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CHARACTERIZATION OF STAPHYLOCOCCI ISOLATED FROM THE DISTRIBUTION CHAIN OF MEAT IN ALGERIA

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ABSTRACT

Staphylococci are ubiquitous bacteria; diseases caused by these germs are highly polymorphic, ranging from benign skin lesions such as boils and paronychia to pathologies which are life-threatening such as septicemia, endocarditis, and pneumonia and nervous system infections.

Our work focuses on the characterization of staphylococci found in the distribution chain of meat, which may be pathogen especially via the digestive tract, for that we have made various biochemical tests for the phenotypic identification and PCR for molecular identification.

The phenotypic results show the presence of some species, among are opportunistic pathogens such as *S. epidermidis*, *S. heamolyticus*, *S.caprea*, *S.hominis* and *S.saprophyticus* with rates of 2 %, 2 %, 2.38 %, 2.38 % and 7.14 % respectively and species responsible for infections and serious foodborne illness such as *S. aureus* with a rate > 12,95 %. The antibiogram of *S. aureus* strains shows their sensitivity to most of antibiotics except penicillin at which all strains are resistant, whereas 40 % of strains are resistant to tetracycline and 20 % are resistant to erythromycin and clindamycin.

Molecular identification based on research *"Nuc"* gene confirms some phenotypic identification which indicate the prevalence of pathogenic strains, but these results need to be confirmed by other techniques such as sequencing, genomic, transcriptomic, and proteomic analysis..

KEYWORDS: *Staphylococcus*, Meat, Phenotypic, Molecular, Characterization

INTRODUCTION

Staphylococcus germs are recognized worldwide as commensally and Pathogenic Agents of humans and animals (Aouati, 2009; Ote et al., 2011), whose natural ecological niches are the nasopharynx and skin. From these sites of Portage, staphylococci can colonize the skin areas especially wetlands (axilla, perineum) (Boulanger et al., 2006). Further these bacteria are commonly found in the environment, their remarkably flexible character makes it possible to survive or develop in many different niches (inanimate surfaces, foodstuffs) (Duquenne, 2010).

In fact of these good capacity to adapt, staphylococci have many virulence factors to infect its host (Hennekinne, 2010) and for each of the key steps leading to infection, staphylococci have developed systems to escape the immune response (Boulanger et al., 2006).

Among those infections, some are benign community infections such as skin furuncles, but others such as septicemia, endocarditis, pneumonia and infections of the central nervous system (corne, 2004) and nosocomial infections in patients with weakened either by immunosuppression or by the presence of foreign material are pathologies which are life-threatening (Lepeul, 2010), and also the occurrence of foodborne illness can be due to ingestion of staphylococcal enterotoxins produced by bacteria in food (corne, 2004).

The treatment of these infections has become more difficult because of the high prevalence of methicillin-resistant strains and the development of the emergence of multidrug-resistant strains to different families of antibiotics (corne, 2004), which increases the duration of hospitalization, morbidity, cost of care treatment and in some cases mortality (Lecornet, 2007), so what are the different characters that allow us to detect staphylococci in media in contact with the men.

Therefore, our work is based on the realization of molecular and phenotypic analysis of *staphylococcus* strains isolated from different food and inanimate surfaces, taking as an example the meat chain distribution.

First, we perform a phenotypic analysis. On the one hand, performing various biochemical tests that will help:

- To characterize the strains of Staphylococci.
- To select strains of human pathogenic staphylococci (*S. aureus*), especially by the coagulase test and ID32 STAPH gallery.

On the other hand, we perform a molecular analysis by using the PCR technique for search thermo nuclease a specific enzyme of *S.aureus.*

In the end, we will compare the results of the molecular analysis of the phenotypic analysis to confirm that the selected strains are strains of *S. aureus*.

Then, we perform an antibiogram by the use of 25 antibiotics to investigate the sensitivity and resistance of *S. aureus* strains selected.

