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Genetic characterization and antimicrobial-resistance of *Staphylococcus aureus* isolated from bovine milk in Tunisia

Running head: *Staph. aureus* in Tunisian bovine milk

M. Ben Said [1, 2], M.S. Abbassi[1], V. Bianchini[3], S. Sghaier[1, 2], P. Cremonesi[4], A. Romanò[3], V. Gualdi[5], A. Hassen[2], M. Luini[3*]

[1] Université de Tunis El Manar, Institut de la Recherche Vétérinaire de Tunisie, 20 rue Jebel Lakhdhar, Bab Saadoun, 1006 Tunis, Tunisie.

[2] Laboratory Wastewater Treatment, and Research Center of Water Technologies, Techno park Borj Cedria, PO Box 273, 8020 Soliman, Tunisie.

[3] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, via Einstein, 26900 Lodi, Italy.

[4] Institute of Agricultural Biology and Biotechnology, National Research Council, via Einstein, 26900 Lodi, Italy.

[5] Piattaforma Genomica, Parco Tecnologico Padano, via Einstein, 26900 Lodi, Italy.

*Corresponding author: Dr. Mario Vittorio Luini

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Sezione di Lodi

Via Einstein, 26900 Lodi, Italy

Phone: +390371439354

Fax: +390371438043

email: mariovittorio.luini@izsler.it

SIGNIFICANCE AND IMPACT OF THE STUDY

This paper describes the characteristics of *Staphylococcus aureus* isolated from bulk tank and individual cow milk in Tunisia. All strains were genotyped by *spa* typing and RS-PCR, a method based on the amplification of the 16S-23S rRNA intergenic spacer region, and multiplex PCRs for 22 virulence genes. A selected sub-sample of strains was also genotyped by multi-locus sequence typing. All strains were tested for antimicrobial resistance. Our study evidences a predominance of strains belonging to Clonal Complex 97. Methicillin-resistant strains were not detected, and overall low level of antimicrobial resistance was reported.

ABSTRACT

Staphylococcus aureus is a major agent of bovine mastitis in dairy herds, causing economic losses in dairy industry worldwide. In addition, milk and milk-products contaminated by *Staph. aureus* can cause harmful human diseases. The aim of this study was to characterize *Staph. aureus* strains isolated from dairy farms in Tunisia. Bulk tank milk (n = 32) and individual cow's milk (n = 130) samples were collected during the period of 2013-2014. Forty-three *S. aureus* isolates were recovered and typed by *spa* typing, 16S-23S rRNA intergenic spacer (RS-PCR) and multiplex PCRs for 22 virulence genes. Antimicrobial resistance was also investigated with a disk diffusion test. A selected sub-sample of 22 strains was additionally genotyped by MLST. Seventeen *spa* types were recovered and t2421 (n = 10), t521 (n = 6), and t2112 (n = 5) were the most common. Fourteen different RS-PCR genotypes grouped into 11 clusters were detected in our study, with predominance of the R^{VI} genotype (n = 24). Eight sequence types were identified and Clonal Complex 97, corresponding to RS-PCR cluster R, was the most common (n = 10), followed by CC1 (n = 4), CC15 (n = 3) and other four accounting for 1 or 2 strains. Different combinations of virulence genes were reported, and enterotoxin genes were present in few strains (*seh*, n = 4; *sea*, n = 2; *sea* and *seh*, n = 2; *sec* and *sel*, n = 2). The majority of strains were resistant only to penicillin, only one strain was found to be multi-resistant and no methicillin-resistant *Staph. aureus* (MRSA) was demonstrated. Our study reported the isolation of CC97 from bovine milk in Tunisia for the first time and confirmed the relevance of this lineage in intramammary infection in cows.

Key words: *Staphylococcus aureus*, bovine milk, genotyping, virulence genes, antimicrobial-resistance, CC97

INTRODUCTION

Staphylococcus aureus is recognized as an important pathogen for both humans and numerous animal species. In dairy herds it causes clinical and subclinical mastitis, a disease affecting milk production industry worldwide, determining important economic losses (Seegers et al., 2003). Spread of infection in a farm usually occurs from cow to cow during the milking process through contaminated machines, equipment, or milker's hands (Petersson-Wolfe et al., 2010). The presence of such pathogen in food producing animals or raw milk is a matter of concern for human health as enterotoxigenic *Staph. aureus* strains are common cause of food intoxication worldwide due to the ingestion of preformed enterotoxins (Kadariya et al., 2014). Although *Staph. aureus* belongs to the commensal bacterial flora of humans and animals, it can become pathogenic through acquisition of virulence factors that damage the host cells. Indeed, *Staph. aureus* can produce a wide range of extracellular toxins and virulence factors, such as Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin (TSST-1), exofoliative toxins (such as ETA and ETB), and several staphylococcal enterotoxins, which represent a risk for humans, being associated with severe infections (Lina et al., 1999; Jarraud et al., 2002).

