.6% in Iran according to the studies [40], and around 9% of *S. aureus* isolates responsible for community-acquired infections in West Bengal of India [41].

In conclusion, the prevalence of MRSA in *S. aureus* isolates circulating in Kabul was quite high (66.3%) when compared with the prevalence rates obtained from other similar studies conducted in neighboring countries. Most of *S. aureus* isolates, especially MRSA, were multidrug-resistant (generally resistant to beta-lactams, macrolides, quinolones, and cotrimoxazole and less often to aminoglycosides, chloramphenicol, and tetracyclines). This may be related to the overuse or the inappropriate use of antimicrobials which was recently reported in Afghanistan [14, 15]. A great diversity of *S. aureus* clones was brought to light using microarray analysis, with a predominance of the Southwest Pacific, the CC22-MRSA-IV TSST-1, and the Bengal Bay clones. This diversity and the clones identified reflect epidemiological links, including travels, migrations, commerce, and international military actions, with South West Pacific, Indian, and Middle Eastern/Arabian Gulf regions, as well as Western Europe, the USA, and Africa. In the future, major efforts have to be made on the monitoring, distribution, and spread of multidrug-resistant bacteria to control their diffusion and reduce the prevalence of MRSA in Afghanistan.

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Authors' contribution Frédéric Laurent and François Vandenesch were involved in the conception of the study, and in analysis and interpretation of data. Haji Mohammad Naimi was involved in conducting the study, statistically analyzing the data, and drafting the manuscript. Camille André was involved in antibiotic susceptibility testing. Céline Dupieux was involved in interpretation of data and revising and editing the manuscript. Michèle Bes, Anne Tristan, and Claude-Alexandre Gustave were involved in the interpretation of DNA microarray. Qand Agha Nazari conducted preliminary identifications of strains. All authors read and approved the final manuscript.

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Data Availability All relevant materials and data supporting the findings of this study are contained within the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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2. Second publication (Short note in preparation): "Molecular characterization and antimicrobial resistance of nasal *Staphylococcus aureus* in the community of Kabul

Introduction

S. aureus, particularly methicillin-resistant *S. aureus* (MRSA), is a major pathogen in both hospital- and community-associated infections. The nasal mucosa is the main *S. aureus* reservoir associated with transmission to other body sites and auto-infections, as well as transmission to other people (280). It has been reported that nasal carriers of *S. aureus* have an increased risk of being infected by this pathogen (281). About 20 to 30% of healthy individuals are persistent carriers of *S. aureus* for periods ranging from a few weeks to many years, and mostly in the anterior nares (282). MRSA nasal carriers among the university students who are in close contact with each other may spread MRSA to hospitalized patients, but also to their family members and to the community.

Recent study concerning *S. aureus* infections diagnosed in Kabul hospitals, highlighted a high rate of methicillin resistance (66.3 %) and presence of multiple virulence and antibiotic resistance genes in *S. aureus* isolates, but no study had been carried out in Afghan community on nasal carriage rate, antibiotic susceptibility patterns, and molecular characteristics of circulating *S. aureus* strains. The present study aimed to assess the nasal carriage rate, antibiotic susceptibility patterns, and associated risk factors of *S. aureus*/MRSA among university students, excluding medical faculties who are an active part of the community.

For this study, we chosed Kabul university students, because it is a large university and students come from different parts of Kabul area and other provinces and they can quite well represent the community of Afghanistan. On the other hand, it was easier to obtain nasal swabs from them after signing the consent form. Samples were taken randomly from the students of different non-medical faculties with no history of antibiotic consumption, hospitalization, admission to a nursing home, or surgery during the last 2 months. Standard microbiological procedures were conducted on nasal samples with minimum delay for culture; confirmatory tests and antibiotic susceptibility testing (AST) were performed in Kabul. The conserved strains were then brought to France for molecular analysis including detection of *mecA*/*mec*C genes, *agr* typing and DNA microarray for identification of clones.

Our finding showed that the nasal carriage rate of *S. aureus* in Kabul students was 33.3% (50/150), and 38% (19/50) of *S. aureus* isolates were identified as MRSA, which highlights a high prevalence

of MRSA nasal carriage compared to similar studies in the region. Various resistance genes were detected in the MRSA isolates including *blaZ*, *ermC*, *msrA*, *mphC*, *aacA-aphD*, *aadD*, *aphA3, sat*, *dfrA*, *tetK*, *tetEfflux*, and *fosB.* Regarding the molecular typing of the MRSA isolates, a wide clonal diversity was observed. CC22-MRSA-IV TSST-1-positive dominated and accounted for 63.2% (12/19) of the isolates. Isolates of this clone were resistant to multiple antibiotics, particularly penicillin, erythromycin, clindamycin, cotrimoxazole, and norfloxacin. This clone also represented 16.9% of HA-MRSA from clinical samples in recent study in Kabul. Out of the 7 MRSA clones isolated in this study, 5 clones accounted for 89.5% of MRSA, were also detected in HA-MRSA in Kabul.

In conclusion we found a high frequency of MRSA nasal carriage in students, and high rates of MRSA transmission between healthcare settings and community in Kabul. Individuals who are harboring these isolates can act as reservoirs and a risk factor for outbreak of MRSA infection. Supporting policies are needed to prevent the spread of MRSA clones between hospitals and community.

Molecular characterization and antimicrobial resistance of nasal *Staphylococcus aureus* in the community of Kabul

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Keywords: Nasal carriage, MRSA clones, antibiotic resistance, molecular typing, Kabul.

Abstract

Objectives: This study aimed to investigate the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) nasal carriage in the community among students at Kabul University and their molecular characteristics.

Methods: Nasal swabs were collected from anterior nares of 150 healthy non-medical students in Kabul. MRSA isolates were then confirmed by *mecA/mecC* PCR and characterized using DNA microarray.

Results: A total of 50 *S. aureus* strains were isolated from the anterior nares of the 150 participants. The prevalence of *S. aureus* and MRSA nasal carriage among Kabul students was 33.3% and 12.7% respectively. Thirty-seven (36.8%) of MRSA isolates and 25.8% of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates were resistant to at least 3 different antimicrobials tested. All MRSA isolates were susceptible to linezolid, rifampicin and fusidic acid. Four clonal complexes (CCs) along with 7 clones were identified. The most commonly identified clone was

CC22-MRSA-IV TSST-1-positive, which accounted for 63.2% (12/19) of MRSA isolates. The SCC*mec* typing showed that most MRSA strains harbored SCC*mec* type IV (94.7%). Sixty-eight (68.4%) of MRSA isolates carried the TSST-1 and 26.3% the PVL genes.

Conclusion: Our findings revealed the relatively high prevalence of MRSA nasal carriers in the community in Kabul, with the predominance of the CC22-MRSA-IV TSST-1-positive clone, and the presence of multidrug resistance (MDR) among these isolates.

1. Introduction

Since methicillin-resistant *Staphylococcus aureus* (MRSA) was first identified in 1961, it has been associated with higher mortality rates and increased lengths of hospital stays as well as associated health care costs [1]. MRSA infections are a serious risk for patients in healthcare settings and a challenge for public health, whereas infections due to community-acquired MRSA (CA-MRSA) seem to be increasing among people in different areas of the world [2]. The most frequent carriage site is anterior nares, which serves as a reservoir for the spread of the pathogen. Nasal carriage has been shown to play a key role in the pathogenesis of *S. aureus* infections in patients undergoing surgery, dialysis, and intensive care unit (ICU) patients [3]. Nasal carriage of *S. aureus* also constitutes a risk factor for the development of skin and soft tissue infections in non-hospitalized and non-diseased subjects [4]. MRSA strains that reside in the anterior nares serve as a reservoir for future infection [5]. In a systematic review, patients colonized with MRSA were four times more likely to develop invasive infection than patients colonized with MSSA [6]. Determination of the prevalence of *S. aureus* nasal carriage, as well as antimicrobial resistance profiles and molecular typing for nasal MRSA isolates in healthy population is beneficial for understanding the epidemiology of community-acquired infections, and supporting infection control measures.

In central Asia, including Afghanistan, studies of MRSA have mainly focused on the prevalence of MRSA, but few data are available about the circulating clones. A recent study concerning *S. aureus* infections diagnosed in Kabul hospitals highlighted a high rate of methicillin resistance (66.3 %) and the presence of multiple virulence and antibiotic resistance genes in *S. aureus* isolates with a predominance of CC22-MRSA-IV TSST-1-positive and ST772-MRSA-V PVL-positive clones [7]. However, no data are available about the prevalence of MRSA carriage and the clones circulating in the community.

The present study aimed to investigate the prevalence of *S. aureus*/MRSA nasal carriage, and the molecular characteristics of MRSA isolates in the community of Kabul through *S. aureus* nasal screening in non-medical students at Kaubul University.

2. Materials and Methods

2.1. Study population

A total of 150 healthy student volunteers from non-medical faculties of Kabul University were randomly enrolled in this study from March to July 2018. Informed consent was obtained from all individual participants included, according to Afghanistan ethical rules. Every student with a history of antibiotic consumption, hospitalization, admission to a nursing home, or surgery during the last 2 months were excluded.

2.2. Identification of *S. aureus* isolates and antimicrobial susceptibility testing

Samples were collected from both anterior nares of volunteers with sterile moistened swabs. Standard microbiological procedures were conducted on nasal swabs with minimum delay for culture. Samples were cultured for 18–24 hours at 37 °C onto blood agar base medium (Oxoid, Basingstoke, United Kingdom) supplemented with 5% sheep blood. Then presumptive *S. aureus* isolates were identified by carrying out confirmatory tests, consisting of Pastorex Staph Plus latex agglutination test (Bio-Rad, Marnes-la-Coquette, France) and coagulase assay.

Antimicrobial susceptibility testing (AST) was performed on Mueller Hinton agar (*Oxoid*) by Kirby Bauer disc diffusion method according to the 2019 guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [8]. The following antimicrobial agents were tested: penicillin G, cefoxitin (used to detect methicillin resistance), erythromycin, clindamycin, norfloxacin, kanamycin, tobramycin, gentamicin, cotrimoxazole, linezolid, fusidic acid, chloramphenicol, fosfomycin, and rifampicin.

2.3. Detection of *mec*A/*mec*C genes and *agr* typing by multiplex PCR

Cellular DNA was obtained from *S. aureus* colonies grown overnight on blood agar plates using DNA Extraction Kit (Promega, USA) in accordance with manufacturer's instructions. The detection of the *mecA* and *mecC* genes and *agr* typing were performed by multiplex PCR using primers already published [9-11].

2.4. Molecular characterization of strains by DNA microarray

The DNA microarray Identibac *S. aureus* genotyping® (Alere Technologies, Jena, Germany) was used as previously described [12]. This microarray allows the detection of 336 different target sequences corresponding to 185 genes and their allelic variants. The assignation of isolates to clonal complexes (CCs) was determined automatically with the Identibac software by comparison of the hybridization profiles to previously typed multi-locus sequence typing reference strains [12].

2.5. Statistical analysis

Statistical analysis was done using SPSS 21 (IBM Inc. Chicago). Chi-square test was used to compare the significance resistance profile between MRSA and MSSA. Binary logistic regression was used to determine the association between MRSA nasal carriage, gender and age. A *P-value* less than 0.05 was considered as statistically significant.

3. Results

During the study period, a total of 50 *S. aureus* isolates, of which 19 (38%) MRSA, were isolated from the anterior nares of the 150 volunteers included in the study. The nasal carriage rate of *S. aureus* was thus 33.3%. Out of the 50 *S. aureus* isolates, 36 (72.0%) were obtained from men and 14 (28.0%) from women. The mean age of carriers was 22 years ([19-38] y, median 22 y). Interestingly, 38% of *S. aureus* isolates (19/50) were identified as MRSA. Therefore the prevalence of nasal MRSA carriage among Kabul University students was 12.7% (19/150). The difference of *S. aureus*/MRSA distribution was not statistically significant according to gender (p=0.84) and age (p=0.18).

The antimicrobial resistance rates of MRSA, MSSA, and *S. aureus* isolates are presented in Table 1. The majority of *S. aureus* isolates were resistant to penicillin (96%). The rates of resistance for other antibiotics were 32% for erythromycin, 8% for clindamycin, 18% for norfloxacin, 30% for kanamycin, 22% for tobramycin, 6% for gentamicin, 18% for cotrimoxazole, 4% for fusidic acid, 2% for chloramphenicol, and 8% for fosfomycin. The resistance rates of MRSA isolates to common antimicrobials were not statistically significant than those of MSSA isolates (Table 1). Interestingly 36.8% (7/19) of MRSA isolates and 25.8% (8/31) of MSSA isolates were resistant to at least 3 classes of antibiotics. All MRSA isolates were susceptible to linezolid, rifampicin and fusidic acid.

Various resistance genes were detected in the 19 MRSA isolates. *blaZ, dfrA and tetK* were the most frequently detected genes in 89.5%, 52.6% and 47.4% of MRSA isolates, respectively. No strain harbored the resistance genes to fusidic acid (*fusB),* to chloramphenicol (*cat*, *fexA*), to linezolid (*cfr*), to mupirocin (*mupR*), and to vancomycin (*vanA).* Out of the 19 isolates phenotypically resistant to methicillin, all harbored the *mecA* gene and no isolate was positive for *mecC* (Table 2). Concerning the staphylococcal chromosomic cassette *mec* (SCC*mec*) element of these MRSA isolates, 18 (94.7%) harbored SCC*mec* type IV, and one (5.3%) harbored SCC*mec* type V.

Regarding the molecular typing of the MRSA isolates, a wide clonal diversity was observed. The 19 MRSA isolates belonged to four different clonal complexes (CCs) and were assigned to seven distinct clones. One CC (CC22) dominated and accounted for 79% of the isolates, and three other CCs (CC5, CC30, and CC88) accounted for 21% (Table 3). The most frequent detected clone was the CC22-MRSA-IV carrying the toxic shock syndrome toxin (TSST-1) gene, which accounted for 63.2% (12/19) of MRSA isolates. Three resistance genes were detected in these isolates: *blaZ* operon in 10/12 (83.3%), *dfrA* in 8/12 (66.7%), and *tetK* in 6/12 (50%). The *egc* cluster (enterotoxins *seg*, *sei*, *sem*, *sen*, *seo*, and *seu*) was detected in all these isolates. The second most frequent clone was the C22-MRSA-IV positive for the Panton-Valentine leukocidin genes, which accounted for 10.5% (2/19) of MRSA isolates. The 2 isolates of this clone were resistant to multiple antibiotics, particularly penicillin, kanamycin, tobramycin, gentamicin, and erythromycin and harbored various resistance genes: *blaZ* operon, *ermC*, *aacA-aphD*, and *dfrA* in all isolates, *aadD* in 1/2. The *egc* cluster was also detected in these 2 isolates (Table 3). Only single isolates of 5 other MRSA clones completed this list, which represented 26.5% of all MRSA isolates and comprised well-known clones such as the CC5-MRSA-IV TSST-1-positive Paediatric clone, the CC30-MRSA-IV PVL-positive Southwest Pacific clone and the CC22-MRSA-IV UK-EMRSA-15 Barnim clone (Table 3). Overall, 5/19 MRSA isolates (26.4%) were PVL-positive and 13/19 (68.4%) TSST-1-positive.

4. Discussion

This original study is the first molecular characterization of MRSA community isolates in Afghanistan and reports the various clones circulating among healthy individuals. In this study, a wide clonal diversity of MRSA was detected with 4 different clonal complexes, along with 7 distinct clones. Molecular analyses showed a predominance of one MRSA clone, representing 63.2% of the isolates: the CC22-MRSA-IV TSST-1-positive. The 6 other clones detected were

present only in 1 or 2 isolates. The results of the present study are consistent with our previous study about clinical isolates of MRSA in Kabul hospitals. Out of the 7 MRSA clones in this study, 5 clones that accounted for 89.5% of the isolates, were also reported in Kabul hospitals [7]. This suggests direct relation between community and hospital MRSA clones in Kabul.

The main limitation of this study is that only MRSA isolates collected from nasal carriers among Kabul university students were included. Therefore, the results do not reflect the global epidemiology of MRSA nasal carriage in Afghanistan. Nevertheless, students are a population without risk factors and the population in our study came from different parts of Afghanistan, therefore represent quite well the Afghan community. In addition, the diversity of strains highlighted by the typing results illustrates the position of Kabul as a center of intense international exchanges, as already described [7].

The prevalence of nasal colonization with *S. aureus* in our study was 33.3%, a higher prevalence than that observed (19.6%) among healthy students of medical and non-medical universities in Urmia, Iran [13]. However, the prevalence of nasal carriage of MRSA in our study (12.7%) was similar to this study (13.1%). Another study in China among medical students showed a *S. aureus* nasal colonization rate of 15.4% and a prevalence of MRSA carriage of 3% [14], which is lower than our findings. In our study, the frequency of MRSA nasal carriage was not significantly different between male and female students (p=0.84), which shows that gender may not be a risk factor for MRSA nasal colonization, while in a study in Iran, [13] authors reported that male was a risk factor for MRSA nasal colonization and argued that it may be related to personal hygiene especially by the female sex. As previousl reported, these data indicate that MRSA colonization varies significantly, from country to country. Put together, these results demonstrate that international data are needed to guide national public health demands.

In this study, three different MRSA clones were identified within CC22. This is a common and widespread clonal complex and different MRSA lineages have emerged from this genetic background. The CC22-MRSA-IV (TSST-1-positive) was the most prevalent clone, detected in 63.2% of all MRSA isolates. In a previous study about MRSA prevalence in clinical samples in Kabul health facilities, this clone accounted for 16.9% of MRSA isolates [7]. This strain is mostly reported in Saudi Arabia and the Middle East region [15]. Its presence in Afghanistan could be related to the large movement of Afghans to Saudi Arabia for pilgrimage each year. The CC22-MRSA-IV PVL-positive clone was detected in 2 MRSA isolates. In our previous study in Kabul, this clone accounted for 7.7% of MRSA clinical isolates [7]. This clone has also been described in

clinical samples from Germany, Australia, England, Ireland, Hong Kong and the United Arab Emirates [16]. One isolate (5.3%) belonged to the CC22-MRSA-IV UK-EMRSA-15 Barnim clone; this is a common and pandemic strain of MRSA that has been found mainly in Western Europe, but also in other parts of the world including some Gulf countries [17]. Two MRSA clones were identified within CC30: the CC30-MRSA-IV PVL-positive Southwest Pacific clone, and the CC30-MRSA-V/VT PVL-positive clone. These lineages were also reported from Iran, United Arab Emirates, Saudi Arabia, Taiwan, Egypt and Europe, both from the community and hospitals [15,16].

One MRSA clone was identified within CC5, the CC5-MRSA-IV TSST-1-positive Paediatric clone. This MDR clone which also harbored the epidermal cell differentiation inhibitor gene (*edinA*) was first reported in hospitals in neonatal patients in Portugal [18], neonates and adults in Brazil [19] and Turkey [20].

One clone was identified within CC88, the CC88-MRSA-IV PVL-positive, which harbored enterotoxin genes and resistance genes only to penicillins and tetracyclines. This clone was reported in healthcare-associated infections in Afghanistan [7], Saudi Arabia [15] and Kuwait [21].

In this study, PVL genes were found in 26.3% of MRSA isolates (n=5), which is similar to another study among healthy adults in Iran [22]. The transmission of such virulent strains in the universities and other crowded places where people are often in close physical contact with each other can contribute to the transmission and the spread of these strains among people.

5. Conclusions

The relatively high frequency of MRSA nasal carriage, especially the virulent TSST-1 and PVL positive clones, among healthy population in Kabul is a reason for concern, since individuals who harbor these isolates can act as reservoirs for outbreak of MRSA infection. Supporting policies for preventing the hospital-community spread of MRSA, and reducing nasal carriage with continued efforts to enhance hygiene among students are necessary.

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Table 1. Antimicrobial resistance rates of *S. aureus*, MRSA and MSSA nasal isolates among students in Kabul, Afghanistan, between March-July 2018 (n=50).

Antibiotic	*S. aureus* (n=50)	MRSA (n=19)	MSSA (n=31)	*P value*
Penicillin G	96	100	93.5	0.26
Erythromycin	32	36.8	29	0.57
Clindamycin	8	10.5	6.5	0.61
Norfloxacin	18	26.3	12.9	0.23
Gentamicin	6	10.5	3.2	0.29
Kanamycin	30	26.3	32.3	0.66
Tobramycin	22	10.5	29	0.13
Cotrimoxazole	18	15.8	19.4	0.75
Linezolid	0	0	0	-
Fusidic acid	4	0	6.5	0.26
Chloramphenicol	2	5.3	0	0.2
Fosfomycin	8	5.3	9.7	0.58
Rifampicin	0	0	0	-

Table 2. Antimicrobial resistance genes identified in MRSA isolates from nasal carriers among students in Kabul, Afghanistan, between March-July 2018 (n=19).

ATB Group	Gene	All isolates n (%)
Beta-lactam resistance	*bla*Z	17 (89.5)
	*mec*A	19 (100)
	*mec*C	0 (0)
Macrolide/lincosamide resistance	*erm*A	0 (0)
	*erm*B	0 (0)
	*erm*C	2 (10.5)
	*lnu*A	0 (0)
	*msr*A	3 (15.8)
	*mph*C	3 (15.8)
Aminoglycoside resistance	*aac*A-*aph*D	2 (10.5)
	*aad*D	1 (5.3)
	*aph*A3	3 (15.8)
	Sat	3 (15.8)
Cotrimoxazole resistance	*dfr*A	10 (52.6)
Fusidic acid resistance	*fus*B	0 (0)
Tetracycline resistance	*tet*K	9 (21.1)
	*tet*M	0 (0)
	*tet*Efflux	4 (81.6)
Chloramphenicol resistance	*Cat*	0 (0)
	Cfr	0 (0)
	*fex*A	0 (0)
Mupirocin resistance	*mup*R	0 (0)
Fosfomycin resistance	*fos*B	3 (15.8)
Vancomycin resistance	*van*A	0 (0)

Table 3. Distribution and characteristics of the 19 MRSA isolates collected from anterior nares of Kabul University students between March-July 2018 (n=19).

CC (number of isolates)	Clone assignment (Alere)[a]	n	% of isolates	*agr* type	PVL	TSST	Enterotoxin genes	Antibiotic resistance genes	Antibiotic resistance[b]
CC5 (1)	CC5-MRSA-IV TSST-1+[c], Paediatric clone	1	5.3	2	Neg	Pos	*sec, seg, sei, sel, sem, sen, seo, seu*	*blaZ, msrA, mphC, aphA3, sat, fosB, tetEfflux*	Pen, Fox, Kan, Ery
CC22 (15)	CC22-MRSA-IV (FNBB+)[d], UK-EMRSA-15/Barnim EMRSA-like MRSA	1	5.3	1	Neg	Neg	*seg, sei,sem, sen, seo, seu*	*blaZ, tetK*	Pen, Fox, Nor
	CC22-MRSA-IV PVL+[e]	2	10.5	1	Pos	Neg	*seg, sei,sem, sen, seo, seu*	*blaZ, ermC, aacA-aphD, aadD(1/2), dfrA*	Pen, Fox, Kan, Tob, Gen, Ery, Nor (1/2), Fos (1/2)
	CC22-MRSA-IV TSST-1+	12	63.2	1	Neg	Pos	*seg, sei,sem, sen, seo, seu*	*blaZ(10/12), dfrA(8/12), tetK(6/12)*	Pen, Fox, Ery (2/12), Cli (1/12), Nor (1/12), Cot (2/12),
CC30 (2)	CC30-MRSA-IV PVL+, Southwest Pacific Clone	1	5.3	3	Pos	Neg	*seg, sei,sem, sen, seo, seu*	*blaz, msrA, mphC, aphA3, sat, fosB, tetEfflux*	Pen, Fox, Kan, Ery, Clin, Nor, Chl
	CC30-MRSA-V/VT PVL+, WA MRSA-124	1	5.3	3	Pos	Neg	*seg, sei,sem, sen, seo, seu*	*blaZ, msrA, mphC, aphA3, sat, tetK, fosB, tetEfflux*	Pen, Fox, Kan, Ery, Nor, Cot
CC88 (1)	CC88-MRSA-IV PVL+	1	5.3	3	Pos	Neg	*sep, sek, seq*	*blaZ, tetK, tetEfflux*	Pen, Fox
Total		19	100.0						

[a] MLST and clone assignments correspond to the comparison of the hybridization profiles on the DNA microarray to previously typed MLST reference strains [9].

[b] Pen, penicillin; Fox, cefoxitin; Kan, kanamycin; Ery, erythromycin; Nor, norfloxacin; Tob, tobramycin; Gen, gentamicin; Fos, fosfomycin; Cli, clindamycin; Cot, cotrimoxazole; Chl, chloramphenicol.

