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VARIABILITY AND ANTIBIOTICS RESISTANCE OF *STAPHYLOCOCCUS* SP FLORA AMONG THE CATTLE CARCASSES

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Abstract: The qualities of meat products are influenced by many factors such as bacteria species mostly implicated in food-borne disease. The aim of this study was to investigate the antibiotic resistance of the coagulase positive and negative *Staphylococcus* and the toxin production of *S. aureus* strains isolated from cattle carcasses collected in the abattoir of Cotonou - Porto Novo. A total of 240 cattle carcasses were sampled by excision at four sites (shoulder, flank, neck and the thigh). Samples were examined for their contamination by coagulase positive and negative Staphylococcus species. The antibiotic resistance profile of all the isolated species was investigated by disc diffusion method. The production of 4 toxins was investigated by immune diffusion method. About 64% of the collected samples were contaminated by 15 different *Staphylococcus* strains. The thigh samples were contaminated by 11 difference species and the *S. hyicus* stains were isolated from only from shoulder samples. The *S. aureus* strains, were resistantat 90% to oxacillin, cefoxitin and penicillin G. No resistant *S. aureus* was recorded with two antibiotics (ciprofloxacin and streptomycin). The coagulase negative strains, were highly resistant to oxy-tetracycline (73.6%) and penicillin G (64.6%). Epidermolysins were produced by 40% (ETA) and 50% (ETB) of *S. aureus* stains and none of them produced LPV and LukE/D. This result reaffirms the potentially critical role that can play commensals in public health.

Keywords: Cattle carcasses, *Staphylococcus sp.*, multidrug Resistance, *S. aureus* toxins, Benin.

INTRODUCTION

Cattle production is one of the major source of meat intended to human consumption in African big cities. Meat and meat products are source of protein, fat, and several functional compounds. A meat that is rich in proteins with a high proportion of essential amino acids and polyunsaturated fatty acids is considered to exhibit good nutritional quality. Meat quality refers to intrinsic attributes critical for the suitability of meat for eating, processing, and storage, including retail display. Therefore, meat quality and it safety are becoming a

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challenging concerns which require the generation of new information and of continuous reevaluation of existing knowledge for meeting market's demands by assuring high quality standards and prevention of recognized risks to human health (Sevi et al., 2016).

The meat hygienic qualities reflect the product's capacity to be safely consumed and they are primarily related to the bacterial load of the product and the presence of chemical residues in the product (Maltin et al., 2003). In meat flesh, these qualities are influenced by many *postmortem* factors such as bacteria species mostly implicated in food-borne disease. Among the bacteria mostly identified bacteria in case of food poisoning we can mention *Salmonella*, Coliforms, and *Staphylococcus*. The ingestion of those bacteria and/or their toxins can cause metabolic problems to the consumer (Baba-Moussa et al., 2010). In addition, bacteria are source of health care taking because of the upsurge of strains resistances to conventional antibiotics (Chambers and DeLeo, 2009).

Staphylococcal food poisoning is an illness caused by the ingestion of contaminated food containing enterotoxins produced by bacteria belonging to this genus. Enterotoxins that exhibit superantigenic activities are heat stable proteins and may not be destroyed even during cooking conditions. *Staphylococcus* classified as coagulase-positive are considered potential food enterotoxin-producing strains (ICMSF, 1983), although, recently, the enterotoxigenic potential of coagulase-negative staphylococci (CNS) species in food poisoning has also been recognized (Veras et al., 2008).Indeed, studies on occurrence and distribution of *S. aureus* in foods and food processing environments are numerous and report variable prevalence (Attien et al., 2013). There is less information available in the literature on the occurrence and distribution of other *Staphylococcus* species, particularly the coagulase negative's, along the meat food product.