Staphylococcus aureus can produce not only a wide range of virulence factors, but it can also acquire the staphylococcal cassette chromosome *mec*, giving rise to methicillin-resistant *Staphylococcus aureus* (MRSA) (Pantosti, 2012). MRSA is an important pathogen in human medicine, but can also colonize and cause infections in a variety of animal species (Pantosti, 2012; Guardabassi et al., 2013). The frequent detection of specific livestock-associated (LA) MRSA clones (primarily CC398) in farm animals and personnel attending them in recent years in several countries is a matter of concern (de Neeling et al., 2007; Spohr et al., 2010; Richter et al., 2012).

Although several studies reported the prevalence and described the genetic lineages of *Staph. aureus* from healthy and hospitalized humans (Kechrid et al., 2010; Ben Slama et al., 2011) and from different animal species in Tunisia (Gharsa et al., 2012a; Gharsa et al., 2012b; Gharsa et al., 2015), few data about prevalence and genotypes of *Staph. aureus* or MRSA from dairy cattle herds in Tunisia are available. Thus, the aim of this study was to characterize *Staph. aureus* strains isolated from bovine milk collected in different regions of Tunisia by antimicrobial susceptibility testing, genotyping techniques (*spa* typing, 16S-23S rRNA intergenic spacer genotyping (RS-PCR), multilocus sequence typing (MLST)) and virulence genes contents determination.

RESULTS AND DISCUSSION

Very few data on the presence and the genetic traits of *Staph. aureus* in bovine milk (either from healthy or diseased cows) in Tunisia are available. Thus, this study was undertaken to fill the gap and to determine genetic lineages and virulence traits of the collected strains.

Amongst the 162 milk samples tested in this study, 43 *Staph. aureus* were recovered. Twenty seven (20.8%) were from individual cows originating from 11 small family farms distributed in six regions of Tunisia and 16 isolates (50%) derived from bulk tank milk collected in various regions of the country (Table 1). Similarly, high rates of *Staph. aureus* occurrence in bovine bulk tank milk have been reported worldwide (Jørgensen et al., 2005; Miranda-Morales et al., 2008; Francoz et al., 2012; Haran et al., 2012).

Several molecular methods have been described for the typing of *Staph. aureus* isolates, such as MLST, *spa* typing, Pulsed-field Gel Electrophoresis (PFGE) or RS-PCR, a genotyping technique based on PCR amplification of the 16S-23S rRNA intergenic spacer region developed by Fournier and colleagues (2008). This method has been successfully applied in Switzerland and Italy to show the genotype's association between virulence gene patterns and clinical properties of *Staph. aureus* intra-mammary infection in cows (Graber et al., 2009; Cremonesi et al., 2015).

In this study all the 43 strains were submitted to *spa* typing and RS-PCR. Seventeen different *spa* types were detected: t2421 (*n* = 10, 23.3%); t521 (*n* = 6, 14%); t2112 (*n* = 5, 11.6%); t114, t254 (*n* = 3, 7%); t127, t4045, t4682, t5428 (*n* = 2, 4.7%); t084, t359, t701, t903, t1201, t2844, t2883, and t10103 (*n* = 1, 2.3%) (Table 1). RS-PCR revealed the presence of 14 different genotypes, grouped into 11 clusters. R^{VI} genotype was the most frequent (*n* = 24, 55.8% of isolates), followed by R^{I} (*n* = 4, 9.3%), both belonging to cluster R, while other genotypes were represented by one or two strains each (Table 1).

One among the strains presenting the same combination of RS-PCR/*spa* type was randomly selected and typed by MLST, for a total of 22 strains further characterized. Eight different sequence types (ST) belonging to 7 clonal complexes were detected: CC97 (*n* = 10, 45.5%), including ST97 (*n* = 7) and ST2826 (*n* = 3), was the most represented, followed by CC1-ST1 (*n* = 4, 18.2%), CC15-ST15 (*n* = 3, 13.6%), CC522-ST522 (*n* = 2, 9.1%), CC6-ST6, CC188-ST188 and CC1153-ST1153 (*n* = 1, 4.5%) (Table 1). Interestingly, ST97 and ST2826 differ by just one nucleotide in *pta* gene (C155T).