[c] Toxic shock syndrome toxin 1; [d] Fibronectin-binding protein B; [e] Panton-Valentine Leukocidin

3. Third publication (Mansuscript in preparation): "Characterization and antimicrobial resistance patterns of *Staphylococcus aureus* strains isolated from livestock in Kabul"

Introduction

S. aureus causes various infections in economically important livestock animals. One of these infections is intramammary infection of dairy cows, which leads to mastitis a major economic burden on the global dairy industry (283). In addition subclinical mastitis or *S. aureus* colonizing the udder is of major concern, because the enterotoxin-producing *S. aureus* strains are associated with food poisoning in humans (284). Many studies showed that the great majority of the livestock isolates of *S. aureus* including MRSA belong to small number of animal-associated clones and may be transmitted to humans and cause human infection or colonisation (43,57,138,285).

As mentioned in the first and second publications, the human infection and colonization with MRSA in Kabul was significant with prevalence of 66.3% and 12.7% respectively. The presence of multiple virulence and antibiotic resistance genes was also prominent in these isolates. More importantly, both studies evidenced a high rate of MRSA transmission between hospitals and community in Kabul.

Up to now, no study has been conducted in regard to *S. aureus* strains of animal origin in Afghanistan.The main purpose of the present study was to investigate the prevalence, molecular characteristics and antibiotic resistance profiles of *S. aureus* isolates from bovine mastitis, as well as sheep nasal carriage in Kabul.

For conducting this study, we chosed the Kabul veterinary clinic, which is a main center for diagnosis and treatment of animals in Kabul, and three big livestock markets that sell sheep to the custormers. Seventeen *S. aureus* strains isolated from bovine mastitis during five months in the veterinary clinic of Kabul, and 189 nasal samples taken randomly from sheep in three different livestock markets in Kabul were collected for this study. Standard microbiological procedures were conducted for primary identification of the strains, followed by confirmatory tests and antibiotic susceptibility testing (AST) in Kabul. The conserved strains were then brought to France for molecular analysis including detection of *mecA*/*mec*C genes, *agr* typing, *spa* typing, and DNA microarray for identification of their gentoypes.

Our result showed that the percentage of MRSA among *S. aureus* clinical isolates obtained from bovine mastitis was 11.8% (2/17). One MRSA isolate was resistant to various antibiotics including penicillin, cefoxitin, cefovecin, kanamycin, tetracycline, erythromycin and enrofloxacin. Thirty five percent (6/17) of the *S. aureus* isolates from bovine mastitis were multi-drug resistant with a high rate of resistance to tetracycline. The presence of PVL, TSST-1, and enterotoxin genes in *S. aureus* isolates obtained from boving mastitis were 35% (6/17), 6% (1/17), and 53% (9/17) respectively. The predominant *spa* type was t021, CC30 (35%; 6/17). The prevalence of *S. aures* in sheep nasal carriage was low 1.1% (2/189), and both *S. aureus* isolates were MSSA. They belonged to *spa* type t5428 and harbored TSST-1, LukM, and two enterotoxin genes.

In conclusion we found the presence of MRSA and enterotoxigenic *S. aureus* strains among the isolates from bovine mastitis and evidence the sharing of MRSA clones in human and livestock. This highlights the importance of setting up efective preventive measures to control the spread of MDR and virulent strains between human and animals and also proper control of dairy products.

Characterization and antimicrobial resistance pattern of *Staphylococcus aureus* strains isolated from livestock in Kabul

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Keyword: *S. aureus,* bovine mastitis, sheep, molecular typing, Kabul.

Highlights

- *S. aureus* isolates from bovine mastitis exhibited high resistance to tetracycline
- MRSA and a considerable number of enterotoxigenic *S. aureus* were present
- There was evidence of spread of MRSA strains between human and livestock

ABSTRACT

Objectives: The aim of this study was to investigate the epidemiology of *Staphylococcus aureus* in livestock in Kabul by determining the prevalence of methicillin-resistant *S. aureus*

(MRSA) in bovine mastitis and nasal carriage in sheep, and characterizing the *S. aureus* strains isolated in these animals.

Methods: A total of 17 *S. aureus* isolates from bovine mastitis obtained from the clinical specimens in veterinary clinic of Kabul and 2 *S. aureus* isolates from nasal carriage in sheep, obtained from livestock markets in Kabul were included in this study. All strains were subjected to antimicrobial susceptibility testing by disc diffusion and molecular typing by *mecA/mecC* PCR, multiplex PCR targeting toxin genes and *spa* typing.

Results: The 17 bovine mastitis isolates belonged to 9 *spa*-types, among which 2 of them were MRSA. Fifty-three percent (9/17) of the isolates harbored one or more enterotoxin genes. Interestingly six isolates were PVL-positive and three isolates were TSST-1-positive. The prevalence of *S. aureus* nasal carriage in sheep was low 1.1% (2 /189, both harboring TSST-1).

Conclusion: The genetic diversity of the isolates and many strains carrying enterotoxin genes is of great concern from public health point of view, because such toxins are associated with human infection or food poisoning cases.

Keywords: *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), bovine mastitis, *spa* typing, Kabul

1. Background

In addition to being a notorious human pathogen, *S. aureus* causes an array of infections in economically important livestock animals including cows, sheep, goats, poultry and rabbits. For example, intramammary infection of dairy cows leading to mastitis is a major economic burden on the global dairy industry [1]. In addition, subclinical mastitis or *S. aureus* colonizing the udder are also worrisom, because the enterotoxin-producing *S. aureus* strains are associated with food poisoning due to production in foodstuffs of the enterotoxins that are heat-stable, and resistant to proteases and environmental conditions [2]. Many studies have shown that the vast majority of *S. aureus* isolates from livestock belong to only small number of animal-associated clones and can be sporadically transmitted to humans [3]. In the recent years, MRSA has been reported with increased frequency from a wide variety of domestic animals including cows, pigs and chickens (4). The potential for animals to act as a source of *S. aureus* zoonotic infections for humans is illustrated by recent descriptions of human infections caused by LA-MRSA [5,6]. In addition, recent evidence implies that some other livestock-associated *S. aureus*

strains may have the capacity to colonise humans [7], and conversely, humans represent a source of new pathogenic strains affecting livestock [8,9]. The most common clonal complex (CC) in bovine mastitis across herds and countries are CC97, CC126, CC133, CC151, CC479 and CC771 [10]. A small proportion of bovine isolates, however, can be grouped with human clones, *e.g.,* CC8, CC20 and CC25, demonstrating that host-association is not absolute [10].
As most livestock in Afghanistan are reared in backyard systems with more direct contact with human than the intensive farming, the possibility and opportunity of cross-transmission cannot be ruled out. The supply of unpasteurized milk directly from farmers into the food chain also increases the occurrence of milk-borne staphylococcal infections or intoxications. The purpose of the present study was to investigate the molecular characteristics of *S. aureus* isolates from bovine mastitis, as well as their antimicrobial resistance profiles, and to determine the frequency of nasal carriage of *S. aureus* among healthy sheep in Kabul livestock markets.

2. Material and Methods

A total of 17 clinical *S. aureus* isolates from bovine mastitis isolated between March and July 2018 in Kabul veterinary clinic. In addition, *S. aureus* nasal screening were performed by obtaining 189 nasal swabs from sheep in 3 different livestock markets in Kabul.

2.1. Isolation and identification of *S. aureus*

Sheep nasal swabs were inoculated in Tryptic soy broth (TSB) (Merck, Germany) at 4°C prior to laboratory analysis. The next day swabs were inoculated onto mannitol salt agar (Oxoid, England) and blood agar plates (blood agar base medium supplemented with 5% sheep blood; Oxoid) and incubated at 35°C for 18-24 hours. Presumptive *S. aureus* isolates were sub-cultured for 18–24 hours at 37ºC onto blood agar, and identification confirmation was carried out by performing Pastorex Staph Plus (Bio-Rad, Marnes-la-Coquette, France) in Kabul Afghanistan, and subsequently by Maldi-Tof in Lyon France.

2.2. Antibiotic susceptibility testing (AST)

AST was performed on Mueller Hinton agar (Oxoid) by Kirby Bauer disc diffusion method according to the 2020 veterinary guidelines of the EUCAST subcommittee on Antimicrobial Susceptibility Testing (VetCAST) [11]. The following antimicrobial agents were tested: penicillin G, cefoxitin and cefovecin (used to detect methicillin resistance), kanamycin,

tobramycin, gentamicin, chloramphenicol, florfenicol, tetracycline, tigecycline, erythromycin, spiramycin, lincomycin, fusidic acid, enrofloxacin, and linezolid.

2.3. PCR based detection of *mecA/mecC* and *spa* types

Detection of the *mecA* and *mecC* genes was performed by multiplex PCR using primers already published [12–14]. *spa* typing was performed by the amplification of the x region of the *spa* gene by PCR, and *spa*-types were assigned by Ridom SpaServer (http://spaserver.ridom.de/staphtype/spa_sequencing.shtml). Detection of toxins was performed by PCR using primers as already described [15,16].

The two MRSA isolates were further characterized by the DNA microarray Identibac *S. aureus* genotyping® (Alere Technologies, Jena, Germany) as previously described [17].

3. Results

The percentage of MRSA obtained from bovine mastitis was 11.8% (2/17). Both MRSA carried *mecA* gene. The first MRSA strain was resistant to various antibiotics including beta-lactams, kanamycin, tetracycline, erythromycin and enrofloxacin; while the second was only resistant to beta-lactams. Out of the 15 MSSA strains isolated from bovine mastitis, 9 (60.0%) were resistant to penicillin, 1 (6.7%) to kanamycin, 1 (6.7%) to tobramycin, 5 (33.3%) to tetracycline, 2 (13.3%) to erythromycin, 1 (6.7 %) to lincomycin, and 2 (13.3%) to enrofloxacin. Four isolates (26.7%) were considered as multi-drug resistant. One MRSA isolate carried enterotoxin gene cluster (*egc*) (*seg, sei, sem, sen, seo and seu*), while the other carried only one enterotoxin gene (*sep*). One MRSA isolate was PVL-positive. In MSSA isolates the PVL gene was present in 33.3% (5/15), TSST-1 gene in 6.7% (1/15), and enterotoxin genes in 46.7% (7/15). Five MSSA isolates carried *egc* (*sem, seu*), and two MSSA isolates carried *egc* (*sed, sem, seo, ser*) (Table 1). The 2 MRSA isolates from bovine mastitis belonged to CC30-MRSA-V/VT and CC88-MRSA-IV PVL-positive clones and of two different *spa* types t021, t6769, respectively. The 15 MSSA isolates belonged to 7 different *spa* types, of which the *spa* type t021 was the most prevalent (5/15; 33.3%), followed by t267 (3/15; 20.0%). Five other *spa* types (t084, t131, t254, t701, and t3750) accounted for 33.3%, and two (13.3%) of them were non-*spa*-typeable.

Out of the 189 nasal swabs from sheep, only 2 *S. aureus* isolates were identified (carriage 1.1%). Both isolates were MSSA and susceptible to all of the antibiotics tested in this study. They belonged to *spa* type t5428 and harbored TSST-1, LukM, and *egc* cluster (*sec, sel*).

4. Discussion

Despite a small collection of *S. aureus* isolates from bovine mastitis and sheep nasal swabs tested in our study, which may not represent the global population of *S. aureus* circulating in dairy herds of Afghanistan, this report provides for the first-time information on the existence of multidrug resistant and virulent clones in livestock in Afghanistan. The percentage of MRSA among *S. aureus* isolates from bovine mastitis was 11.8%, which was slightly higher than in similar studies in Afghanistan's neighboring countries such as China: 7.1% [18], and Iran: 5% [19]; however due to low number of *S. aureus* isolates included in this study, it should be confirmed by further studies, and by including a high number of *S. aureus* isolates from bovine mastitis in Kabul. In the present study a high frequency of resistance was observed among *S. aureus* isolates from bovine mastitis especially for tetracycline; this is likely related to the widespread use of tetracyclines in animal husbandry in Afghanistan, which likely select and promote the dissemination of resistant strains in animals, and humans in contact with animals. The two MRSA clones identified (C88-MRSA-IV PVL-positive, *spa* type t6769; CC30-MRSA-V/VT, *spa* type t021) had been already identified in human HA-infection and colonization in our previous studies in Kabul. Close contact between human and livestock (especially dairy cattle) likely explain the transmission of MRSA between human and animals and may explain why this MRSA clone was detected both in humans and animals.

We identified a very low nasal carriage (1.1%) rate of *S. aureus* in sheep in Kabul. No data about *S. aureus* carriage in sheep in Afghanistan was available before the present study, neither at the local nor at the national scale. In a report from Iran, a higher rate (14.1%) of *S. aureus* sheep nasal carriage was reported and all of them were MSSA [21]. The sheep studied herein mostly belonged to nomads, who sell their livestock in animal markets in Kabul and other Afghanistan's provinces. Their livestock graze in the mountains and deserts, and have less contact with other animals and humans. This could explain why the nasal carriage rate of *S. aureus* among sheep in our study was very low. However, these data should be confirmed by further studies in livestock farms and such studies in other provinces of Afghanistan.

5. Conclusions

These data show that MRSA and enterotoxigenic *S. aureus* strains are present among the isolates responsible for bovine mastitis in Kabul. In order to protect consumers' health, the multiresistance and the virulence profiles in MRSA but also in some MSSA highlights the

importance of proper control of dairy products. Efforts have to be made in the responsible use of antimicrobials, surveillance, prevention and control of the spread of antibiotic-resistant or virulent bacteria from animals to humans.

Declarations

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Ethical Approval: Not applicable

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Table 1. Distribution and characteristics of 19 *S. aureus* isolates obtained from bovine mastitis and sheep nasal carriage in Kabul.

Animal source	Sample	*spa* type	Clonal complex associated with spa-type	Number of isolates	% of isolates	*mecA*	LukM	PVL	TSST-1	Enterotoxin genes	Antibiotic resistance*
Cow	Milk	t021	CC30	1	5.3	Pos	Neg	Neg	Neg	*seg, sei,sem, sen, seo, seu*	Pen, Fox, Cef, Kan, Tet, Ery, Enr
Cow	Milk	t6769	CC88	1	5.3	Pos	Neg	Pos	Neg	*Sep*	Pen, Fox, Cef
Cow	Milk	t021	CC30	5	26.3	Neg	Neg	Pos	Neg	*sem, seo*	None
Cow	Milk	t084	CC15	1	5.3	Neg	Neg	Neg	Neg	Neg	Pen, Tet
Cow	Milk	t131	CC97	1	5.3	Neg	Neg	Neg	Neg	*Neg*	Pen, Tet
Cow	Milk	t254	CC15	1	5.3	Neg	Neg	Neg	Neg	*Neg*	Pen, Kan, Tob, Tet, Lin
Cow	Milk	t267	CC97	3	15.8	Neg	Neg	Neg	Neg	*Neg*	Pen (2/3), Tet (2/3)
Cow	Milk	t3750	Unknown	1	5.3	Neg	Neg	Neg	Neg	*Neg*	Pen
Cow	Milk	t701	CC6	1	5.3	Neg	Neg	Neg	Pos	*Neg*	Pen
Cow	Milk	Unknown	Unknown	2	10.5	Neg	Neg	Neg	Neg	*sed, sem, seo, ser*	Pen, Ery, Enr
Sheep	nasal swab	t5428	Unknown	2	10.5	Neg	Pos	Neg	Pos	*sec, sel*	None

* Pen: penicillin; Fox: cefoxitin; Cef: cefovecin; Kan: kanamycin; Tob: tobramycin; Tet: tetracycline; Ery: erythromycin; Enr: enrofloxacin; Lin: lincomycin.

PVL: Panton valentine leukocidin; TSST-1: Toxic shock syndrome toxin-1

4. Conjugative transfer of *cfr*-carrying plasmids from CoNS to *S. aureus* strains

4.1. Introduction

Antibiotic resistance is one of the biggest public health challenges of our time. In this regard, the World Health Organization (WHO) has recently published a list of "high priority" bacteria, including bacteria belonging to the species *S. aureus* and in particular MRSA, for which new antibiotics are needed and there is an urgent need to control the emergence and spread of resistance (286). Most *S. aureus* strains contain different types of MGEs, including plasmids, transposons, insertion sequences, lysogenic bacteriophages, and pathogenicity islands, that facilitate the acquisition of genes encoding mechanisms of resistance against antimicrobials, biocides, and heavy metals (287).

Oxazolidinones, as the most recent family of anti-staphylococcal antibiotics, are one of the last resorts for the treatment of MRSA infections; linezolid is the most widely used antibiotic in this family. It acts as a protein synthesis inhibitor by binding to the ribosomal PTC on the bacterial 23S ribosomal RNA (rRNA), thereby inhibiting transition of the aminoacyl-tRNA to the A site and protein synthesis by preventing formation of the fMet-tRNA-30S ribosome-mRNA initiation complex (179). Unfortunately, its use is currently on the rise, especially since generic forms are available, and has been accompanied by the emergence of resistances (18,288). Resistance in *S. aureus* as well as CoNS is most often caused by a G-to-T substitution at position 2576 of the 23S rRNA (289). Other mutations in the 23S rRNA (G2447T, T2500A, and C2534T) have been identified in clinical and laboratory-derived staphylococcal isolates (290,291), as well as mutations in the L3, L4, and L22 ribosomal proteins (292,293). Another important mechanism involves the acquisition of the plasmidic *cfr* gene (chloramphenicol/florfenicol resistance), which encodes an adenine methyltransferase that modifies adenosine at position 2503 in the 23S rRNA (294). Its ability to be transmitted between different strains or species represents a global concern. At the moment the emergence of resistance is worrisome. Linezolid remains active against >98% of staphylococcal isolates, with resistance identified in 0.05% of *S. aureus* and 1.4% of CoNS (183).

The *cfr*-positive CoNS are increasingly isolated in hospitals probably due to high consumption of linezolid followed by spread and dissemination. The *cfr* gene has been detected in clinical staphylococcal isolates related to hospital outbreaks in different countries of Europe and in the

USA (186,189,208,292). Linezolid resistance mediated by the *cfr* gene represents a global concern due to its dissemination among multi-resistant nosocomial pathogens such as MRSA and *Enterococcus*. CoNS, in particular *S. epidermidis*, have been largely considered to act as a genetic reservoir for other pathogenic bacteria (184). Recently there have been several reports of the acquisition of plasmids that encode the *cfr* resistance in MRSA, including the pandemic ST22-MRSA-IV clone (295). *cfr*-positive plasmids acquisition may most often happens by conjugation, which is a highly evolved and efficient mechanism facilitating DNA transfer in bacteria (212). At the moment, it is pivotal to determine the risk of conjugative dissemination of *cfr* among clinical isolates to understand and prevent the spread of this resistance. Until now, only few *in vitro* studies have determined the frequency of *cfr* transmission to staphylococci based on conjugative transfer by filter mating which are describe below.

Cafini *et al.,* evaluated the conjugative transmission of *cfr*-positive *pSCFS7*-like plasmids (40 kb) from two *S. epidermidis* ST2 strains (SE45 and SE50) isolated in Spanish hospitals, to clinical MRSA and *Enterococcus* spp. (184). They showed that the SE45 strain was able to transfer the *cfr* gene to all strains tested with a mean frequency of 8.57×10^{-5} transconjugants per recipient cell, while transmission from SE50 was observed only to a few strains and with less efficacy. Of note, no transmission was observed to *Enterococcus spp*. isolates.

In a study by Dexi Li *et al.*, the authors isolated a multi resistant plasmid *pWo28-3* (60.6 kb) in *S. sciuri* harboring *cfr* and *optrA* genes. The conjugative transfer of this plasmid from *S. sciuri* as the donor to *S. aureus* RN4220 and *E. faecalis* JH2-2 as the recipients failed (200).

In an epidemiological study, Laurent Dortet *et al.*, during long term outbreak of LR-CoNS in a tertiary care hospital in France, found 7% (9/130) of *S. epidermidis* isolates harboring *cfr*-positive plasmids namely *p-cfr-PBR-A* (38.8 kb) and *p-cfr-PBR-B* (40.2 kb). They showed that conjugative transfer of both plasmids from *S. aureus* ST2 and ST5 as donors to a wild type of *S. capitis* (*S. capitis* DAM)) as recipient, were successful (208).

In Italy Andrea Brenciani *et al.*, evaluated the transferability of two *cfr*-positive plasmids namely: *pSP01* (76.9 kb), and *pSP01.1* (49 kb) based on filter-mating experiments; both plasmids were successfully transferred from two strains of *S. epidermidis* donors to a *S. aureus* strain with a conjugation frequency, 2.8×10^{-8}, and 4.5×10^{-6} respectively, but not to *E. faecalis* JH2-2 (127).

Similarly, the transfer capabilities of plasmid *p12-00322* (38.9 kb) was assessed by filter-mating with a *S. epidermidis* strain as a donor, to two clinical strains of *S. epidermidis* and *Enterococcus*

faecalis OG1RF as recipients; the transfer of *cfr* gene was successful with a frequency of 2.8 x 10^{-7} and 6.4 x 10^{-7} transconjugants per donor cell, respectively (186).

Lastly, a preliminary work conducted in the team "Pathogénie des staphylocoques" Centre International de Recherche en Infectiologie, Lyon France (unpublished data), focused on conjugative transfer of the same *pSA737* (39.3 kb) plasmid carrying *cfr* gene from *S. aureus* of three different genetic backgrounds (CC8-MSSA, ST8-MRSA-IV Lyon Clone, ST72-MSSA) and one *S. epidermidis* ST2 strain (isolated from University hospital of Nantes) to 9 most prevalent clones of linezolid-susceptible *S. aureus* circulating in France. The objective of this study was to determine wether certain clones were more capable to transfer the plasmid or wether certain recipient clones were more competent to acquire this plasmid. The results showed that all 4 donor strains transferred successfully the *cfr*-carrying plasmid to ST8-MRSA-IV USA300 and CC398-MRSA-X (Table 3). Conversely, for the recipient strains ST72-MSSA, CC398-MSSA, CC30-MSSA and CC80-MRSA-IV, no transmission was observed, regardless of the donor strains. Finally, some recipient strains accepted transfer of the *cfr*-carrying plasmid only from certain donor strains and not from others. The plasmid transfer rate was largely variable from 10^{-5} to 10^{-9}. Finally, the recipient strain ST8-MRSA-IV USA300 showed the highest conjugation frequency (around 10^{-5}) with all donor strains.

Table 3. Conjugative plasmid transfer rate from the 3 *S. aureus* and 1 *S. epidermidis* strains resistant to linezolid (*cfr*-positive) isolated from patients in the University hospital of Nantes as donors, and 9 circulating *S. aureus* clones in France as recipients.

Souches donneuses *cfr* +		Clones souches receveuses								
Souche donneuse	Espèce	ST72 SASM	ST8-MRSA-IV USA300	CC398-MRSA-X	CC8-MRSA-IV Lyon Clone	CC22-MRSA-IV	CC5-MRSA-IV	CC30-MSSA	CC80-MRSA-IV	CC398-MSSA
ST20162102	CC8 MSSA	0	$6{,}3\ 10^{-5}$	$1{,}9\ 10^{-5}$	$2{,}7\ 10^{-8}$	$3{,}1\ 10^{-8}$	$8\ 10^{-9}$	0	0	0
ST20161992	ST8-MRSA-IV Lyon Clone	0	$5\ 10^{-5}$	$2{,}5\ 10^{-5}$	0	$1{,}2\ 10^{-7}$	0	0	0	0
ST20151386	ST72 MSSA	0	$3{,}5\ 10^{-5}$	$5{,}6\ 10^{-6}$	$1{,}2\ 10^{-5}$	$9{,}1\ 10^{-7}$	0	0	0	0
ST20161991	*S.epidermidis* ST2	0	$1\ 10^{-5}$	$7{,}3\ 10^{-6}$	$1{,}3\ 10^{-5}$	0	$4\ 10^{-8}$	0	0	0

This part of our work was the continuation of the above study and aimed to determine the risk of linezolid resistance spreading mediated by the *cfr* gene in French isolates. We investigated (i) the capability of *S. epidermidis* strains to transfer different *cfr*-carrying plasmids to *S. aureus* strains with different genetic backgrounds, (ii) the comparative ability of *S. aureus* clones to receive the *cfr*-carrying plasmids, (iii) the transmission capability of each plasmid via conjugation, and (iv) the stability and fitness cost of these plasmids in bacterial host. The objective is to know if certain *S. epidermidis* clones or strains circulating in France, acting as *cfr*-carrying plasmid reservoir, are more able to transfer the linezolid resistance or certain recipient clones of *S. aureus* circulating in France are more at risk to acquire this resistance from *S. epidermidis*; if certain *cfr*-carrying plasmid are more transmissible. Finally, we planned to evaluate the fitness cost imposed by these plasmids in various *S. aureus* clones circulating in France, and the stability of these plasmids in various *S. epidermids* and *S. aureus* genetic backgrounds.

