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Characterisation of *Staphylococcus aureus* strains isolated from mastitis bovine milk in Argentina

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The study reported in this Research Communication was conducted to characterise *Staphylococcus aureus* isolates recovered from mastitic bovine milk from dairy herds in Argentina. A total of 829 mastitic milk samples, both clinical and subclinical, were collected from 21 farms by veterinarians and submitted to the laboratory for testing from which 229 *S. aureus* isolates were recovered, an isolation rate of 28·1%. These isolates were tested for susceptibility to the antibiotics penicillin, erythromycin and clindamycin. Of the 229 isolates, 53 (23·1%) were resistant to penicillin, 31 (13·5%) to erythromycin and 28 (12·2%) to clindamycin. All isolates were negative for the *mec*A, *mec*C and *pvl* genes by PCR. Southernblot hybridisation revealed that the *erm*C gene was located on plasmid bands. Eighty isolates were randomly selected from the 229 for further characterisation. Restriction analysis of chromosomal DNA with Cf9I followed by PFGE of the 80 isolates revealed 23 distinct pulsotypes at 80% similarity. Seven major types (A, B, N, P, S, T, U and V) accounted for 68·7% of these isolates and 12 pulsotypes (A, B, F, G, J, K, M, N, P, S, T and U) occurred on more than one farm indicating genetic diversity within the farms. MLST of a representative isolate from dominant types identified the STs 97 705, 746, 2102 and 2187 with ST97 being the most predominant. Antibiotic susceptibility testing showed that 53·7% of the 80 randomly selected isolates were resistant to at least one of the three antibiotics tested. To our knowledge, this study represents the first large scale molecular studies on *S. aureus* isolates from dairy farms in Argentina.

Keywords: *Staphylococcus aureus*, bovine mastitis, antimicrobial resistance, molecular typing.

Bovine mastitis is a disease with enormous economic implications. *Staphylococcus aureus* is one of the most common causative agents of bovine mastitis responsible for up to 40% of all mastitis cases in some geographical areas (Tenhagen et al. 2006). Staphylococcal mastitis causes both subclinical and clinical intrammamary infections (IMI) which may persist through successive lactations. Mastitis caused by *S. aureus* has a lower cure rate than for most other causes. This can be explained by any of several characteristics of this organism such as acquired antimicrobial resistance and intracellular presence avoiding antibiotic treatment (Barkema et al. 2006). Various molecular techniques have been used to study the epidemiology of *S. aureus* in mastitis including pulse field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) (Kadlec et al. 2015). PFGE is considered to be the 'gold standard' because of its discriminatory power and reproducibility. Although *S. aureus* is the most prevalent cause of bovine intramammary infection in Argentina (Neder et al. 2011), no large scale molecular studies on *S. aureus* isolates from dairy farms have been conducted. To our knowledge, there are no published reports regarding the distribution of bovine *S. aureus* strains in Argentina as determined by molecular methods such as PFGE and MLST. This study was conducted to determine the occurrence and distribution of *S. aureus* and MRSA strains from mastitic cows from various herds in Argentina and to characterise these isolates with particular reference to their genotypic and antibiotic resistance patterns.

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Materials and methods

Origin of the isolates

A total of 829 milk samples were obtained from milk samples of cows with subclinical or clinical mastitis. These samples were collected by veterinarians from 21 farms in four different provinces and scattered over 16 cities (Fig. S1). Two hundred and twenty nine *S. aureus* isolates were recovered. These isolates were characterised based on their antibiotic susceptibility patterns and 80 were randomly selected for further characterisation.

Biochemical and molecular identification

Milk samples were collected aseptically from quarters of infected cows and were initially screened using the California Mastitis Test. Positive samples were transported to the laboratory on ice for further processing. Isolation of *S. aureus* was performed using standard procedures. Briefly, 10 µl from each milk sample was spread on a Columbia Blood Agar plate and incubated at 37 °C for 24 h. Presumptive *S. aureus* colonies were identified by standard microbiological tests which included Gram-staining, catalase and coagulase reactions, the oxidation and fermentation of mannitol, maltose and trehalose, and Voges-Proskauer. Isolates were also subjected to a specific PCR that targets the thermonuclease (*nuc*) gene specific for *S. aureus* (Table S1). Briefly, *S. aureus* cells from a broth culture were pelleted and treated with lysostaphin to release genomic DNA. A 257 bp product was obtained for the *nuc* gene following PCR amplication with the primers 5′ GCGATTGATGGTGATACGGTT 3′ and 5′ AGCCAAGCCTTG ACGAACTAA AGC 3′ located within the *nuc* gene (Brakstad et al. 1992). A total of 35 PCR cycles were run following DNA denaturation at 94 °C for 30 s, primer annealing at 57 °C for 30 s and DNA extension at 72 °C for 1 min.