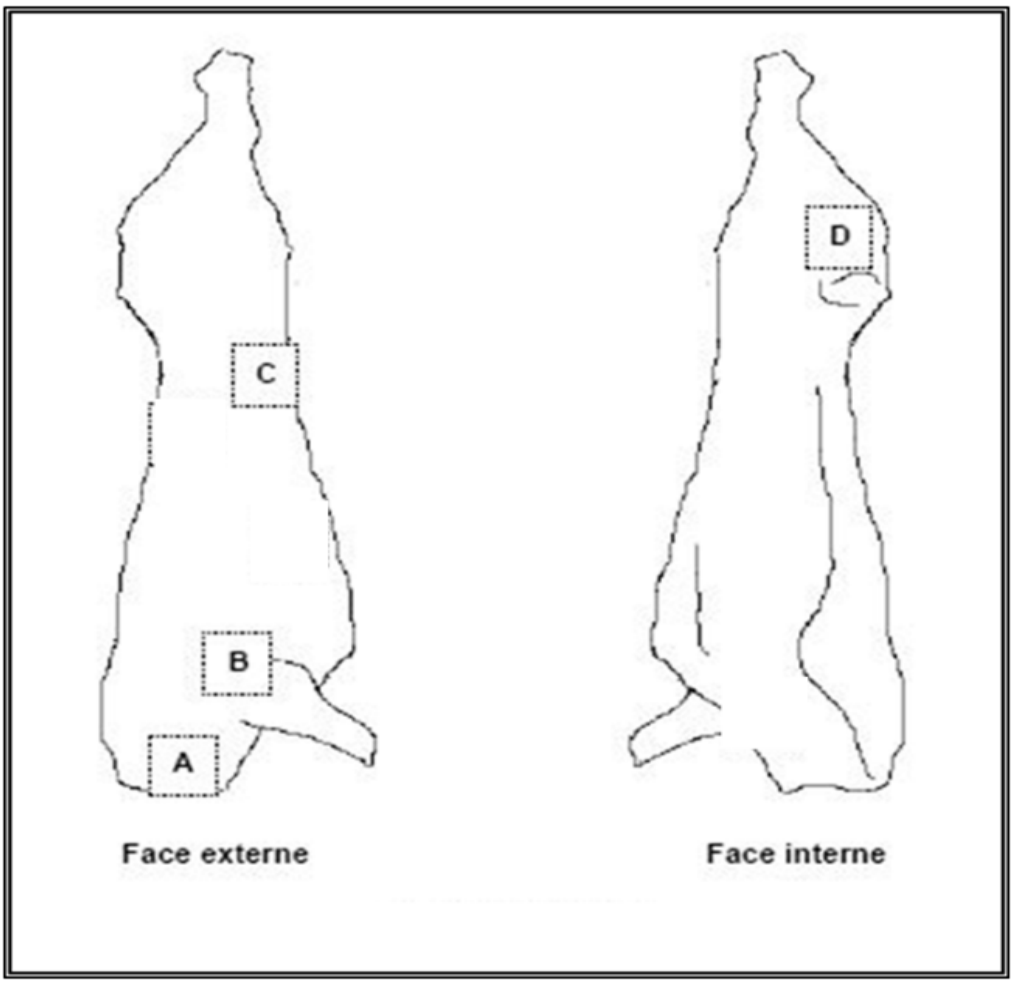
The aim of this study was not only to investigate the antibiotics resistance of the coagulase positive and negative *Staphylococcus* from cattle carcasses but also to carry out the some toxins produced by the identified *S. aureus* strains.

MATERIAL AND METHODS

Samples collection

The samples of cattle carcasses were collected in the slaughterhouses of Cotonou -Porto Novo, the biggest of Benin. On a randomly selected carcasses, foursites (neck, shoulder, flank and the thigh) were collected (Figure 1). Per week, samples were collected once at each site on 5 different carcasses. For each collection site, a part of muscle (5 cm^2) of the carcasses was collected with a sterilized scalpel using a template cutting surface. The samples were

collected in sterile Falcon tubes and then carried to laboratory in icebox at ~ 4 °C. For the whole study, a total of 240 samples from 60 different cattle carcasses and 60 samples of each collection site were collected.



A: neck, B: the thorax in the shoulder portion (shoulder), C: the outer face of the side (Flank), D: the inner face of the thigh.