4.2. Materials and and method

4.2.1. Conjugative transfer of *cfr*-carrying plasmids from *S. epidermidis* strains to various clones of *S. aureus* circulating in France

Selection of strains

Five LRSE clinical isolates carrying different *cfr*-positive plasmids were selected for this study:
(1) A *S. epidermidis* isolate (ST2014-0255) belonging to ST2 and carrying *p12-02300* plasmid isolated in the university hospital of Strasbourg in 2014
(2) A *S. epidermidis* isolate (ST2016-1991) belonging to ST2 and carrying *pSA737* plasmid isolated in the university hospital of Nantes in 2015
(3) A *S. epidermidis* isolate (ST2018-0691) belonging to ST5 and carrying *p-cfr-PBR-B* plasmid isolated in a tertiary care university teaching hospital in southern suburb of Paris in 2010
(4) A *S. epidermidis* isolate (ST2018-0678) belonging to ST2 and carrying *p-cfr-PBR-B* plasmid); isolated in tertiary care university teaching hospital in Paris in 2010
(5) A *S. epidermidis* isolate (ST2018-0680) belonging to ST2 and carrying p-*cfr-PBR-A* plasmid isolated in tertiary care university teaching hospital in Paris in 2010
The recipient strains selected, belonged to nine clones of *S. aureus* with various genetic backgrounds circulating in France: ST8-MRSA-IV USA300 (ST2018-0363), CC5-MRSA-IV (ST2017-1473), CC398-MRSA-X (ST2011-2959), CC80-MRSA-IV (ST2011-0372), CC8-MRSA-IV Lyon clone (ST2017-0975), CC22-MRSA-IV (ST2018-0136), ST72-MSSA (ST2018-0281), CC398-MSSA (ST2017-0060), and CC30-MSSA (ST2017-0008).
In order to separate the donor and recipient cells from transconjugants during the conjugation experiments, their susceptibility and resistance profile were used as a choice criterion to be able to select it with selective media containing antibiotics (please see below). All the donor strains were *cfr*-positive and so resistant to linezolid/chloramphenicol (cross-resistance) but susceptible to tetracycline or erythromycin. Conversely all recipient strains were suceptible to linezolid/chloramphenicol but resistant to tetracycline or erythromycin.

4.2.2. Study of the transmission capability of different *cfr*-carrying plasmids

Selection of strains

In order to evaluate the transmission rate of each *cfr*-carrying plasmid, all the plasmids studied have been transferred to the same *S. epidermidis* strain (ST2020-0560) to obtain transconjugant derivatives in the same genetic background, but harboring a panel of *cfr*-positive plasmids. For conducting this experiment, all five strains of *S. epidermidis* carrying *cfr* plasmids already mentioned in the section 4.2.1, were selected as donors. The unique recipient strain selected was a linezolid susceptible *S. epidermidis* (LSSE) belonging to ST2 isolated from a cutaneous abscess in a patient in the university hospital of Lyon in 2020.

In the first step, all *cfr*-carrying plasmids were successfully transferred to the recipient strain by filter mating experiments; thus, five transconjugant derivatives of this same recipient strain were obtained (TCs 1-5). In the second step, in order to study the transmission capability of each plasmid, independently of the genetic background, all five transconjugants of this same strain were then used as donor to the six *S. aureus* strains belonging to various genetic background: ST8-MRSA-IV USA300 (ST2018-0363), CC5-MRSA-IV (ST2017-1473), CC398-MRSA-X (ST2011-2959), CC80-MRSA-IV (ST2011-0372), CC22-MRSA-IV (ST2018-0136), and ST72-MSSA (ST2018-0281). The donor strain harboring the 4 different *cfr*-positive plasmids was resistant to linezolid/chloramphenicol but susceptible to tetracycline; while all the six recipient strains were susceptible to linezolid/chloramphenicol but resistant to tetracycline.

4.2.3. Preparation of selective media

In order to separate the transconjugants from donor and recipient strains, selective media were prepared. The Tryptic Soy Agar (TSA) medium (Difco, USA) containing chloramphenicol (32 mg/L) supplemented with tetracycline (8 mg/L) or erythromycin (32 mg/L) was used to select *cfr*-positive staphylococci resistant to linezolid (with cross-resistance to chloramphenicol) obtained by conjugation (184).

Preparation of TSA

Suspend 20 g of TSA in 500 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes.

Preparation of stock solutions of antibiotic

- Tetracycline: 25 mg/mL
- Erythromycin: 50 mg/mL
- Chloramphenicol: 50 mg/mL
- Heat the TSA in water bath until it is completely melted.
- Add the antibiotic to the TSA (Table 4) when the temperature of the TSA is reached around 55 °C.
- Pour 19 mL per petri plate.

Table 4. Volume of initial antibiotic solution to be added in TSA medium.

ATB	Concentration of ATB in the medium (mg/L)	Initial ATB in 1mL (mg/L)	Final volume of ATB in 500mL (µL)	Final volume of ATB in 250mL (µL)
Tetracycline	8	25000	160	80
Erythromycin	32	50000	320	160
Chloramphenicol	32	50000	320	160

ATB, antibiotic

4.2.4. Conjugative plasmid transfer by filter mating

Bacterial conjugation is a natural process that allows horizontal transmission of DNA from one bacterium to another. While gram-negative bacilli are able to transfer the plasmids in liquid suspensions, conjugative plasmid transfer requires intimate cell-to-cell contacts between the donor and the recipient through filter mating for staphylococci. Here we describe the protocol of conjugative plasmid transfer by filter mating that we performed (Fig. 15). To ensure the accuracy and reproducibility, three independent conjugation experiments were performed for each donor and recipient strains.

Day 1

- Grow the donor and recipient strains onto Columbia Agar plate with 5% sheep blood (COS) and incubate at 37ºC for 18-24 hours.

Day 2

- Inoculate each donor and recipient strains in TSB medium and incubate at 37ºC for 18-24 hours.

Day 3

- Adjust the optical density (OD_{600}) of the overnight cultures to 1 (+/- 0.1) by using fresh TSB medium. Add 1 mL of phosphate-buffered saline (PBS) in Eppendorf tubes of 2 mL and mix with 0.5 mL of the donor and 0.5 mL of the recipient culture.
- Transfer the mixture of bacteria onto a 0.45 µm filter membrane using a vacuum pump system.
- Put the filter membrane on a Columbia agar plate with 5% sheep blood agar. Incubate at 37 °C for 18-24 hours.

Day 4

- Take out the filter membrane from the plate and suspend in 10 mL PBS. Vortex for one minute to collect all the bacteria attached on the filter membrane.
- Make a serial 10-fold dilution of the bacterial suspension by using fresh TSB. Plate 100 µL of the undiluted and 10^{-2} diluted samples on TSA plates supplemented with 32 mg/L chloramphenicol and 8 mg/L tetracycline or 32 mg/L erythromycin for the selection of transconjugants. Plate 100 µL of the diluted suspension (10^{-5}) onto TSA plates with 8 mg/L tetracycline or 32 mg/L erythromycin to count the total number of recipients. Plating is done with automatic spiral plater (Interscience, Puycapel France). Incubate the plates at 37 °C for 24-48 hours

Day 5

- Count the total number of recipients on the TSA plates supplemented with 8 mg/L tetracycline or 32 mg/L erythromycin.

Day 6

- Count the total number of transconjugants on the TSA plates supplemented with 32 mg/L chloramphenicol and 8 mg/mL tetracycline or 32 mg/mL erythromycin.
- Calculate transfer rates (conjugation frequency): number of transconjugants/number of recipient cells x dilution factor.

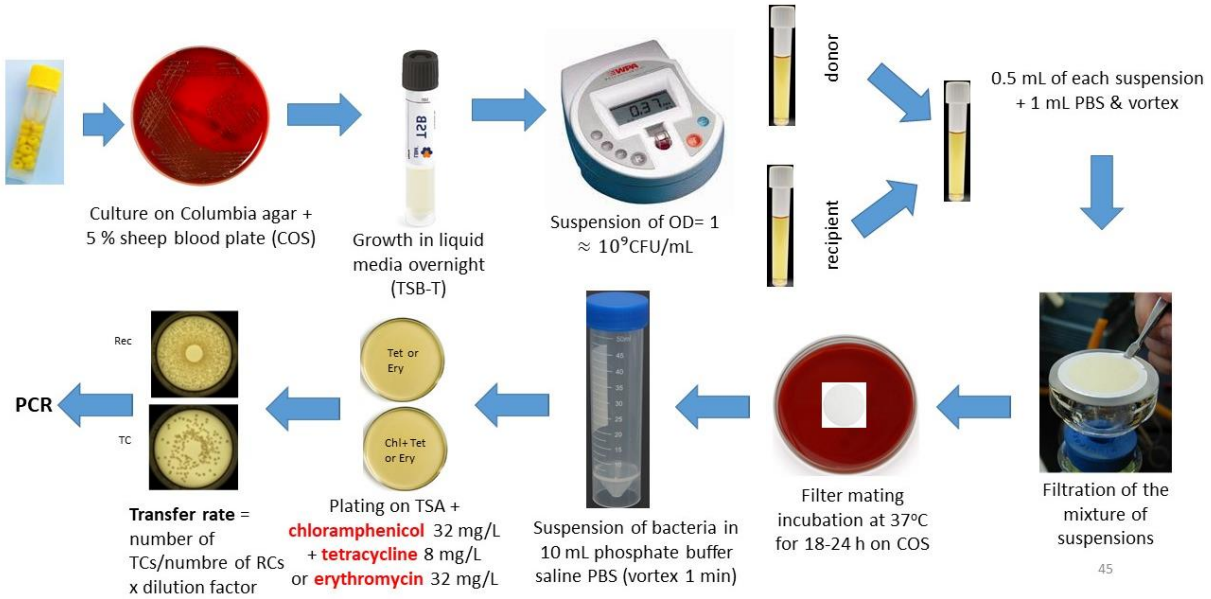

Fig 15. Protocol of conjugative plasmid transfer by filter mating.

- Determine the antimicrobial susceptibility profile of the transconjugants by disk diffusion method (296). The susceptibility profile of the transconjugants must be identical to the recipient, except for linezolid/chloramphenicol and other compounds affected by the *cfr* gene.
- Conserve each transconjugant at -80ºC in cryotube for future works.

Note: A Pastorex Staph Plus® (Biorad) test (rapid latex particle agglutination test for the identification of *S. aureus* strains from colonies) was performed on the *S. aureus* transconjugants. In order to check the acquisition of the *cfr* gene, a PCR targeting this gene was carried out.

4.2.5. Confirmation of *cfr* gene acquisition by PCR

In order to confirm the transfer of *cfr* gene to all transconjugants, 2-3 colonies of each transconjugant were picked from agar plates and suspended in 100 µL Quick Extract TM DNA (Epicentre, réf: QE 09050). These bacterial suspensions were then placed in a thermocycler for the DNA extraction program (30 min at 65 °C, then 20 min at 98 °C). DNA extracts were then stored at 4 °C or -20 °C for long storage or later use. Then 1 µL of the DNA extract was used as a template in the PCR that was carried out with two *cfr*-specific primers, TGA AGT ATA

AAG CAG GT and ACC ATA TAA TTG ACC A, under the following conditions: 2 min at 94°C; 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 1 min.

Both positive control (*cfr*-positive *S. aureus*) and negative control (*cfr*-negative *S. aureus*) are used in PCR experiment.

4.2.6. Determination of linezolid MIC of the transconjugants

In order to verify the functionality of *cfr* gene in all transconjugants and the role of plasmids or strains in linezolid MIC variations, the MIC determination assays using E-test (bioMerieux, Marcy-l'Etoile, France) were conducted. The MIC values were interpreted according to the 2020 guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (296).

4.2.7. Determination of plasmid fitness effects

In order to determine whether the acquisition of plasmids have a fitness cost for transconjugants, the initial recipient strains and their transconjugants were grown in TSB in 96-well microplate at 37°C for 20 hours. The microplates were then placed in the microplate reader (TECAN, Switzerland) that measures the optical density (OD) at the wavelength of 600 nm every 15 minutes, and orbital shaking on 3mm dimension for 15 seconds. To ensure the accuracy and reproducibility, three independent experiments for each initial recipient and their transconjugant derivatives, performed in triplicate. By measuring the OD curves, the average of generation time (doubling time) of each recipient and its transconjugant derivatives have been calculated and compared. Statistical analysis was carried out using the Mann-Whitney U test, and a *p value* of less than 0.05 was considered statistically as significant.

Day 1

- Grow the strains onto Columbia Agar plate with 5% sheep blood (COS) and incubate at 37°C for 18-24h

Day 2

- Inoculate 4-5 colonies from Columbia Agar plate with 5% sheep blood (COS) into a tube containing TSB and incubate at 37°C for 18-24h

Day 3 (Plating)

- Inoculate a new TSB from the TSB prepared on Day 2 by adjusting the OD to 0.03 - 0.05.
- In order to maintain sufficient humidity in the microplate, fill the surrounding wells of the plate with 100µL of sterile water,
- Add 100µL of sterile broth in several wells as negative control (at least 3 wells)
- Add 100µL of bacterial suspension of each strain in at least 3 wells for each

Day 4

To calculate the generation time for each replicate:

- Subtract the OD values of negative controls (without inoculated bacteria)
- Plot the OD curve as function of time
- Measure the slope µ of the exponential growth phase (by dividing the difference of the y-coordiantes of 2 points of the curve line by the difference of the x-coordinates of those same two points)
- Calculate the doubling time = ln(2)/µ

4.2.8. Determination of plasmid stability under non-selective condition

Plasmid persistence in the absence of positive selection was determined by various factors including plasmid stability. To evaluate the plasmid stability in the transconjugants under non-selective condition over time, we selected all transconjugant derivatives of two initial strains ST8-MRSA-IV USA300 (ST 2018-0363) and *S. epidermidis* ST2 (ST 2020-0560) having received different plasmids. In order to conduct the experiment, the following protocol (plasmid loss assay) was used:

- Culture the transconjugants from cryotubes onto Columbia Agar plate with 5% sheep blood (COS), or selective medium (TSA) supplemented with chloramphenicol 32 mg/L at 37°C overnight.
- Inoculate 5-6 colonies of each plasmid containing strain in TSB and incubate at 37 °C overnight.
- Sub-culture every 18-24 h by diluting a 9 µl aliquot into 9 mL (1:1000) of TSB.

- After every 5 passages, make an aliquot of 1 mL of the subculture in a sterile Eppendorf tube, then transfer 100 µL into 900 µL of TSB to be serially diluted from 10^{-1} to 10^{-5} and conserve the remaining 900 µL of the aliquot at -80ºC for future tests.
- Plate 50 µL of 10^{-5} dilution onto Columbia Agar plate with 5% sheep blood (COS) and spread the sample over the surface of COS using sterile spreader to obtain a clear indication of colony numbers, and incubate at 37°C for 1-2 days.
- Replicate this plate onto the TSA without antibiotic (control) and then TSA with antibiotic (chloramphenicol 32 mg/L) with the aid of colony stamp already sterilized by autoclave and incubate at 37°C for 2 days.
- The next day, compare the colonies which grew on non-selective media but did not grow on selective media (sign of *cfr*-positive plasmid loss), and calculate the percentage of plasmid loss rate by dividing the total number of colonies on selective media to the total number of colonies on non-selective media multiplied by 100.
- Check the *cfr*-positive plasmid loss by antibiotic susceptibility testing and PCR

Note: We verified that the use of chloramphenicol (32mg/L) in selective media has no impact on the growth and CFU reduction for linezolid resistant strains.

4.3. Results

4.3.1. Conjugative transfer of various *cfr*-carrying plasmids from *S. epidermidis* strains to linezolid-susceptible *S. aureus* clones by filter-mating experiments

The outcome of filter-mating experiments and the frequency of plasmid transfer between 5 donor *S. epidermidis* and 9 recipient *S. aureus* strains are shown in Table 5. Plasmid transer was not observed between all couples. When observed the rate of plasmid transfer were highly variable depending to the donor-recipient couples: 10^{-4} to 10^{-9} transconjugants per recipient cells. The results of this experiment showed that transfer of *cfr*-positive plasmids occurred from all 5 donor strains to the recipient strain ST8-MRSA-IV USA300; conversely some recipient strains accepted transfer of the *cfr*-positive plasmid only from certain donor strains and not from the others. Of note, no detectable transfer to CC5-MRSA-IV, CC80-MRSA-IV, and CC398-MSSA was observed whatever the donor strains. The *S. epidermidis* ST2 strain (ST2014-0255) presented a higher efficacy to transfer the plasmid *p12-02300* to *S. aureus* strains with different genetic backgrounds. Interestingly, *S. epidermidis* ST5 strain (ST2018-0691) carrying the plasmid *p-cfr-PBR-B* was more able to transfer the plasmid to different genetic backgrounds of *S. aureus*, compared to the *S. epidermidis* ST2 strain (ST2018-0678) carrying the same plasmid. For this latter strain, we observed the transmission of the *p-cfr-PBR-B* plasmid in only one of the experiments and at a very low rate (6.9×10^{-9}).

Table 5. Conjugative plasmid transfer rates from five donor strains of *S. epidermidis* resistant to linezolid (*cfr*-positive) to nine *S. aureus* strains with different genetic backgrounds circulating in France, by three independent experiments.

Donor strains *cfr*-positive		**Recipient strains *cfr*-negative**									
Species/clone	***cfr*-postive plasmid**	**No of experiments**	**CC5-MRSA-IV**	**CC398-MRSA-X**	**CC22-MRSA-IV**	**ST72-MSSA**	**ST8-MRSA-IV USA300**	**CC80-MRSA-IV**	**CC8-MRSA-IV Lyon clone**	**CC398-MSSA**	**CC30-MSSA**
ST 2018 0680 (SE ST2)	***p-cfr-PBR-A***	1	0	$9.7\ 10^{-7}$	$2.7\ 10^{-8}$	$2.4\ 10^{-8}$	$2.2\ 10^{-6}$	0	/	/	/
		2	0	$2.5\ 10^{-6}$	$5.9\ 10^{-9}$	$2.4\ 10^{-8}$	$9.5\ 10^{-6}$	0	/	/	/
		3	0	$5.6\ 10^{-7}$	$7.1\ 10^{-9}$	$9.2\ 10^{-9}$	$4.6\ 10^{-6}$	0	/	/	/
ST 2018 0678 (SE ST2)	***p-cfr-PBR-B***	1	0	0	0	0	0	0	0	0	0
		2	0	0	0	0	$6.9\ 10^{-9}$	0	0	0	0
		3	0	0	0	0	0	0	0	0	0
ST 2018 0691 (SE ST5)	***p-cfr-PBR-B***	1	0	0	$1.3\ 10^{-8}$	$4.5\ 10^{-9}$	$8.9\ 10^{-8}$	0	$1.2\ 10^{-6}$	0	0
		2	0	0	$5.1\ 10^{-8}$	$9.5\ 10^{-9}$	$8.1\ 10^{-8}$	0	$8.0\ 10^{-9}$	0	0
		3	0	0	$2.1\ 10^{-8}$	$7.3\ 10^{-9}$	$9.8\ 10^{-8}$	0	$2.1\ 10^{-9}$	0	0
ST 2014 0255 (SE ST2)	***p12-02300***	1	0	$5.4\ 10^{-6}$	$3.2\ 10^{-8}$	$7.8\ 10^{-8}$	$6.0\ 10^{-6}$	0	$5.6\ 10^{-5}$	0	$1.3\ 10^{-7}$
		2	0	$6.0\ 10^{-6}$	8.9 10-9	$7.3\ 10^{-8}$	$8.2\ 10^{-6}$	0	$1.4\ 10^{-4}$	0	0
		3	0	$8.0\ 10^{-7}$	$8.5\ 10^{-9}$	$4.4\ 10^{-8}$	$5.3\ 10^{-6}$	0	$6.9\ 10^{-5}$	0	$3.4\ 10^{-7}$
ST2016 1991 (SE ST2)	***pSA737***	1	0	$3.7\ 10^{-6}$	0	0	$1.2\ 10^{-5}$	0	$7.3\ 10^{-5}$	0	0
		2	0	$5.2\ 10^{-7}$	0	0	$4.4\ 10^{-6}$	0	$5.1\ 10^{-6}$	0	0
		3	0	$1.3\ 10^{-5}$	0	0	$1.3\ 10^{-4}$	0	$5.2\ 10^{-4}$	0	0

0, No plasmid transmission detected between donor and recipient couples; SE, *S. epidermidis;* ST, sequence type

4.3.2. Study of the transmission capability of different *cfr*-carrying plasmids

The transmission capability of 4 different *cfr*-carrying plasmids (in case of *p-cfr-PBR-B* plasmid, one carried by *S. epidermidis* ST2 and another by *S. epidermidis* ST5) from the same donor strain of *S. epidermidis* (transconjugants 1-5) to six *S. aureus* strains with different genetic backgrounds, is presented in Table 6. The plasmids *pSA737* and *p12-02300* were transmitted to all six recipients at a relatively higher transfer rates (around 10^{-4}) for ST8-MRSA-IV USA300 strain; at moderate transfer rates for CC22-MRSA-IV, ST72-MSSA, and CC398-MRSA-X strains (ranged between 10^{-4} to 10^{-7}); and at lower transfer rates for CC80-MRSA-IV and CC5-MRSA-IV strains (ranged between 10^{-7} to 10^{-9}). The plasmid *p-cfr-PBR-A* was also transmitted to all six recipients, but at a very low transfer rate to strains belonging to CC5-MRSA-IV and CC80-MRSA-IV (10^{-9} in one round of the experiments and no transfer in the others). The transfer rate of this plasmid was relatively moderate (10^{-5}) for ST8-MRSA-IV USA300 and CC398-MRSA-X, and low (10^{-6} to 10^{-8}) for ST72-MSSA and CC22-MRSA-IV strains. Out of six recipients, the plasmid *p-cfr-PBR-B* transmitted only to three recipient strains, namely ST8-MRSA-IV USA300, CC22-MRSA-IV, and ST72-MSSA, with a transfer rate of 10^{-7} to 10^{-8}.

Table 6. Plasmid transmission capability from the same donor strain (*S. epidermidis* ST2, ST2020-0560) to six different *S. aureus* strains circulating in France, by three independent conjugation experiments.

Donor strain (*cfr*-positive)		**Recipient strains (*cfr*-negative)**						
Transconjugants	***cfr*-positive plasmids**	**No of Experiments**	**CC5-MRSA-IV**	**CC398-MRSA-X**	**CC22-MRSA-IV**	**ST72-MSSA**	**ST8-MRSA-IV USA300**	**CC80-MRSA-IV**
ST 2020 0560-TC1	***p-cfr-PBR-A* from SE ST 2018 0680**	1	0	$2.6\ 10^{-5}$	$1.3\ 10^{-6}$	$2.0\ 10^{-6}$	$6.8\ 10^{-5}$	0
		2	0	$1.9\ 10^{-5}$	$8.9\ 10^{-8}$	$4.4\ 10^{-7}$	$2.6\ 10^{-5}$	0
		3	$3.0\ 10^{-9}$	$3.0\ 10^{-5}$	$2.1\ 10^{-6}$	$1.9\ 10^{-6}$	$7.2\ 10^{-5}$	$3.1\ 10^{-9}$
ST 2020 0560-TC2	***p-cfr-PBR-B* from SE ST 2018 0678**	1	0	0	0	0	0	0
		2	0	0	$2.4\ 10^{-7}$	$1.4\ 10^{-8}$	$6.5\ 10^{-7}$	0
		3	0	0	$1.9\ 10^{-8}$	$1.6\ 10^{-8}$	$2.5\ 10^{-7}$	0
ST 2020 0560-TC3	***p-cfr-PBR-B* from SE ST 2018 0691**	1	0	0	$3.0\ 10^{-8}$	0	$9.0\ 10^{-8}$	0
		2	0	0	$2.1\ 10^{-8}$	$2.5\ 10^{-8}$	$4.7\ 10^{-7}$	0
		3	0	0	$2.3\ 10^{-8}$	$1.9\ 10^{-8}$	$2.4\ 10^{-7}$	0
ST 2020 0560-TC4	***p12-02300* from SE ST 2014 0255**	1	$1.9\ 10^{-9}$	$4.7\ 10^{-5}$	$2.7\ 10^{-7}$	$1.8\ 10^{-6}$	$1.1\ 10^{-4}$	$3.5\ 10^{-9}$
		2	$7.9\ 10^{-9}$	$7.1\ 10^{-6}$	$1.8\ 10^{-7}$	$2.9\ 10^{-7}$	$1.3\ 10^{-4}$	0
		3	$2.6\ 10^{-8}$	$6.0\ 10^{-6}$	$3.7\ 10^{-7}$	$1.3\ 10^{-6}$	$1.6\ 10^{-4}$	$3.3\ 10^{-9}$
ST 2020 0560-TC5	***pSA737* from SE ST 2016 1991**	1	$2.7\ 10^{-8}$	$1.5\ 10^{-4}$	$1.4\ 10^{-6}$	$1.8\ 10^{-5}$	$5.1\ 10^{-4}$	$2.1\ 10^{-8}$
		2	$3.0\ 10^{-7}$	$1.3\ 10^{-4}$	$2.3\ 10^{-6}$	$2.6\ 10^{-6}$	$3.3\ 10^{-4}$	$9.9\ 10^{-9}$
		3	$2.3\ 10^{-8}$	$5.6\ 10^{-5}$	$3.1\ 10^{-7}$	$1.2\ 10^{-5}$	$3.1\ 10^{-4}$	$3.3\ 10^{-9}$

0, No plasmid transmission detected between donor and recipient couples; SE, *S. epidermidis;* ST, sequence type; TC, transconjugant

4.3.3. Determination of effect of plasmid acquisition on bacterial fitness

The fitness cost was assessed by comparing the doubling time prior and after conjugation for original recipients and their transconjugants. Eight original recipient strains and their 27 transconjugant derivatives were tested. The mean doubling time of recipients varied from 28.6 minutes (ST72-MSSA) to 38.7 minutes (CC30-MSSA). The fitness cost observed after transconjugation was highly variable, depending to plasmids and *S. aureus* recipient strains (Fig.16). More than half of the transconjugants (17/27) did not show any significant fitness cost compared to the recipient before acquisition, while 10/27 of transconjugants showed statistically significant fitness cost (*i.e.,* they showed a significant increase in doubling time ranging from 1 to 6 min). None of the plasmid demonstrated a systematic fitness cost in all genetic backgrounds. In the same way, the highest fitness costs, when existing in a recipient, were not related to the acquistion of the same plasmid (*e.g, p12-02300* in CC22-MRSA-IV strain, *p-cfr-PBR-A* in CC398-MRSA-X strains, *pSA737* in ST72-MSSA strains.