All this work was done at the laboratory of Microbiology Faculty of Sciences, University Hassiba Ben Bouali Chlef and service of medical bacteriology at the Pasteur Institute in Algiers.

MATERIALS AND METHODS

Bacterial Strains

All of this work has been performed on strains isolated from different origins in contact with humans (red meat, white meat, environment, tables, hands manipulator ... etc.).

Strains isolated from different origins are stored at -20 °C in LB broth with 50% (v/v) glycerol until the final characterization.

A total of 115 *S. aureus* isolates were cultured and identified on the basis of established morphological and biochemical tests. The Ability to produce coagulase was determined by tube test employing human plasma. Two reference strains of bovine mastitis were included in this collection: *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923.

The Oligonucleotides

The primers used for amplification of *nuc* gene were having the following pairs with sequence as described in the following table.

Table 1: Primer Sequences

Primer	Sequence 5'→3'	Length
primer 1(*nuc*F)	GCG ATT GAT GGT GAT ACG GTT.	21pb
primer 2(*nuc*R)	AGC CAA GCC TTG ACG AAC TAA AGC.	24Pb

Source: Kuzma et al. (2003)

The oligonucleotide primers generally provided in lyophilized form and must be diluted to a final concentration of 10 µM.

Phenotypic Identification

0.5 ml of each strain retained (on glycerol nutrient broth) was inoculated in 5 ml of nutrient broth, after 24 hours of incubation a second seeding is performed on broth Chapman and Incubated at 37 ° C for 24h to 48h, these isolates were cultured on Columbia sheep blood agar plates . And they were presumptively identified as *S. aureus* according to the following scheme: fresh status, Gram-positive cocci, catalase-positive and coagulase-positive, haemolytic on Columbia sheep blood agar. The presumptive identification was confirmed by API ID32 Staph (BioMerieux).

The identified strains were maintained on agar deep at ambient temperature and in cryotube at - 20 ° C in LB broth with 50% (v/v) glycerol.

Antibiotic Sensitivity

Antibiotic resistance was determined by disk diffusion method (Bio-Rad, France), MH agar (Bio-Rad, France) according to the recommendations outlined by the CA-SFM 2007 (Committee of the Antibiogram of the French Society for Microbiology) (Soussy, 2008). An inoculum of 10^6 CFU / ml was prepared for each bacterial strain and the MH medium was inoculated by swabbing, after 18-24 hours of incubation at 36 ° C in an ambient atmosphere, reading and interpretation of antibiograms were made by the "OSIRIS ®" software (Bio-Rad).

MOLECULAR IDENTIFICATION

DNA Extraction

The DNA of each suspected strain was extracted according to standard protocol (Konate, 2001). Briefly, 1-2 colonies were collected from nutritive agar plates, resuspended in 500 µl of 10 mM Tris, 1 mM EDTA buffer, pH 8.0 and incubated at 100°C for 15 min and then at 37°C for 30 min with 80 µg/ml final concentration of lysostaphin, and subsequently treated with proteinase K, 100 mg/ml for 10 min at 55°C. Proteinase K was then inactivated by heating at 95°C for 15 min.

The DNA was then precipitated harvested, quantified by agarose gel electrophoresis and then stored at -20 °C.

PCR Amplification

PCR detection of target for a fragment of 270 base pairs (bp) specific to *nuc* gene of the *S. aureus* was performed using primers previously described (Morot-Bizot, 2006; Bohach, 2008).

Two reference strains of Pasteur institute: 25923 (S. aureus, *nuc gene* positive) and 29213 (*S. aureus*, *nuc gene* positive) were used as controls.

The PCR reaction is carried out in a final volume of 50 µl. Sample containing: Sterile distilled water (28.5µl), Tris-HCl buffer (5µl), dNTP (6 µl), primer1 (0.5 µM) primer2 (0.5 µM) Taq polymerase (1.5 U), MgCl2 (3.5 µM), DNA (30 ng).