Figure 1. Cattle carcasses sampling sites

Microbiological analysis

Once at the laboratory, 5cm^2 of each carcasses sample were homogenized in 25 ml of sterile bacteriological peptone (Oxoid, England) and then incubated at 37°C for 1 to 3 h (Akoachere et al., 2009). To perform the isolation of *Staphylococcus* strains, 0.1 ml of serial decimal dilutions were plated in duplicate on Chapman Agar (Difco, France) medium and incubated at 37°C for 48 h. To obtain pure cultures, subcultures were alternately made on Brain-Heart infusion and selective Chapman agar.

Staphylococcal species identification

With the previously culture obtained, the staphylococcal species were identified using the standard microbiological methods (Akoachere et al., 2009). First, suspected *Staphylococcus* colony was sub-cultured on Mueller-Hinton agar and then use for performing subsequent Gram staining, catalase, agglutination (Slidex Staph Plus) and coagulase (with rabbit plasma) test (Cheesbrough, 2004). Finally, the strains were analyzed by API Staph (bio Mérieux, France) according to the manufacturer instructions.

Susceptibility to antibiotics

The antimicrobial susceptibility to 20 antibiotics was determined by the disc diffusion method of Kirby-Bauer on agar Mueller-Hinton as recommended by the Antibiogram Committee of the French Microbiology Society (SFM, 2015). After 24 h at 37°C, inhibition zone was measured. The 20 tested antibiotics were Chloramphenicol (30 µg), penicillin G (10 µg), azithromycin (15 µg), oxacillin (1 µg), gentamicin (10 µg), erythromycin (15 µg), cefoxitin (30 µg), fosfomycin (50 µg), kanamycin (30 µg), amoxicillin+clavulanic acid (30 µg), teicoplanine (30 µg), oxy-tetracycline (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (25 µg), amikacin (30 µg), tobramycin (10 µg), fusidic acid (10 µg), rifampicin (30 µg) and streptomycin (10 µg).

***Staphylococcus aureus* toxin detection**

The production of three toxins produced by pathogenic *S. aureus* was performed by radial gel immunodiffusion. So, the production of Panton-Valentine Leukocidin (PVL), Luk A-Luk B and epidermolysins A (ETA) and B (ETB) were evidenced from 18 h Yeast Casamino-acid Pyruvate (YCP) medium sub-culture bacterial supernatants (Gauduchon et al., 2001). The supernatant was use to perform a radial gel immunodiffusion in 0.6% (wt/vol) agarose with component-specific rabbit polyclonal and affinity-purified antibodies (Gravet et al., 1998).

Data analysis

The collected data were process by the Microsoft Excel Spreadsheet. The software Graph Pad Prism 5 is use for the comparison tests of positive isolates in various collection site; the Student T test, and the Fischer's test were used for lower number series. $P<0.05$ was considered statistically significant.

RESULTS

Globally, about 64% (154/240) of the collected samples were contaminated by *Staphylococcus* strains. However it was recorded a light, but not significant, variability (from

60 to 70%) of contamination rate according to the collection site (Figure 2). Thus, 70% of the neck samples were contaminated by *Staphylococcus* spp. against 60% for thigh samples.

