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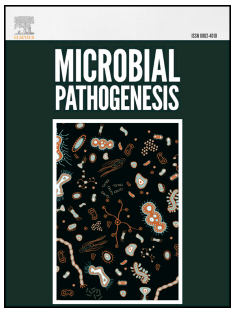

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Molecular epidemiology of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolated from milk of cows with subclinical mastitis.

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ABSTRACT

Bovine mastitis has been a concern for dairy herd for decades. The adaptation capacity of one of the main species responsible for this disease, *Staphylococcus aureus* (*S. aureus),* plays a pivotal role in this issue. The aim of this study was to establish a molecular and phenotypic profile of 285 *S. aureus* strains isolated from milk of subclinical mastitis cows from 18 different farms in São Paulo State using *spa* typing, multilocus sequence typing (MLST), pulsed field gel electrophoresis (PFGE), *agr* cluster (I, II, III and IV) typing, PCR for genes including enterotoxins (*sea, seb, sec, sed, see, seg, seh, sei*), toxic shock syndrome toxin (*tsst*-1), and Panton-Valentine leucocidin (*pvl*), as well as *in vitro* resistance assays for 12 antibiotics. The results showed a wide variety of strains with a high toxigenic potential; concomitantly, *sec*, *seg* and *seh* were prevalent. In addition, we observed a predominance of the *spa* types t605 (ST 126, CC126) and t127 (ST1, CC1) and the unusual presence of t321 causing bovine mastitis, which has been previously reported only in swine. The most frequent ST were ST126 (70.5%) and ST1 (10.5%). Regarding PFGE, we observed four major groups and six profile patterns. The highest resistance was observed for streptomycin (9.5%), followed by tetracycline (3.5%), clindamycin (9.3%), and erythromycin (2.8%). The *tsst*-1 gene was detected in 36.8% of isolates and *pvl* was not observed. One hundred and thirty-six (47.7%) isolates possessed *agr* type II, followed by types III (20%) and I (8.1%), with type IV not being detected. We observed that the same *spa* type could result in different PFGE profiles, so the exclusive use of *spa* type sequences can lead to incorrect interpretations regarding the spread of clones in an epidemiological context.

1. Introduction

Staphylococcus aureus is one the most important pathogens causing mastitis [1] and is a public health concern due to the presence of at least one gene encoding for enterotoxin production in most isolates [2]. Also, some of these staphylococcal enterotoxins (SEs) may play an important role in the pathogenesis of mastitis [3], such as toxic shock syndrometoxin-1 (TSST-1) and Panton-Valentine leucocidin (PVL) [4,5].

Resistance to β-lactam antibiotics has been also reported as one of the main problems related to the persistence of mastitis [6,7]. Multidrug resistance is also reported to be increasing. Wang et al. [8] found that 69% of the *S. aureus* isolates collected from milk of animals with bovine mastitis were resistant to more than 10 drugs used in the treatment of mastitis.

S. aureus isolates collected from dairy cattle with mastitis have been studied by various molecular methods such as multi locus sequence typing (MLST), pulsed filed gel electrophoresis (PFGE), and *agr* cluster and *spa* typing, which allow for the characterization of this pathogen through the comparison of sequences or fragments described in different parts of the world; these techniques can also be used to trace, dispersal routes and demonstrate the global spread of clones [9,10]. Many studies have shown that only a few specialized clones are responsible for most cases of mastitis on a single farm and some of these clones may have a wide geographical distribution [9,11].

Due variability and diversity in nucleotide sequences and even in genetic loci, MLST has over 2,400 “sequence types” (ST). Most STs are grouped into a limited number of clonal complexes (CCs) which appear to be distributed worldwide. The predominant CCs in *S. aureus* are CC1, CC5, CC8, CC15, CC22, CC30, CC45, CC59, CC80, CC97, and CC121 [12].

Therefore, molecular studies of *S. aureus* isolated from dairy farms can provide information about the dispersion of clones around the world in order to determine which of the most common circulating clones are causing disease.

The aim of the present work was to assess the molecular-epidemiological relationships between MSSA isolated from milk of cows with mastitis through *spa* typing, MLST, PFGE, and *agr* typing and to verify the presence of virulence factors and drug resistance.