PFGE

Strain relatedness was analysed by PFGE of total DNA restricted with the Cfr9I enzyme (Fermentas Life Sciences, Burlington, ON, Canada) using the protocol of Mulvey et al. (2001) with minor modifications. First, *Cfr9I* was used instead of *SmaI* because we initially thought that ST398 isolates would be present among the isolates and this ST is insensitive to *Sma*I digestion owing to the methylation of the *Sma*I site. Secondly, an incubation time of 4 h at 37 °C was used instead of 2 h at 37 °C. Electrophoresis was performed with CHEF DR-III apparatus (Biorad) using switch times of 5·3 to 34·9 for 18 h at 6·0 V/cm and 14 °C in TBE 0·5X. The gel was stained in 0·5 mg/l of ethidium bromide and photographed with a UV transilluminator. The band patterns were analysed using the Bionumerics software version 6.6 (Applied Maths, Belgium). Dendograms were generated from similarity matrixes calculated with the Dice coefficient, and patterns were clustered by the unweighted-pair group method with arithmetic averages using an optimisation and tolerance of 1·5%. The definition of a PFGE cluster was based on a similarity cut-off of 80% which corresponds to the Tenover et al. (1995) criterion of four to six bands differences between related isolates.

Antibiotic susceptibility tests

Susceptibility tests were performed by the agar diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2013). The antibiotics tested were penicillin (10 µg, ≤28 mm), oxacillin, (1 µg, ≤10 mm), cefoxitin (30 µg, ≤21 mm), erythromycin (15 µg, ≤13 mm), and clindamycin (2 µg, ≤14 mm). These antibiotics belong to the β-lactam, macrolide and lincosamide classes, those most commonly used in Argentina to treat subclinical and clinical mastitis, and for dry cows. All antibiotic discs were purchased from Laboratorio Britania, Buenos Aires, Argentina.

Detection of antimicrobial resistance genes

Antibiotic resistance genes were detected by PCR (See online Supplementary Table S1 for the PCR conditions and primers used).

Detection of Panton-Valentine leucocidin genes

The presence of the *pvl* gene was determined by PCR (See online Supplementary Table S1).

MLST

MLST was performed as described by Enright et al. (2000). Briefly, the seven housekeeping genes *arc*C, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, and *yqi*L were amplified by PCR using the Platinum Taq DNA polymerase High Fidelity (Invitrogen). The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and used as templates for sequencing of both strands at IRIC, Montréal, Canada. Sequence types (STs) were assigned by comparison to the *S. aureus* online database (http://www.mlst.net) using Bionumerics software version 6.6 (Applied Maths, Belgium).

Plasmid DNA extraction

Plasmid DNA was extracted from early stationary phase cells in prewarm TSB at 37 °C by alkaline lysis using the plasmid midi-Kit (Qiagen) with minor modifications. Buffer P1 was supplemented with 100 µl lysostaphin 0·5 mg/ml and the cell suspension was incubated for 1 h at 37 °C. Subsequent isolation steps were then followed according to the manufacturers recommendations.

Southernblot hybridisation analysis

Plasmid DNA was digested with 50 U of Hind III (New England Biolabs) for 1 h at 37 °C and migrated for 3 h at

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45 V in 0·8% agarose. After migration, the digested plasmids were transferred to positively charged nylon membranes (Roche Diagnostics, Mannheim, Germany) using a vacuum blotter model 785 (Bio-Rad). The membranes were probed with digoxigenin labelled PCR products for the genes *erm*A, *erm*B, *erm*C, *lnu*A, *mef*A and *msr*A genes using the PCR DIG probe synthesis kit (Roche Diagnostics).

Results and discussion

Resistance to the beta lactam penicillin, was detected in 53 (23·1%) isolates, similar to penicillin resistance of 16% reported in the USA (Haran et al. 2012). European studies have reported a more heterogeneous profile with resistance rates ranging from 2 to 70% (Vintov et al. 2003). For macrolides, resistance to erythromycin (13·5%) and clindamycin (12·2%) was also detected. These resistance rates are somewhat similar to findings by Kumar et al. (2010) who reported resistance rates of 20·9 and 12·2% for erythromycin and clindamycin, respectively (Kumar et al. 2010). However, our results are in contrast to Wang et al. (2008) who reported rates of 93·1% for erythromycin and 36·1% for clindamycin. This variations could be explained by the different management strategies and antimicrobial use patterns for mastitis treatment. In Argentina for example, in addition to beta-lactams, macrolides are frequently used for mastitis treatment. The PVL gene was not detected in any of the isolates. None of the isolates were identified as MRSA, tested by cefoxitin disk diffusion and PCR detection of *mecA* or *mecC*. The molecular and genotypic profiles of these isolates are reported in Table 1 and Fig. 1. Forty-three of these isolates (53·7%) were susceptible to at least one antimicrobial. Multidrug resistance, defined as intermediate or complete resistance to three or more classes of antimicrobials, was observed in 2 (2%) of these isolates.