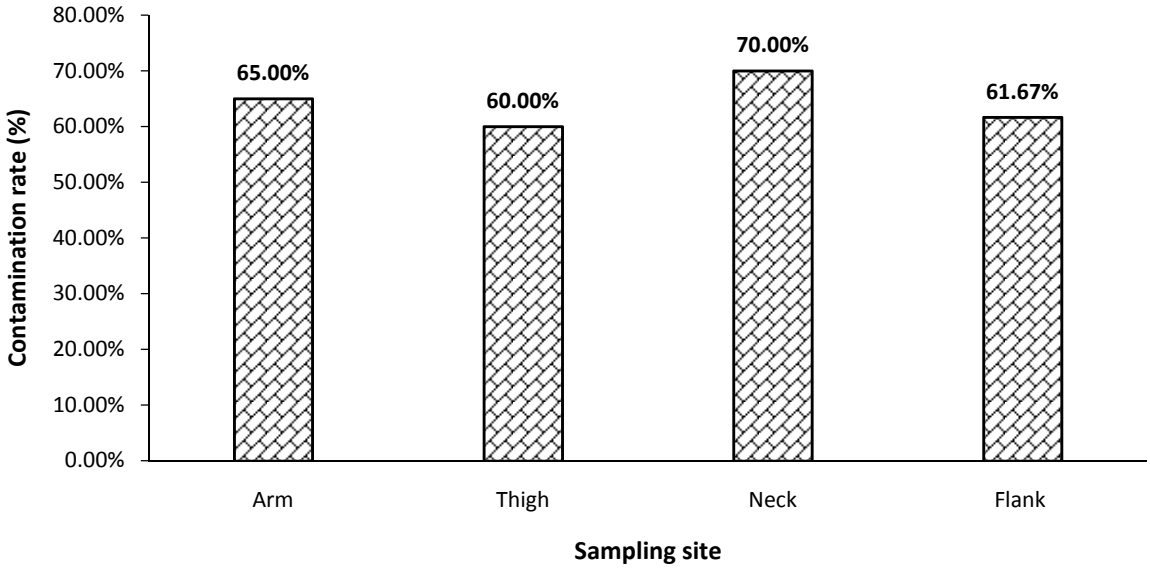
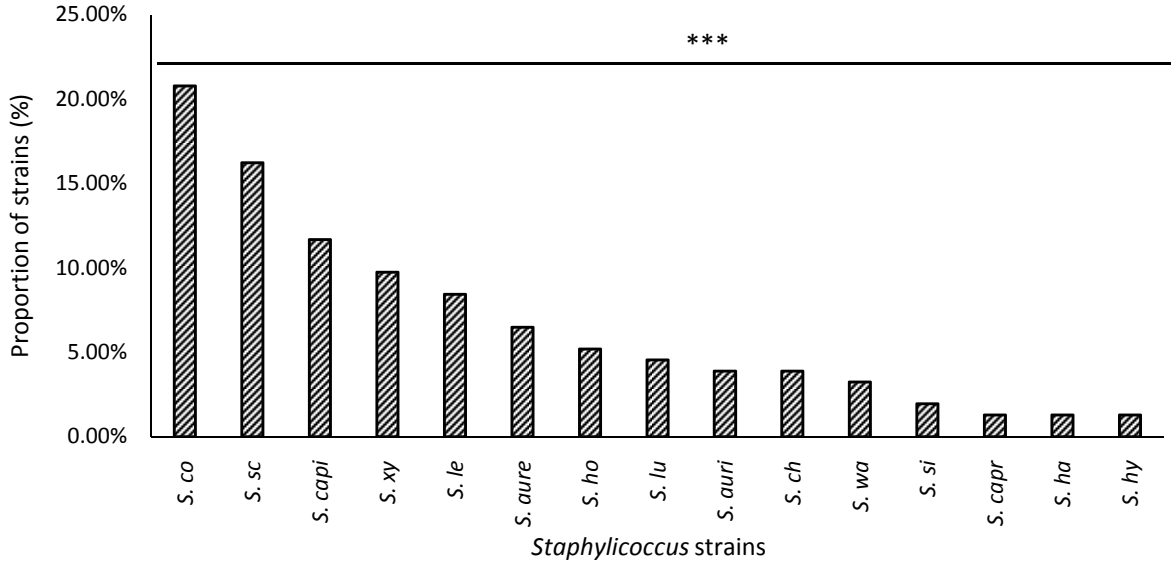


Figure 2. Global contamination rate of the cattle carcasses by *Staphylococcusspp*. according to the collection site.