2. Materials and Methods

2.1. Samples

A total of 4,908 quarter milk samples from 1,313 cows were analyzed, 2,118 samples (43.2%) were diagnosed as subclinical mastitis, obtained from 18 different dairy farms in São Paulo State, Brazil. Cows with subclinical mastitis presented no physical symptoms, absence of lumps or flakes, and no abnormal color or consistency of milk from the first three or five streams of milk were observed, but samples had high cell counts. The diagnosis of each quarter was based on the California Mastitis Test (CMT), according to Schalm and Noorlander [13], and confirmed by somatic cell counting (SCC), defined as a cow-level SCC above 200,000 cells/mL using flow cytometry (Somacount 300, Bentley Instruments, Chaska, MN). A milk sample from each positive quarter based on the CMT and CCS tests were plated onto blood agar plates (Oxoid Brasil Ltda, São Paulo, Brazil) and incubated at 37°C for up to 72 h. The colonies were identified based on morphology, Gram staining, and catalase, coagulase, and DNase activities [14]. Two hundred and eighty-five *S. aureus* isolates (13.5%, n = 285/2,118) were obtained and molecular confirmation was performed using multiplex

PCR, looking for a species-specific staphylococcal nuclease (*nuc*) gene, as well as the staphylococcal methicillin-resistance genetic determinant (*mecA*) [15].

2.2. PCR testing for genes encoding staphylococcal virulence factors

The Minispin Kit (GE Healthcare, Little Chalfont, UK) was used for DNA extraction according to the manufacturer's instructions. We performed PCR for the detection of staphylococcal super-antigens *(Sags)* genes [16,17], and *lukF*/*S-PV* [18]. *S. aureus* USA 100 (*lukF*/*S-PV*), ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), FRI 361 (*sed*, *seg*, *sei*), ATCC 27664 (*see*), FRI 137 (*seh*), and ATCC 51650 (*tsst*-1) were used as positive controls and ultrapure water was used as the negative control.

2.3. Molecular typing of *S. aureus* isolates

All isolates were tested for the *spa* gene (http://spaserver.ridom.de) and the *agr* allotype [19]. MLST (http://www.mlst.net) was performed on just one isolate for the more frequent *spa* type. PFGE of genomic DNA, digested with the macro-restriction *Sma*I enzyme under standardized Pulsenet electrophoresis conditions, was performed on 10 randomly selected strains, isolated from four different farms. A dendrogram of percentage similarity was calculated to define clonality by PFGE using a similarity value of 80% as a cut-off, which is considered the gold standard [20]. The band profiles were compared according to Bionumérics (BioNumerics software, 5.0) and Tenover et al. [21].

2.4. Antimicrobial susceptibility testing and detection of resistance genes

Antimicrobial susceptibility was performed using the disk-diffusion agar method, according to recommendations of the Clinical and Laboratory Standards

Institute (CLSI), [22]. The drugs tested were oxacillin, cefoxitin, tetracycline, erythromycin, clindamycin, gentamicin, tobramycin, streptomycin, trimethoprim-sulfamethoxazole, ciprofloxacin, cephalothin, and vancomycin. The detection of resistance genes was investigated in resistant isolates by PCR, using specific primers such as *mecC* (cefoxitin and oxacillin), *ant(4')* (tobramycin), *aac(6')-Ie-aph(2")-Ia* (gentamicin), *aph(3')-IIIa* (kanamycin and amikacin), *tet (K), tet(L),* and *tet(M)* (tetracycline), *erm(A), erm(B), erm(C),* and *msrA* (erythromycin), and *grl (A)* and *gyr (A)* (ciprofloxacin) [23-30]. Sanger sequencing was performed for the *grl (A)* and *gyr (A)* genes.

3. Results

3.1. Typeability and diversity of *spa* types

All 285 strains were confirmed as *S. aureus* using a PCR assay, but none were identified to be MRSA. As can be observed in Table 1, we found 23 different *spa* types (t605, t127, t458, t521, t342, t318, t693, t177, t11659, t021, t2164, t1192, t7335, t114, t6811, t6980, t002, t3324, t559, t138, t321, t456, t2066); the most frequent were t605, occurring in 201 isolates (70.5%), and t127, found in 30 isolates (10.5%).

