RESEARCH ARTICLE

Genetic characterisation of staphylococci of food-producing animals in Senegal. PVL detection among MSSA

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Abstract

Background: Food-producing animals can be a vehicle for staphylococcal species as well as their virulence and antimicrobial resistance genes. This work aimed to analyse the diversity of staphylococcal species in food-producing animals in Dakar/Senegal, and to determine the antimicrobial resistance phenotype/genotype and virulence factors of recovered isolates. Nasal samples of 149 cows and 199 chickens (348 animals) were collected from one slaughterhouse and a local market respectively, and were inoculated on selective media for staphylococci recovery. For *S. aureus* isolates, molecular typing (*spa*-type, MLST) was performed by PCR/sequencing, and the presence of 27 virulence genes (exfoliative and toxic shock toxins, PVL, haemolysins and enterotoxins) as well as the gene *scn* were analysed by PCR. Susceptibility to twelve antibiotics was studied by disc-diffusion method for all staphylococci; the resistance genes involved were screened by PCR.

Results: *Staphylococcus* spp. was present in 3 and 26.8% of chicken and cow nasal samples, respectively. Seven *S. aureus* isolates and forty isolates of other staphylococcal species were identified. *S. aureus* isolates were recovered from cow (*n* = 6) and chicken (*n* = 1) samples, belonging to four genetic lineages: t084/ST15 (*n* = 1); t10579/ST291 (*n* = 3); t355, t4690/ST152 (*n* = 2); and t6618/ST6 (n = 1). All *S. aureus* were methicillin-susceptible, penicillin-resistant (*blaZ*), and two of them were also tetracycline-resistant [*tet*(K)]. All the isolates carried at least one of the virulence genes tested. The PVL genes were detected in three ST15 and ST152 isolates. They all harboured haemolysins encoding genes and lacked the *scn* gene. The other staphylococci recovered were *S. sciuri* (*n* = 16), *S. simulans* (*n* = 11), *S. hyicus* (*n* = 5), *S. haemolyticus* (*n* = 4), *S. chromogenes* (*n* = 3), and *S. hominis* (n = 1); they were all methicillin-susceptible and 27.5% tetracycline-resistant [*tet*(K) and *tet*(L)].

Conclusions: A low prevalence of *S. aureus* was detected among food-producing animals, all susceptible to methicillin. However, the presence of virulence genes (*lukF/lukS-PV*, *eta*, *tst*, *sea* and *see*) is worrisome to the extent that they could be transferred to derived food and therefore, to humans.

Background

Staphylococcus species are common colonizers of skin and mucous membranes of humans and different animal species, but can become opportunistic pathogens causing skin and soft tissues infections (SSTIs) and mastitis, among others [1]. Furthermore, they are current contaminants of animal-derived food, being responsible for food intoxication [2]. *S. aureus* in particularly can express a large variety of pathogenicity factors, such as the staphylococcal enterotoxins, toxic shock syndrome toxin (TSST-1), and Panton-Valentine leucocidin (PVL), among others [3]. In fact, PVL is the most important toxin produced by *S. aureus*; it destroys membranes of host defence cells and erythrocytes by the synergetic action of two specific proteins named LukS-PV and LukF-PV [4]. PVL is though involved in severe skin infections, haemolysis, leucocyte destruction and necrosis [4] . PVL-positive methicillin susceptible *S. aureus* (MSSA) is considered endemic in the African continent [5]. The toxin has been detected worldwide in MSSA

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and methicillin resistant *S. aureus* (MRSA) isolates of diverse ecosystems, including humans (clinical or community-associated isolates) [6], wildlife [7], farm animals and animal-derived food [8]. The presence of the toxin in such different environments and in MRSA is a concern for public health and food safety, mainly for African regions, especially in those areas in which access to healthcare is limited. Moreover, *S. aureus* strains adapted to humans carry an innate immune evasion cluster (IEC) system that protects them against the human immune system [9]. The IEC consists of several genes, the combination of which gives a determined IEC type. The gene *scn*, since present in all the IEC types, is considered to be a marker-gene for the detection of the IEC [9]. The presence of this system in a *S. aureus* isolate would suggest a human origin.