The PCR reaction is conducted according the following programme: initial denaturation cycle (95C ° / 10 min), 35 cycle (denaturation 94C ° / 1 min, hybridization 57C ° / min and 0.5 Extension 72C ° / 1.5 min), a cycle of Extension final (72C° / 5 min) (Brakstad et al., 1992).

Analysis of PCR products by agarose gel electrophoresis: All amplification products were separated by electrophoresis on agarose gel 1.5% stained with ethidium bromide (0.5µg/ml) in Tris-acetate-EDTA (TAE) 0.5 X Photos of gels were taken under UV.

RESULTS AND DISCUSSIONS

In our study 158 samples were analyzed, the samples in the most common are materials (≈ 38%), followed by red meat (≈ 28%) in second position, the hands of manipulators come in 3rd position with (≈ 18%), and finally in last, white meats which are only nearly 16% of the samples.

Among 158 samples inoculated into broth Chapman, 132 samples (83.54%) were positive, and 26 samples (16.46%) were negative.

Isolation

For our 132 isolates, 112 isolates (84.84%) were with a color change of the medium and 20 isolates (15.16%) did not induce the shift of the Chapman medium. On this medium, *S. aureus* causes acidification of mannitol (turns yellow), unlike most other staphylococci (Avril et al**.,** 2000). It should be noted that the positive mannitol fermentation is often regarded as a presumptive test for pathogens, but it is not sufficient (Guiraud, 1998).

After Gram staining and observation under an optical microscope with immersion (objective × 100), of the 132 isolates, 130 isolates (98.48%) appear as purple hulls in pairs or in clusters and are grapes, which characterizes the genus Staphylococcus (Bakhoum, 2004; Federighi, 2005), and tow isolate (1.52%) appears as bacillus colored pink.

So the 130 isolates suspected of being staphylococci which are immobile and have no ciliature locomotion (Guiraud, 1998).

Catalase Test

The 130 isolates were catalase positive, we have the appearance of a seething after contact of bacterial colony with hydrogen peroxide. This test is used to differentiate Staphylococcus (positive catalase) and Streptococcus (negative catalase) (Ferron, 1989).

Oxidase Test

This test differentiates between Micrococcus and Staphylococcus (Ferron**,** 1989). Of the 130 isolates there is a color change OX discs in 16 isolates (12.3%), therefore they are oxidase positive and no color change in 114 isolates

(87.7%), so are oxidase negative this directs us to staphylococci that do not have this enzyme to catalyze the oxidation of an organic substrate by oxygen (Guiraud, 1998).

ONPG Test

Of the 114 isolates tested 48 isolates (42.1%) were ONPG positive therefore they have the B-galactosidase, enzyme which hydrolyzes lactose into glucose and galactose, 66 isolates (57.9%) do not possess this enzyme and are ONPG negative.

Study of Carbohydrate Metabolism

Glucose fermentation is a differential characteristic, important for the elimination of the Micrococcus genus (Guiraud, 1998).

Our results for the remaining 114 isolates was observed 3.5% of isolates do not ferment glucose and 8.77% was suspicious micrococci, because the fermentation of glucose is made only the aerobic zone of the tube at the top, while the fermentation of glucose by 87.71% of isolates appears over the entire height of the tube above the anaerobic zone characterizes the genus Staphylococcus (Morot-Bizot, 2006).

Reaction to Methyl Red

For the 100 isolates we have 48 isolates RM +, which explains the production of acid which results in the appearance of red, and 52 isolates RM- shows a lack of acid production revealed by the absence of color change remaining yellow. staphylococci have a varied response to methyl red, Sometimes they ferment glucose through mixed acids which release a large amount of organic acids in the medium which lowers the pH (<4.5), methyl red guard the red color (Ferron, 1989).

The Hemolysin

The production of β hemolysin is more common among strains of coagulase positive, conversely to the α hemolysin is more common in the strains of coagulase negative (Franco et al., 2008). Test results demonstrate that the 100 isolates studied were 32% isolates who have the hemolysin β represented by a halo of total net hemolysis around the colonies, and 28% which has α hemolysin represented by incomplete hemolysis, and 40% who have δ hemolysin represented by the absence of hemolysis.