In our study several RS-PCR genotypes were found, but the cluster R was most prevalent (65.1%). This result is different if compared with the European ones, where recently it was shown that *Staph. aureus* belonging to RS-PCR cluster B (CC8) predominates in several

countries, including Switzerland and Italy (Cosandey et al., 2016). Eight different *spa* types were shown to be associated with cluster R (t521, t1201, t2112, t2421, t2844, t4045, t4682, t10103), but there is evidence that all of them could belong to CC97. Clonal complex 97 is known to be one of the *Staph. aureus* lineages associated with cattle, and especially with bovine mastitis (Sung et al., 2008; Hasman et al., 2010; Hata et al., 2010). However, it was also described in other hosts, such as human, sheep, and pigs (Battisti et al., 2010; Shepheard et al., 2013). To the best of our knowledge this is the first report of the occurrence of the CC97 from bovine isolates in Tunisia. However, it was previously described in a case of bacteremia in a child and in the nares of one healthy sheep (Kechrid et al. 2010; Gharsa et al., 2012b). In the African continent CC97 was only reported in the nasal cavity of healthy pigs in Senegal (Lozano et al., 2016).

Despite the fact that CC97 is one of the animal associated lineages frequently carrying the *mecA* gene, responsible for the methicillin resistance, and it represents one of the LA-MRSA clones (Battisti et al., 2010; Pantosti, 2012), none of the strains belonging to cluster R-CC97 isolates was here recognized as MRSA. Overall, no MRSA were found in our study and, except for penicillin, few antimicrobial resistances were observed. This is in agreement with the evidence that in African countries MRSA colonization of animals is very low (Lozano et al., 2016). However, as only one colony per each positive sample was examined and no selective enrichment was performed, the presence of MRSA in some of them might have passed unnoticed.

Thirteen (30.2%) isolates were susceptible to all antimicrobial tested in this study, while the remaining were resistant to penicillin (n = 27, 62.8%) or penicillin and tetracycline (n = 2, 4.7%). One strain (2.3%) showed a pattern of multiple resistances to penicillin, tetracycline, streptomycin and clindamycin (Table 1). In animal staphylococci, resistance to penicillin, tetracycline, streptomycin and clindamycin are mainly encoded by *bla*Z, *tet*(k) or *tet*(M), *ant*(6)-Ia or *str*, also known as *aad*(6) and *lnu*A/*lnuB*/lsa(B) genes, respectively (Wendlandt et al. 2013). Concerning vancomycin, the results of the disk diffusion test (inhibition zone > 25 mm) does not completely exclude that some strains may had a reduced susceptibility as those strains can be revealed only by broth dilution or agar screen test (CLSI, 2013).

The high level of penicillin-resistance detected in the present work is in agreement with the study of Nam et al. (2011) in Korea and with the results reported form human isolates in Tunisia (Ben Slama et al., 2011). This high level of penicillin-resistance detected in our study could be explained by the frequent use of this drug to treat bovine bacterial infections, but it can also be due to inadequate dosage or duration of treatment. Overall, the finding of low

resistance rates compared to other countries (Oliver and Murinda, 2012; Li et al., 2015) could be explained by the low use of antibiotics in Tunisia or could be linked to the type of dairy farming. Indeed, factors contributing to spread of the infection are high density of animals, high milk production and animal introduction from other dairies, while in the present study small family farms, which represents well the Tunisian dairy cattle reality, were investigated.

Besides cluster R-CC97, a number of other genotypes (represented by few strains each) were reported and some of them were already described in Tunisia. Clonal complex 1 was previously observed in *Staph. aureus* isolates from donkey nares (Gharsa et al., 2012a) and from children patients (Kechrid et al., 2010), while CC6, CC15 and CC188 were found in nasal samples of pets (Gharsa et al., 2015). Overall, CC1 is known to be a common lineage among human isolates (Monecke et al., 2011); however, transmission of this lineage (CC1) from animals to humans is possible (Franco et al., 2011). CC15 is mainly associated with human isolates, while CC6, CC188 and CC1153 are rarely reported both in human and animal isolates (Smith et al., 2014). Two strains belonged to CC522, a lineage mainly reported from goats and in less extent from sheep (Shepheard et al., 2013). Interestingly, these two strains harbored s*ec* and *sel* genes, enterotoxins that usually predominate in sheep and goat isolates (Morandi et al., 2009), and one of them was classified as RS-PCR genotype BW^{II}, a common genotype in goats (personal data), suggesting a possible transfer of strains between species. In different cases in the investigated farms small ruminants were also reared, supporting the hypothesis of the inter-species transmission of the strains.