In general, studies about molecular epidemiology of staphylococci in African countries are being mainly performed in hospital environments [10, 11] and in the food sector [12], but there is scarce data related to farm animals [8]. Furthermore, those studies essentially focus on *S. aureus* species and most of them are performed in the Northern, Central and Southern regions of Africa [8]; consequently, data from West Africa is scarce, especially regarding Senegal. In Senegal, a study was carried out in 2012 on *S. aureus* from pigs and pig farmers, which highlighted the predominance of the clonal complexes CC152 and CC15, the low rate of resistance to methicillin and the frequent detection of PVL toxin [13], and a few years ago a review revealed that CC398, an emergent Livestock-associated *S. aureus* lineage in Europe [2, 14–17], was almost absent in animals and food in Africa [8]. In the said context, this study attempts to provide new information on molecular diversity, antimicrobial resistance and virulence determinants for *S. aureus* and other staphylococcal species in other food-producing animals (such as cow and chicken,) in Senegal (West Africa), and proposes an analysis of the potential occurrence of the lineage CC398 in the area.

Results

Staphylococcus species detection

Staphylococcus spp. were present in 3 and 26.8% of chicken and cow nasal samples, respectively. *S. aureus* was detected in seven of the tested animals (six cows and one chicken) (Table 1), whereas other staphylococcal species were found in cows (*n* = 35 isolates) and chickens (*n* = 5 isolates) (Table 2). One *Staphylococcus* isolate was obtained from all positive samples, except for one cow sample with two isolates (*S. aureus* and *S. hyicus*). A total of 47 staphylococci were recovered: *S. aureus* (*n* = 7), *S. sciuri* (*n* = 16), *S. simulans* (*n* = 11), *S. hyicus* (*n* = 5), *S. haemolyticus* (*n* = 4), *S. chromogenes* (*n* = 3), and *S. hominis* (n = 1).

Staphylococcus aureus isolates: antimicrobial resistance, molecular typing and virulence

All seven *S. aureus* isolates were susceptible to cefoxitin and were therefore considered as MSSA. The six isolates of cow origin showed resistance for penicillin (with *blaZ* gene) and two of them also to tetracycline (with *tet*(K) gene). The *S. aureus* isolate of chicken origin showed susceptibility to all antimicrobials tested.

Five *spa*-types were detected among the *S. aureus* isolates, associated with four sequence-types (STs): t084/ST15 (*n* = 1); t10579 /ST291 (*n* = 3); t355, t4690 /ST152 (*n* = 2); and t6618 /ST6 (n = 1) (Table 1). All the isolates were negative for the clonal complex (CC) 398 specific PCR.

The genes encoding for PVL were detected in 2 out of 7 MSSA isolates (28.6%), specifically in t355/ST152 and t4690/ST152 isolates from chicken and cow origins, respectively. The *eta* and *tst* virulence genes were found in isolates of cow origin: 1) *eta* gene in one isolate t084/ST15; and 2) *tst* gene in three t10579/ST291 isolates. The enterotoxin genes *sea* and *see* were present in one isolate of lineage t6618/ST6 recovered of a cow. All the isolates hosted haemolysin encoding genes. In addition, all *S. aureus* isolates lacked the *scn* gene (IEC-negative). This data is summarised in Table 1.

Table 1 *S. aureus* from nasal samples of healthy cows and chicken: phenotypic and genotypic characteristics

| Origin | Strain | ST/CC | Spa-type | Antimicrobial resistance | | Virulence genes | scn |
				Phenotype[a]	Genotype		
Cow	C10068	ST6/CC6	t6618	PEN-TET	*blaZ, tet*(K)	*sea, see, hla, hlb, hld*	negative
Cow	C10067	ST15/CC15	t084	PEN-TET	*blaZ, tet*(K)	*eta, hla, hld*	negative
Cow	C10064	ST291	t10579	PEN	*blaZ*	*tst, hla, hlb, hld, hlg*	negative
Cow	C10066	ST291	t10579	PEN	*blaZ*	*tst, hla, hlb, hld, hlg*	negative
Cow	C10063	ST291	t10579	PEN	*blaZ*	*tst, hla, hlb, hld, hlg*	negative
Cow	C10065	ST152/CC152	t4690	PEN	*blaZ*	*lukF/lukS-PV, hla, hlb, hld, hlg*	negative
Chicken	C10056	ST152/CC152	t355	Susceptible	–	*lukF/lukS-PV, hla, hlb, hld, hlg*	negative