Coagulase

Of the 100 isolates belonging to the genus Staphylococcus, 22 isolates were identified as coagulase positive by highlighting the free coagulase using sterile rabbit plasma, which represents a rate of 22% of all isolates staphylococci. *Staphylococcus* that produces this enzyme is identified as *S. aureus* (Avril et al**.,** 2000), the remaining isolates represent a rate of 78% belong to other species of coagulase-negative staphylococci. If the coagulase test allows the identification of approximately 99% of strains of *S. aureus*, some do not produce free coagulase and species identification in this case is achieved by other tests.

API Staph

Of the 100 isolates we characterized 22 strains were identified as *S. aureus* by highlighting coagulase, but the analysis of these strains by the gallery APIstaph confirms that we have only 14 strains representing a rate of 63,63% of all

coagulase-positive strains and 14% of all isolated strains of staphylococci were identified as *S. aureus*, while the other eight strains representing a rate of 37.37% on all strains coagulase positive, and a rate of 86% over the whole of staphylococci isolated and identified as other staphylococci. The results show the presence of some species, among are opportunistic pathogens such as *S. epidermidis*, *S. heamolyticus*, *S.caprea*, *S.hominis* and *S.saprophyticus* with rates of 2 %, 2 %, 2.38 %, 2.38 % and 7.14 % respectively.

The Antibiogram

The analysis of the 100 strains for susceptibility shows that they are sensitive to most antibiotics except for someone like penicillin which 38 strains (38%) were resistant to this antibiotic, 10 strains (10%) were resistant oxacillin, 56 strains (56%) were resistant to tetracycline, 48 strains (48%) were resistant to erythromycin and 2% is resistant to cefoxitin See Figure 1.