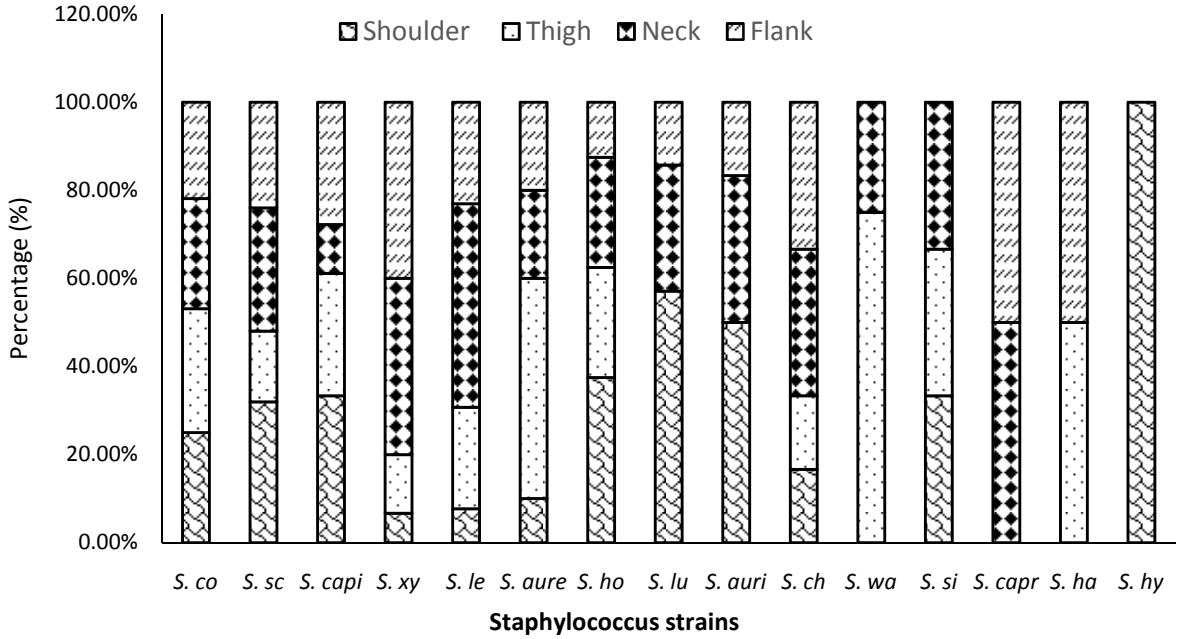
At total of 15 *Staphylococcus* strains (*S. aureus, S. auricularis, S. capitis, S. caprae, S. chromogenes, S. cohnii ssp cohnii, S. haemolyticus, S.hominis, S. hyicus, S. lentus, S.lugdunensis, S. sciuri, S. simulans, S. warneri* and *S. xylosus*) were isolated at different proportion. So, independently to the collection site, *S. cohnii ssp cohnii* was the most isolated (20.78%) strains whereas *S. caprae*, *S. hyicus* and *S. haemolyticus* were the less (~1%) isolated (Figure 3). *S. aureus* is the single coagulase positive species identified among the 15 species.



*S. co: S. cohnii ssp cohnii, S. sc: S. sciuri, S. capi: S. capitis, S. xy: S. xylosus, S. le: S. lentus, S. aure: S. aureus, S. ho: S.hominis, S. lu: S.lugdunensis, S. auri: S. auricularis, S. ch: S. chromogenes, S. wa: S. warneri, S. si: S. simulans, S. capr: S. caprae, S. ha: S. haemolyticus and S. hy: S. hyicus; ***: p<0.0001*

Figure 3. Global distribution of the *Staphylococcus* strains isolated from cattle carcasses collected in biggest slaughterhouses of Benin.