[a]*PEN* penicillin, *TET* tetracycline

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Table 2 Non-aureus staphylococcal species in nasal samples of healthy cows and chicken: phenotypic and genotypic characteristics

Origin	Species	Antimicrobial Resistance	
		Phenotype[a]	Genotype
(n° of positive animals)	(n° of isolates)	(n° of isolates)	(n° of isolates)
Cow (35)	S. sciuri (16)	TET (2)	tet(K) (2)
		TET (1)	tet(K)
		Susceptible (13)	–
	S. simulans (8)	TET (2)	tet(K) (2)
		Susceptible (6)	–
	S. haemolyticus (3)	SXT (1)	dfrG
		Susceptible (2)	–
	S. chromogenes (3)	Susceptible (3)	
	S. hyicus (5)	TET (1)	tet(K)
		Susceptible (4)	–
Chicken (5)	S. simulans (3)	PEN-TET (1)	blaZ, tet(K)
		TET-SXT (2)	tet(L) (2), dfrK (2)
	S. haemolyticus (1)	TET-SXT	tet(K), tet(L), dfrK
	S. hominis (1)	ERY-TET	tet(L), msr(A)/msr(B)

[a]PEN penicillin, TET tetracycline, ERY erythromycin, SXT trimethoprim/sulfamethoxazole

Non-aureus staphylococci: antimicrobial resistance phenotype and genotype

Among the 40 non-S. aureus isolates, 32.5% showed resistance to at least one antimicrobial agent tested. The following resistance rates and genotypes were observed: tetracycline [27.5%; tet(K), tet(L)], trimethoprim/sulfa-methoxazole (SXT) (10%; dfrG, dfrK), penicillin (2.5%; blaZ), erythromycin [2.5%; msr(A)/msr(B)] and clinda-mycin (2.5%). It should be noted that resistance to tetra-cycline was mediated only by tet(K) gene for the isolates from cow origin, and by either tet(K) or tet(L) for the isolates recovered from chicken; furthermore, dfrG was present in one isolate from cow, whereas dfrK was de-tected in isolates of chicken origin.

Discussion

Farm animals are a source for staphylococcal species as well as for their resistance genes and virulence factors [28]. The transmission to humans could occur either through direct contact or via animal derived food [1], hence the importance of analysing staphylococci from food-producing animals.

In this study, the frequency of detection of S. aureus was low in cows (4%) and chickens (0.5%). A similar study from Nigeria showed a rate of 2.6% in cattle from slaugh-terhouses [29]. A higher detection rate was observed in other animals intended for human consumption, such as

pigs in Senegal (12.3%) [13] as well as goat and sheep, ac-cording to studies carried out in Tunisia [28, 30].

Among the S. aureus detected in our work, the most fre-quently detected lineage was ST291, followed by ST152; however, a similar study performed in Senegal on isolates of pigs and pig farmers showed a predominance of the line-ages ST15 and ST152, containing the PVL genes [13]. The sequence type ST291 is a ST398 double locus variant, which encodes two specific subunits, sauI-hsdS1 and sauI-hsdS2, located in GIα and GIβ genomic islands respectively, whereas CC398 isolates encode a single sauI-hsdS1, located in GIα [31]. Furthermore, sauI-hsdS1 of ST291 showed 60% nucleotide similarity to the CC398 sauI-hsdS1; conse-quently, the CC398 specific PCR cannot identify ST291 iso-lates as part of the CC398 cluster [25, 31], as was the case in our study. The lineage ST291 has been previously de-scribed as the major lineage in cattle with mastitis in Egypt [32]; they were all MSSA harbouring scn and PVL genes, unlike our isolates. The lineage ST15, mainly associated to MSSA isolates, frequently harbours PVL and enterotoxins [5] and is highly prevalent in African countries, according to the findings of healthcare institutions [33, 34]. Neverthe-less, this lineage has also been found in animals (cattle, poultry and donkeys) [3, 8, 30]. The clonal complex CC152 was reported as one of the major clonal complexes in many African countries (healthcare environment) (Madagascar, Morocco, Cameroon, Gabon, Niger, Nigeria, Ghana, Mali and Senegal) [13, 33]. The lineage ST152 is sporadically as-sociated to community-associated (CA) MRSA in some European countries, whereas ST152-MSSA is a particularly frequent clone in Western and Central Africa [33, 35]. PVL is the most important toxin secreted by S. aureus and is involved in severe skin infections and life-threatening dis-eases. This toxin is found all over the world, mainly among CA S. aureus isolates [6, 35]. Nonetheless, it was also de-scribed in isolates from farm/wild animals (linked to the lineages ST5, ST8, ST15, ST80, ST152, and ST121) [36–38] and animal-derived food (linked to the lineages ST8, ST121, and ST152) [3, 39]. PVL is very frequently har-boured by MSSA isolates in Africa, where PVL-positive S. aureus is considered endemic [5]. In this work, all the PVL-positive isolates were MSSA (28.6% of S. aureus detected), contrary to the results obtained from healthy sheep in Tunisia, showing only PVL-positive MRSA (6.8% of S. aur-eus detected) [40]. In a previous study performed on pigs in Senegal, 38.4% of the S. aureus isolates harboured the PVL toxin, being 78.6% of them MSSA [13]. Contrarily to the above mentioned studies highlighting the recurrence of PVL-positive S. aureus among animals intended for human consumption in Africa, the absence of PVL was noted among S. aureus recovered from donkeys for meat con-sumption in Tunisia [30]. Furthermore, other virulence en-coding genes were detected among the isolates (sea, see, eta, tst, hla, hlb, hld and hlg). The presence of staphylococcal

