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Etiology, Antimicrobial Susceptibility of Udder Pathogens Phenotypic and Genotypic Characterization of *Staphylococcus aureus* Involved in Bovine Mastitis in Algeria

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Abstract: Mastitis is an inflammation of the mammary gland with local and or symptoms that occasionally result in a systemic infection. This disease has a profound impact on animal welfare and milk quality. The aim of this research was to investigate the microbial etiology and the occurrence of antimicrobial resistance. Moreover, difference between staphylococci antimicrobial resistance isolated subcliniques cases and clinical cases was investigated. In total, 213 quarter milk samples were collected from 56 dairy cows at 6 farms from February 2011 to February 2012. The quarter milk samples were bacteriological analyzed and scored using California Mastitis test. The most frequently isolated bacteria were the enterobacteriacea, staphylococci and streptococci, the presence of resistance was evaluated in all specific udder pathogens. Staphylococci antimicrobial resistance was performed by detection of *mecA* gene and the Pantin Valentine leukocidin coding gene (*pvl*) by PCR. The most common isolated of 250 bacteriological diagnoses were Enterobacteria (45.76%) *Staphylococcus aureus* (38.9%), coagulase-negative staphylococci (2.7%) and Streptococcus (12.43%) in milk samples from clinical cases (47.87%) and subclinical cases (52.12%) infected cows. All retained *Staphylococcus aureus* species contained *gyr* gene. Overall the *S. aureus* isolates were sensible to antimicrobials compounds. Staphylococcus, *Escherichia coli and Enterobacter cloacae* were the most frequently isolated pathogens. The testing of Staphylococcus antimicrobial resistance isolated subclinical and clinical cases showed the absence of resistance to antimicrobials agent.

Key words: Mastitis, milk, quality, *S. aureus*, gram negative bacteria, PCR, mecA, pvl

INTRODUCTION

Mastitis, the most expensive disease of dairy cows, continues to be a persistent problem in the dairy industry (Barkema *et al*., 2009; Le Marechal *et al*., 2011). Mastitis, inflammation of the mammary gland with local and or general symptoms that occasionally result in a systemic infection can be caused by a wide range of microorganisms including gram-negative and gram-positive bacteria (Le Marechal *et al*., 2011). This disease is considered to be the most frequent and most costly production disease in dairy herds of developed countries (Fourichon *et al*., 2001; Benhamed *et al*., 2011). Mastitis is one of the dominant pathological dairy farming actually decreased milk production per cow due to the prevalence of clinical and subclinical mastitis. The main etiology is infectious. It results in the majority of cases by cell type inflammatory response involving an increase in the cell concentration in milk, cell counts in the diagnosis of mastitis is essential and the results should be according to the results of the CMT (California Mastitis test).

Bovine mastitis produces a wide variety of problems in the dairy farm. The treatment of this disease is based on the use of antibiotics which are not always effective. These drugs are also responsible for the presence of residues in the milk and the increase of antibiotic-resistant strains. Probiotic products were proposed as a valid alternative to antibiotic therapies and are also useful for the prevention of infectious syndromes (Espeche *et al*., 2012). *Staphylococcus aureus* is the most predominant contagious pathogen responsible for clinical and subclinical infections in lactating cows (Dego *et al*., 2002).

Antimicrobials compounds are an important tool in mastitis control programs. Therefore, surveillance of antimicrobial resistance is important to ensure optimal results of antimicrobial use and minimize the risk for selection and spread of antimicrobial resistance (Myllys *et al*., 1998). The bacterial species targeted in this study are pathogens frequently isolated from clinical and subclinical mastitis cases (Riekerink *et al*., 2008; Piepers *et al*., 2007). The purpose of this study was to investigate the microbial etiology to determine the antimicrobial resistance of the isolates from the selection

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of dairy herds in Oran Region West Algeria. Surveys of *Staphylococcus aureus* and Gram negative bacteria antimicrobial resistance isolated subcliniques cases and clinical cases were occurred.