Concerning virulence factors, various virulotypes were observed (Table 1). Different from previous studies (Akineden et al., 2001; Jørgensen et al., 2005), few strains (n = 10) encoded for enterotoxins: only *seh* in four (9.3%), only *sea* in two (4.7%), and the combinations *sea-seh* and *sec-sel* in two strains each (4.7%). Genes coding for toxins usually associated with human diseases, such as *tst* (responsible for toxic shock syndrome), *eta* and *etb* (responsible for the staphylococcal scalded-skin syndrome), or *lukS-lukF* (coding for the PVL, a cytotoxin that causes leukocyte destruction and tissue necrosis), were rarely or never detected (*tst*, n = 2, 4.7%; *eta-etb*, n = 2, 4.7%; *lukS-lukF*, n = 0). Similarly, low rates of these toxins were reported either in animal or human isolates in Tunisia (Ben Slama et al., 2011). As previously reported, we observed in two isolates the concomitant occurrence of *sec-sel-tst*, confirming their genetic linkage in the pathogenic island (SaPI2) (Orden et al., 1992). Genes encoding the bicomponent leucotoxin *lukE-lukD* were observed in all but one isolates (n = 42, 97.7%), and 29 also harbored *lukM* (67.4%). The high rates of *lukE-lukD* and *lukM* found in our study are in agreement with other reports (Fueyo et al., 2005; Schlotter et al., 2012). Cell-wall

associated protein genes were detected in almost all strains (*clfA-cna*, *n* = 43, 100%; *fmtB*, *n* = 42, 97.7%) and 22 strains (51.2%) were positive for at least one gene of the immune evasion cluster (IEC). This cluster encoded proteins relevant for the host immune evasion that target specifically human immune responses (Sung et al., 2008). However the frequency of *Staph. aureus* isolates containing the *scn* and others IEC system genes is high (51.2%), suggesting that this complex could be involved also in bovine immune response.

In conclusion, our study reports for the first time the predominance of *Staph. aureus* belonging to CC97 isolated from bovine milk in different areas of Tunisia, agreeing with the findings reported worldwide. MRSA strains were not detected in our collection, and low frequencies of antibiotics resistance was reported, except for penicillin. Despite the occurrence of low rates of genes encoding enterotoxins, exfoliative toxins and toxic shock syndrome toxin, these strains might represent a health threat to humans.

MATERIALS AND METHODS

Samples and bacterial isolates

Milk samples were collected during the period of October 2013-March 2014 from the bulk milk tanks of 32 farms. Additionally, 130 milk samples (from other 22 farms) aseptically collected during controls for subclinical mastitis from pool of quarters of individual apparently healthy cows were considered. The 54 farms investigated in this study are small family farms (number of milking cows ranging from 4 to 10) distributed in the areas of Tunisia where the majority of dairy cow herds are localized. These farms produce milk for own consumption, for transformation to fresh cheese or for sell to milk factory.

After collection, milk samples were chilled and transported to the laboratory and immediately processed. One hundred microliters of the samples were inoculated onto Mannitol salt agar (Bio-Rad, Hercules, CA, USA) and incubated for 48h at 37°C. From each positive sample, one presumptive *Staph. aureus* colony was selected and identified by mannitol fermentation, Gram staining, catalase, ability to coagulate rabbit plasma (bioMérieux, Marcy l'Etoile, France), and DNase activities. *Staph. aureus* identification was then confirmed by PCR targeting the *nuc* gene (Cremonesi et al., 2005).

Antimicrobial susceptibility testing

Using the standard disk diffusion method in accordance with the Clinical and Laboratory Standards Institute recommendation (CLSI, 2013), antimicrobial susceptibility of the isolates was determined against (charge in µg/disk): penicillin (10), oxacillin (1), cefoxitin (30)

tetracycline (30), streptomycin (10), chloramphenicol (30), fusidic acid (10), teicoplanin (30) and vancomycin (30). Double-disk diffusion test (D-test) with erythromycin (15 µg) and clindamycin (2 µg) was performed on all isolates to detect inducible clindamycin resistance (Fiebelkorn et al., 2003).

spa typing

spa typing was performed as described by Shopsin et al. (1999). The polymorphic X region of the Staphylococcal Protein A gene was amplified by PCR, and sequences data were analyzed using Ridom Staph-Type software (www.spaserver.ridom.de) which detects *spa* repeats and assigns *spa* types.