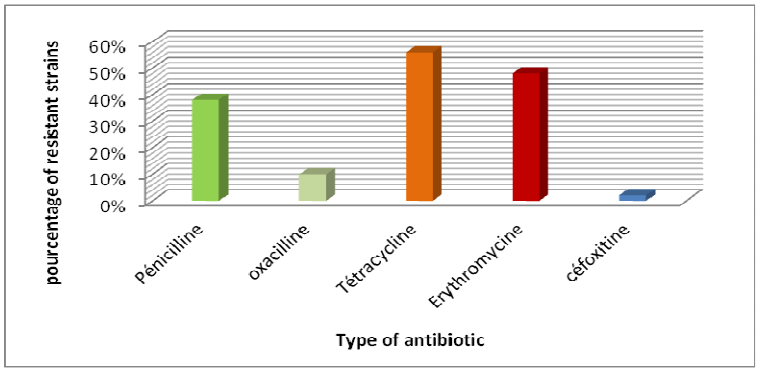

Figure 1: Percentage of Strains Resistant to Various Antibiotics

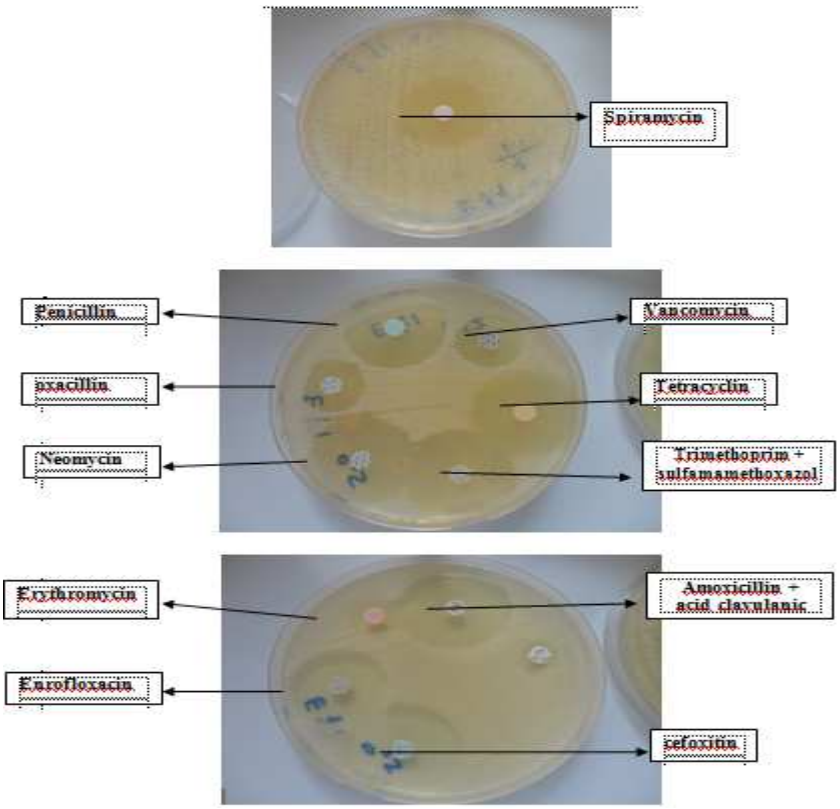

Figure 2: Example of an Antibiogram

The mechanism of resistance to penicillin based on the synthesis, by the bacteria, of an enzyme called β-lactamase or penicillinase which hydrolyzes the β-lactam ring of penicillins and makes them inactive (Lowy, 2003). Resistance to oxacillin (methicillin resistance or) is related to the modification of the target of the β-lactam with a new and insensitive, penicillin binding protein PBP2a, encoded by the gene *mecA* (Aouati, 2009) and the Mechanisms of resistance to macrolides (erythromycin, spiramycin) include a target modification, the efflux systems and the inactivating enzymes (corne, 2004).

The antibiogram of *S. aureus* strains shows their sensitivity to most of antibiotics except penicillin at which all strains are resistant, whereas 40 % of strains are resistant to tetracycline and 20 % are resistant to erythromycin and clindamycin.

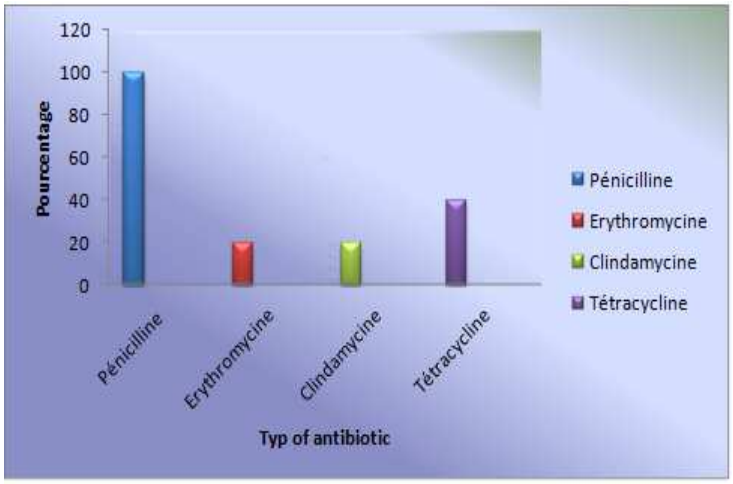

Figure 3: Percentage of *S. aureus* Strains Resistant to Various Antibiotics

Investigating Resistance to Methicillin

After the implementation of MRSA screening test , The absence of a bacterial culture on the Oxacillin Screen Agar medium showed that the strain of *S. aureus* suspected methicillin-resistant is a MSSA (*methicillin sensitive S.aureus*).

MRSA are generally found in hospitals, as opposed to MSSA which is the group of communal staphylococci (Aouati, 2009).