MATERIALS AND METHODS

Study design: A diagnostic sample size of 56 dairy cows in both cases (clinically infected or subclinically infected) at 6 dairy farms from February 2011 to February 2012 in the Oran Region West Algeria. The quarter milk samples were bacteriological analyzed and scored using California Mastitis test. The number of clinical or subclinical infected cases was calculated to be included in the study per farm. During the sampling researchers registered data about the cows and the herd by using a specified questionnaire; breed of the cow, lactation number, date of lasted calving, milk yield at lasted monthly milk recording and presence of teat lesions were recorded as were number of cows in the herd and if automatic milking systems was used.

Milk samples: Udder quarter milk from the dairy cows were collected in aseptic conditions and were analyzed by California Mastitis test (Schalm *et al*., 1971). The CMT-reaction was graded from 1-4. The scores are ranked according to an increase in viscosity where the highest viscosity (CMT4). Samples were stored in cold at 4°C until bacteriological analysis (Getahun *et al*., 2008; Benhamed *et al*., 2011).

Bacteriological analysis: Milk samples were analyzed in Applied Microbiology Laboratory, Faculty of Sciences, Oran University. Milk samples were cultured on several media on blood (5% sheep blood) agar plates, Chapman agar and Drigalski agar, incubated at 37°C for 24 h. Growth on the plates was confirmed by additional laboratory tests in accordance. *S. aureus* was identified by means of typical colony and cells morphology, catalase reaction by coagulase reaction using rabbit plasma (Quinn *et al*., 1994) (coagulase positive) or Pastorex (agglutination test) (Bio-rad, France) and biochemical characterization using the Api-Staph System (Biomerieu, France). Strains expressed phenotypic resistance to cefoxitin confirmed by polymerase chain reaction detection of the *mecA* gene, typing of the accessory gene regulator (agr) and detection of control specific gene *Gyr* of *Staphylococcus aureus*. Coagulase-negative staphylococci were identified by typical colony and cell morphology and coagulase reaction. Streptococci were determined by colony and cell morphology, catalase and CAMP reaction and Api Strep. Gram-negative bacteria were confirmed by Gram-straining and typical colony and cell morphology. The test of API 20 E (Biomerieu, France) was applied. A milk sample was classified as positive if at least one Colony-Forming Unit (CFU) of *S. aureus* and *Streptococcus agalactiae* was isolated. For other bacteria such as Gram negative bacteria, the calculated CFUs were needed for positive significance. Samples were classified as contaminated if growth of major udder pathogen and if the CMT was high. The sample would be diagnosed as positive for growth of the major udder pathogen. The predominant pathogenic bacteria, *Staphylococcus aureus* and Gram negative bacteria were tested for susceptibility to antimicrobial agents (Benhamed *et al*., 2011).

Susceptibility testing: Susceptibility testing was performed by Disk (Bio-rad, France) Diffusion Method on Muller-Hinton agar plates. Testing was performed according to recommendation of Ca-SFM-veterinarian 2012 (Committee on Antimicrobial Company Information French Microbiology). The antibiotics tested were Penicillin G (PG-6 μg), Kanamyin (K-30 IU), Gentamicin (Gm-15 μg), Tetracyclin (Te-30UI), Erythromycin (E-15UI), Lincomycin (L-15 μg), Pristinamycin (PT-15 μg), Chloramphenicol (C-30 μg), Pefloxacin (Pef-5 μg), Fosfomycin (Fos-5 μg), cefoxitin (Fox-30 μg), Fusidic Acid (FA-10 μg), Vancomycin (VA-30 μg), Oxacillin (Ox 5 cmg), Amikacin (10 mcg), Carbenicillin (cB 100 μg), Ciprofloxacin (5 mcg) and Amoxicillin + Clavunique acid (AMC 10/20 mcg). For testing susceptibility in staphylococci, 2% NaCl was added to the broth and to Muller-Hinton agar plates. Control strains, *S. aureus* ATCC 43300, *S. aureus* ATCC 25923 and *E. coli* 25922 were tested in parallel with each batch of isolates (Smyth *et al*., 2001). The susceptibility to the cefoxitin of *S. aureus* was confirmed by polymerase chain reaction of the *mecA* gene.

Bacterial DNA extraction: DNA of each strain of *Staphylococcus aureus* was extracted according to the standard protocol (Sambrook *et al*., 1989). The collected DNA was precipitated, described by electrophoresis on agarose gel and then stored at -20°C.