enterotoxins (SEs) in bovine isolates is worrisome since, as the literature shows, SEs are detected more often in cows with mastitis than in healthy cows [41]. Furthermore, SEA is the enterotoxin most frequently reported in food (encoded by *sea* gene) and the main cause of staphylococcal food poisoning (SFP) in many countries [42]; it is generally detected in meat, poultry and milk, among others. Nevertheless, SEE (encoded by *see* gene) is rarely reported in food and food-producing animals, although it was involved in some cases of SFP outbreaks in France [42]. Interestingly, none of our isolates carried the genes which encode the toxins SEC or SED, described as the most recurrent in bovines [43]. The presence of such virulent factors in *S. aureus* from food-producing animals, especially in African countries like Senegal, is a big concern for public health to the extent that in some cases, the animals are raised in the houses or sold in open markets, where they are in contact with people and retail food products. This easily results in the dissemination of staphylococcal virulence genes in different niches of the community. The lack of *scn* gene in our isolates suggests their being of animal origin, as expected, thus discarding a potential human origin (by handlers during slaughter).

In addition, other species were detected, the most prevalent being *S. sciuri* and *S. simulans,* followed by *S. hyicus*, *S. haemolyticus*, *S. chromogenes* and *S. hominis*. The coagulase-negative species mentioned above seem to be frequent in cattle and poultry samples [36, 44, 45]. Increasingly considered as opportunistic pathogens for humans and animals [44], coagulase-negative staphylococci are thought to be a reservoir for important resistance genes that could be transferred to *S. aureus* isolates [45], hence the importance of their surveillance.

Regarding the antimicrobial resistance, the phenotypes and genotypes observed in this study are frequent among *S. aureus* isolates from food-producing animals and animal-derived food [12, 28, 36, 38]. Similar phenotypes were previously observed among pig isolates of the same country [13]. Resistance to at least one antimicrobial agent was evidenced in 32.5% of the non-*S. aureus* isolates tested (tetracycline, SXT, penicillin, erythromycin and clindamycin), maybe due to the very frequent use of beta-lactams, tetracyclines, lincosamides and sulphonamides in the veterinary sector (food-producing animals and pets) [46].

Conclusion

A relatively low prevalence of *S. aureus* has been observed in nasal samples of food-producing animals (chickens and cows) in Senegal, with *S. aureus* being MSSA in all cases. Nevertheless, all the *S. aureus* isolates detected harboured at least one virulence gene (especially PVL and enterotoxins genes), which could be a concern for food-safety and public health, particularly in

a developing country with areas in which access to medical care is difficult and limited.

Methods

Sample collection

From May to July 2017, nasal samples of 149 cows and 199 chickens (348 animals) were taken with aseptic swabs in the General Society of Slaughterhouses of Senegal (SOGAS) and a local market, respectively. In all cases, nasal samples were obtained from dead animals, just after they were sacrificed to human consumption as part of routine work in the slaughterhouse and the market.