A significant fitness cost was observed in all transconjugants of ST72-MSSA strain, two transconjugants of CC22-MRSA-IV, one transconjugant of CC398-MRSA-X, one transconjugant of CC80-MRSA-IV, and one transconjugant of CC30-MSSA. Conversely, the transconjugants related to the clones ST8-MRSA-IV USA300, CC5-MRSA-IV, and CC8-MRSA-IV Lyon clone did not show any significant fitness cost after acquisition of different plasmids.

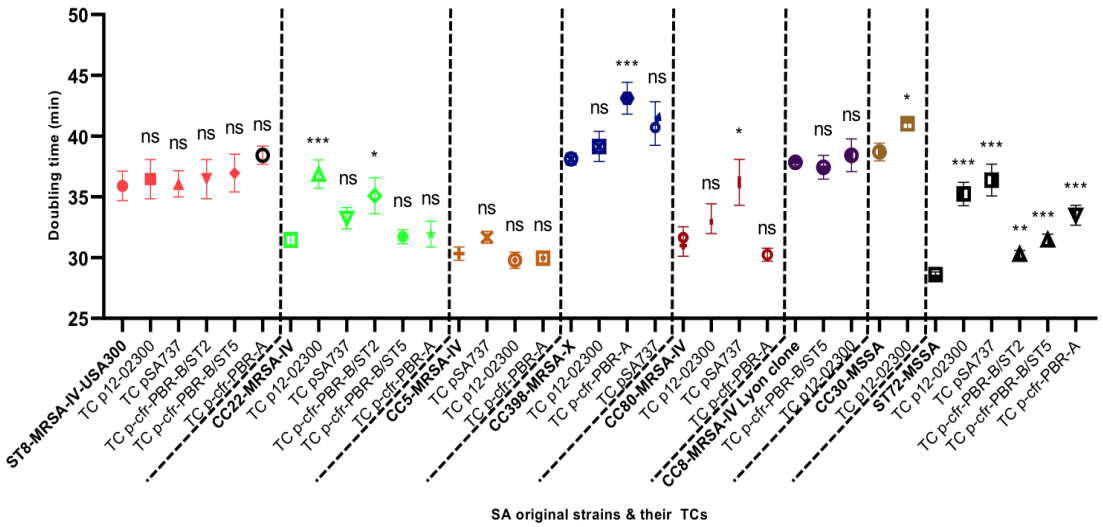

Fig 16. Evaluation of fitness cost of *cfr*-carrying plasmids by comparison of the doubling time in the transconjugants versus the recipient strains (3 independent experiments for each triplicates). Statistical analysis was carried out using Mann-Whitney U test. *, P<0.05; **, P< 0.001; ***, P< 0.0001; ns, no significant difference ; SA, *S. aureus* ; TC, transconjugant

4.3.4. Confirmation of *cfr* gene acquisition by PCR

One transconjugant derivative of each donor-recipient couple obtained in this study, were screened for the presence of *cfr* gene by PCR. All of them were found to be positive, and the PCR test confirmed that *cfr*-carrying plasmids were successfully transferred to the recipients tested.

4.3.5. Determination of linezolid MIC conferred by acquisition of *cfr*-carrying plasmids

In the present study, the linezolid MIC of the 8 clinical susceptible *S. aureus* strains used as recipients ranged from 1-2 mg/L. The linezolid MIC of the transconjugant derivatives of these 8 strains after acquisition of various *cfr*-carrying plasmids were determined and ranged from 8 to 48 mg/L, *i.e.,* 4- to 48-fold higher comparing to the original susceptible strains (Table 7). The increases in MICs are highly variable. Thus, the increase in linezolid MICs for *p-cfr-PBR-A* transconjugants ranged from 8 to 48 µg/mL, from 12 to 16 µg/mL for *p-cfr-PBR-B*-ST2 transconjugants, from 12 to 32 µg/mL for *p-cfr-PBR-B*-ST5 transconjugants, from 8 to 48µg/mL for *p12-02300* transconjugants, and from 8 to 32 µg/mL for *pSA737* transconjugants. In addition, the increase in linezolid MICs conferred by the plasmids was also variable according to the genetic background of transconjugants. The lowest MIC (8 µg/mL) was seen for all three CC398-MRSA-X transconjugants whatever the plasmid acquired, and higher MICs were observed with the same transconjugated plasmids in all other transconjugants belonging to the other CC tested. The transfer of the same 3 plasmids impacted differently the linezolid MICs in ST72-MSSA (12, 16, 24 and 32 µg/mL) or in CC5-MRSA-IV (16, 48 and 48 µg/mL). Intriguingly, we also noticed that the transconjugation of *p-cfr-PBR-B/ST2* in the CC22-MRSA-IV, ST72-MSSA, ST8-MRSA-IV USA300 presented linezolid MICs of 12, 16 and 12 µg/mL respectively, whereas the same strains when acquired *p-cfr-PBR-B/ST5* (the same plasmid but transferred from a different donor strain) presented respectively linezolid MICs of 32, 12 and 16 µg/mL.

Table 7. Determination of linezolid MIC of transconjugants according to the acquired *cfr*-carrying plasmids or genetic background of original recipients (µg/mL).

cfr-carrying plasmids	***S. aureus* various genetic backgrounds**							
	CC5-MRSA-IV	**CC398-MRSA-X**	**CC22-MRSA-IV**	**ST72-MSSA**	**ST8-MRSA-IV USA300**	**CC80-MRSA-IV**	**CC8-MRSA-IV Lyon clone**	**CC30-MSSA**
Original strain without *cfr*-plasmid	1.5	1.5	1.5	2	2	2	2	1
p-cfr-PBR-A	48	8	8	24	16	16	-	-
p-cfr-PBR-B/ST2	-	-	12	16	12	-	-	-
p-cfr-PBR-B/ST5	-	-	32	12	16	-	24	-
p12-02300	48	8	12	12	16	12	16	16
pSA737	16	8	32	32	16	16	-	-

-, No transconjugants obtained

4.3.6. *cfr*-carrying plasmids stability under non-selective conditions

The protocol of plasmid loss assay describe above (section 4.2.8) was applied for all transconjugants of the two different recipient strains namely *S. epidermidis* ST2020-0560 (ST2) and *S. aureus* ST2018-0363 (USA300). Plasmid loss rate in the transconjugants, after ten passages in TSB non-selective medium at 37°C overnight was confirmed by PCR. The results showed that plasmid stability in the transconjugants in non-selective condition was highly variable (Fig. 17). Except *S. epidermidis* ST2 which could maintain efficiently the plasmid *p12-02300,* all other transconjugants lost the *cfr*-carrying plasmid but at various rates. The plasmid loss rate in the ST8-MRSA-IV USA300 strain was 84.7% for the plasmid *p-cfr-PBR-B/ST2*, 60% for the plasmid *p12-02300,* 100% for the plasmid *p-cfr-PBR-B/ST,* 90.5% for the plasmid *p-cfr-PBR-A,* and 91.6% for the plasmid *pSA737.* While in the *S. epidermidis* ST2, the loss rate was 86.1% for the plasmid *p-cfr-PBR-B/ST5*, no loss (0%) for the plasmid *p12-02300*, 82.3% for the plasmid *p-cfr-PBR-B/ST5,* 35.2% for the plasmid *p-cfr-PBR-A,* and 42.9% for the plasmid *pSA737.*

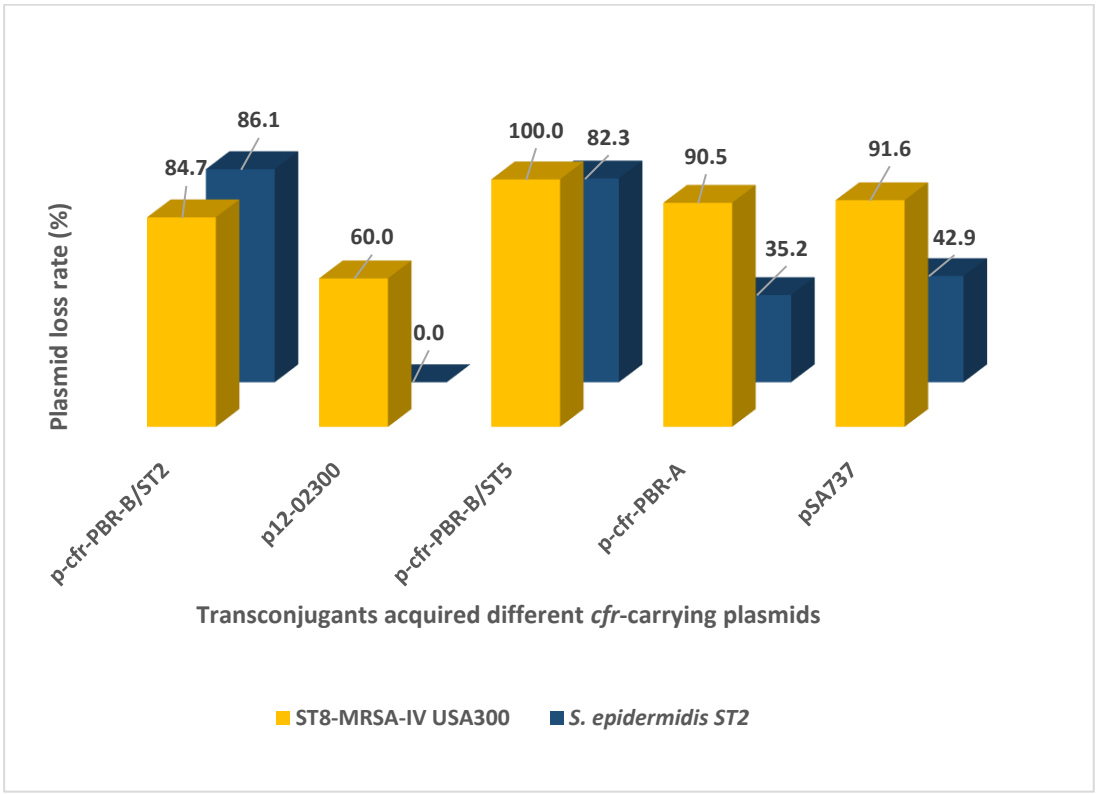

Fig 17. Plasmid loss rates for two strains having acquired different *cfr*-carrying plasmids, after ten passages in non-selective medium (TSB).

4. Discussion

This work was divided into two axes. In the first part, we investigated the molecular features and antibiotic resistance profile of *S. aureus*/MRSA strains isolated from clinical samples, from nasal colonization, as well as from animal infection and colonization in Kabul. In the second part, we explored the conjugative transferability and receptivity of *cfr*-carrying plasmids from *S. epidermidis* strains to *S. aureus* strains by filter-mating method, as well as plasmid fitness cost and stability.

5.1. Epidemiology, molecular characterization, and resistance profile of MRSA isolates from Afghanistan

5.1.1. Antibiotic resistance profile and molecular characterization of *S. aureus* strains isolated in hospitals in Kabul, Afghanistan

This study examined the prevalence and molecular characteristics of 98 *S. aureus* (33 MSSA and 65 MRSA) isolates from patients obtained during six months in two main hospitals of Kabul. This work was the first molecular study of clinical isolates of *S. aureus* in Afghanistan, and revealed that various MDR and virulent MRSA clones circulate in Kabul. Our results showed that the rate of MRSA among *S. aureus* clinical isolates was high (66.3%) when compared with the prevalence rates obtained from other similar studies conducted in our neighboring countries and in the region, such as Pakistan (297), Iran (298), Saudi Arabia (299), and Turkey (300). Our study showed a great diversity in *S. aureus* clones (13 distinct MRSA and 14 MSSA clones). The genotyping results revealed a predominance of three MRSA clones, comprising 44% of the isolates: Southwest Pacific clone (CC30-MRSA-IV PVL-positive), CC22-MRSA-IV TSST-1-positive clone, and the Bengal Bay clone (ST772-MRSA-V PVL-positive). The overall prevalence of PVL among MRSA and MSSA isolates was 70.8% and 36.4% respectively. This high prevalence and the diversity of PVL-positive isolates could be linked to the import of different strains via international exchanges. In the studies in neighboring countries the prevalence of PVL was generally lower: from 0 to 12.7% in Turkey (301), from 7.4 to 55.6% in Iran (83), and around 9% in West Bengal India (302). Most of *S. aureus*, especially MRSA, were MDR (generally resistant to beta-lactams, macrolides, quinolones, cotrimoxazole, and less often to aminoglycosides, chloramphenicol, and tetracyclines). This may be linked to the overuse or inappropriate use of antimicrobials which

was recently reported in Afghanistan (110,113). The diversity in the clinical *S. aureus* strains in Kabul reflect epidemiological links including travel, migration, commerce, and international military missions with Southwest pacific, India, Middle Eastern/Arabian Gulf region, as well as Europe and USA. Most of these MRSA clones were also found in the healthy people in the community in Kabul. As in all countries, major efforts have to be made toward infection control and the prevention of futher spread of MDR bacteria, as well as prudent use of antimicrobials agents in Afghanistan.

5.1.2. Nasal carriage of methicillin-resistant *Staphylococcus aureus* among students at Kabul University: Prevalence, phenotypic and molecular characterization

This work examined the prevalence and molecular characteristics of MRSA sampled from 150 healthy students in Kabul University; reports various clones circulating among healthy individuals in Kabul.

From 150 nasal swabs, we had a 33.3% (50/150) and 12.7% (19/150) recovery of *S. aureus* and MRSA, respectively, a higher prevalence of *S. aureus*/MRSA compared to similar studies among students conducet in Iran (303), and China (304). In our study, the frequency of MRSA nasal carriage was not significantly different between male and female, which may indicate that gender is not a risk factor for MRSA nasal colonization.

Of 19 MRSA, 7 distinct clones with predominance of CC22-MRSA-IV TSST-1-positive were detected, which showed that like clinical strains, there is a wide clonal diversity among MRSA community strains isolated from healthy people in Kabul. The results of the this study showed that almost all of the identified MRSA clones, have also been reported from clinical samples in our first study in Kabul (305). This indicates the widespread dissemination of MRSA strains between healthcare settings and the community, and obviously it is a matter of great concern.

One of the main limitation of this study is the low number of samples and MRSA strains collected from nasal carriers among Kabul university students. Therefore, the results do not likely reflect the global epidemiology of MRSA nasal carriage in Kabul or Afghanistan. However, the students are a population without risk factors and therefore represent well the healthy community. Moreover, as colonization is classically depends on the students comes from different regions of Afghanistan, this population is likely representative in a certain way of the Afghan diverse *S. aureus* clones. On the other hand, the diversity of strains highlighted by the genotyping results, also illustrates the position of Afghanistan as a center of intense

international exchanges, as in the previous study (305). The genetic diversity of the identified MRSA clones in our study showed an epidemiologic link to other countries close to Afghanistan or far from it such as: Iran, China, Gulf countries, Turkey, Western Europe, USA, Australia, and Egypt. These epidemiological links could be related to the large movement of Afghans to Saudi Arabia for pilgrimage each year, commerce with China, and the large migratory movements of Afghans to Iran, Gulf region, Europe, USA, and Australia.

The relatively high frequency of MRSA nasal carriage among university students in Kabul, harboring various multiresistant genes and virulence markers (such as PVL and TSST-1) is becoming critical and requires more attention. The existence of such MDR and virulent strains among healthy population in the universities and other crowded places where people are more often in close physical contact with each other can directly facilitate the spread of these strains to the community, and can act as reservoirs for outbreak of MRSA infections. Supporting policies for reducing nasal carriage and continued efforts to enhance hygiene among students are necessary to slow down the transmission of MRSA in the community.

5.1.3. Characterization and antimicrobial resistance patterns of *S. aureus* strains isolated from livestock in Kabul

A total of 17 *S. aureus* including 2 MRSA isolates from bovine mastitis in Kabul veterinary clinic during five months, while only 2 MSSA isolates out of 189 sheep nasal swabs, have been studied. However, this small collection of *S. aureus* isolates tested in our study may not represent the entire population of *S. aureus* circulating in dairy herds of Afghanistan. Anyway, this report provides for the first time reliable information about the existence of multidrug resistant and virulent clones in livestock in Kabul. In this study the percentage of MRSA among *S. aureus* isolates from bovine mastitis was 11.8%, which was higher than similar studies in the neighboring countries such as China (306) and Iran (307). In the current study a high frequency of resistance was observed among *S. aureus* isolates from bovine mastitis against tetracycline, and could be explained by widespread use of tetracycline compounds in veterinary care and animal husbandry in Afghanistan. This high selective pressure could further select and spread such strains between human and animals. The two MRSA clones (CC30 and CC88) identified in this study, had already been identified in human infections and colonization in our previous study in Kabul. The close contact between human and livestock in Afghanistan may create the

potential for transmission of microorganisms between human and animals. Of note, 52.9% of the isolates harbored one or more enterotoxin genes, which is also a point of concern.

We found a very low nasal carriage (1.1%) rate of *S. aureus* in sheep in Kabul, but we couldn't compare our results to other similar studies at the local or national level, due to the lack of such data in Afghanistan. While in a similar report from Iran, a higher rate (14.1%) of *S. aureus* sheep nasal carriage was reported and all of them were MSSA (91). The sheep studied in our report mostly belonged to nomads, who sell their livestock in animal markets in Afghanistan. Their livestock are grazing on the mountains and deserts, having less contact with other animals and human. This may explain why the nasal carriage rate of *S. aureus* among sheep in our study was very low. However, this hypothesis should be confirmed by further studies in animal farms and also other provinces of Afghanistan.

Finally, among all three studies conducted about resistance profile and genotypes of *S. aureus* strains isolated from human infection and colonization and bovine mastitis in Kabul, we found a high rate of MRSA in human infection and colonization. The diversity of the clones in human infection and colonization as well as in diary cattle infection, the presence of virulence determinants and various resistance genes among these isolates, and the connection of certain clones between health facilities and the community as well as livestock in Kabul, is a matter of concern.

5.2. Conjugative transfer of *cfr*-carrying plasmids from CoNS to *S. aureus* strains

Even if the number of donor and recipient strains tested as well as the diversity of plasmids harboring *cfr* gene are limited and would deserve to be extended, our work on conjugative transfer of *cfr* gene highlighted interesting data about the efficiency of *cfr*-carrying plasmids transfer from donors (*S. epidermidis* strains) to recipients (*S. aureus* strains) as well as the impact on linezolid MICs in recipient.

Based on the strains tested in the present study, our results suggest that certain clones, *e.g*., ST8-MRSA-IV USA300, could be more at risk for acquisition of *cfr*-carrying plasmids from *S. epidermidis*, while others, *e.g.,* CC398-MSSA, did not accept any of *cfr*-carrying plasmids. Finally, other clones (CC5-MRSA-IV and CC80-MRSA-IV, see table 5 and 6) acquired *cfr*-plasmids only from a few donors with a lower conjugation frequency. It has been already showed that the non-transfer condition or low transfer rate of plasmids in gram-positive bacteria

is due either to a restriction modification system or similar mechanisms (which acts like an immunity system in prokaryotes that induce an alteration of foreign DNAs (243,244)) or the plasmid fitness cost (248). On the other hand, despite they harboured the same plasmid (*p-cfr-PBR-B)*, the *S. epidermidis* ST2018-0691 strain belonging to ST5 was more capable to transfer the plasmid *p-cfr-PBR-B* to the various *S. aureus* genetic backgrounds tested in comparison to *S. epidermidis* ST 2018-0678 belonging to ST2. In a similar study, conducted by Cafini *et al.*, the transfer of *cfr*-positive *pSCFS7*-like plasmid was successful from *S. epidermidis* ST2 to all MRSA strains with a mean transfer rate of 8.6 x1 0^{-5} transconjugants per recipient cells, while the other *S. epidermidis* ST2 strain was able to transfer the plasmid to only few recipients and with less efficacy (184). In the same way, Brenciani *et al.* showed that the transfer of *cfr* gene from two *S. epidermidis* strains to one *S. aureus* strain was successful but with different frequencies (127). Our results extended the data already published and confirmed that the genetic background of donors plays a key role in the transfer rate of a given *cfr*-positive plasmid. This difference in the transfer efficacy of *cfr*-positive plasmid from *S. epidermidis* strains belonging to different MLST group to various genotypes of *S. aureus* may be due to different genetic background of donor/recipient and a higher copy number of plasmids (308,309).

In order to assess the transmission capability of each plasmid independently to the donor strain, we were able to transfer all the plasmids studied to the same ST2 genetic background by using *S. epidermidis* ST2020-0560 as unique recipient. Then, we have been able to evaluate the transfer rate from the various transconjugants carrying these 4 different plasmids in a unique and fixed genetic background (a single clinical strain) to six different clones of *S. aureus* through conjugative plasmid transfer by filter mating. Our results indicated that all plasmids are transferable but the number of recipient strains for which transconjugates were obtained, and the rate of transfer are highly variable independently to the donor strain. This data highlights, this time, the role of genetic background of recipient in the conjugative plasmid transfer efficacy as well. Interestingly, we did not observe any transfer of *p-cfr-PBR-A* plasmid from *S. epidermidis* ST2 (ST 2018-0680) to CC5-MRSA-IV and CC80-MRSA-IV genetic backgrounds, while the *S. epidermidis* ST2020-0560 strain, that belongs also to ST2, was able to transfer the *p-cfr-PBR-A* plasmid to the two MRSA clones mentioned above (even if the transfer rate is low). This result suggests that the ability to transfer plasmid varied within ST. It advocates a larger number of strains to be tested to be able to draw definitive conclusions.

In addition, our experiments demonstrate that some ST2 *S. epidermidis* strains (such as ST2020-0560) mobilize and transfer more easily the plasmid *p-cfr-PBR-A/B* at a higher rate

than other *S. epidermidis* (such as ST2018-0680, ST 2018-0678 or ST2018-0691). The conclusion is the same for plasmid *p12-02300* transfer from the ST2020-0560 used as donor strains compared to the "natural" clinical strains harboring the two plasmids, *i.e.*, ST2016-1991 and ST 2014-0255 respectively. Moreover, we observed identical conjugative transfer of *p-cfr-PBR-B* plasmid from the *S. epidermidis* transconjugant strains (ST2020-0560) to three *S. aureus* strains with different genetic background wether the plasmid was originated from ST2018-0678 or ST2018-0691. Based on literature review, plasmids play a role in their own transfer/transferability as they usually encode their own replication-initiation (Rep) proteins, and increased plasmid copy number; this would be an important factor in the evolving conjugation frequency which is regulated by the plasmids itself (262,309).