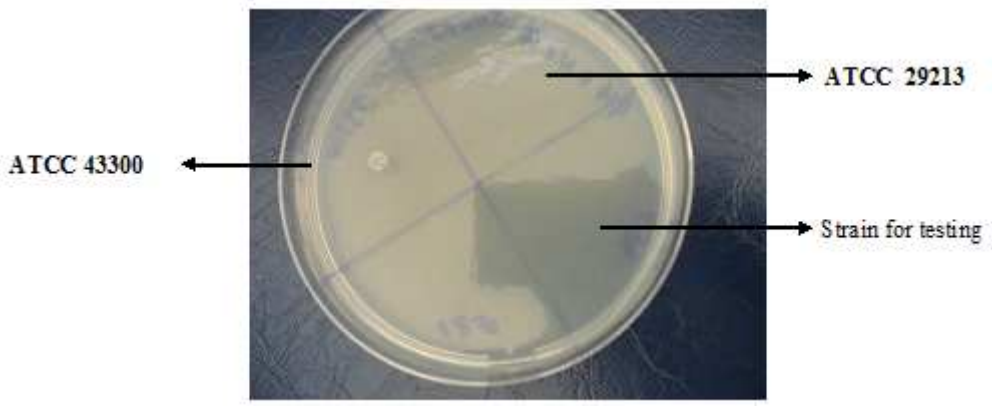

Figure 4: A Screening Test to Oxacillin

PCR Molecular Identification

Molecular analysis of 23 strains of staphylococci isolated from different environments by amplification of "nuc" gene coding thermonuclease. A DNA fragment of 270 bp (approximately) (Brakstad et al., 1992) was amplified from lysed cells of staphylococci. This molecular technique (PCR) showed the presence of this gene in 12 *S. aureus* strains which is detected by the presence of a DNA band of 270 bp (approximately) comparable with the positive control, which means that these strains are able to synthesize thermonuclease as a virulence factor in contrast to other CNS (coagulase-negative staphylococci) strains that were PCR negative, these results were compatible with other studies (Brakstad et al., 1992; Turkyilmaz and Kaya, 2006).

Direct detection of the nuc gene encoding the S. thermonuclease aureus has shown a 100% correlation with the results of API STAPH and 63.63% with the coagulase test.

While strains: *S. epidermidis, S.saprophyticus, S.haemolyticus, S.hyicus , S.chromogenes , S. lentus, S. xylosus,* showed an absence of amplification of this gene. However, other studies have showed that certain strains of the CNS (coagulase-negative staphylococci) also developing a nuclease (Jarvis and Wynne, 1969).