Detection of *mecA* gene, *GyrA* gene and *pvl* gene by PCR: The confirmation of *Staphylococcus aureus* species was performed on the basis of standard biochemical tests. The isolates were further characterized by molecular analysis amplifying the gene *gyr* typing of the accessory gene regulator (Brakstad *et al*., 1992). A duplex PCR for the simultaneous fragment 533 base pairs (bp) specific *mecA* gene and another 280 bp fragment of the gene *GyrA* were used to prove the *S. aureus* species. The pathogenicity and virulence of *S. aureus* is associated

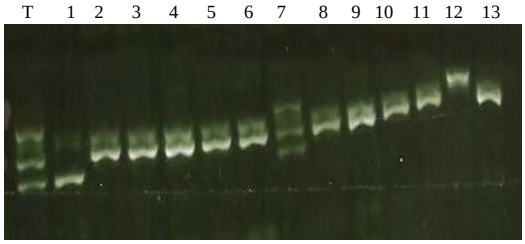

Fig. 1: Agarose gel electrophoresis of Polymerase Chain Reaction (PCR) amplification of *agr1*/2/3/4 gene

with the capacity of this organism to produce several virulence factors including Panton-Valentine Leukocidin (PVL) (Shittu *et al*., 2011). In addition the Pantin-Valentine Leukocidin coding *pvl* gene was detected by simple PCR using specific fragment 433 pb (Sung *et al*., 2008).

Typing of *agr* gene (accessory gene regulator): Using PCR multiplex for search simultaneous fragment agr type (agr1; 440, agr2; 550, agr3; 300, agr4; 650), the research has been carried out for strains of *S. aureus* (Fig. 1). The fragment used to define the type of agr *S. aureus* isolates. All amplification products were separated by electrophoresis on agarose gel 1.5% stained with Ethidium bromide (0.5 μg mL^{-1}) in Tris-Borate-EDTA TBE (at a rate of one to two drops added). Photographs of gels were taken under UV device (Gel Doc) (Sambrook *et al*., 1989).

RESULTS AND DISCUSSION

Descriptive data: The diagnostic of 54 cows from 6 herds (1-20 cows/herds), satisfying the selection criteria, CMT has allowed us to reveal a rate of (56.41%) of mastitis in dairy herds included in the study. Clinical examination showed a rate of 52.12 and 47.87% cases of subclinical and clinical mastitis, respectively (Table 1). These results are nearly similar to those mentioned by Bakken (1981) and El-Seedy *et al*. (2010) which found that incidence of subclinical mastitis was 38.2%. Study visiting showed that overall the farms had mechanical milking (Table 2). The cows were mainly of Holstein (85%) or the Jersey (7.40%) breeds, the rest (7.60%) were cross-breeds. Mean daily milk yield was between (7-12 L day^{-1}).

Distribution of udder pathogens: At least one microbial species involved in mastitis cases was isolated from 120 (56%) of 213 quarter samples. The distribution of microbial diagnoses is shown in Table 3. The distribution of the most commonly isolated bacteria considering only bacteriological positive samples were: a rate of 41.68% which proved to be Gram-positive cocci with catalase reaction, coagulase and thermonuclease (Table 4).

Table 1: Distribution of bacteriological diagnoses from quarter milk samples from clinical or subclinical mastitis cases

	Data of strains					
Strains	Clinical case (%)	Subclinical case (%)	Winter (%)	Spring (%)	Summer (%)	Autumn (%)
S. aureus	59.58	40.42	26.00	17.78	40.00	16.22
SCN	0.00	100.00	2.33	40.00	47.67	10.00
Streptococcus sp.	15.55	84.45	45.90	0.00	45.00	9.10
Gram (-) bacteria	81.18	18.82	35.00	17.50	30.00	17.50

Table 2: Distribution of bacteria species (%) isolated from cow in different farms in the area of Oran

Farms	Gram (-) bacteria	*S. aureus*	*Strep* sp.	SCN
F1	100.00	0.00	0.00	0.00
F2	29.05	64.70	0.00	6.25
F3	28.70	57.60	13.07	0.00
F4	39.92	50.08	0.00	10.00
F5	38.45	30.76	30.76	0.00
F6	38.45	30.76	30.76	0.00