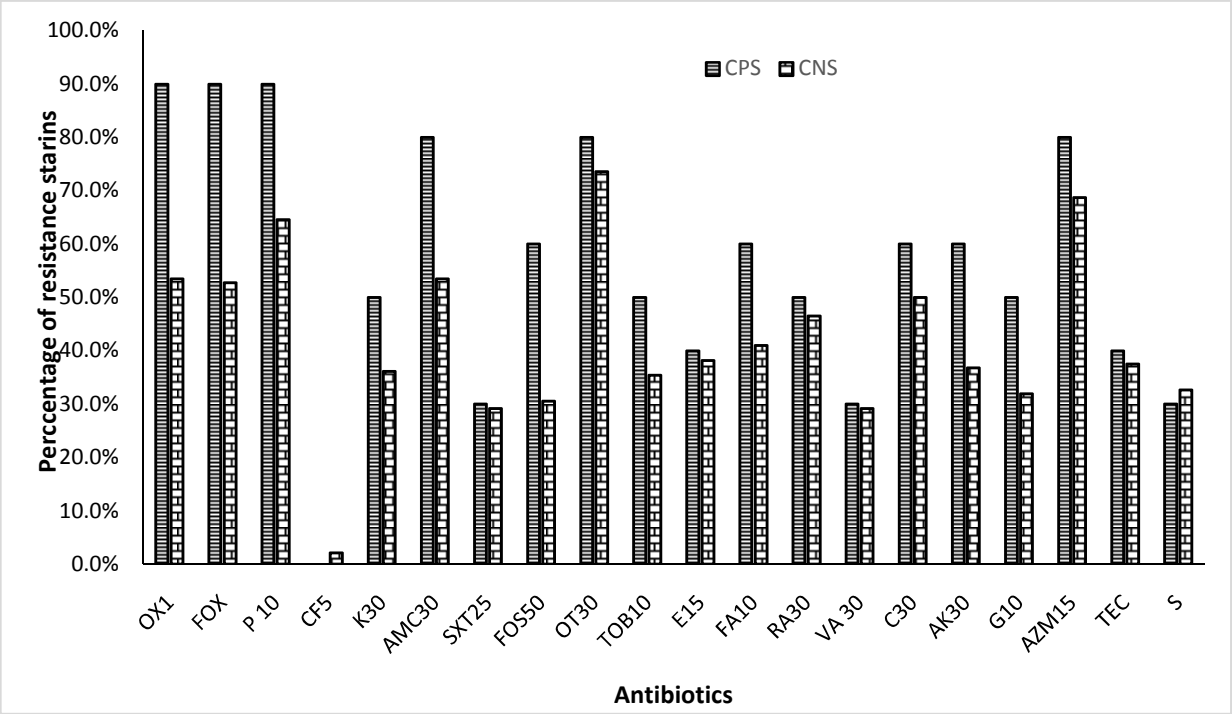
The kind and proportions of strains varies according to the collection site (figure 4). Thus, among the identified species, the thigh samples were contaminated by 11 difference species. In addition, the *S. hyicus* stains were isolated from shoulder samples. Globally, there is not significant distribution difference considering a specie from a collection site to another (p >0.05). The coagulase positive strains were isolated in all the 4 collection site with the highest level recorded at the thigh (50%) and the lowest at the shoulder (10%).



S. co: S. cohnii ssp cohnii, S. sc: S. sciuri, S. capi: S. capitis, S. xy: S. xylosus, S. le: S. lentus, S. aure: S. aureus, S. ho: S.hominis, S. lu: S.lugdunensis, S. auri: S. auricularis, S. ch: S. chromogenes, S. wa: S. warneri, S. si: S. simulans, S. capr: S. caprae, S. ha: S. haemolyticus and S. hy: S. hyicus.

Figure 4. Contamination rate of *Staphylococcus* species according the connection sites.

All the coagulase positive (*S. aureus*) and negatives (*S. cohnii ssp cohnii, S. sciuri, S. capitis, S. xylosus, S. lentus, S.hominis, S.lugdunensis, S. auricularis, S. chromogenes, S. warneri, S. simulans, S. caprae, S. haemolyticus* and *S. hyicus*) strains isolated in our study were resistant at different proportion to the tested antibiotics (Figure 5). For *S. aureus* strains, the highest resistance rate recorded was 90% with oxacillin, cefoxitin and penicillin G. No resistant *S. aureus* was recorded with two antibiotics (ciprofloxacin and streptomycin). With coagulase negative strains, the highest resistance levels were observed when using oxy-tetracycline (73.6%) and penicillin G (64.6%) (Figure 5). For all the tested antibiotics, the isolated coagulase negative strains resistance level was not significantly different to those recorded with *S. aureus* (p>0.05).