Altogether, the results obtained suggests that the interspecies plasmid transfer within staphylococci could be affected by different parameters such as: plasmid type, genetic background of donors and recipients. Other parameters have been proposed in the literature including plasmid copy number, genetic relatedness, plasmid fitness cost, and restriction modification system. These data suggest that, when a new *cfr*-positive *S. epidermidis* is emerging, it is of interest to evaluate its capacity to transfer *cfr*-positive plasmid in the MSSA /MRSA clones that are circulating in the same hospital, at the regional or national scale. Such risk assessment could be helpful to guide or drive the public health actions/decisions/measures.

We also explored the impact of the acquisition of different *cfr*-positive plasmids on the level of linezolid resistance. Based on our data, the transfer of *cfr*-carrying plasmids has been associated with a 4- to 48- fold increase in the linezolid MIC in transconjugants, supporting the notion that *cfr* is an important contributor to the resistance in *cfr*-positive strains. The data of the experiments performed in the present study highlighted variations in the linezolid MIC value according both to plasmid types and genetic background of the strains. According to LaMarre *et al*., the association of *cfr* with multicopy plasmid, and the efficient *cfr* transcription due to plasmid-derived promoters in the surrounding environment of *cfr* gene and its idiosyncratic transcription pattern, are the main factors contributing to increased activity of *cfr* methyltransferase. They suggested that linezolid MIC variation could be attributed to the genetic background of the host, and different copy number of *cfr*-positive plasmids. In addition they hypothesized that linezolid efflux or enzymatic modification may affect the MIC variations (317).

Finally, we have evaluated the fitness cost of the acquisition of *cfr*-positive plasmids in all 27 *in vitro* transconjugant derivatives obtained in the present study in comparison to their original

recipient strains without plasmids. This cost appears to be highly transconjugant dependant. After acquisition of the *cfr*-carrying plasmids, around 37% (10/27) of transconjugants showed a significant increase in their generation time, while the remaining 63% (17/27) didn't show any significant change. For one strain (ST72-MSSA genetic background) we found a significant fitness cost after acquisition of any of the five plasmids; on the contrary, for the other strains, the fitness cost was neither plasmid-specific nor strain-specific. These results are in agreement with Vogwill and Maclean, for whom it is not possible to predict the evolution of the same plasmid in different bacterial clones and the fitness costs of different plasmids in the same strain (264). Some studies suggest that, for minimizing plasmid fitness costs on bacterial host, in most cases, transcriptional regulation by the host protein, or specific regulators on the plasmids to control the transcription of their own genes are activated (264,311). This hypothesis is in agreement with the fact that the same plasmid does not impact the linezolid MICs at the same level which could be related to a differential expression of the plasmidic *cfr* gene from one strain to another. Conversely, if the transconjugants are less fit (such as all transconjugant derivatives of ST72-MSSA in this study), they will not resist the fitness effects, will grow more slowly under non-selective conditions (*i.e* in the absence of antibiotic), and finally will gradually lose their plasmids which may allow them to outgrow the resistant strains. In addition, in order to evaluate the stability of plasmids in the transconjugants, we select all transconjugant derivatives of two clinical strains, namely *S. aureus* ST 2018-0363 (ST8-MRSA-IV USA300) and *S. epidermidis* ST2 (ST2020-0560). The two strains were chosen because transconjugants were obtained for all *cfr*-positive plasmids. Data provided demonstrate that persistence and stability of the four plasmids tested was variable according to the plasmids and the strains. Based on literature, the plasmid persistence in the population depends on plasmid stability. For instance, plasmid replication control mechanisms are important determinants of plasmid stability as they ensure the presence of sufficient plasmid copies in the cell prior to cell division (312). Conversely, the formation of plasmid multimers can lead to a decrease in the number of heritable plasmid units during cell division, hence reducing the chance of successful segregation and consequently reducing the plasmid persistence over time (313,314). In our study the plasmid loss rate was lower in *S. epidermidis* ST2 strain than ST8-MRSA-IV USA300. The limited number of strains tested is a clear limitation of the data. Nevertheless, according to Reyes-Lamoth *et al.* and Simson *et al*., the segregation of low-copy plasmids during cell division typically relies on partition systems that actively distribute plasmid copies into the daughter cells, which have been found both in gram-negative and gram-positive bacteria

including staphylococci (315,316). In the absence of a partition system, successful plasmid segregation depends on the multi-copy state and the physical distribution of the plasmids inside the cell. So, the better stability of plasmids in transconjugant derivatives from *S. epidermidis* versus *S. aureus* observed in the present study, may be associated with a more efficient partition system in the distribution of plasmid copies into the daughter cells, in *S. epidermidis*. This higher stability in *S. epidermidis* could also be one of the reasons why *S. epidermidis* strains seems to be a major reservoir of *cfr*-carrying plasmids in the hospital environment compared to *S. aureus* by assuring the maintenance of plasmid in presence or absence of selective pressure. In our experiment, one transconjugant derivative of *S. epidermidis* harboured the plasmid *p12-02300,* did not lose this plasmid at any rate after ten passages in non-selective medium; while in *S. aureus* even if this plasmid was lost in a certain rate, it appeared as more stable than other plasmids. Conversely, the most unstable plasmid was *p-cfr-PBR-B* both in *S. epidermidis* and *S. aureus* transconjugants tested. Both data suggest that the stability is also plasmid dependant. According to literature review, the higher stability of the plasmid *p12-02300* may be associated to better control of its encoded genes on the production of sufficient copy numbers of the plasmid and its physical distribution inside the cell (262,315). In addition a lower stability, such as for *p-cfr-PBR-B*, may be due to its relatively larger size (40.2 kb) and/or possibly to low copy numbers of this plasmid within the host cell, but more importantly the co-existence of various other resistance genes that may have significant fitness cost (262,312,315). These hypotheses would deserve to be confirmed by further experiments using our complete set of strains and transconjugants. It would be of interest to extend our experiments to the complete set of isolates and transconjugants by evaluating the impact of the copy numbers of each *cfr*-positive plasmid and their size on the plasmid transfer rate and the plasmid lost rate.

Finally, it's worth mentioning that, with the expanded use of linezolid, especially since the availability of cheap generic forms, more exposure of pathogens to this agent will occur, which may lead to the selection of *cfr*-positive strains and emergence of clinical isolates with resistance or decreased susceptibility to linezolid.

However, all of these findings should be confirmed by including greater number of *S. aureus* and *S. epidermidis* strains in such studies in the future.

6. Conclusion and perspectives

Several important results were obtained in this work, which are summarised below and based on these findings, further studies are proposed on both axes.

6.1. Epidemiology, molecular characterization, and resistance profile of *S. aureus*/MRSA isolates from Afghanistan

This work was the first deep molecular study of clinical isolates of *S. aureus* in Afghanistan, integrating data both from hospital, community and veterinary settings. The epidemiological data obtained, demonstrate for the first time the high clonal diversity among *S. aureus*/MRSA isolates from human infection and colonization, as well as animal infection in Kabul. Some MDR and virulent clones are found to be spreading between health-care settings, community as well as livestok. This raises major public health concerns, and highlighting the need for more systematic surveillance. The diversity of the clones identified likely reflect epidemiological links, including travels, migrations, commerce, and the stay of international military forces, with/from Indian and Middle Eastern/Arabian Gulf regions, South West Pacific, as well as Western Europe, USA and Africa.

The prevalence of MRSA and virulent clones in clinical samples and healthy carriers obtained in Kabul was quite high in comparison to the neighboring countries. Despite great diversity, three clones were predominant namely: the Southwest Pacific clone (CC30-MRSA-IV PVL+), the CC22-MRSA-IV TSST-1 and the Bengal Bay (ST772-MRSA-IV PVL+) clones.

In regard to the animal strains in Kabul, the rate of MRSA and enterotoxigenic *S. aureus* strains isolated from bovine mastitis in our study was higher in comparison to the published literature. The presence of some MDR and virulent strains in cattle, and dissemination of such strains between animals and human in Kabul are of great public health concern.

The results of this thesis limited to Kabul, suggest major efforts have to be made on the prudent use of antibiotics and the prevention of the spread of MDR and virulent strains between health facilties, community and animals in Afghanistan. It would be also very important to screen patients for MRSA nasal carriage upon admission to the hospitals in Kabul and other provinces of Afghanistan, in order to isolate and decolonise the positive cases, enhance hygiene and taking other precautionary measures are necessary to help prevent the MRSA auto-infections and cross

infections. Supporting policies for reducing nasal carriage and continued efforts to enhance hygiene among students are necessary to slow down the transmission of MRSA in the community.

Finally, further expanded studies covering an extensive *S. aureus*/MRSA population from human infection and colonization as well as animals that are in close contact with human, collected in Kabul and other major cities of Afghanistan are recommended. These studies will give more comprehensive information about the genetic lineages, virulence and MDR profiles of colonizing and infecting strains and their dissemination and depth of the problem from public health point of view.

6.2. Conjugative transfer of *cfr*-carrying plasmids from CoNS to *S. aureus*

Our study showed the important role of LRSE as a reservoir of *cfr* gene and the efficient conjugative transfer of *cfr*-carrying plasmids from *S. epidermidis* to *S. aureus* with different genetic backgrounds. In our study the conjugative plasmid transfer seems to be influenced by different parameters: the genetic background of donors and recipients, and the nature of plasmid. Among recipients, the ST8-MRSA-IV USA300 genetic background seems to be more at risk of acquisition of *cfr*-carrying plasmids from LRSE. The plasmids *pSA737* and *p12-02300* appears more transmissible than *p-cfr-PBR-A* and *p-cfr-PBR-B.* In our study, the plasmid fitness cost and stability were highly variable in the transconjugants and not related neither to plasmid types nor to the genetic background of recipients. Some plasmids were more stable in non-selective conditions than other plasmids which may give them a higher epidemic potential. Interestingly the studied *cfr*-carrying plasmids were more stable in *S. epidermidis* than *S. aureus* in non-selective conditions which may confer *S. epidermidis* a potential to act as a reservoir of linezolid resistance. It's worth mentioning that resistance to linezolid and other oxazolidinones among *S. aureus* is still rare, and studies show a direct relation between linezolid overuse and emergence of linezolid resistance strains. Responsible use of linezolid, efficient surveillance of plasmid encoded resistance, and determination of plasmid transfer rate and stability will permit to evaluate the dissemination of such plasmid, the risk of their spread from environmental or microbiotal reservoir to pathogenic bacteria.

Based on the various important findings in this thesis, it would be interesting now to further expand this study and include other major clones of *S. aureus* and *cfr*-postive LRSE. In order

to expand further our knowledge about the risk of horizontal transfer of *cfr*-carrying plasmids, it would be also important to investigate the conjugative transfer capacity of *cfr*-carrying plasmids in the presence of subinhibitory concentrations of linezolid and inside biofilm, two environmental conditions that may promote their selection and dissemination. For further assessment of plasmids' stability and fitness cost in bacterial host, it is mandatory to extend the number of strains belonging both to *S. epidermids* and *S. aureus* and even to other staphylococcus species. It would be also of interest to study the genomic sequences of good-recipients, low- and non-recipients, in order to explore the mechanisms (such as restriction modification system), by which some strains prevent acquisition of the *cfr*-carrying plasmids, or acquired them at very low conjugation frequency. It would be also important to quantify the copy numbers of plasmids in the *cfr*-positive strains by quantitative PCR (qPCR) to investigate the relation between plasmid copy number and transfer efficiency, MIC value, fitness cost, and plasmid stability. Finally, it would be crucial to map the genetic environment of *cfr* genes on plasmids, and investigate their relation for MIC variations, stability, and transmission capability.

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ANNEX 1

Determination of antimicrobial susceptibility patterns in *Staphylococcus aureus* strains recovered from patients at two main health facilities in Kabul, Afghanistan

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Determination of antimicrobial susceptibility patterns in *Staphylococcus aureus* strains recovered from patients at two main health facilities in Kabul, Afghanistan

Haji Mohammad Naimi*, Hamidullah Rasekh, Ahmad Zia Noori and Mohammad Aman Bahaduri

Abstract

Background: *Staphylococcus aureus (S. aureus)* is a major pathogen implicated in skin and soft tissue infections, abscess in deep organs, toxin mediated diseases, respiratory tract infections, urinary tract infections, post-surgical wound infections, meningitis and many other diseases. Irresponsible and over use of antibiotics has led to an increased presence of multidrug resistant organisms and especially methicillin resistant *Staphylococcus aureus* (MRSA) as a major public health concern in Afghanistan. As a result, there are many infections with many of them undiagnosed or improperly diagnosed. We aimed to establish a baseline of knowledge regarding the prevalence of MRSA in Kabul, Afghanistan, as well as *S. aureus* antimicrobial susceptibility to current available antimicrobials, while also determining those most effective to treat *S. aureus* infections.

Methods: Samples were collected from patients at two main Health facilities in Kabul between September 2016 and February 2017. Antibiotic susceptibility profiles were determined by the disc diffusion method and studied using standard CLSI protocols.

Results: Out of 105 strains of *S. aureus* isolated from pus, urine, tracheal secretions, and blood, almost half (46; 43.8%) were methicillin-sensitive *Staphylococcus aureus* (MSSA) while 59 (56.2%) were Methicillin-resistant *Staphylococcus aureus* (MRSA). All strains were susceptible to vancomycin. In total, 100 (95.2%) strains were susceptible to rifampicin, 96 (91.4%) susceptible to clindamycin, 94 (89.5%) susceptible to imipenem, 83 (79.0%) susceptible to gentamicin, 81(77.1%) susceptible to doxycycline, 77 (77.1%) susceptible to amoxicillin + clavulanic acid, 78 (74.3%) susceptible to cefazolin, 71 (67.6%) susceptible to tobramycin, 68 (64.8%) susceptible to chloramphenicol, 60 (57.1%) were susceptible to trimethoprim-sulfamethoxazole, 47 (44.8%) susceptible to ciprofloxacin, 38 (36.2%) susceptible to azithromycin and erythromycin, 37 (35.2%) susceptible to ceftriaxone and 11 (10.5%) were susceptible to cefixim. Almost all (104; 99.05%) were resistant to penicillin G and only 1 (0.95%) was intermediate to penicillin G. Interestingly, 74.6% of MRSA strains were azithromycin resistant with 8.5% of them clindamycin resistant. Ninety-six (91.4%) of the isolates were multi-drug resistant.

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Conclusions: There was a high rate of Methicillin resistance (56.2%) among *S. aureus* strains in the samples collected and most (91.4%) were multidrug resistant. The most effective antibiotics to treat *Staph* infections were vancomycin, rifampicin, imipenem, clindamycin, amoxicillin-clavulanic acid, cefazolin, gentamicin and doxycycline. The least effective were azithromycin, ceftriaxone, cefixim and penicillin. We recommend that, where possible, in every case of *S. aureus* infection in Kabul, Afghanistan, Antibiotic susceptibility testing (AST) should be performed and responsible use of antibiotics should be considered.

Keywords: MRSA, Kabul health facilities, Antimicrobial susceptibility

Background

Staphylococcus aureus is a major pathogen implicated in skin and soft tissue infections [1]. Multidrug resistance in *Staphylococci* is an increasing problem in clinical practice especially methicillin-resistant *S. aureus* (MRSA) strains. These strains are resistant to most of the antimicrobial agents, and isolates with reduced susceptibility and resistance to vancomycin, which is the last drug for the treatment of MRSA infections [2]. These multidrug resistant strains may cause severe infections with a high rate of mortality. In vitro susceptibilities of MRSA strains, especially those from community-acquired infections, to clindamycin, macrolides, quinolones, tetracyclines, and trimethoprim-sulfamethoxazole have frequently been reported [3, 4]. Strains of MRSA, which had been largely confined to hospitals and long-term care facilities, are emerging elsewhere in the community. The changing epidemiology of MRSA bears striking similarity to the emergence of penicillinase-mediated resistance in *S. aureus* decades ago [5]. One of the best choices of treatment of MRSA is to treat with clindamycin and fluoroquinolones such as ciprofloxacin, but recent studies showed that susceptibility of this microorganism is also decreasing to clindamycin and fluoroquinolones [6, 7].

In a study in southern districts of Tamilnadu, India [8], the prevalence of MRSA strains isolated from clinical and carrier samples were 37.9%. Almost all clinical MRSA strains (99.6%) were resistant to penicillin, 93.6% to ampicillin, and 63.2% towards gentamicin, co-trimoxazole, cephalexin, erythromycin, and cefotaxime. All MRSA strains (100%) of carrier screening samples had resistance to penicillin and 71.8% and 35.9% respectively were resistant to ampicillin and co-trimoxazole. However, all strains of clinical and carrier subjects were sensitive to vancomycin. In this study, it was concluded that the determination of prevalence and antibiotic susceptibility patterns of MRSA would help the treating clinicians for first line treatment in referral hospitals.

A study in a hospital in Turkey aimed to determine the susceptibility patterns of *Staphylococcus aureus* strains to various antimicrobials, 50.2% were resistant to methicillin. All strains were susceptible to vancomycin, teicoplanin, quinupristin/dalfopristin, and linezolid. It was found that 53.4% MRSA strains were erythromycin resistant, and 39.6% showed constitutive clindamycin resistance. In this study they identified the high rate of methicillin resistance among *S. aureus* strains in their hospital [9].

In Afghanistan the widespread use of antibiotics has led to increase in the number of multidrug resistant organisms including MRSA [10, 11]. A study in Afghanistan, showed that there is a significant amount of overuse and abuse of antimicrobials in primary health care clinics that may lead to problem of antimicrobial resistance [12]. Another study at a US military hospital in Bagram Airbase in Afghanistan, found that Afghan patients often carry multidrug-resistant (MDR) bacteria compared to US citizens treated in this hospital. Their findings suggested the need for effective infection control measures at deployed hospitals where both soldiers and local patients are treated [13].

Indeed, some strains have become resistant to practically all of the commonly available antibiotics in Afghanistan. That is why the physicians mostly prescribe new antibiotics in order to get positive results without knowing the susceptibility patterns of causative bacterial agents [11]. There is no study regarding the prevalence of MRSA which is a multidrug resistant bacteria and its susceptibility patterns to most common antibiotics in Afghanistan, which causes severe infections with a higher mortality rate both community and hospital acquired infections. The aim of this study is to assess the prevalence of MRSA, as well as determining antimicrobial susceptibility patterns of *S. aureus* strains to common antibiotics available in Kabul, Afghanistan. The results of this study would help physicians in Kabul to know the prevalence of MRSA and to help them change their treatment protocols, and to know the importance of bacteriological culture and antibiotic susceptibility testing (AST). It would also be helpful for the Ministry of Health of Afghanistan to pay more attention to diagnostic labs and the role of bacteriological culture and AST to provide better treatment outcomes and responsible use of antibiotics. The findings would also emphasize the importance of local surveillance in generating relevant local resistance data that can guide empiric therapy.

Methods

This longitudinal study was conducted in the Microbiology Laboratory of the Faculty of Pharmacy of Kabul University between September 2106 and February 2017. Presumptive isolates from various clinical samples were brought from two main health facilities of Kabul, to the microbiology lab of the Faculty of Pharmacy. All of the isolates were collected from clinical specimens obtained from hospitalized patients. The standard microbiological procedures were conducted with minimum delay for culture, confirmatory tests and AST. We selected two main health facilities in Kabul, because they have standard microbiology labs and perform most of the bacteriological cultures and identification in Kabul. Confirmatory tests were carried out for diagnosis of *S. aureus strains*, by inoculating presumptive isolates onto Blood agar base medium (Oxoid, England) to which 5% sheep blood was added. All cultured media were incubated at 37 °C for 18–24 h under aerobic condition. The suspected isolated colonies were subjected to Gram's staining, Catalase test, Coagulase test, and Mannitol fermentation on Mannitol Salt agar (Oxoid, England) [14]. Confirmed *S. aureus* isolates were subjected to AST by Kirby Bauer disc diffusion method as per Clinical Laboratory Standards Institute (CLSI) guidelines [15] on Muller Hinton agar (Oxoid, England) for 20 antimicrobials such as: penicillin G (P,1unit), amoxicillin-clavulanic acid (AMC, 30μg), oxacillin (OX, 1μg), azithromycin (ATH, 15μg), erythromycin (E, 15μg), cefazolin (CZ, 30μg), ceftazidime (CAZ, 30μg), cefoxitin (FOX, 30μg), cefixim (CFM, 5μg), ceftriaxone (CRO, 30μg), ciprofloxacin (CIP, 5μg), trimethoprim-sulfamethoxazole (SXT, 1,25/23,75μg), gentamicin (CN, 10μg), tobramycin (TOB, 10μg), doxycycline (DO, 30μg), imipenem (IMI, 10μg), clindamycin (CD, 2μg), vancomycin (VA, 30μg), chloramphenicol (C, 10μg) and rifampicin (RP, 5μg).

The growth suspension for AST was prepared in 5 ml Normal saline solution and the turbidity was adjusted to match that of 0.5 McFarland standards to obtain approximately the organism number of 1×10^6 colony forming units (CFU) per ml. Antibiotic discs were placed after 15 min of inoculation to Muller Hinton agar seeded with each isolate and were incubated for 18–24 h at 35–37 °C. The diameter of the zone of inhibition around the disc was measured using sliding metal caliper. For accuracy, during the antibiotic screens, three independent replicates were performed. The susceptibility of all isolates were determined against different classes of antibiotics as follows:

For detection of MRSA we applied two definitions: [1] inhibition zone less than or equal to 23 mm on Mueller Hinton Agar (MHA) with 30 μg cefoxitin disc seeded with growth suspension of *S. aureus* isolates adjusted to 0.5 McFarland standards at 37 °C for 18-24 h [16]; [2] inhibition zone on MHA containing 2% NaCl with 1μg oxacillin disc less than or equal to 10 mm seeded with growth suspension of *S. aureus* isolates adjusted to 0.5 McFarland standards at 30 °C for 18-24 h [17].

For detection of Multi Drug Resistance, we used the definition of *Magiorakos* et al. [18] as non-susceptibility to at least one agent in three or more antimicrobial categories.

Statistical analysis and quality assurance

The reliability of the study findings was guaranteed by implementing quality control measures throughout the whole processes of laboratory work. We used two strains of *S. aureus* as control. *S. aureus* ATCC 29213 a *mecA* negative strain, and *S. aureus* ATCC 43300 a *mecA* positive strain; both confirmed with standard PCR as reference methicillin-sensitive *S. aureus* (MSSA) and MRSA strains respectively using the DNA amplification instrument Mastercycler gradient (Eppendorf, Germany).

The statistical analysis was done using SPSS version 19. Binary logistic regression was used to determine the association between *S. aureus* infection, gender and age. Multivariate logistic regressions were used to control confounding factors. A *P-value* less than 0.05 was considered as statistically significant.

Results

Of 105 strains of *S. aureus* isolated from various types of pus, urine, tracheal secretions and blood, 46 (43.8%) were MSSA while 59 (56.2%) were MRSA. All strains (105; 100%) were susceptible to vancomycin. Almost all (104; 99.05%) were resistant to penicillin G and only 1 (0.95%) was intermediate to penicillin, for further information please refer to Table 1.

We did not find any strain of MSSA to be resistant to clindamycin and only 6.5% were intermediate to clindamycin, while 8.5% of MRSA strains were resistant to clindamycin. Susceptibility to azithromycin was low in both MSSA (52.2%) and MRSA (23.7%). MSSA vs MRSA isolates showed a higher susceptibility to amoxicillin + clavulanic acid, 2nd and 3rd generation of cephalosporins, aminoglycosides, imipenem, ciprofloxacin, rifampicin, and co-trimoxazole, for further information please refer to Table 2.

The difference of MRSA infection was not statistically significant according to gender ($p = 0.42$). Of 59 MRSA strains isolated, 44 (74.6%) were from males while 15 (25.4%) from females. According to category of age, the prevalence of MRSA was 39.0% in ages between 1 and 17 years, 39.3% in ages between 18 and 40 years and 66.7% in ages between 41 and 75 years old. The difference of MRSA distribution was not statistically significant according to age ($p = 0.50$), and health facility ($p = 0.95$).