Table 3: Distribution (n, %) of bacteriological diagnoses from quarter milk samples from cows with clinical or subclinical mastitis

Diagnosis	Clinical mastitis	Subclinical mastitis	Total (n, %)
Staphylococcus aureus	58 (15.63%)	83 (22.37 %)	144 (38.98%)
Coagulase-negative staphylococci	0 (0%)	10 (2.7%)	10 (2.7%)
Streptococcus uberis	0 (0%)	21 (5.66%)	21 (5.66%)
Streptococcus agalacteae	7 (1.88%)	17 (4.58%)	24 (6.46%)
Escherichia coli	20 (25%)	60 (16.17%)	80 (47.05%)
Klebseilla sp.	2 (5.39%)	15 (4.04%)	17 (10%)
Proteus sp.	0 (0%)	8 (2.15%)	8 (4.70%)
Enterobacter cloacae	5 (1.34%)	37 (9.91%)	42 (23.33%)
Serratia sp.	0 (0%)	8 (2.15%)	8 (4.70%)
Other coliform bacteria	5 (1.34%)	10 (2.69%)	15 (8.82%)
Other bacteria	0 (0%)	2 (0.53%)	2 (0.53%)
Total	97 (26%)	274 (73.85%)	371

Table 4: Resistance rate of Gram (-) bacteria strains isolated in this study

Antimicrobial drug	Rate of susceptibility	Rate of resistance
Penicillin	8.57	91.42
Oxacillin	22.22	77.77
Cefoxitin	8.57	91.42
Kanamycin	85.71	14.28
Gentamycin	100.00	0.00
Acide nalidixique	100.00	0.00
Tetracycline	45.71	54.28
Ciprofloxacin	8.57	91.42
Pefloxcin	5.71	94.28
AMC	0.00	100.00
Carbenicillin	88.57	11.42

These tests allowed us to assign the species *Staphylococcus aureus* 38.9% and a reduced rate of *Staphylococcus* coagulase negative 2.7% (Table 5). These results were confirmed by Apistaph galleries test and confirmed by PCR by the presence of the *gyr* gene which was a specifique to revel *S. aureus* species, all retained species contained *gyr* gene (Fig. 2). Although, the spectrum of causative pathogens in intramammary infections continues to change, *Staphylococcus aureus* is still considered one of the most common etiological agents associated with clinical and subclinical infections in lactating cows by Esmat and Bader (1996) and El-Seedy *et al*. (2010).

Table 5: Characterization and rate of presence of different genes tested of *S. aureus*

		Rate of presence of genes											
		pvl		mecA		agr 1		agr2		agr3		GyrA	
Species	No. of strains	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%	Nb	%
Strains of *S. aureus*	11	1	9.09	0	0.0	0	0.0	10	90.91	1	9.09	11	100

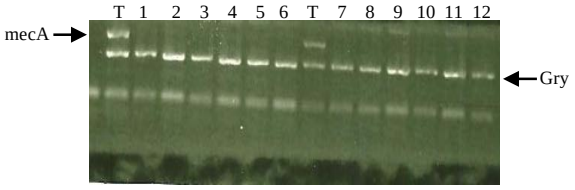

Fig. 2: Agarose gel electrophoresis of Polymerase Chain Reaction (PCR) double amplification of *mecA* and *GyrA* gene

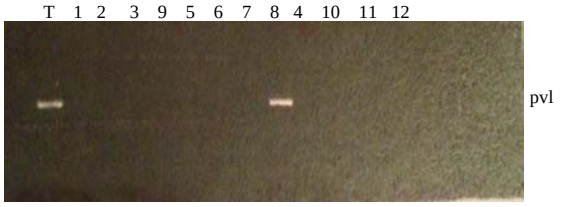

Fig. 3: Agarose gel electrophoresis of Polymerase Chain Reaction (PCR) double amplification of *pvl* gene

Precise identification of *S. aureus*-infected cows is important for successful implementation of a mastitis control program. Therefore, according to the phenotypic, biochemical properties as well as by amplification of the *gyr* gene, all of the isolates obtained in this study were identified as *S. aureus* (Saei, 2012).