16S-23S rRNA intergenic spacer genotyping (RS-PCR)

The amplification of the 16S-23S rRNA intergenic spacer region was performed as previously reported by Fournier et al. (2008). PCR products were analyzed by the miniaturized electrophoresis system DNA 7500 Lab Chip (Agilent Technologies, Santa Clara, CA, USA). This system separates pieces of DNA according to their size resulting in a plot of corresponding peaks (electropherogram). Genotypes were defined by calculating the corresponding Mahalanobis distance of informative peak sizes and by comparing it to those of the prototype strains using the "Mahalanobis distance of *Staph. aureus* genotypes" software (Syring et al., 2012).

Multilocus sequence typing (MLST)

MLST was performed as previously described (Enright et al., 2000) on one representative strain per *spa* type and RS-PCR genotype (n = 22). Allele numbers and sequence type (ST) were assigned by the MLST database (http://saureus.mlst.net). Clonal analysis of the STs was performed using the clustering algorithm e-BURST (http://eburst.mlst.net), which divides MLST data sets into groups of related isolates and predicts the founding genotype of each clonal complex.

PCR for virulence factors

All isolates were tested by PCR for the presence of virulence-associated genes applying primers and protocols previously described. The following virulence factors were investigated: enterotoxins (from *sea* to *see*, from *seg* to *sel*; Akineden et al., 2001; Cremonesi et al., 2005), toxic shock syndrome (*tst*) and exfoliative toxins (*eta*, *etb*) (Akineden et al.,

2001), PVL (*lukS-lukF*; Lina et al., 1999), leucocidin M (*lukM*) and leucotoxin ED (*lukE-lukD*) (Jarraud et al., 2002). Cell-wall associated protein encoding genes *clfA* (clumping factor A; Akineden et al., 2001), *cna* (collagen-binding protein; Zecconi et al., 2005), and *fmtB* (cell-wall protein; Sung et al., 2008) were also searched, as well as *scn*, *chp* and *sak* genes belonging to the IEC (Sung et al., 2008).

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CONFLICT OF INTEREST

No conflict of interest declared.

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Table 1. Origin and characteristics of the 43 *S. aureus* strains recovered from bovine milk.

Farm ID	Source*	Region	Genotyping					Virulence profile												Resistance profile‡
			RS-PCR		*spa* type	MLST†	Enterotoxins	Toxins			Leucocidins, leucotoxins			Cell-wall- associated proteins			Immune evasion cluster			
			Cluster	Genotype				*eta*	*etb*	*tst*	*lukED*	*lukM*	*lukSF*	*clfA*	*cna*	*fmtB*	*chp*	*scn*	*sak*	
01	ICM	Morneg	R	R^{VI}	t2421	CC97-ST97	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
01	ICM	Morneg	R	R^{VI}	t2421	nd	-	-	-	-	+	-	-	+	+	+	+	+	-	PEN
02	BTM	Beja	R	R^{VI}	t2421	nd	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
03	BTM	Tunis	R	R^{VI}	t2421	nd	-	-	-	-	+	-	-	+	+	+	-	-	-	NR
04	BTM	Tunis	R	R^{VI}	t2421	nd	-	-	-	-	+	-	-	+	+	+	-	-	-	PEN, TET, CLI, STR
05	ICM	Manouba	R	R^{VI}	t2421	nd	-	-	-	-	+	-	-	+	+	+	+	-	-	NR
05	ICM	Manouba	R	R^{VI}	t2421	nd	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
05	ICM	Manouba	R	R^{VI}	t2421	nd	-	-	-	-	+	-	-	+	+	+	-	-	-	NR
06	BTM	Menzelbouzelfa	R	R^{VI}	t2421	nd	-	-	-	-	+	-	-	+	+	+	+	+	+	PEN
07	BTM	Slimen	R	R^{VI}	t2421	nd	-	-	-	-	+	-	-	+	+	+	-	-	-	PEN
08	ICM	Morneg	R	R^{VI}	t2112	CC97-ST97	-	-	-	-	+	+	-	+	+	+	+	-	-	PEN
08	ICM	Morneg	R	R^{VI}	t2112	nd	-	-	-	-	+	+	-	+	+	+	+	-	-	PEN
08	ICM	Morneg	R	R^{VI}	t2112	nd	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
08	ICM	Morneg	R	R^{VI}	t2112	nd	*seh*	-	-	-	+	+	-	+	+	+	-	+	+	PEN
08	ICM	Morneg	R	R^{VI}	t2112	nd	-	-	-	-	+	-	-	+	+	+	-	-	-	PEN
08	ICM	Morneg	CB	CB	t254	CC15-ST15	-	-	-	-	+	+	-	+	+	+	+	+	-	NR
09	ICM	Morneg	R	R^{VI}	t521	CC97-ST97	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
09	ICM	Morneg	R	R^{VI}	t521	nd	-	-	-	-	+	-	-	+	+	+	-	-	-	PEN
10	ICM	Beja	R	R^{VI}	t4682	CC97-ST97	-	-	-	-	+	+	-	+	+	+	+	+	+	PEN, TET
10	ICM	Beja	R	R^{VI}	t4682	nd	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
10	ICM	Beja	R	R^{VI}	t4045	nd	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
11	ICM	Kairouan	R	R^{VI}	t10103	CC97-ST97	-	-	-	-	-	+	-	+	+	-	+	+	+	PEN