3.2. Clonal lineages

The most frequent ST was ST126, occurring in 70.5% of isolates, followed by ST1 (10.5%), belonging to CC 126 and 1, respectively. The Ridom Staph Type software also identified five strains belonging to CC 30, with the *spa* types t138 (n= 1) and t318 (n= 4), and three strains identified as t177 belonged to CC1.

148

149

150 **3.3. *Pulsed field gel electrophoresis* (PFGE)**

151 We performed PFGE on ten strains, randomly selected, with eight being
152 t605/ST126 and two being t693/ST1. The isolates were subdivided into six PFGE
153 patterns, clustered into four dominant PFGE types.

154 The strains analyzed by PFGE were from four different farms (Figure 1).
155 According to Tenover et al. [22], only three strains from farm I were closely related
156 with, the patterns showing variations in two to three bands. It is interesting to note that
157 strain 18 was closely related to strain 08 (90.2%) but had a different *spa* type and
158 MLST, yet the same *agr* cluster. Equally, strains 128 and 89 were closely related
159 according to PFGE and harbored the same differences but were isolated from different
160 farms. We also observed six unrelated PFGE profiles considering the same *spa* type on
161 different farms, such as t605.

162

163 **3.4. *agr* cluster typing**

164 Type II *agr* was the most frequent, occurring in 136 (47.7%) out of 285 strains,
165 followed by type III (n= 57,20%) and type I (n= 23, 8.1%). Type IV *agr* was not found
166 and 69 (24.2%) did not belong any group.

167

168 **3.5. Virulence factors**

169 Regarding virulence factors, the *tsst-1* gene was found in 36.8% (n= 105) of
170 isolates. The SE gene distribution was highly variable, since all isolates (n= 285) were
171 positive for at least one of these genes. The most frequent were *seg*, occurring alone or
172 in different combinations in 275 (96.5%) isolates, followed by *seh* (n= 250, 87.7%), *sec*

(n= 227, 79.6%), *sei* (n= 44, 15.4%), *sea* (n= 31, 10.9%), and *sed* (n= 10, 3.5%). The *seb* and *see* genes were not observed, nor was the *pvl* gene.

3.6. Antimicrobial susceptibility testing and detection of resistance genes

As observed in Table 2, streptomycin resistance was the most prevalent (9.5%), followed by tetracycline (3.5%), clindamycin (3.2%), cefoxitin (1.4%), and ciprofloxacin (1.1%) resistance. Resistance to cephalothin, trimethoprim, and tobramycin was observed in just one isolate each. All 285 strains were sensitive to vancomycin, oxacillin, kanamycin, and gentamicin.

Among the isolates with intermediate resistant to erythromycin (n= 8), four were positive for the *mrs*A gene, but the *erm*A, *erm*B, and *erm*C genes was not noted. All isolates resistant to tetracycline presented with *tet*(K), while the *tet*(M) and *tet*(L) genes was not observed. All three isolates resistant to ciprofloxacin was positive for both the *grl (A)* and *gyr (A)* genes; Sanger sequencing was performed to identify mutations in amino acids, but none were observed. The *ant*(4') gene, responsible for tobramycin resistance, was identified in intermediate and resistant isolates. The *mec*A, and *mec*C genes were not noted in cefoxitin-resistant isolates.

4. Discussion

The interest in MSSA has increased in recent years since it may also be involved in important infections and could help to explain the occurrence and development of different successful strains of MRSA. There are few data regarding MSSA genetic strains in food products of animal origin and derivatives such as milk [10].

We found high diversity in *spa* types (n= 23) in 285 isolates of MSSA associated with subclinical mastitis. Aires-de-Sousa et al. [31] and Silva et al. [10] also reported the prevalence of t605 and t127 in Brazil. Said et al. [32] observed the MSSA *spa* type

t605 in Canada, and t127 has already been found in Switzerland [33] and Korea [34] in isolates from bovine mastitis.

As far as we know, this is the first report of the detection of *spa* type t321 isolates in milk samples from cows with subclinical mastitis. Previously, it has only been isolated from humans, and swine [35-37], showing that the host specificity of the pathogen may be questioned.