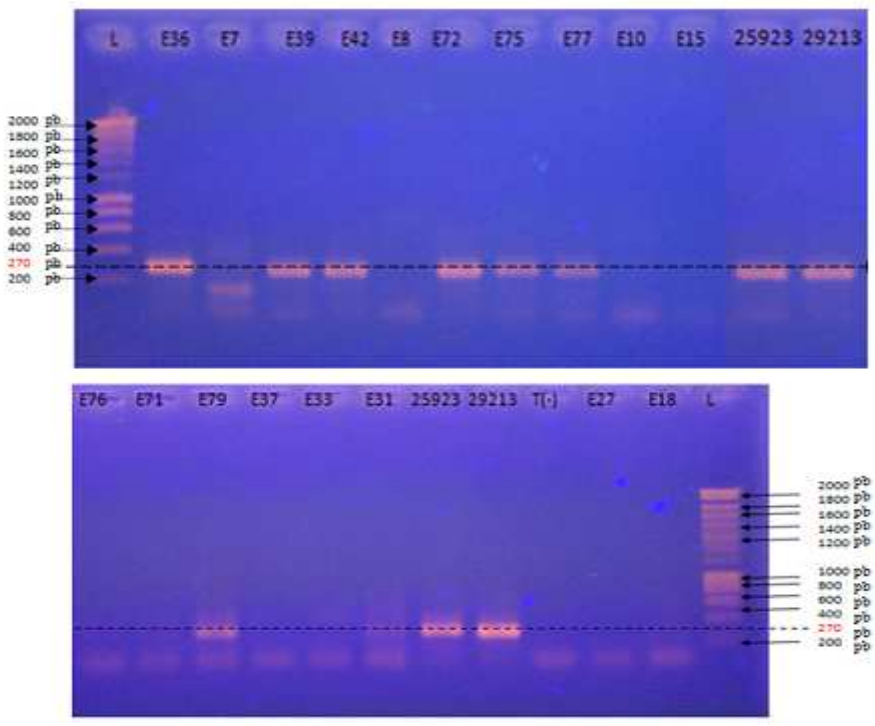

Figure 5: Examples of Electrophoresis of Amplicons of *"nuc"* Gene on Agarose Gel

While other studies (Barry et al., 1973; Zarzour and Belle 1978) showed that nuclease produced by *S. aureus* is unique and thermostable. while, the nuclease produced by SCN, is thermolabile when tested by the method of Lachica et al. (1971). [163].

The Activity of thermonuclease (TNase) is a characteristic of S. aureus, although it is not specific to him. However, the data reported by Brakstad and Maeland (1993) have shown that the "nuc" gene coding for the (thermonuclease) TNase in *S. aureus* has a specific sequence.

Thus, our data indicate that the amplification of the nuc gene by PCR has the potential to identify isolates of *S. aureus* only.

CONCLUSIONS

Staphylococci are ubiquitous bacteria that are frequently found on the skin and in the nostrils of man. *S. aureus* is both a commensal organism and a major pathogen of man, involved in a variety of pathologies including 1-5% of community infections and up to 30% of hospital-acquired infections (nosocomial infections). This is one of the major causative agents of superficial and deep suppurative infections and syndromes related to the action of toxins.

The main risk factors for infection are nasal carriage and any breach of the mucocutaneous barrier promoting the penetration of the germ.

The aim of this investigation was to evaluate the presence of staphylococci in the distribution chain of meat. The microbiological, biochemical and molecular tests that we have used have enabled us to isolate and identify some isolates of *Staphylococcus.*

Staphylococci are classified according to the production of free coagulase, which allows us to distinguish coagulase positive species such as *S. aureus* and coagulase-negative species such as *S. epidermidis.*

In our approach, the characters retained were those that were necessary and essential to good identification, we have started first by identifying the family, then genus then species. 130 strains were isolated from the media in contact with humans (red meat, white meat, rack, knife, table ...) and then analyzed and characterized.

22 strains were coagulase positive. biochemical study of these 22 strain by the API STAPH gallery, we were used to select 14 strains of *S. aureus*, whereas the molecular identification by amplification of the *" nuc"* gene of thermonuclease confirms that 12 strains have this gene. For this purpose, this test allows a reliable, fast and specific confirmation of *S. aureus*.

This study also revealed another important observation about multidrug resistance of these isolates to antibiotics. Although the molecular mechanisms of this resistance are unknown at this stage, it would be useful to pursue this investigation. The one hand, the antibiotic resistance is alarming, considering that the multi-resistant germs can be easily transmitted to humans, and their treatment with antibiotics would be ineffective. it should also be noted the possibility of transfer of resistance genes to other pathogenic strains. It is therefore important that current practices should be reviewed especially at the hygiene and the judicious use of antibiotics. On the other hand it would be very useful to serve as prophylaxis.

Today *S.aureus* is considerably studied in all scientific areas: molecular biology, food microbiology, medicine...

However, because of its continual expansion, each study analyzing a particular situation can not only the following and comprehension its evolution, but also to define strategies to fight in hospitals (nosocomial infections), agro-food industries or even a country. The PCR method has proved more effective for the identification of Staphylococcus. It is therefore important to encourage the use of this technique in epidemiological investigations in order to obtain specific and rapid results. However, these findings require confirmation by other techniques such as sequencing, genomics, transcriptomics, proteomics, because the detection of the gene does not mean that it is functional but it can be swich-off.

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