12	BTM	Slimen	R	R^{VI}	t4045	CC97-ST2826	-	+	+	-	+	+	-	+	+	+	+	+	+	PEN
13	BTM	Tunis	R	R^{VI}	t2844	CC97-ST2826	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
14	ICM	Beja	R	R^{VI}	t1201	CC97-ST2826	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
15	ICM	Raouede	R	R^{I}	t521	CC97-ST97	-	-	-	-	+	-	-	+	+	+	-	-	-	NR
16	ICM	Beja	R	R^{I}	t521	nd	-	-	-	-	+	+	-	+	+	+	+	-	-	PEN
16	ICM	Beja	R	R^{I}	t521	nd	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
16	ICM	Beja	R	R^{I}	t521	nd	-	-	-	-	+	+	-	+	+	+	-	-	-	PEN
17	BTM	Ben Arous	J	J^{I}	t254	CC15-ST15	-	-	-	-	+	+	-	+	+	+	+	+	-	PEN, TET
18	BTM	Slimen	J	J^{I}	t254	nd	-	+	+	-	+	-	-	+	+	+	+	+	-	NR
19	BTM	Tunis	J	J	t084	CC15-ST15	-	-	-	-	+	-	-	+	+	+	+	+	-	NR
20	BTM	Menzelbouzelfa	BA	BA	t127	CC1-ST1	*sea*, *seh*	-	-	-	+	+	-	+	+	+	-	+	+	PEN
21	BTM	Tunis	BA	BA	t127	CC1-ST1	*sea*, *seh*	-	-	-	+	-	-	+	+	+	-	+	+	PEN
22	ICM	Beja	CA	CA	t114	CC1-ST1	*seh*	-	-	-	+	+	-	+	+	+	+	-	-	NR
22	ICM	Beja	CA	CA	t114	nd	*seh*	-	-	-	+	+	-	+	+	+	-	-	+	NR
22	ICM	Beja	C	C	t114	CC1-ST1	*seh*	-	-	-	+	+	-	+	+	+	-	+	+	NR
23	BTM	Tunis	F	F	t701	CC6-ST6	*sea*	-	-	-	+	-	-	+	+	+	-	+	+	PEN
24	BTM	Zaghouan	F	F^{I}	t903	CC1153-ST1153	-	-	-	-	+	+	-	+	+	+	-	+	+	PEN
25	BTM	Zaghouan	S	S	t2883	CC188-ST188	*sea*	-	-	-	+	+	-	+	+	+	-	+	+	PEN
26	ICM	Sousse	AJ	AJ	t5428	CC522-ST522	*sec*, *sel*	-	-	+	+	+	-	+	+	+	-	-	-	NR
26	ICM	Sousse	BW	BW^{II}	t5428	CC522-ST522	*sec*, *sel*	-	-	+	+	+	-	+	+	+	-	-	-	NR
27	BTM	Manouba	BL	BL	t359	CC97-ST97	-	-	-	-	+	+	-	+	+	+	+	+	-	NR

*BTM: bulk tank milk; ICM: individual cow's milk. †CC-ST; nd: not done. ‡CLI: clindamycin, PEN: penicillin, STR: streptomycin, TET: tetracycline, NR: not resistant.