Isolation and identification of staphylococci strains

Tubes of 5 ml of Brain Heart Infusion (BHI) broth (+NaCl 6.5%) were inoculated with the nasal swabs and then incubated at 37 °C for 24 h. After growth, bacterial culture was distributed on plates of mannitol-salt-agar (Conda, Madrid/Spain), Baird Parker (Becton Dickinson, Heidelberg/Germany) and oxacillin-resistance-screening-agar-base (Oxoid, Hampshire/England) for *S. aureus* and MRSA recovery. Non-*aureus* staphylococci were also identified and characterised. Up to two colonies/plate with staphylococcal morphology were isolated and subjected to Dnase agar test (Conda, Madrid/Spain) and identification by matrix-assisted laser desorption/ ionization time of flight (MALDI-TOF) mass spectrometry (Bruker, Massachusetts/USA).

Antibiotic susceptibility and resistance genes detection

For all staphylococci identified, susceptibility to penicillin (10 units), cefoxitin (30 μg), gentamicin (10 μg), tobramycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), clindamycin (2 μg), ciprofloxacin (5 μg), linezolid (30) and SXT (1.25 + 23.75 μg) was analysed by disk-diffusion method [18]. In addition, susceptibility to streptomycin (30 μg) was also tested [19]. Antimicrobial resistance genes were determined by PCR, according to the resistance phenotype of the isolates: penicillin (*blaZ*), tetracycline [*tet*(K), *tet*(L), and *tet*(M)], macrolides [*erm*(A), *erm*(B), *erm*(C), *msr*(A)/*msr*(B)], and trimethoprim (*dfrA*, *dfrD*, *dfrG*, *dfrK*) [20–23] .

Molecular typing and virulence genes study in *S. aureus* isolates

Spa-typing and Multilocus sequence typing (MLST) were performed for *S. aureus* strains by polymerase chain reaction (PCR) and sequencing and the *spa*-type, sequence type (ST), and clonal complex (CC) were determined as previously described [24]. In addition, a specific PCR was performed for the livestock-associated CC398 lineage detection [25]. The presence of the genes encoding the PVL (*lukF/lukS-PV*), exfoliative toxins (*eta*

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and *etb*), toxic shock syndrome toxin (*tst*), haemolysins (*hla, hlb, hld, hlg* and *hlgv*) and SEs (*sea, seb, sec, sed, see, seg, seh, sei, sej, sek, sel, sem, sen, seo, sep, seq, ser,* and *seu*) was screened by PCR [14, 26, 27]. The gene *scn,* was also tested for *S. aureus* isolates [14]. Positive and negative control strains of the University of La Rioja were included in all PCR reactions.

Abbreviations
BHI: Brain Heart Infusion; CA: Community-Associated; CC: Clonal Complex; IEC: Immune Evasion Cluster; MALDI-TOF: Matrix-Assisted Laser Desorption/ Ionization Time Of Flight; MLST: Multilocus Sequence Typing; MRSA: Methicillin-Resistant *S. aureus*; MSSA: Methicillin-Susceptible *S. aureus*; PCR: Polymerase Chain Reaction; PVL: Panton-Valentine Leucocidin; SEs : Staphylococcal Enterotoxins; SFP: Staphylococcal Food Poisonning; SSTIs: Skin and Soft Tissues Infections; ST: Sequence Type; SXT: Trimethoprim/ Sulfamethoxazole; TSST: Toxic Shock Syndrome Toxin

Authors' contributions
CT conceived and designed the study. MD, CGMD and BH designed and participate in the sampling procedure. OMM, LRR and MD performed laboratory works. OMM and CT interpreted the results and do the first writing of manuscript. All authors have revised and approved the manuscript.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
In this study nasal samples were taken from animals during routine processing at the slaughterhouse and the market, once they were dead. No animal was sampled alive or sacrificed for the purpose of this study. Thus, according to the Spanish National law for the protection of animals (RD53/ 2013) that transcribes the EU directive 2010/63/UE, no ethics committee approval was deemed necessary. Furthermore, ethic committee approval was not deemed necessary based on the Article 7.1 (on the recommendations for animal welfare) and the Article 7.8 (on the use of animals in research and education) of the OIE Terrestrial Animal Health Code followed by Senegal.
At the time of processing the entity responsible for the animals was the slaughterhouse and authorization for sample collection was obtained from the directing board of the slaughterhouse. Also, the chicken sampling was authorized verbally by the owner which is responsible for the animals.

Consent for publication
Not Applicable.

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

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