One limitation in this study was related to performing other molecular characterizations only in the most frequent *spa* types (t605 and t127), this included 81% of all isolates. MLST characterization was performed, and most of the clones were ST126, corresponding to 70,5% of the isolates, followed by ST1 (10.5%), belonging to CC 126 and 1, respectively. The Ridom Staph Type software also identified five strains belonging to CC 30, but these were *spa* types t138 (n= 1) and t318 (n= 4). One strain was identified as t177 belonging to CC1; it is important to note that this CC is most commonly isolated from humans [38,39]. The population of *S. aureus* seems to be highly clonal, as suggested by Musser et al. [40]. As noted in other studies [12, 41, 42], these strains can be represented by several clonal complexes, suggesting that there is no link between the specific genotype of MLST and the propensity to cause disease [43].

In PFGE results, we observed different profiles in isolates within the same *spa* type. According to Tenover et al. [21], a clone is a group of related isolates belonging to the same PFGE pattern; therefore, strains of the same *spa* type cannot be called a clone. Also, the presence of the same profiles carrying different molecular markers and virulence genes can explain the difficulty in identifying effective medicines against this pathogen [44]. According to Feil et al. [43], the gain and loss of virulence genes carried out through moving elements play important roles in determining the virulence of an

isolate. The movement of these genes can occur so rapidly that the presence or absence is only weakly related to clonal stability.

In summary, we did not find a good correlation between PFGE, *spa* typing, and MLST and showed that these methods have incomparable discriminatory power. Therefore, we strongly suggest that *spa* typing can be useful to complement genotyping results in studies to compare bovine mastitis isolates; however, there is no greater specificity when used alone.

One of the most well-known regulatory systems involved in the expression of virulence genes in *S. aureus* is the *agr* group. Studies have shown that *agr* type I is more prevalent in humans than in animals [45]. Conversely, *agr* II has been found more frequently in animals [10, 46, 47]. These results were similar to those of our study, with positivity of 47.7% for *agr* II (n= 136/285), followed by 20% for type III (n= 57/285) and 8.1% for type I (n= 23/285). The presence of *agr* type IV was not detected, and 69 (24.2%) strains were negative for all known types. Melchior et al. [46] suggested that *agr* type II isolates are better adapted to the dairy environment than *agr* type I isolates.

Regarding virulence factors, the *tsst-1* gene was found in 36.8% (105/285) of isolates, which was similar (37.5%) to the result found by Nader Filho et al. [48], also in Brazil. On the other hand, Sá et al. [49] did not observe this gene in *S. aureus* isolated from cows with mastitis from the same region as that investigated in the current study, but their study was performed 13 years ago, which suggests that there may have been a recent diversification of strains or the spread of a new clone into this region, demonstrating the genetic evolution of *S. aureus* towards a more virulent strain.

S. aureus strains can encode more than one enterotoxin gene simultaneously; over 50% of the isolates assessed showed this property [50,51]. In the current study, all 285 strains were positive for at least one gene, and the combination *sec+seg+seh* was

the most frequent, occurring in 56.8% of isolates. Zschock et al. [50] analyzed milk from cows with mastitis and also observed that some strains of *S. aureus* can encode several genes, with 26.9% carrying two genes. According to these authors, the simultaneous production of different types of enterotoxins can increase the toxigenic effect, suggesting that this co-production may play an important role in mastitis. Enterotoxin A (SEA) is one of the most frequently observed enterotoxins [52], although the literature shows highly variable results in the prevalence of *S. aureus* enterotoxin genes, depending on the kind of food and the biovar investigated [53]. In the current study, we found 31 (10.8%) isolates encoding the *sea* gene. On the other hand, Hata et al. [54] evaluated this gene in isolates from mastitic milk, which it was not found. As in our study, the *pvl* gene was not observed by Aires-de-Sousa et al. [31].