OX1: oxacillin (1 µg); FOX: cefoxitin (30 µg), P1: penicillin G (10 µg); CF5: ciprofloxacin (5 µg), K30: kanamycin (30 µg), AMC30: amoxicillin +clavulanic acid (30 µg), SXT25: trimethoprim sulfamethoxazole (25 µg), FOS50: fosfomycin (50 µg), OT30: oxy-tetracycline (30 µg), TOB10: tobramycin (10 µg), E15: erythromycin (15 µg), FA10: fusidic acid (10 µg), RA30: rifampicin (30 µg), VA 30: vancomycin (30 µg), C30: Chloramphenicol (30 µg), AK30: amikacin (30 µg), G10: gentamicin (10 µg), AZM15: azithromycin (15 µg), TEC: teicoplanine (30 µg), S: streptomycin (10 µg), CNS: Coagulase Negative *Staphylococcus*, CPS: Coagulase Positive *Staphylococcus*.

Figure 5. Antibiotics resistance profile of coagulase negative and positive *Staphylococcus* isolated from cattle carcasses.

Epidermolysins were produced by 40% (ETA) and 50% (ETB) of *S. aureus* stains isolated from the cattle carcasses. None of the strains produced LPV and LukE/D (Table 1).

Table 1. Toxins produced by *Staphylococcus* strains isolated from cattle carcasses.

Investigated Toxins	**Percentage of production (%)**
LPV	0
LukE/D	0
ETA	40
ETB	50

DISCUSSION

Meat microbial quality is important in the prevention of food poisoning. Due to its high content in protein, fresh meat are good subtract for various microbial (pathogenic or not) development. Thus, in this study, it was observed a contamination rate of 64% by *Staphylococcus* ssp. among the meat samples collected directly on the cattle carcasses were isolated from bovine carcasses. These proportion include both coagulase positive and negative species. The high contamination rate by *Staphylococcus* strains is a potential food poisoning risk factor. However, similarly, some authors reported 10% to 72% of *Staphylococcus* strains in beef (Olsson et al., 2000; Shale et al., 2005).

Among the isolated strains of *Staphylococcus*; the coagulase positive strains were lower (6.49%) than coagulase negative ones (93.51%). In the same way, it was shown a dominance of primary coagulase negative *Staphylococcus* during a study conducted on street meat (~80%) sold in Ivory Coast (Attien et al., 2013) and in farm animals (~77%) sampled in South Africa (Adegoke and Okoh, 2014). The slightly lower proportion reported in the previous can be due by the fact that those authors conducted their investigation on cooked meat ready to be eaten. Indeed, the cooking processed act by reducing the bacterial amount.

Fifteen *Staphylococcus* species have been identified and *S. cohnii* species is the majority (20.78%) followed by *S. sciuri* (16.23 %) and *S. capitis* (11.69%) (Figure 3). This number of species his higher than the 11 reported in the street meat. The variation between these two results can be explain by the bactericidal effect of temperature. Indeed, the street meet are currently in contact with a heat source that contribute to destroy some microorganism such as *Staphylococcus* spp. The distribution of the isolated species widely varies according to the collection site. Thus, the figure 4 shows that each site hosts a group of specified species without strict specificity. Height (*S. cohnii ssp cohnii*, *S. sciuri, S. capitis, S. xylosus, S. lentus, S. aureus, S. hominis* and *S. chromogenes*) are present in the arm, neck, thigh and wing. Species such as *S. lugdunensis*, *S. auricularis* and *S. simulans* were not isolated in the samples of thigh whereas *S. warneri* is absent in arm and flank samples. A study conducted by Shale et al. (2005) reported that the main staphylococcal species containing the meat during slaughtering *S. lentus, S. sciuri* and *S. xylosus.* The isolation of those species indicated a high contamination level of the cattle's carcasses in the slaughtering houses. An additional source of contamination is the one coming probably from the slaughtering staff. This kind of contamination indicated the bad precaution adopted by the staff and can include *S. auricularis*, *S. capitis* and *S. warneri* (Shale et al., 2005) and eventually pathogenic strains. It

then clear that the wide variety of staphylococcal flora observed in cattle carcasses result from the existence of multiple sources of contamination. These can include, inter alia, slaughtering environment, the direct contact of carcasses with utensil or operators hands (Benaissa et al., 2014).