Specimen-wise distribution showed that MSSA vs MRSA in blood was (44% vs 56%), in ear pus (50% vs 50%), in pus from other sites of the body (44% vs 56%), in urine (33% vs 67%), and in tracheal secretions (50% vs 50%). The specimen

Table 1 Antimicrobial susceptibility patterns of *S. aureus* strains to different antimicrobial agents

Classes of ATB	Antibiotics	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Penicillines	Penicillin G		1 (1.0)	104 (99.0)
	Amoxicillin + Clavulanic acid	81 (77.1)		24 (22.9)
	Oxacillin	49 (46.7)		56 (53.3)
Macrolides	Erythromycin	38 (36.2)	4 (3.8)	63 (60.0)
	Azithromycin	38 (36.2)	4 (3.8)	63 (60.0)
2nd and 3rd generation of Cephalosporins	Cefazolin	78 (74.3)	10 (9.5)	17 (16.2)
	Cefixim	11 (10.5)	5 (4.8)	89 (84.8)
	Cefoxitin	49 (46.7)		56 (53.3)
	Ceftriaxone	37 (35.2)	39 (37.1)	29 (27.6)
	Ceftazidime	6 (5.7)	12 (11.4)	87 (82.9)
Quinolones	Ciprofloxacin	47 (44.8)	5 (4.8)	53 (50.5)
Sulfonamides	Cotri-moxazole	60 (57.1)	9 (8.6)	36 (34.3)
Aminoglycosides	Gentamicin	83 (79.0)	5 (4.8)	17 (16.2)
	Tobramycin	71 (67.6)		34 (32.4)
Tetracycline	Doxycycline	81 (77.1)	10 (9.5)	14 (13.3)
Carbapenems	Imipenem	94 (89.5)	1 (1.0)	10 (9.5)
Lincosamides	Clindamycin	96 (91.4)	4 (3.8)	5 (4.8)
Polypeptides	Vancomycin	105 (100.0)		
Divers	Rifampicin	100 (95.2)		5 (4.8)
	Chloramphenicol	68 (64.8)	30 (28.6)	7 (6.7)

Table 2 Comparative susceptibility of MRSA *and* MSSA strains to different antimicrobial agents

Classes of ATB	Antibiotics	MSSA (%)	MRSA (%)	*P-value*
Penicillines	Penicillin G	0	0	
	Amoxicillin + Clavulanic acid	97.8	61	**0.0001**
Macrolides	Erythromycin	52.2	23.7	**0.002**
	Azithromycin	52.2	23.7	**0.002**
2nd and 3rd generation of Cephalosporins	Cefazolin	97.8	55.9	**0.0001**
	Cefixim	19.6	3.4	**0.001**
	Ceftriaxone	71.7	6.8	**0.0001**
	Ceftazidime	8.7	3.4	**0.005**
Quinolones	Ciprofloxacin	60.9	32.2	**0.0001**
Sulfonamides	Cotri-moxazole	69.6	47.5	**0.004**
Aminoglycosides	Gentamicin	95.1	66.7	**0.001**
	Tobramycin	82.6	55.9	**0.004**
Tetracyclines	Doxycycline	71.7	81.4	0.478
Carbapenems	Imipenem	100	81.4	**0.008**
Lincosamides	Clindamycin	93.5	89.8	0.063
Polypeptides	Vancomycin	100	100	
Divers	Rifampicin	100	91.5	**0.043**
	Chloramphenicol	63	66.1	0.933

A *p*-value less than 0.05 was considered statistically significant and are in boldface

wise distribution of MSSA and MRSA was not significantly different ($p = 0.96$).

In males the percentage of MSSA was 31 (41.3%) versus MRSA 44 (58.7%), and in females, MSSA 15 (50%) versus MRSA 15 (50%). The difference of MRSA distribution was not significant according to gender ($p = 0.52$).

Multi-drug resistance (MDR) pattern of *S. aureus*

Eighty-eight (83.8%) of the isolates were multi-drug resistant. Multi-drug resistant strains ranged from resistance to three classes of antibiotics (11, 10.48%) to 9 classes of antibiotics (1, 0.95%). The highest rate of MDR were observed for 4–5 classes of antibiotics (28, 26.67%). Details of resistance to different antibiotics are described in Table 3.

Discussion

In our study, methicillin resistant *S. aureus* was found to be 56.2%. There is no previous information regarding prevalence of MRSA in Afghanistan. In West Asia, MRSA prevalence ranges from 12% to 49.4% in six different hospitals of Saudi Arabia [19]. In European countries, MRSA rates varied from 0.6% in Sweden to 40.2–45% in Belgium, Greece, Ireland, Italy, and the United Kingdom [20, 21], because the use of antibiotics are much more controlled in these countries. In Turkey, the proportion of MRSA were reported to be 50.2% [9] which is similar to European countries. In a study performed in 17 different regions of Russia, methicillin resistance among *S. aureus* strains was between 0% and 89.5% [22] which is very diverse. In a systemic review in Iran, the prevalence of MRSA was determined to be approximately 56.5% (ranged between 50 and 60%) [23], which is similar to our findings and the similarity would be due to irresponsible use of antibiotics in both countries.

We found that the prevalence of MRSA among patients in our study to be 56.2% which is higher compared to findings of a similar study conducted in Peshawar Pakistan, which is very close to Afghanistan. In that, study the researchers examined 280 isolates of *S. aureus* recovered from hospitalized patients, and indicated that 36.1% of *Staphylococci* were detected as MRSA [24]. There was also a significant difference between gender and MRSA infections. In our study, 74% of MRSA isolates were from males. As compared to the study from Pakistan, 34% of MRSA infections were from males. According to age in both studies the prevalence of MRSA infections were higher among elderly in Pakistan and Afghanistan 60.71% and 66.7% respectively, which is a known risk factor for MRSA infections, however in both studies it was not statistically significant. The prevalence of MRSA infection in the present study did not vary significantly by gender ($p = 0.42$), age group ($p = 0.50$), specimen ($p = 0.96$) and health facility ($p = 0.95$). This is in agreement with earlier reports by *Geyid* et al. [25] indicating that gender and age are not risk factors for the acquisition or colonization of MRSA.

In our study, despite the high prevalence of MRSA, there was no isolate with reduced susceptibility to vancomycin, however we could not include other new antibiotics like teicoplanin, linezolid and quinupristin/dalfopristin in our study to assess their efficacy as well, because these antibiotics are not included in the licensed and official medicine list of Afghanistan and therefore are not available in Afghanistan [26].

In this study, it was observed that 8.5% of the MRSA strains were resistant to rifampicin and clindamycin and 16.9% were resistant to imipenem; this is probably because these antibiotics are not widely used in the treatment of *Staph* and other bacterial infections in clinics in Afghanistan and are mostly effective in the treatment of sensitive G+ and G- bacteria. Most of the MRSA isolates were resistant to multiple other antimicrobial agents like cefixim (96.6%), ceftazidime (93.2%).

Interestingly, ceftriaxone, which is widely used in Kabul and other provinces of Afghanistan, we found that 44.1% of MRSA strains were resistant to this antibiotic and 49.2% intermediate and only 6.8% were susceptible. This is an alarming sign, which highlights widespread use of this antibiotic and other similar broad spectrum antibiotics in clinical settings and increased resistance toward third generation cephalosporins. In general, elevated rates of multidrug resistance may emerge from diverse isolates of *S. aureus* under antimicrobial pressure or as a result of widespread person to person transmission of multidrug resistant isolates [27]. In our study, although imipenem resistance was detected in 81.4% MRSA strains, no resistance was detected in MSSA strains. In this study, cefazolin, gentamicin and ciprofloxacin were found to be more effective on MSSA than MRSA strains.

Interestingly 8.5% of MRSA strains were resistant to clindamycin, while there was no resistant strain of MSSA to clindamycin. We found that 6.5% of MSSA strains to be intermediate to clindamycin. Our findings support the previous study conducted by Frank, et al. [28] that clindamycin is effective for the treatment of infections caused by *Staphylococci*, or for patients allergic to beta-lactam agents [18, 29]. It is a good alternative to the treatment of both MSSA and MRSA infections.

Conclusions

The prevalence of MRSA strains obtained in this study was high (56.2%) when compared with the prevalence rates obtained from other similar studies conducted

Table 3 Percentage of resistance patterns of *S. aureus* isolates to different number of antibiotics

Antibiotic	Resistant strains	
	No of *S. aureus*	%
P	2	1.91
P,CIP	1	6.67
P,CAZ	3	
P,CFM	2	
P,TOB	1	
P, CAZ, CFM	7	10.48
P, CAZ, CIP	1	
P,E,ATH	1	
P,C,CFM	1	
P,CAZ,SXT	1	
P,CAZ,CFM,DO	1	4.76
P,CAZ,E,ATH	1	
P,TOB,E,ATH	1	
P,CIP,E,ATH	1	
P,CIP,CAZ,CFM	1	
P,TOB,CFM,E,ATH	1	12.38
P,DO,C,CAZ,CFM	2	
P,CAZ,CFM,E,ATH	2	
P,SXT,DO,CAZ,CFM	2	
P,CAZ,OX,FOX,CFM	2	
P,CIP,CAZ„E,ATH	1	
P,CAZ,OX,FOX,AMC	1	
P,TOB,OX,FOX,AMC	1	
P,DO,CFM,E,ATH	1	
P,CIP,CAZ,CFM,E,ATH	3	8.57
P,CRO,CAZ,OX,FOX,CFM	2	
P,TOB,CAZ,CFM,E,ATH	1	
P,CIP,CRO,CFM,E,ATH	1	
P,SXT,DO,CIP,CAZ,CFM	1	
P,CIP,CAZ,OX,FOX,CFM	1	
P,CRO,CAZ,OX,FOX,CFM,AMC	2	7.62
P,SXT,TOB,CAZ,CFM,E,ATH	1	
P,TOB,DO,CAZ,CFM,E,ATH	1	
P,CIP,CRO,CAZ,CFM,E,ATH	1	
P,SXT,CAZ,CFM,CZ,E,ATH	1	
P,SXT,TOB,G,CIP,CAZ,CFM	1	
P,CAZ,OX,FOX,CFM,E,ATH	1	
P,CIP,CAZ,OX,FOX,CFM,E,ATH	8	13.33
P,SXT,CAZ,OX,FOX,CFM,E,AT	2	
P,RP,C,CAZ,OX,FOX,CFM,CZ	1	
P,CAZ,OX,FOX,CFM,E,ATH,AMC	1	
P,SXT,TOB,CIP,CAZ,OX,FOX,CFM	1	
P,SXT,TOB,CRO,CAZ,OX,FOX,CFM	1	
P,CIP,CRO,CAZ,OX,FOX,CFM,E,ATH	2	13.33
P,CIP,OX,FOX,CFM,CZ,E,ATH,AMC	1	
P,SXT,CIP,CAZ,OX,FOX,CFM,E,ATH	3	
P,SXT,TOB,CAZ,OX,FOX,CFM,E,ATH	1	
P,SXT,CIP,CRO,CAZ,CFM,E,ATH,AMC	1	
P,SXT,TOB,C,CIP,CAZ,OX,FOX,CFM	1	
P,SXT,RP,CAZ,OX,FOX,CFM,E,ATH	1	
P,DO,CIP,CAZ,OX,FOX,CFM,E,ATH	1	
P,SXT,CRO,CAZ,OX,FOX,CFM,E,ATH	3	
P,E,ATH,CFM,FOX,OX,CAZ,CIP,G,TOB	1	1.91
P,AMC,E,ATH,CFM,FOX,OX,CAZ,CIP,DO	1	
P,E,ATH,AMC,CFM,OX,FOX,CAZ,CIP,G,TOB	1	4.76
P,E,ATH,CFM,OX,FOX,CAZ,CIP,DO,G,SXT	1	
P,AMC,CZ,CFM,FOX,OX,CAZ,CRO,CIP,SXT,TOB	1	
P,E,ATH,CFM,FOX,OX,CIP,CD,C,SXT,TOB	1	
P,E,ATH,CZ,CFM,FOX,OX,CAZ,CRO,CIP,TOB	1	
P,E,ATH,AMC,CFM,FOX,OX,CAZ,CRO,G,TOB,SXT	1	1.91
P,AMC,CZ,CFM,FOX,OX,CAZ,CRO,CIP,G,RP,TOB	1	
P,AMC,E,ATH,CFM,FOX,OX,CAZ,CRO,CIP,DO,RP,SXT	1	1.91
P,AMC,E,ATH,CZ,CFM,FOX,OX,CAZ,CRO,CIP,G,TOB	1	
P,E,ATH,CFM,FOX,OX,CAZ,CRO,CIP,CD,DO,G,TOB,SXT	1	0.95
P,AMC,E,ATH,CZ,CFM,FOX,OX,CAZ,CRO,CIP,G,IM,TOB,SXT	5	5.71
P,AMC,E,ATH,CZ,CFM,FOX,OX,CAZ,CRO,CIP,RP,IM,TOB,SXT	1	
P,AMC,E,ATH,CZ,CFM,FOX,OX,CAZ,CRO,CIP,DO,G,IM,TOB,SXT	1	2.86
P,AMC,E,ATH,CZ,CFM,FOX,OX,CAZ,CRO,CIP,CD,G,IM,TOB,SXT	2	
P,AMC,E,ATH,CZ,CFM,FOX,OX,CAZ,CRO,CIP,CD,C,G,IM,TOB,SXT	1	0.95
Total	105	100.0

P Penicillin G, *CIP* Ciprofloxacin, *AMC* Amoxicillin-Clavulanic acid, *C* chloramphenicol, *FOX* Cefoxitin, *E* Erythromycin, *ATH* Azithromycin, *G* Gentamicin, *VAN* Vancomycin, *SXT* Trimethoprim-Sulfamethoxazole, *CD* Clindamycin, *CFM* Cefixim, *CZ* Cefazolin, *OX* Oxacillin, *CAZ* Ceftazidime, *CRO* Ceftriaxone, *IM* Imipenem, *TOB* Tobramycin, *RP* Rifampicin, *DO* Doxycycline

elsewhere. Most of *S. aureus* strains especially MRSA strains were multidrug-resistant and fortunately no isolate was resistant to vancomycin, the drug of choice for treating multidrug resistant MRSA infections. Isolates showed a higher susceptibility to vancomycin, clindamycin, rifampicin, imipenem, amoxicillin + clavulanic acid, cefazolin, gentamicin, and doxycycline. The least effective were azithromycin, ceftriaxone, cefixim and penicillin.

Good infection control practices such as strict hand washing, identifying and treating MRSA carriers, as well as prudent use of antimicrobial agents is recommended. Further, genotypic studies are needed to characterize resistant strains of *S. aureus*.

Abbreviations
AST: Antibiotic susceptibility testing; ATCC: American Type Culture Collection; CFU: Colony Forming Unit; CLSI: Clinical and Laboratory Standards Institute; FMIC: French Medical Institute for Children; MDR: Multi drug resistant; MRSA: Methicillin resistant *Staphylococcus aureus*; MSSA: Methicillin sensitive *Staphylococcus aureus*

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Availability of data and materials
All relevant materials and data supporting the findings of this study are contained within the manuscript.

Authors' contributions
HMN has made substantial contributions to the conception and design of the study, analysis and interpretation of data and has given final approval to submit the manuscript to be published. HR was involved in conducting the study; drafting the manuscript; and statistically analyzing the data. AZN was involved in culture of strains and antibiotic susceptibility testing. MAB brought samples from health facilities to Microbiology Lab of faculty of Pharmacy and conducted preliminary identifications of strains. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by the Academic Council of the Faculty of Pharmacy and Research Committee of Kabul University with approval numbers: FoP.124, 12/7/2016 and KURC.115, 03/08/2016. The research committee of KU is considered the local research review board. According to the decision of local research review board, verbal consent from all participants was also obtained for specimens to be used in this study due to the illiteracy of some participants. However, this study was conducted on specimens from patients and not directly on patients. The specimens for this study were collected from French Medical Institute for Children (FMIC) and Kabul Central Laboratory (KCL), which are two main health facilities in Kabul.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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ANNEX 2

Characterizing the molecular epidemiology of *Staphylococcus aureus* across and within fitness facility types

Mark Dalman, Sabana Bhatta, Nagashreyaa Nagajothi, Dipendra Thapaliya, Hailee Olson, Haji Mohammad Naimi and Tara C. Smith

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Characterizing the molecular epidemiology of *Staphylococcus aureus* across and within fitness facility types

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Abstract

Background: *Staphylococcus aureus* is a common bacterium found in the nose and throat of healthy individuals, and presents risk factors for infection and death. We investigated environmental contamination of fitness facilities with *S. aureus* in order to determine molecular types and antibiotic susceptibility profiles of contaminates that may be transmitted to facility patrons.

Methods: Environmental swabs ($n = 288$) were obtained from several fitness facilities ($n = 16$) across Northeast Ohio including cross-fit type facilities ($n = 4$), traditional iron gyms (n = 4), community center-based facilities ($n = 5$), and hospital-associated facilities ($n = 3$). Samples were taken from 18 different surfaces at each facility and were processed within 24 h using typical bacteriological methods. Positive isolates were subjected to antibiotic susceptibility testing and molecular characterization (PVL and *mec*A PCR, and *spa* typing).

Results: The overall prevalence of *S. aureus* on environmental surfaces in the fitness facilities was 38.2% (110/288). The most commonly colonized surfaces were the weight ball (62.5%), cable driven curl bar, and CrossFit box (62.5%), as well as the weight plates (56.3%) and treadmill handle (50%). Interestingly, the bathroom levers and door handles were the least contaminated surfaces in both male and female restroom facilities (18.8%). Community gyms (40.0%) had the highest contamination prevalence among sampled surfaces with CrossFit (38.9%), traditional gyms (38.9%), and hospital associated (33.3%) contaminated less frequently, though the differences were not significant ($p = 0.875$). The top *spa* types found overall were t008 (12.7%), t267 (10.0%), t160, t282, t338 (all at 5.5%), t012 and t442 (4.5%), and t002 (3.6%). t008 and t002 was found in all fitness facility types accept Crossfit, with t267 (25%), t548, t377, t189 (all 10.7%) the top *spa* types found within crossfit. All samples were resistant to benzylpenicillin, with community centers having significantly more strains resistant to oxacillin (52.8%), erythromycin (47%), clindamycin (36%), and ciprofloxacin (19%). Overall, 36.3% of isolates were multidrug resistant.

Conclusions: Our pilot study indicates that all facility types were contaminated by *S. aureus* and MRSA, and that additional studies are needed to characterize the microbiome structure of surfaces at different fitness facility types and the patrons at these facilities.

Keywords: Athletic fitness facilities, *S. aureus*, MRSA, Multi-drug resistant, Northeast Ohio, CrossFit, Gym

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Background

Staphylococcus aureus is an opportunistic pathogen that colonizes asymptomatically the skin and nares of approximately 1 in 3 people worldwide [1, 2]. *S. aureus* is found in hospital settings, and with the emergence of antibiotic resistance, can cause life threatening infections. Recent studies have suggested that, in the United States, over 80,000 invasive infections and 11,000 yearly deaths are attributed to methicillin-resistant *S. aureus* (MRSA) [3]. While the number of invasive MRSA infections has declined slowly over the past decade, our group has identified reservoirs of MSSA (methicillin-susceptible *S. aureus*) and MRSA in humans, animals, and the environment [4–11]. Although colonization with *S. aureus* is usually asymptomatic, it is a risk factor for active infection [12] and enhances the ability of an individual to transmit *S. aureus* to fomite surfaces [13].

One location that has been associated with an increased risk of both infection and colonization with *S. aureus* is the athletic fitness facility [14, 15]. Over 50 million individuals in the United States support approximately 30,000 fitness facilities, and the number continues to rise [14]. MRSA infections have been linked to contamination of athletic facilities due to shared exercise equipment [15] and towels [16]. Facility-associated MRSA infections have also been documented in both professional and collegiate athletic teams [17–19], and even in high school athletes [20, 21]. The link between fitness facilities and contaminated surfaces is unclear [22], but Markley and colleagues [15] found that approximately 10% of gym surfaces were contaminated with *S. aureus* and these contaminated fomites may serve to spread *S. aureus*. However, not all fitness facility types and surfaces may warrant the same scrutiny.

In the present study, we assessed the prevalence of *S. aureus* and MRSA across several fitness facility types to characterize the microbial environment and potential for *S. aureus* transmission, to identify potential risks associated with certain areas, environmental conditions, or types of exercise equipment, and to provide a more inclusive study addressing exercise facility type as a facilitator of *S. aureus* transmission.

Methods

Facility sampling

We performed point prevalence microbiological surveys at 16 fitness facilities in Northeast Ohio. The facilities were convenience-sampled with the surfaces selected as the most commonly hand-touched areas in the building. Environmental swabs were obtained from CrossFit type facilities ($n = 4$), traditional free weight gyms ($n = 4$), community center facilities ($n = 5$), and hospital –associated facilities ($n = 3$) with the written permission of the owners. Traditional free weight gyms only have weights for lifting and do not offer any other services such as a pool or daycare. Community center facilities were usually larger facilities with a wide range of services from yoga, gyms for basketball or volleyball, swimming pools, daycare services, rock climbing walls, and food preparation. Hospital-associated facilities were linked to a hospital or clinic and had weights, swimming pool, and guided care for rehabilitation purposes, in addition to providing membership access to the general public. CrossFit facilities involved highly intensive, varied movement exercises covering several sports movements, and were required to self-identify as a CrossFit facility for inclusion purposes.

The sampling technique of surfaces was as previously described [7]. Briefly, a sterile Swiffer® cloth was used to wipe down a 9 square inch environmental surface for 10 s using a new set of gloves for each surface as to reduce cross contamination. Samples were then placed in a labeled, individual sterile Whirl-Pak™ bag (Nasco, Fort Atkinson, WI) and stored on ice until returning to the lab for processing within 3 h of acquisition.

Bacterial culture and identification

Samples were processed as previously described [23]. Samples were reconstituted in 50 mL of sterile 0.1% peptone broth and massaged for 25 s to enhance bacterial recovery. Subsequently, this peptone solution was transferred to a 50 mL aliquot of sterile, 2X Baird Parker Broth (BPB) solution with tellurite enrichment (Sigma products-Sigma Aldrich, St. Louis, MO) and allowed to incubate at 37 °C for 24 h. After incubation, 1 μL inoculums were streaked onto Baird Parker Agar (BPA) with EY tellurite enrichment and selective MRSA agar plates (BBL CHROMagar MRSA, Becton, Dickinson and Company), and allowed to incubate for 48 h at 37 °C. Potential *S. aureus* colonies (black colonies) and presumptive MRSA colonies (mauve colonies on CHROMagar) were then plated on Columbia colistin- nalidixic acid agar with 5% sheep's blood (CNA; Ramel). Plated CNA were allowed to grow at 37 °C for 24 h. Colonies were confirmed using a series of biochemical assays including: catalase, coagulase, and *S. aureus* latex agglutination (Pastorex Staph-Plus, Bio-Rad, Hercules, CA). Confirmed *S. aureus* isolates were stored at − 80 °C with a single colony used for antibiotic susceptibility testing (AST) and subsequent molecular analyses.

Molecular characterization

Positive *S. aureus* isolate genomic DNA was isolated using Wizard Genomic DNA preparation kit (Promega, Madison, WI). Polymerase chain reaction (PCR) was used to amplify the presence of methicillin resistance gene (*mec*A) and PVL genes (*luk*S, *luk*F) [24, 25]. Furthermore, Staphylococcus protein A (*spa*; FOR 5'-GAAC

AA-CGTAACGGCTTCATCC-3′ and REV 5'-CAGC AGTAGTGCCGTTTGCCT) was used for molecular typing [26–28]. Ridom StaphType software was used to assign *spa* types (v2.2.1; Ridom GmbH, Wurzburg, Germany). The Based upon Repeat Pattern (BURP) algorithm was used to group *spa* types based on their genetic proximity [29], as well as Bionumerics software (version 7.6.2). Only *spa* typing was conducted, since previous studies have found high congruence and discriminatory power compared to MLST sequence data [28, 30, 31]. A positive (USA300) and negative control were used for all biochemical and molecular assays.

Antimicrobial susceptibility testing (AST)

All *S. aureus* isolates were subjected to AST by VITEK 2 system (bioMerieux, Durham, NC; Version R06.01) using AST-GP71 cards according to manufacturer's and Clinical Laboratory Standards Institute Standards (CLSI, 2012). A (0.5–0.63 OD) bacterial suspension in 0.45% saline was prepared for each sample tested. AST-GP71 cards test for: benzylpenicillin, oxacillin, tetracycline, erythromycin, ciprofloxacin, moxifloxacin, minocycline, clindamycin, trimethoprim-sulfamethoxazole, quinupristin/ dalfopristin, gentamicin, levofloxacin, linezolid, daptomycin, vancomycin, rifampin, tigecycline, and nitrofurantoin. Resistance to ≥3 class of antibiotics was considered as multi-drug resistant (MDR) [32].

Environmental factors

Temperature and relative humidity measurements were collected at all locations at a central point away from any external door or HVAC vent. Temperature and relative humidity measurements were collected at the end of sampling (~ 45–60 min) to ensure that the temperature and relative humidity were indicative of the gym facility, and not a carryover from transport or previous location. Total patron membership numbers and cleaning regimens were also collected via a self-reported questionnaire.

Statistical analysis

Association of variables was tested by Pearson's Chi-square test in addition to Fisher's exact test for outcome. For all analyses, P was set at 0.05 with all tests carried out using SAS software (Ver. 9.3, SAS Institute Inc., Cary, NC). Minimum spanning tree was conducted using Bionumerics software (7.6.2).