The increase in multidrug-resistant *S. aureus* isolated from bovine mastitis is a serious problem, with increasing morbidity and costs related to this disease. In addition, the indiscriminate use of antibiotics can lead to their accumulation in food, which can ultimately affect human health [55]. Since antimicrobial therapy is one of the main tools for the control of mastitis caused by *S. aureus*, antibiogram assays can indicate the best treatment for each case of mastitis [56]. According to our results, 230 (80%) isolates were sensitive to all drugs tested. For the resistant and intermediate strains, we tested the presence of genes responsible for resistance to tetracycline (*tet*K, *tet*L, and *tet*M), erythromycin (*erm*A, *erm*B, *erm*C, and *mrs*A), tobramycin (*ant*4), and ciprofloxacin (*glr,* and *gyr*). The resistance of strains to tetracycline was confirmed by the presence of the *tetK* gene, and both genes responsible for resistance to ciprofloxacin were present. Regarding the strains with intermediate resistance to erythromycin, only four were positive for the *erm*A gene and no gene encoding resistance for tobramycin was found. The discrepancy observed between the genotype and phenotype in the four strains with

intermediate resistance, which did not harbor any erythromycin resistance genes, was also observed by Goudarzi et al. [57], where nine *S. aureus* isolates were resistant to erythromycin, but did not carry any of the tested erythromycin resistance genes. The authors presumed that other variants of *erm* genes, the efflux pump (*msrB*), and a high rate of horizontal gene transfer were involved in this finding [58-60].

We sequenced both *glr*A and *gyr*A (responsible for resistance to ciprofloxacin), which present in three isolates, but no mutations were observed. This resistance could occur due to another mechanism such as the expression or overexpression of efflux pumps that can actively remove antibacterial agents from the cell [61].

The current study clearly shows that only performing molecular profile analyses in *S. aureus* isolated from mastitis is not enough to determine the pathogenic potential, but it did help to obtain insights about the population structure of *S. aureus* in São Paulo State. Due to a wide variety of genotypic profiles, we couldn't describe a predominant profile of *S. aureus* on these farms. Our data suggest that there is no link between the virulence and molecular profiles of *S. aureus* associated with mastitis in cows, as highly variable profiles were found.

Additionally, due to the discrepancies observed between the different molecular typing techniques such as PFGE, MLST, and *spa* typing in the current study, we question the exclusive use of *spa* typing, since strains with the same type may have no phylogenetic relationship, as shown by PFGE. On the other hand, *spa* typing can be used for screening purposes, as it is inexpensive, and portable. Moreover, we noted some t321 strains causing bovine mastitis, which had previously been isolated only from pigs and humans.

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Conflict of Interest

All authors of this manuscript have no conflict of interest to declare.

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Table 1. Distribution of virulence genes regarding the molecular characterization of *S. aureus* strains, isolated from the milk of cows with subclinical mastitis.

Spa types		*agr* types				virulence genes						
	N	*agrI*	*agrII*	*agrIII*	*agr*NT	*tsst*	*sea*	*sec*	*sed*	*seg*	*seh*	*sei*
t605	201	9	125	18	49	73	24	171	9	194	177	28
t127	30	0	1	21	8	10	3	22	0	29	26	1
t342	7	0	1	6	0	0	0	6	0	7	7	2
t458	7	7	0	0	0	7	0	3	0	7	6	2
t693	6	0	5	0	1	1	0	4	0	6	5	1
t521	6	3	0	0	3	4	1	4	1	6	5	2
t318	4	0	2	2	0	1	1	2	0	4	3	3
t177	3	0	0	2	1	1	0	3	0	3	3	0
t11659	3	0	0	0	3	0	1	1	0	3	3	0
t002	2	1	1	0	0	2	0	0	0	2	2	2
t021	2	0	0	2	0	0	0	0	0	2	2	0
t2164	2	0	1	0	1	1	1	2	0	2	2	2
t6811	2	0	0	0	2	0	0	2	0	1	2	0
t114	1	0	0	0	1	0	0	1	0	1	1	0
t1192	1	0	0	1	0	1	0	0	0	1	1	0
t138	1	1	0	0	0	0	0	1	0	0	0	0
t2066	1	0	0	1	0	1	0	1	0	1	0	1
t321	1	0	0	1	0	0	0	0	0	1	1	0
t3324	1	0	0	1	0	1	0	0	0	1	1	0
t456	1	1	0	0	0	1	0	1	0	1	0	0
t559	1	0	0	1	0	0	0	1	0	1	1	0
t6980	1	0	0	1	0	0	0	1	0	1	1	0
t7335	1	1	0	0	0	1	0	1	0	1	1	0
total	285	23	136	57	69	105	31	227	10	275	250	44

NT: not typable.