The isolated strains (coagulase positive and negative) displays a variability of sensitivity against the 20 tested antibiotics. Thus, concerning the coagulase negative *Staphylococcus*, the highest resistance level was observed with oxy-tetracycline (73.6%). This resistance proportion observed corroborate those reported, for tetracycline, by several authors (Werckenthin et al.,2001; Aarestrup, 2000). Apart of oxy-tetracycline, about 80% of the staphylococcal strains are reported to be resistance to penicillin G (Ciupek et al., 2002). In our study, 64.6% of the coagulase negative strains were resistance to penicillin. In most case, this antibiotic displays high resistance levels due to it wild and bad utilization. With it inefficacity to control bacterial infection, penicillin is less and less use nowadays. The reduction of its use in self-medication induce the decrease of the high resistance level previously recorded (Erskine et al., 2004).

Sixty one percent (53.5%) of the coagulase negative *Staphylococcus* and 90% of *S. aureus* are resistance to methicillin. Our results concerning this antibiotic is slightly lower than those reported among the clinical isolated coagulase negative *Staphylococcus* (Koksal et al., 2009). This different can be due by the fact that the clinical isolated strains are most in contact with the molecule than the food isolates. However, the proportion is scary for the food because it is reported that methicillin resistant *Staphylococcus* strains began to develop resistance to many antibiotics (quinolone antibiotics macrolide group, aminoglycosides, tetracycline, trimethoprim-sulfamethoxazole, clindamycin and chloramphenicol) widely used to control staphylococcal infection (Drozenova and Petras, 2000; Huang et al., 2003; Jain et al., 2004; Knauer et al., 2004) such as food poisoning.

A significant resistance was observed for aminoglycosides, glycopeptides and macrolides. Thus, the resistance rate of 35% and 38% were respectively recorded for vancomycin and teicoplanin. These two antibiotics have long been considered as a last resort molecules to overcome multi-drugs resistant staphylococcal infections (Mayhall, 2004).The resistance to these molecules appear higher for the coagulase negative species than for the coagulase positives'. However, the emergence and high proportion of such resistance in *S. aureus* and coagulase negative *Staphylococcus* was reported in several studies (CDCP, 2004; Palazzo et al., 2005).

Furthermore no resistance was observed with the Coagulase Positive strains to ciprofloxacin (quinolone) and streptomycin. The two antibiotics are also less activeagainst SCN. This interesting sensitivity observed could be due to their rational and moderate use in veterinary medicine.

Among the investigated *S. aureus* toxins, only epidermolysins were produced. This found suggest a contamination from the skin of the operators hands. Then, the considerable number of *S.aureus* producing epidermolysins in the cattle carcasses samples can be explained by slaughtering contamination. In fact, after killing the animal, the carcasses are generally separate from the animal skin which constitutes a selective filter for microorganisms. After removing the skin, the consumed parts become exposed to the operator's manual contamination during the cleaning process. However, considering the multiple forms and sites of staphylococcal infections, it is likely that these bacteria are well equipped to sense environmental conditions and to regulate expression of virulence factors (Gravet et al., 2001).

CONCLUSION

This study demonstrated the presence of 15 staphylococcal strains mainly coagulase negative's, in fresh meat from the slaughterhouse. Some of the *S. aureus* strains isolated are able to produce epidermolysins A and B. The resistance patterns against the tested antibiotics are alarming because of the high rates recorded. Meanwhile, the resistance to methicillin continues to be the key marker for antibiotic resistance of *Staphylococcus*. This result also reaffirms the critical role of commensals in public health. Thus, the dangers associated with coagulase negative staphylococcus may be aggravated by the notorious increases of this antibiotic resistance. It will therefore be useful to investigate a wide range of toxins that can be produces by both coagulase positive and negative strains, particularly enterotoxins.

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