Results

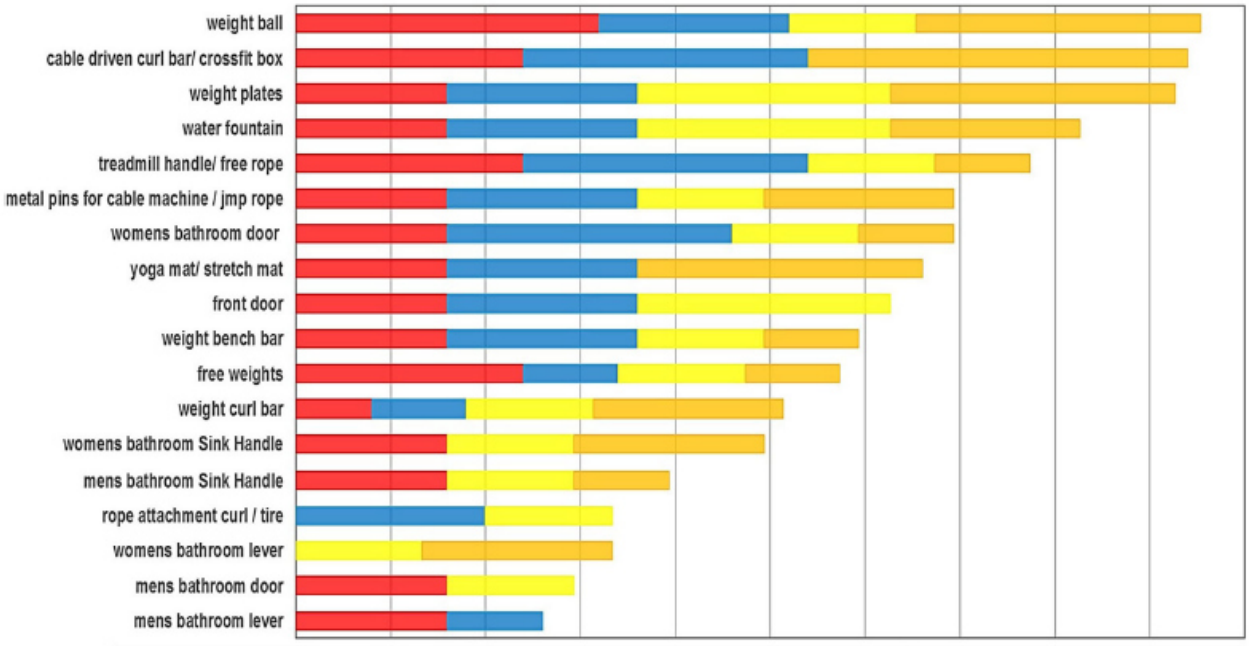
Prevalence of *S. aureus*

A total of 288 environmental samples (fitness facility surface samples) were collected from 16 fitness facilities in Northeast Ohio. A total of 110 sites were identified as *S. aureus*-positive. The overall prevalence of *S. aureus* for all locations was 38.2% (110/288) with a prevalence of 26.7% (77/ 288; ± 1.65% SE) and 11.5% (33/ 288; ± 3.95% SE) for MSSA and MRSA respectively (Table 1). There was an average of 27.5 ± 3.69 (Mean ± SE) positive isolates per site type. Table 1 shows the distribution of *S. aureus* across multiple fitness facility types sampled. We found similar *S. aureus* contamination across fitness facilities buildings (community: 40.0%, 36/90; traditional: 38.9%, 28/72; CrossFit: 38.9%, 28/72; and hospital-associated: 33.3%, 18/54). The prevalence of MSSA was significantly higher compared to MRSA ($p = 0.024$), while the total number of contaminated surfaces was not significantly different across fitness facility types (Table 1; $p = 0.875$). The highest prevalence of *S. aureus* was observed on the weight ball (62.5%, 10/16) and cable-driven curl bar/ CrossFit box (62.5%, 10/16), followed by weight plates (56.3%, 9/16), treadmill handle/free rope (50.0%, 8/16), and water fountain (50.0%, 8/16) (Fig. 1). Interestingly, based on *mec*A presence, MRSA contamination was higher in community-associated fitness facilities (52.8%, 19/36) compared to hospital-associated (5.56%, 1/18), CrossFit (14.3%, 4/28), and traditional fitness facilities (32.1%, 9/28) (Table 1; $p = 0.001$).

MRSA = Methicillin resistant *S. aureus*. MSSA = Methicillin susceptible *S. aureus*.

Table 1 Prevalence of *S. aureus* (MRSA and MSSA) by fitness facility type

Traditional			Community			Hospital			Crossfit		
Total positive isolates n=28 4 Facilities sampled			Total positive isolates n=36 5 Facilities sampled			Total positive isolates n=18 3 Facilities sampled			Total positive isolates n=28 4 Facilities sampled		
Facility	MRSA (n)	MSSA (n)	Facility	MRSA (n)	MSSA (n)	Facility	MRSA (n)	MSSA (n)	Facility	MRSA (n)	MSSA (n)
A	0	10	E	4	6	J	0	7	M	1	7
B	5	3	F	7	2	K	0	6	N	3	4
C	1	4	G	1	6	L	1	4	O	0	7
D	3	2	H	5	0				P	0	6
			I	2	3						
	9 (32.1%)	19 (67.9%)		19 (52.8%)	17 (41.2%)		1 (5.60%)	17 (94.4%)		4 (14.3%)	24 (85.7%)



	mens bathroom lever	mens bathroom door	womens bathroom lever	rope attachment curl / tire	mens bathroom Sink Handle	womens bathroom Sink Handle	weight curl bar	free weights	weight bench bar	front door	yoga mat/ stretch mat	womens bathroom door	metal pins for cable machine / jmp rope	treadmill handle/ free rope	water fountain	weight plates	cable driven curl bar/ crossfit box	weight ball
Community Center	40%	40%	0%	0%	40%	40%	20%	60%	40%	40%	40%	40%	40%	60%	40%	40%	60%	80%
Traditional Gym	25%	0%	0%	50%	0%	0%	25%	25%	50%	50%	50%	75%	50%	75%	50%	50%	75%	50%
Hospital associated	0%	33%	33%	33%	33%	33%	33%	33%	33%	67%	0%	33%	33%	33%	67%	67%	0%	33%
CrossFit	0%	0%	50%	0%	25%	50%	50%	25%	25%	0%	75%	25%	50%	25%	50%	75%	100%	75%

Fig. 1 *S. aureus* percent contamination for each fitness facility and surface type

Under facility type (Traditional, Community, Hospital, and Crossfit), each facility (A through P) had 18 samples taken and only positive MRSA or MSSA are reported here.

Facilities A through P represent unique facility addresses.

Molecular characterization of *S. aureus* isolates

Molecular typing of the *spa* gene was performed on all confirmed *S. aureus* isolates, in addition to examination of presence of the *mec*A and PVL genes. A total of 38 unique *spa* types were identified among 110 isolates with the most common 14 *spa* types present in at least three or more surfaces. The most common *spa* type present was t008 (12.7%; 14/110), followed by t267 (10.0%; 11/110), t160, t282, t338 (all at 5.45%; 6/110), t012, t442 (both at 4.55%; 5/110), t002, t026, t334 (all at 3.64%; 4/110), t148, t189, t377, and t548 (all at 2.73%; 3/110) (Table 2; Fig. 2). Of 33 MRSA isolates, 33.3% were t008. The most common *spa* types found in each fitness type were t267 (25.0%; 7/28; CrossFit), t012 (22.2%; 4/18; hospital- associated), t008 (22.2%; 8/36; community center), and t008 and t016 were tied for the most common in the traditional free-weight gym (17.9%; 5/28).

The prevalence of the *mec*A gene among *S. aureus* isolates was 30.0% (33/110). MRSA was isolated from 14.3% (4/28) of isolates in CrossFit facilitates, 5.6% (1/18) of hospital-associated facilities, 52.8% (19/36) of community facilities, and 32.1% (9/28) of traditional gym facilities (Table 2). There was a significant difference observed between fitness facility types ($p = 0.001$). There were only three isolates (2.7%; 3/110) that were PVL-positive and were correspondingly found only in community facilities.

Antibiotic susceptibility profile

All *S. aureus* isolates were subjected to antibiotic susceptibility testing. Cumulatively, 37 isolates (33.6%) were resistant to erythromycin, 33 (30.0%) were resistant to oxacillin, 29 (26.4%) were resistant to clindamycin, 9 (8.2%) were resistant to tetracycline, 11 (10.0%) were resistant to ciprofloxacin, 11 (10.0%) were resistant to levofloxacin, and 4 (3.6%) were resistant to minocycline (Fig. 3). Forty isolates (36.3%) were MDR- *S. aureus*. The 4 isolates that demonstrated intermediate resistance to vancomycin were found within community (1), CrossFit (2), and hospital (1) -associated facilities. Community-associated facilities had significantly more oxacillin (52.8%; 19/36; $p = 0.001$), levofloxacin (22.2%; 8/36; $p = 0.021$), clindamycin (36.1%; 13/36; $p = 0.025$), erythromycin (47.2%; 17/36; $p = 0.054$), and ciprofloxacin (19.4%; 7/36; $p = 0.056$) resistant strains compared to hospital, crossfit, and traditional facility types.

Table 2 *S. aureus spa* typing by fitness facility location

Crossfit Gym			Hospital-Associated			Community Associated			Traditional Gym		
%	n	type	%	n	type	%	n	type	%	n	type
25%	7	t267	22%	4	t012	22%	8	t008	18%	5	t008
11%	3	t548	11%	2	t002	11%	4	t282	18%	5	t160
11%	3	t377	11%	2	t026	11%	4	t267	11%	3	t442
11%	3	t189	11%	2	t148	6%	2	t338	7%	2	t026
7%	2	t084	11%	2	t338	6%	2	t442	7%	2	t062
7%	2	t282	6%	1	t008	6%	2	t922	7%	2	t2726
7%	2	t334	6%	1	t073	3%	1	t002	4%	1	t002
7%	2	t4371	6%	1	t160	3%	1	t012	4%	1	t003
4%	1	t338	6%	1	t216	3%	1	t051	4%	1	t164
4%	1	t346	6%	1	t334	3%	1	t1154	4%	1	t179
4%	1	t360	6%	1	t688	3%	1	t148	4%	1	t1954
4%	1	t688		18		3%	1	t179	4%	1	t216
	28					3%	1	t2414	4%	1	t276
						3%	1	t242	4%	1	t334
						3%	1	t334	4%	1	t346
						3%	1	t345		28	
						3%	1	t7540			
						3%	1	t774			
						3%	1	t7954			
						3%	1	t8504			
							36				

Environmental factors

Temperature and relative humidity measurements were collected at all locations, as were patron membership numbers. The mean facility temperature across all facilities was 21.5 °C (70.7 °F ± 2.4) with a range of 18.3 to 23.3 °C (Data not shown; $p = 0.156$). The average relative humidity was 47.6% ±3.8 with no significant difference between facilities (Data not shown; $p = 0.708$). Patron membership was highest in community centers (7496 ± 4327), with hospital associated facilities at 2400 ± 1053, traditional gyms at 1350 ± 724, and CrossFit at 103 ± 27 (Mean ± SE). However, the patronage difference was not statistically significant across facilities sampled (Data not shown; $p = 0.22$). All facilities provided access to hand sanitizer stations except for 2 CrossFit facilities (50%) and 1 community center (20%).

Discussion

This study examined the prevalence and molecular characteristics of *S. aureus* and MRSA sampled from 288 gym surfaces collected from 16 gyms across four different types of gym facilities in Northeast Ohio. From 288 samples, 110 were positive *S. aureus* isolates and we had a 26.7% (77/288) and 11.5% (33/288) recovery of *S. aureus* and MRSA, respectively. We found similar *S. aureus* prevalence across community, traditional, CrossFit, and hospital-associated fitness facilities (40.0% vs 38.9% vs 38.9% vs 33.3%, respectively) (Table 1). However, our prevalence rates were higher than those identified previously [15–21]. The prevalence rate difference may be attributed to fitness facility and/or patron type. As athletes and athletic personnel generally have a significantly higher incidence of both infections and over a ten-fold higher number of antibiotic prescriptions per year compared to the general public, many infections may remain unmonitored or unrecognized [18, 33, 34]. Despite their continual occurrence, there has been little effort to identify and monitor contaminated surfaces and the role they may play in transmission, until recently [35–39]. Though our study found similar rates of contamination across all fitness facilities that mirror common human carriage rates, the incidence of both *S. aureus* and MRSA is higher in our environmental contamination study than what has been observed in the literature, such as in school settings, playgrounds, and beaches [4–8, 15, 22, 40]. Despite other environmental

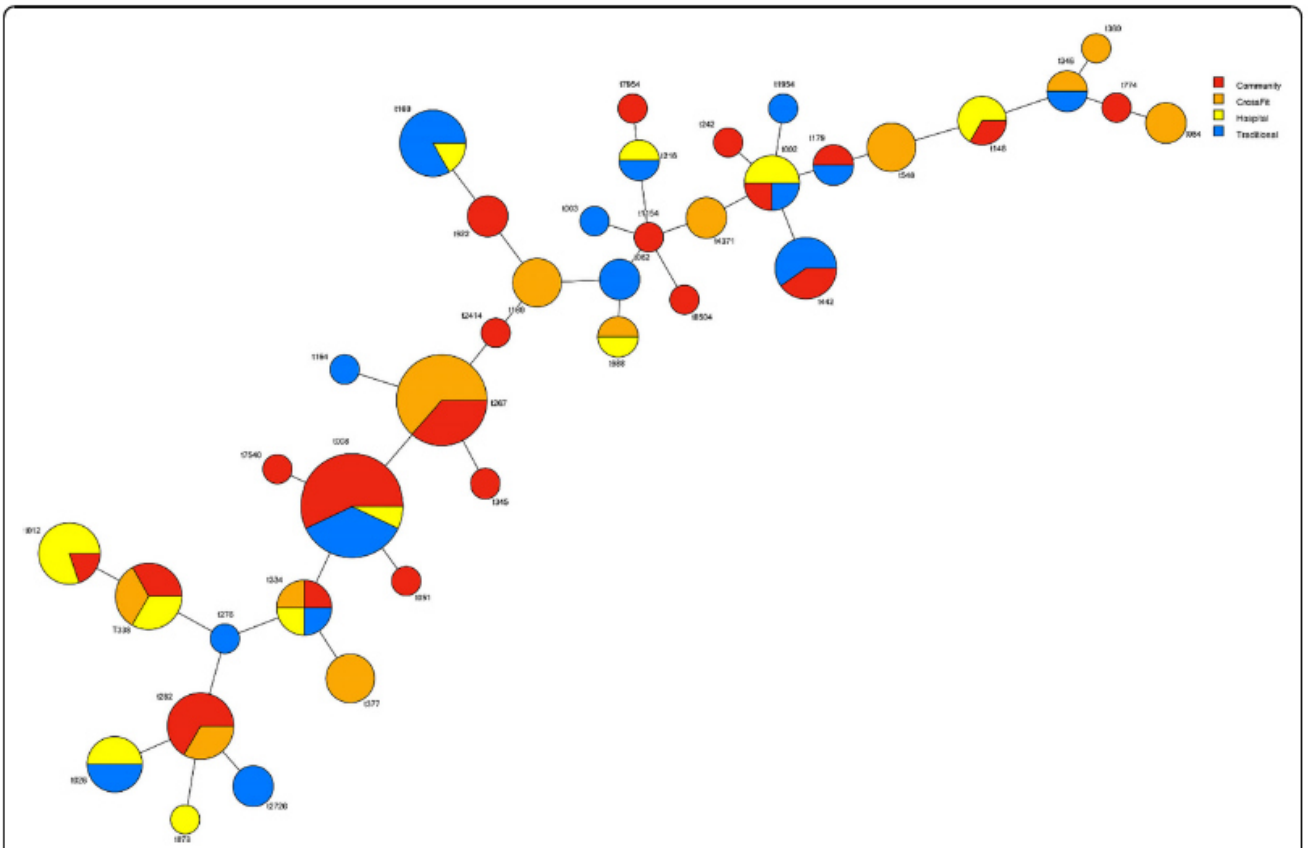


Fig. 2 BURP clustering of *spa* typing from *S. aureus* isolates. Color represents fitness facility type with percent of circle equal to percent total of *spa* type in relation to total positive *S. aureus* isolates identified

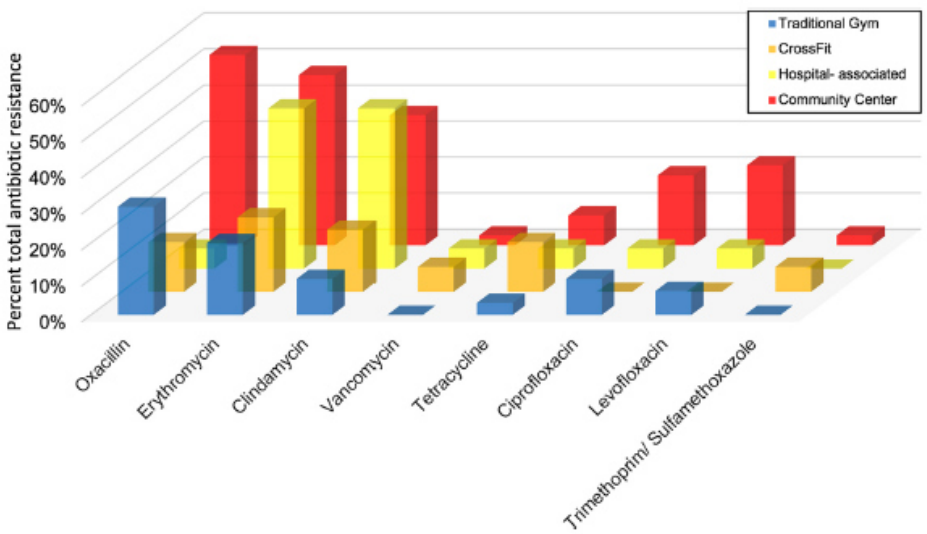


Fig. 3 Percent total antibiotic resistance of positive *S. aureus* isolates by fitness facility location. *All vancomycin resistance is intermediate

contamination studies, studies of fitness facilities have received attention only recently.

For example, Ryan and colleagues [22] found zero presence of *S. aureus* in gym facility surfaces suggesting that all transmission was entirely via-person to person contact or at least ruling out that gyms were reservoirs of *S. aureus*. Markley and colleagues [15] sampled 16 different surface types at one large community center and found that 10% (10/99) of samples were contaminated with MSSA only. Due to the significantly limited size and scope of the study (one facility type was examined), their reported incidence may be underestimated. Prior to these studies, 5 players (9%) on the 2003 St. Louis Rams football team were found to have MRSA infections [18]. Interestingly, they found zero nasal carriers and/or environmental reservoirs of MRSA, but did grow MSSA from whirlpool water and a gel-applicator stick used for taping ankles, suggesting that fomite surfaces have the potential to harbor and transmit *S. aureus*. Although Ryan and colleagues [22] surveyed three facilities (college, high school, and private gyms) before and after cleaning regiments, they found zero presence of MSSA and MRSA from their 240 samples. Their results may be attributed to differences in bacterial isolation and cultivation. Almost 40% of the population are carriers of *S. aureus* [41], thus, it is surprising that these studies found very few contaminated surfaces, while contamination in a hospital setting is sufficiently ubiquitous to sound alarm [42–46]. For example, a hospital study found that 76% of skin and soft tissue infections were of *S. aureus* etiology and 59% of those were attributable to MRSA [47]. Of those presenting MRSA, 99% of isolates were community-associated (CA-MRSA), pointing to acquisition of MRSA strains from outside of the hospital setting, such as a gym facility. Likewise, hospital ward high touch surfaces areas were highly contaminated with *S. aureus* concentration increasing by almost 80% over a 4-h period despite the use of hypochlorite. The addition of Quaternary Ammonium Compound surfactants (QAC) did drastically decontaminate surfaces to almost 10% of their original bacterial load count, pointing to potential bacterial decontamination strategies to reduce transmission [46]. Additionally, through the use of sequencing techniques, small amounts of biomass collected from gym facilities were sufficient to identify community bacteria, as well as staphylococcal species present on athletic surfaces in the gym, with the composition modulated by interacting with human skin [48]. Though our study did not track personnel or patron *S. aureus* carriage or microbiome composition, provenance of contamination will be key for future studies addressing the movement, transmission, and potential antibiotic-resistant reservoirs of fitness facilities.

The increased prevalence of *S. aureus* on fitness facility surfaces may also be a result of environmental co-evolution. As *S. aureus* can tolerate high osmolarity stress (high saline environments), the production of sweat at gym facilities can even be extrapolated to other high intensity situations such as war and combat, which may select for *Staphylococcal* species in the environment or individual [49]. With ease of horizontal gene transfer, the acquisition of antibiotic resistance may be enhanced as a result. The incidence of MRSA in athletes is almost triple what is observed in the general population. Thus, it is not surprising that we observed higher contamination rates on gym surfaces than other surfaces [50].

We found a total of 38 unique *spa* types with t008 (14; 12.7%) and t267 (11; 10.0%) being the most common (Table 2; Fig. 2). Interestingly, some loosely identified livestock associated strains such as t548 and t338 were found in Crossfit, community, and hospital-associated strains but not in strains isolated from traditional gyms (Fig. 2). t548 is associated to upper Midwestern and northwestern regions of the US, including Ohio. Since it was found in hospital associated gyms, the line between the original provenance of *S. aureus* strains and their site of contamination is becoming increasingly fluid. Approximately 8.2% (9/110) of strains were categorized as livestock-associated. A total of 6 isolates were t338 and were found in hospital, Crossfit, and community fitness facilities. Three isolates (2.7%) were t548 and found solely within Crossfit facilities. Additionally, t012 was the most common strain type found in hospital-associated facilities (4; 22.0%) and community fitness centers (1; 3.0%), but not traditional or Crossfit facilities. t012 is known to be less prevalent as the age of the individual increases [51, 52]. However, its increased incidence in hospital- associated facilities may be a result of rehabilitation of both older and younger patients. Conversely, t002 was found in hospital-associated (2; 11.0%), community (1; 3.0%), and traditional facilities (1; 4.0%). t002 is often found in nursing homes and in older patients [53]. As such, the hospital-associated facilities also had strains associated with older patients. These results suggest that age demographics may play a significant role in strain isolates found in various fitness facilities. We found t002 on the weight ball and weight bench bars in hospital, traditional, and community facilities. As it has the possibility to be present in cases of bacteremia [52, 54], it is important to be careful in regards to lifting weights with any cuts present, which could contaminate gym equipment.

Looking across gym facilities, Crossfit had the most diverse range of *spa* types (Table 2; Fig. 2). Of the 10 *spa* types found in Crossfit facilities, 6 were community-associated (t267, t377, t084, t282, t334, and t4371), two have been found in livestock (t548 and t338), and two have been associated in hospital settings (t189 and t346). Though other facilities sampled had some similar variations of these associations, none were as diverse as Crossfit

types. This may be a result of the comparative lack of lifting machine structure in Crossfit gyms and/ or the wide range of people that attend them [55, 56]. Furthermore, if common cleaning regimens are not followed, high hand-touch surfaces may harbor and easily disseminate pathogenic, antibiotic resistant bacteria to other people via hand contact, as hands are recognized as a primary mode of transmission of many diseases (Fig. 3), [38, 45, 57].

To the best of our knowledge, this is the first study to evaluate systematically different gym facility types for contamination and molecular typing of *S. aureus*. The strength of our study resides within our large sample size both across fitness facility types, as well as across fitness surfaces sampled. In addition, we also carried out molecular genotyping of *S. aureus* isolates, further strengthening our epidemiological study of *S. aureus* contamination on athletic gym surfaces. However, there are limitations to our study that included convenience sampling in Northeast Ohio fitness facilities only. In addition, we only sampled surfaces at one point in time, we did not sample surfaces after a cleaning regimen, and we did not sample and type isolates found on patrons and facility employees. Future studies should evaluate the patron microbiome, as well as the collective microbiota impact on fomite contamination and transmission. Our results indicate not only the presence of putatively dangerous isolates of *S. aureus,* but also that increased cleaning regimens and enhanced hygiene practices should be followed in fitness facilities as is practiced in the hospital or work place.

Conclusions

Of 288 surfaces swabbed from 16 different facilities (traditional gyms, community centers, hospital-associated facilities, and CrossFit facilities), 38.2% (110/288) of surfaces were positive for *S. aureus*. 30.0% of all isolates were mecA-positive with community-associated fitness facilities containing the most mecA (17.3%) compared to traditional, CrossFit, and hospital-associated fitness centers (8.20, 3.64 and 0.91%, respectively). t008 was the most common spa type present across all gym facility types. All fitness locations were contaminated with both *S. aureus* and MRSA, and 36.4% of all positive isolates were multidrug-resistant.