Table 2. Susceptibility to antimicrobial agents and detection of associated genes in *S. aureus* strains, isolated from milk of cows with subclinical mastitis.

Antibiotic/gene	Susceptible (N)	Intermediate (N)	Resistant (N)
Oxacillin	285	0	0
Cefoxitin *(mecA, mecC)*	281	0	4 (0/0)
Tetracycline *(tetK, tetM, tetL)*	275	0	10 (10 *tetK*)
Tobramycin [*ant(4')- Ia*]	283	1 (*ant 4'*)	1 (*ant 4'*)
Erythromycin *(erm cl, msrA)*	277	8 (4 *mrsA*)	0
Streptomycin	258	0	27
Gentamicina *aac(6')-Ie-aph(2")-Ia*	285	0	0
Clindamycin	276	0	9
Trimethoprim- sulfamethoxazole	284	0	1
Cephalothin	284	0	1
Ciprofloxacin *(grlA), gyrA)*	282	0	3 (3 *grlA, gyrA*)
Vancomycin	285	0	0
Kanamicym *aph(2")*	285	0	0

Note: cl: cluster

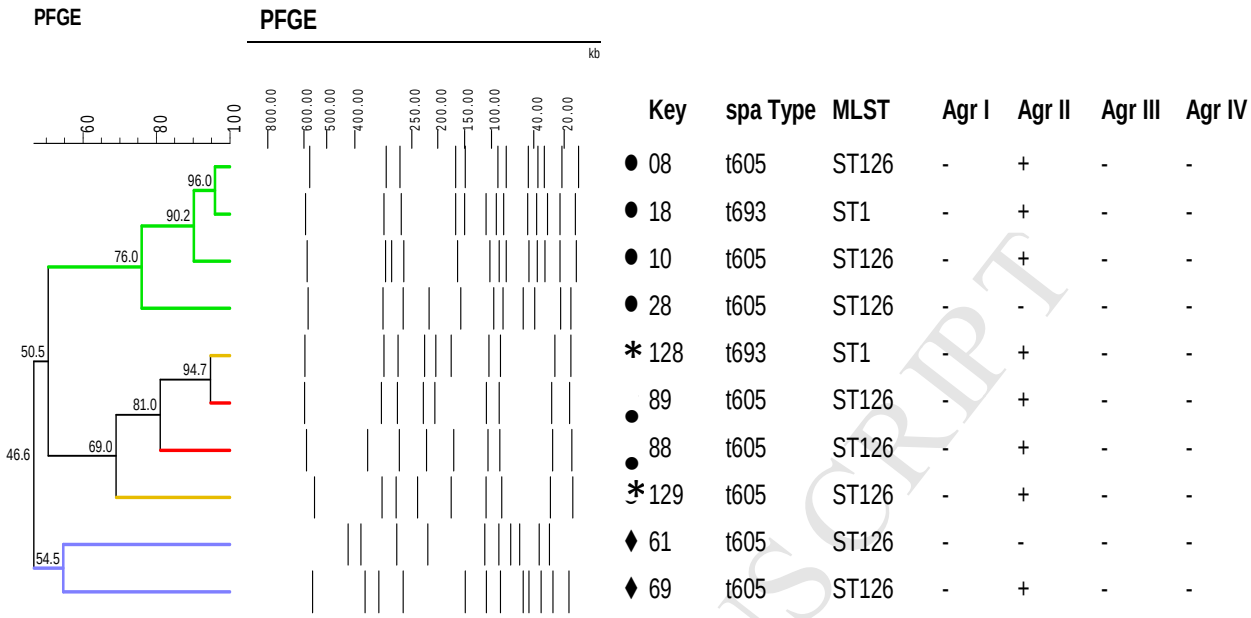

Figure 1. Dendrogram of pulsed-field gel electrophoresis (PFGE). PFGE types were determined by the unweighted pair group methodology based on Dice coefficients (BioNumerics software, 5.0), with 1.25% band position tolerance and optimization of 0.5%. A similarity coefficient of 80% was selected to define PFGE types. ● Farm I, * Farm II, • Farm III, ♦ Farm IV

Highlights

-unusual case of spa type t321 causing mastitis in cows

-Discrepancy in profiles patterns among typing techniques

- *spa typing* is not good molecular marker

-Prevalence of spa types t605 and t127