Detection of pvl-luk toxin by amplification of the *pvl* gene from extracted DNA of the *S. aureus* strains reveled that positive amplification of the 533 pb fragment of *pvl* gene from the extracted DNA of 1 strains from 11, this strain had the following profiles agr3, mecA-, pvl+ (Fig. 3). This finding was comparable to the study of Sung *et al.* (2008). One isolate was positive for gene encoding the components of the Pantin-Valentine-Leukocidin (pvl-luk) this results are similar to those obtained by Monecke *et al.* (2011) and Shittu *et al.* (2011). A rate of 12.43% of *Streptococcus* species with *Streptococcus agalactiae* (54) and (46%) of *Streptococcus uberis* confirmed by Camp-reaction. Gram negative bacteria species are found in all cases with the rates of 45.76%, these results are similar to those observed by Lam.

In clinical mastitis the percentage of *S. aureus* and Gram negative bacteria isolates presented, respectively 59.58 and 81.18% of total bacterial as major pathogen followed by *Streptococcus* sp. (15.55%) and the absence of SCN. These results are nearly similar to those mentioned by Esmat and Bader (1996) and El-Seedy *et al.* (2010).

S. aureus, *E. coli* are the most frequently bacteria isolated from bovine mastitis and are considered the most important etiological agents for this infection. In the early studies, *S. aureus* was isolated in a small number of subclinical mastitis cases (Vassiliadou, 1991; Deinhofer and Pernthaner, 1995; Contreras *et al.*, 1999). However, in the present investigation, it was identified in 40.42% of the subclinical mastitis isolates (Table 3).

Streptococcus sp. (100%) were the most bacterial agent associated with subclinical mastitis cases (Table 3). These data are in agreement with the results obtained by several researchers, Ochoa-Zarzosa *et al.* (2008). This study showed that CNS species are the agents most commonly involved in subclinical mastitis in the herds tested in Oran area. This finding are consistent with other studies realized in other country such as Brazil and Egypt (Contreras *et al.*, 1995, 1999; Bedidi-Madani *et al.*, 1998).

Antimicrobial susceptibility: Antimicrobial susceptibility testing reported a high susceptibility of *S. aureus* strains to antimicrobial agents which was confirmed by PCR by the absence of *mecA* gene. The resutts showed absence of *mecA* gene for all *S. aureus* strains which were phenotypically susceptible to cefoxitin and oxacillin. Results shown in Fig. 1 showed the absence of the *mecA* gene from extracted DNA of *S. aureus* strains tested, this result confirmed the antibiogram results for susceptibility to methicillin. Whereas, the gentamicin and kanamycin were the most active against all GNB isolated in this study. Although, tetracyclin had a low activity (around 45.71%) against most Gram Negative Bacteria (GNB). All selected GNB showed a high resistance to pefloxcin, ciprofloxacin, cefoxitin and penicillin. These findings were comparable to other studies realized in Korea and Poland (Lee *et al.*, 2007; Malinowski *et al.*, 2006). Recently, a great deal of attention has been paid to GNB because of extensive antibiotic resistance in some species that poses a serious threat to public health (O'Mahony *et al.*, 2005; Lockhart *et al.*, 2007). The environment and animals on dairy farms could serve as important reservoirs of pathogenic and commensal bacteria (Straley *et al.*, 2006) which often cause udder

infection in dairy cattle. Coliform bacteria isolated from mastitis may reflect the general resistance situation in the herd and can be considered more as indicator bacteria than as specific pathogens of the udder (Lockhart *et al*., 2007).

CONCLUSION

The results indicate that several bacteria species were found in mastitis cases. The predominant species were *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Escherichia coli* and *Enterobacter cloacae*. Identification of *Staphylococcus aureus* was confirmed by detection of different genes *gyrA*, *agr* typing. One strains of *Staphylococcus aureus* contain PVL-Luk toxin by presence of *PVL* gene. The antimicrobial susceptibility testing showed that *Staphylococcus aureus* isolates from Oran Region West Algeria exhibited high susceptibility to all antimicrobial agents tested. The absence of *mecA* gene in all strains of *Staphylococcus aureus* tested.

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