Abbreviations

AST: antibiotic susceptibility testing; BURP: Based upon Repeat Pattern; CA-MRSA: community-associated Methicillin resistant *Staphylococcus aureus*; HVAC: Heating, ventilation, and air conditioning; MDR: multi-drug resistant; MLST: Multilocus sequence typing; MRSA: Methicillin resistant *Staphylococcus aureus*; MSSA: methicillin-susceptible *S. aureus*; PVL: Panton–Valentine leukocidinPCRPolymerase chain reaction; QAC: Quaternary Ammonium Compound surfactants

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Availability of data and materials

All relevant materials and data supporting the findings of this study are contained within the manuscript. Raw data can be requested from the corresponding author.

Authors' contributions

MD was involved in all aspects of research design, implementation, and writing the manuscript. SB, NN, HO, DT, and HMN were involved in sample acquisition and/or molecular work. TCS was involved in manuscript revision and overall study design. All authors were involved in drafting and approving the final manuscript, and give final approval for the manuscript.

Ethics approval and consent to participate

Not applicable. However, access to facilities was through written consent by owner.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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ANNEX 3

Prevalence and Risk Factors of Hepatitis B, Hepatitis C and HIV Viruses Among People Who Use Drugs (PWUD) in Kabul, Health Care Facilities

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Prevalence and Risk Factors of Hepatitis B, Hepatitis C and HIV Viruses Among People Who Use Drugs (PWUD) in Kabul, Health Care Facilities

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Abstract

Background: Human immunodeficiency virus (HIV), hepatitis B (HBV) and C (HCV) viruses infect millions of people around the world. People who use drugs (PWUD) are at high risk of such viral diseases and could be the source of these infections to other people. Shared needle, unsafe extra-marital sexual contact, skin popping and other risky behaviors are well-known risk factors for the prevalence of these infections among PWUD worldwide. There is no valid information regarding the danger and rate of the above-mentioned viral infections and associated risk factors among PWUD referred to healthcare facilities in Kabul, Afghanistan for the treatment and support.

Objectives: The main objective of this study was to determine the prevalence and risk factors of HBV, HCV, and HIV among PWUD in Kabul, healthcare facilities.

Methods: This study was conducted in 7 public and private healthcare facilities at the primary care level in Kabul, which provides social support and medical care to PWUD. All patients who referred to the healthcare facilities from May 2016 to October 2016 and signed the consent were included in this study.

Results: Out of 410 PWUD, 15 (3.7%) were positive for the HBV surface antigen (HBsAg), 45 (11%) were positive for HCV antibody, and one (0.2%) was positive for HIV antibody. Among the risk factor variables studied, the administration of drugs by injection was considered the most important for HCV (OR= 3.80, P< 0.01) and viral infections among PWUD (OR= 5.40, P< 0.01).

Conclusions: This study draws attention to the high prevalence and spread of viral hepatitis among PWUD in Kabul, Afghanistan. The prevalence of HCV and HBV was higher among PWUD compared to general populations, and drug use via injection was an important risk factor for transmitting viral infections. Based on our results, active preventive programs focusing on educational campaigns targeted at the youth populations should be undertaken in Afghanistan. Further studies, especially among PWUD living without shelter in Kabul and other major cities of Afghanistan, are recommended in order to better analyze the dangers among drug addicts in Afghanistan.

Keywords: Hepatitis B, Hepatitis C, HIV, People Who Use Drugs (PWUD), Kabul

1. Background

Hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) infections constitute serious healthcare problems and infect millions of people around the world. HBV and HCV infections can result in chronic liver diseases, including cirrhosis and hepatocellular carcinoma, while HIV infection can result in severe opportunistic diseases (1).

Illegal drug use has become an increasingly major problem in Afghanistan due to widespread armed conflict resulting in extensive internal and external displacement and social and economic upheavals since 2001 (2). The population of Kabul, the capital of Afghanistan, has increased to approximately 3 million since 2001 because of returning refugees (3, 4). External displacement is significant, particularly in refugee populations moving to neighboring countries, including Iran and Pakistan. Both countries harbor higher drug user populations and higher prevalence of HBV, HCV, and HIV among people who use drugs (PWUD), especially people who inject drugs (PWID) (5-7). As a result, when the populations return to Afghanistan, they spread illegal drug use to others.

Estimates say that about 1.6 million Afghans are PWUD, fostered by readily available opium and heroin and most of them are men. The Afghan government has space for only about 2,300 of them in its drug treatment facilities

around the country and Kabul harbors a large number of PWUD. At these treatment facilities, in addition to stopping drug abuse, the goal of the treatment is to return people to productive lives in their family, workplace, and community. During their 3 - 6-month treatment, the patients receive medication therapy, counselling, and psychotherapy (8).

Most of the PWUD in Kabul live collectively in open areas without any shelters. This lifestyle increases their risky behaviors such as poor hygiene, close contact, shared needles, skin popping, and unsafe extra-marital sexual contact, and not only exposes them to transmissible infections such as HBV, HCV, and HIV, but also makes them the main source of transmission of such viral infections to other people. These observations raised concerns that PWUD and accompanying high-risk behaviors are increasing in Afghanistan and the risk of HBV, HCV, and HIV epidemic would have the potential to be high in the future (9,10). Additionally, a study carried out on chronic intranasal users of the drug in the USA showed that it has implications for potential transmission of HCV via contact with contaminated nasal secretions (11), which is also common among PWUD in Kabul. The PWUD are a potential source of infection and may represent a serious threat to the community. Therefore, it is extremely important to identify and track infected persons in order to prevent further transmission of these infections by preventive measures and appropriate controls. There are no major differences between in-treatment and out of treatment PWUD in terms of risky behaviors and sociodemographic characteristics in Kabul. However, out of treatment PWUD may differ in terms of continuous contact with infected persons and the prevalence of viral infections (10).

A recent survey conducted by Afghanistan National AIDS Control Program (NACP) in 2012 revealed 12,541 PWUD were living in Kabul. Unsafe injection drug use, including shared needles and other injecting equipment, is considered the key route of HIV, HBV and HCV transmission in Afghanistan. Based on available data, the HIV epidemic in Afghanistan seems to be low and HIV affects mainly PWUD, and are among a key population at higher risk of contracting HIV (12). In a study from 2005 to 2010 covering 1,696 PWUD from some provinces of Afghanistan such as Kabul showed a range of 36.0% - 36.6% seroprevalence of HCV and 5.8% - 6.5% hepatitis B surface antigen (HBsAg) positive (13, 14).

2. Objectives

Little data are available on hepatitis B, hepatitis C, and HIV prevalence, and associated risk behaviors among PWUD who use different methods or a combination of drug use methods in Afghanistan, especially in Kabul. The primary objective of this study was to determine the prevalence of HIV, HBV, and HCV infections among PWUD treated at health facilities of Kabul and to identify the associated risk factors and behaviors that make them exposed to these infections. The impact of this study will be to inform policymakers and program managers at the Ministry of Public Health of Afghanistan and related stakeholders about HBV, HCV, and HIV infection rates among PWUD and the associated risky behaviors in Kabul. In addition, this study might prove useful for developing proper treatment and prevention guidelines and future educational programs.

3. Methods

3.1. Subjects

The current cross-sectional study was conducted in 7 main public and private healthcare facilities treating PWUD from May 2016 to October 2016 in Kabul, Afghanistan. These centers provide social support and medical care for PWUD. Four hundred and ten male users of drug treated at these healthcare facilities were included in this study. Users of drugs are almost male in Kabul and Afghanistan and no female users were referred to these health facilities during the time of the study. The eligible participants were all the PWUDs admitted to these health facilities, with no previous vaccination against HBV (while there is no effective vaccine against HCV and HIV) and were able to provide informed consent. Before data collection, this study was approved by the Research Committee of Kabul University and the Academic Council of the Ministry of Higher Education of Afghanistan, as well as the Ministry of Public Health of Afghanistan. The participants were interviewed, using a questionnaire that included sociodemographic, travel or displacement histories, the past and current drug use, method of drug use, history of blood transfusion, and unsafe extra-marital sexual behaviors.

3.2. Serological Analysis

Five mL of the blood sample was collected from each participant and was centrifuged at 5000 RPM for five minutes at the microbiology laboratory of the Faculty of Pharmacy of Kabul University in order to isolate plasma for serological tests.

First rapid immunochromatographic tests (ICT) for HBsAg (Standard Diagnostics Inc. Korea), anti-HCV (SD, Korea), and anti-HIV-1/HIV-2 (SD, Korea) were performed with 100% sensitivity and 99.5% specificity as mentioned by the manufacturer. For diagnostic accuracy, all samples identified positive by ICT were confirmed by ELISA Kits (General Biologicals Corporation, Kaohsiung, Taiwan). The patients

who were diagnosed seropositive for HBV, HCV, or HIV were referred for further investigation and treatment.

3.3. Statistical Analysis

The overall prevalence of viral infections among PWUD was calculated with a 95% confidence interval (CI) with descriptive analysis, reported in numbers and percentages. The chi-square test was used to evaluate the prevalence of viral infections (HIV, HBV, and HCV) among those of various ages, marital status, and economic status, and duration, method of drug use, previous prison residence, and unsafe extra-marital sexual contact. The crude odds ratios (OR) were estimated by univariate analysis to observe the association of each variable with viral infection. The adjusted OR was generated to determine the independent contribution of risk factors to viral infection, using multivariate logistic regression models. All statistical tests were two-tailed and a level of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 19 (SPSS Inc, Chicago, IL, USA) software.

4. Results

All 410 PWUD were male aged between 18 - 65 years (mean 31.5, median 30.0); 212 (51.7%) aged between 18 - 30, 144 (35.1%) aged between 31 - 40, and 54 (13.2%) aged between 41 - 65 years old. Among them, 55 (13.4%) were PWID and 355 (86.6%) were non-injection users of drugs (smoking, eating, sniffing, and chewing). Their past history of addiction ranged from 73 days to 30 years (mean 7.2 years, median 6 years), and 277 of them (67.6%) were in a lower economic status (income less than 200 USD per month to feed themselves and their family). Among them, 156 (38%) were single, 252 (61.5%) married, and 2 (0.5%) were divorced. Also, 219 (53.4%) of them were illiterate, 154 (37.6%) had primary level education, and 25 (6.1%) had secondary education, and 12 (2.9%) had higher education. Among them, 303 (73.9%) were jobless before consuming drugs and after drug addiction, all of them had lost their job. Moreover, 352 of the participants (85.9%) were using heroin, 31 (7.6%) were using methamphetamine crystals, 26 (6.3%) were using opium, and 1 (0.2%) was using marihuana. In addition, 236 (57.6%) of them have started drug use in Afghanistan, 159 (38.8%) of them have started drug use in Iran, and 15 (3.7%) of them have started drug use in other countries such as; Pakistan, countries to the North of Afghanistan and even Europe. Further sociodemographic details and other information about study participants are described in Table 1.

Table 1. Sociodemographic and Other Characteristics of Study Population

Variable	Values, No. (%)
Age	
18 - 30	212 (51.7)
31 - 40	144 (35.1)
40 - 65	54 (13.2)
Marital status	
Single	156 (38.0)
Married	252 (61.5)
Divorced	2 (0.5)
Education	
Illiterate	219 (53.4)
Primary	154 (37.6)
High school	25 (6.1)
Higher education	12 (2.9)
Duration of drug use, year	
< 1	43 (10.5)
1 - 5	141 (34.4)
> 5	226 (55.1)
Drug	
Heroin	352 (85.9)
Crystal methamphetamine	31 (7.6)
Opium	26 (6.3)
Method of use	
Injection	25 (6.1)
Smoke	350 (85.4)
Other methods	5 (1.2)
Injection + other methods	30 (7.3)
Transfusion	
Yes	12 (2.9)
No	398 (97.1)
Reason of using drug	
Friendship	369 (90)
Social problems	4 (1)
Medical problems	37 (9)
Country of starting drug use	
Afghanistan	236 (57.6)
Iran	159 (38.8)
Other countries	15 (3.7)

Fifteen PWUD (3.7%, 95% CI 1.8 - 5.5) were HBsAg positive, forty-five (11.0%, 95% CI 7.9 - 14.0) were anti-HCV positive and one anti-HIV positive (0.2%, 95% CI -0.2 - 0.7); one person was co-infected with HIV and HCV (0.2%, 95% CI -0.2 - 0.7) and two persons were co-infected with HCV and HBV (0.5%, 95%

CI -0.2 - 1.2). Further details are summarized in Table 2.

In univariate and multivariate analysis of HIV and HBV, we did not find any significant association between different variables and HIV, HBV infection among infected and uninfected persons. Owing to that the number of HCV infected persons was higher, we performed a multivariate analysis and adjusted for other variables. We found that shared needles and the use of drugs by injection independently associated with HCV infection (OR = 5.40, CI: 2.60 - 11.23). Due to the small number of HIV and HBV infections, we could not separately calculate risk factors for these infections.

Finally, we analyzed the associated risk factors for viral infection (HIV, HBV, and HCV infection). By univariate analysis, the use of drugs by injection, history of unsafe extra-marital sexual contact, the history of prison was significantly associated with viral infection, but in multivariate analysis, the use of drugs by injection remained significantly associated with viral infection, which is mostly related to HCV infection (OR= 3.57, CI: 1.76 - 7.24). Further details are summarized in Table 3.

As injection drug use is already a known risk factor for HIV, HBV, and HCV infections, we analyzed some factors that may predict injection drug use as well. In the univariate analysis, joblessness, location of starting drug use in Iran and other countries, previous prison and starting drug use with a duration of two years or more were significantly associated with injection drug use. After multivariate analysis, joblessness, location of starting drug use in other countries, and previous prison remained significantly associated with injection drug use. Further details are summarized in Table 4.

5. Discussion

In this study, among PWUD in Kabul, 189 (42.4%) of them have started drug use outside Afghanistan, especially Iran (38.8%). This highlights the role of external displacement and as a result bringing this habit back to Afghanistan. The remaining 236 (57.6%) have started drug use in Afghanistan, which shows a great danger of becoming a drug addict in the country nowadays. All subjects of the study population were male because almost drug addicts are male in Afghanistan and it was not possible to compare females with males in this regard.

There was just one case of HIV infection by detection of anti-HIV antibody, but this finding is not representative of PWUD living in Kabul without shelter. Other vulnerable population groups and those having risky healthy behaviors were also not included in this study and may harbor higher prevalence of HIV than the study group; however, this is consistent with the World Health Organization's latest report on HIV (15). It can be concluded that Afghanistan is in the early stage of an HIV epidemic and the main route of transmission remains shared needle among PWID. On the other hand, this study was conducted on drug user populations referred to hospitals by their families or by themselves. While the prevalence of risk factors, especially shared needles among PWID living on the streets or under bridges crossing Kabul River, in very bad hygienic conditions, is higher and the prevalence of viral infections among them could be higher too, which is not reflected in this study.

In the current study, 3.7% were HBsAg and all of them were using drugs by smoking. This finding may indicate the role of other risk factors other than PWID; however, owing to a small number of positive HBV cases in our study, we could not conclude smoking as a specific risk factor. Moreover, 11% were anti-HCV positive and this was seen both among PWID and non-injecting drug users, this finding potentially anticipates that HCV transmission and incidence among PWUD may increase in the near future and also could be the main source of HCV transmission to other people. In a review article concerning the prevalence of HBV and HCV among all available Afghanistan populations (2), the prevalence of HBV was 1.9% and HCV was 1.1%. Our findings suggest that the prevalence of HBV and especially HCV is ten times higher among PWUDs than the general population.

Among risk factors, injection drug use was related to viral infection and mostly for HCV infection, which is a known risk factor for HCV transmission (16). This finding is similar to a study conducted in Kashan, Iran (17). The results of this study showed no association of HBV and HCV infection with age, economical condition, unsafe extra-marital sexual contact, previous prison, and the duration of drug use; however, shared needles were significantly associated with HCV infection.

In our study, the associated factors for injection drug use that promoted viral transmission and infection included; joblessness, previous prison, and the location of starting drug use in other countries, remained significant. This study is worthwhile for the Ministry of Public Health of Afghanistan and related stakeholders. This study has some strengths and limitations. This was the first study among PWUD admitted in different Kabul health facilities, with the purpose to interview and collect blood samples from a considerable number of the PWUD that can represent all PWUD under treatment and social support. One of the limitations of this study was the lack of information about the history of injection use or shared syringe among PWUDs; also we could not interview and collect blood samples from drug user living without shelters on streets and under bridges in very bad hygienic conditions.

Table 2. Prevalence of HIV, HBV, and HCV Among PWUD by Method of Drug Use

Virus	Situation	Drug User Category, No. (%)			Total, No. (%)
		Smoke	Injection	Other	
HCV	Positive	26 (57.80)	19 (42.20)	0 (0.00)	45 (100.00)
HBV	Positive	15 (100)	0 (0.00)	0 (0.00)	15 (100.00)
HIV	Positive	0 (0.00)	1 (100.00)	0 (0.00)	1 (100.00)
Viral infection (Either HIV, HBV or HCV)	positive	38 (67.85)	18 (32.15))	0 (0.00)	56 (100.00)
Co-infection					
HIV + HCV	Positive	0 (0.00)	1 (100.00)	0 (0.00)	1 (100.00)
HCV + HBV	Positive	2 (100.00)	0 (0.00)	0 (0.00)	2 (100.00)

Table 3. Univariate and Multivariate Analysis of Risk Factors for HCV, HBV, and HIV by Binary Logistic Regression

Variable	Univariate OR, CI (95%)	P Value	Multivariate OR, CI (95%)	P Value
Age				
18 - 30	1.0			
31 - 40	1.64 (0.89 - 3.01)	0.10		
> 40	1.16 (0.47 - 2.87)	0.73		
Marital status				
Single	1.0			
Married	0.84 (0.47 - 1.50)	0.56		
Divorced	5.78 (0.35 - 95.75)	0.22		
Economic status				
Lower[a]	1.0	0.11		
Middle[b]	0.59 (0.30 - 1.13)			
Duration of use	1.022 (0.97 - 1.07)	0.38		
Method of use				
Smoke	1.0			
Injection	3.99 (2.07 - 7.69)	< 0.01	3.57 (1.76 - 7.24)	< 0.01
Other methods	0.00	0.99		
Previous prison				
No	1.0			
Yes	1.84 (1.00 - 3.39)	0.05		
Unsafe extra-marital sexual contact				
No	1.0			
Yes	2.03 (1.15 - 3.60)	< 0.01	1.78	0.06

[a] Income between 200 - 500 USD per month.
[b] Income less than 200 USD per month.

5.1. Conclusions

Our study showed that the prevalence of HCV and HBV are higher among PWUD in Kabul than the normal population and the rate of HIV infection is lower among them. The use of drugs by injection was significantly associated with HCV infection among PWUD. While further studies, especially among PWUD living without shelter in Kabul and other major cities of Afghanistan, are recommended to better analyze the dangers among drug addicts. Based on our results and given the fact that adequate programs against drug use are scarce in Afghanistan, active preventive drug abuse programs focusing on educational cam-

Table 4. Univariate and Multivariate Analysis of Associated Factors for Drug Injection Use by Binary Logistic Regression

Variable	Univariate OR, CI (95%)	P Value	Multivariate OR, CI (95%)	P Value
Occupation				
Employed	1.0			
Jobless	2.27 (1.04 - 4.98)	0.04	2.92 (1.20 - 7.11)	0.02
Place of starting drug use				
Afghanistan	1.0			
Iran	2.21 (1.19 - 4.10)	0.01	1.27 (0.63 - 2.56)	0.50
Other[a]	12.34 (4.06 - 37.57)	< 0.01	7.46 (1.99 - 28.03)	< 0.01
Previous prison				
No	1.0			
Yes	4.79 (2.65 - 8.67)	< 0.01	3.57 (1.85 - 6.86)	< 0.01
Duration of drug use, y				
< 2	1.0			
2 - 10	4.53 (1.36 - 15.11)	0.01		
> 10	4.67 (1.29 - 16.92)	0.02		

[a]Pakistan, countries to the north of Afghanistan, and even Europe.

paigns and targeted at young people should be undertaken. It is a fact that intravenous injection of drugs, especially among drug addicts who are jobless and imprisoned, as well as sharing of injection equipment are associated with dangerous viral infections and should be highlighted in future public educational and informational campaigns.

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Footnotes

Authors' Contribution: Haji Mohammad Naimi made substantial contributions to the conception and design of the study, analysis, interpretation of data and given final approval to submit the manuscript to be published. Hamidullah Rasekh was involved in conducting the study, drafting the manuscript, and statistically analyzing the data. Sayed Hamid Mousavi was involved in writing reports and drafting the manuscript and English revision of the manuscript. All authors read and approved the final manuscript.

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Patient Consent: Before participating in this study, the participants were asked to read the consent form containing all the necessary information about the study and make an informed decision whether they want to participate in this study. The consent forms signed by each participant are available.

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Haji Mohammad NAIMI

***Staphylococcus aureus* : Caractérisation moléculaire des isolats Afghans et étude du transfert conjugatif de la résistance au linézolide**

1. Caractérisation moléculaire des isolats de *S. aureus* en provenance d'Afghanistan

Staphylococcus aureus (SA) est une cause majeure d'infections chez l'Homme et de nombreux animaux. L'un des principaux objectifs de cette thèse a été d'étudier les caractéristiques moléculaires et le profil de résistance aux antibiotiques des souches d'infection et de colonisation de SA/SA résistant à la méticilline (SARM) chez l'Homme et le bétail à Kaboul. Les souches ont été identifiées par des méthodes phénotypiques conventionnelles, et l'antibiogramme réalisé par diffusion. Les souches de SARM ont ensuite été caractérisées à l'aide de puces à ADN. Parmi les isolats de SA provenant d'infections humaines dans 2 hôpitaux de Kaboul entre Janvier et Juin 2017, la prévalence des SARM était élevée (66,3 %). Les 98 isolats de SASM et SARM appartenaient à 12 complexes clonaux et 27 clones distincts. La prévalence du portage nasal de SA et du SARM dans la communauté, plus précisément chez des étudiants (hors médecine) de l'Université de Kaboul, était respectivement de 33,3% et 12,7%. Les 19 isolats de SARM appartenaient à 4 clones. Enfin, les études réalisées chez l'animal ont montré qu'environ 11,8% des souches de SA responsables de mammite bovine à Kaboul étaient résistantes à la méticilline (2/17) et que la prévalence du portage nasal de SA chez les moutons était très faible (1,1 %). Globalement, la caractérisation moléculaire des isolats a mis en évidence une grande diversité génétique et la présence de multiples gènes de virulence et de résistance parmi les souches de SA/SARM circulant dans les établissements de soins, la communauté et le bétail à Kaboul.

2. Transfert conjugatif du gène de résistance au linézolide *cfr*

Le linézolide est un antibiotique de recours pour le traitement des infections à SARM. L'objectif de ce travail a éte d'étudier, i) le risque de transfert conjugatif de la résistance au linézolide médiée par le gène plasmidique *cfr,* de souches de *S. epidermidis* (SE) vers des souches de SA de divers fonds génétiques, et ii) le coût de ce transfert pour les souches receveuses et la stabilité des plasmides. Cinq souches de SE portant 4 plasmides différents (*pSA737, p12-02300, p-cfr-PBR-A, p-cfr-PBR-B*) portant le gène *cfr*, et des souches sensibles appartenant à 9 clones majeurs de SA circulant en France ont été respectivement utilisées comme souches donneuses et receveuses. Les réusltats obtenus montrent que certains clones, comme le clone ST8-MRSA-IV USA300, présentaient un risque plus important d'acquisition des plamides proteur de gène *cfr*. La souche SE ST5 avait une plus grande capacité à transférer le plasmide *p-cfr-PBR-B* à différentes souches receveuses que la souche ST2 portant ce même plasmide. Certains plasmides, comme *pSA737* et *p12-02300,* étaient transférables à la plupart des souches receveuses testées. Les coûts de l'acqusition des plasmides portant le gène *cfr* étaient variables pour une même souche et entre les plasmides eux-mêmes. Enfin, il apparaît que les plasmides *cfr*+ étaient systématiquement plus stables dans les SE que dans les SA après des subcultures itératives sans pression de sélection. Au final, nos résultats ont montré que la résistance au linézolide, médiée par *cfr*, peut être transférée efficacement par conjugaison de SE aux clones pathogènes majeurs de SA circulant en France mais que le transfert est très variable en fonction des souches donneuses et receveusess ainsi que des plamides considérés.

MOTS-CLES

Staphylococcus aureus, SARM, caractérisation moléculaire, Afghanistan, linézolide, *cfr*, transfert plasmidique, fitness.

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