PFGE revealed a high rate of genetic diversity within these isolates. Twelve pulsotypes occurred on more than one farm with pulsotypes A, N, P, T and U most widely distributed. A majority of the isolates (38·7%) originated from two farms and these farms shared six pulsotypes with two pulsotypes (S and U) common to both farms. These two farms are located in different provinces. Interestingly, clonal spread of *S. aureus* mastitis isolates has been previously described at both the farm and regional level (Fessler et al. 2010). MLST of a representative isolate from each subpulsotype (for example an isolate was taken from U1, U2, U3, etc) within the dominant pulsotypes assigned them to ST 97, 705, 746, 2102 and 2187. Pulsotypes U and N belonged to ST97 and represented 31·2% of the total isolates, making this ST the most dominant. In addition, pulsotype P (ST746) and pulsotype S (ST746) were also found on more than one farm. The ST 97, 746 and 2187 were 48·7% of the typed isolates and these ST are part of the clonal complex 97 (CC97), a dominant lineage found in mastitis cases worldwide (Wang et al. 2015). This is in agreement with previous findings that few specialised clones with broad geographic distribution are responsible for most mastitis cases (Smith et al. 2005). The ST 705 and 2187 have been reported in Japan (Hata et al. 2010) and Canada (El Haddad et al. 2014) respectively. Pulsotype V belonged to ST2102 and this pulsotype was found in one farm. This ST has been reported in a Spanish hospital (Torres-Sangiao et al. 2012); it belongs to the CC30, a dominant lineage in community and hospital associated *S. aureus* infections. It is reasonable to hypothesise that the detection of this ST was due to human contamination as we did not find any reports in the literature on the involvement of this ST in bovine mastitis.

In this study, PFGE was more discriminatory than MLST. Although pulsotypes with similarities of 80% and above belonged to the same ST, this ST was also shared among other pulsotypes with no similarity. For example, pulsotype U and N belonged to ST97, pulsotype P and T belonged to ST746 and pulsotype A and B belonged to ST705 (Fig. 1). Although a PFGE cut-off of 90% or more would have affected the distribution of pulsotypes among farms leading to fewer strains being shared among the farms, we believe that the 80% cut-off represents the true genetic relatedness among the isolates. In fact, most of the isolates within each pulsotype differed by six bands or fewer in agreement with the criterion of Tenover et al. (1995) for 1 to six band differences for interpreting strain relatedness. For example, a cutoff of 90% or higher would have separated subpulsotypes A2 and A3 from A1 even though they differed by two bands only.

Antimicrobial susceptibility testing revealed that there was no uniformity in susceptibility patterns within pulsotypes. Forty-three of the selected isolates (53·7%) were susceptible to all the antibiotics tested, similar to the susceptibility rates of 58% reported from the USA (Haran et al. 2012).

The *erm*(C) gene was located on plasmid bands in three isolates. The *erm* genes were detected alone or in different combinations (Table 1) in agreement with earlier reports that detected these genes in mastitic isolates (Wang et al. 2008). The *bla*Z gene encoding resistance to beta-lactams was detected in 32 (40%) of the 80 isolates. We found that among isolates carrying the *bla*Z gene, only 2 (6·2%) were susceptible to penicillin.

A majority of the resistance isolates were in pulsotypes that were found in more than one farm suggesting that resistance was pulsotype linked. In fact, the largest pulsotype in this study, pulsotype U was susceptible to penicillin, an indication that penicillin resistance was pulsotype dependent. Interestingly, a link between penicillin susceptibility and pulsotypes has been reported (Bagcigil et al. 2012). A multi-resistance phenotype was observed in 2 (2·5%) isolates.

In summary, our data demonstrate that *S. aureus* isolates from mastitis cases in Argentina are genetically diverse and widely distributed across multiple farms consistent

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Table 1. Characterisation of the 80 randomly selected *S. aureus* isolates.

			Antibiotic susceptibility pattern					Resistance gene profile							
PFGE type	MLST ST	Isolate	PEN	OXA	FOX[†]	ERY	CLI	*blaZ*	*mecA/C*	*ermA*	*ermB*	*ermC*	*lnuA*	*mefA*	*msrA*
A1	705	V124	R	S		R	S	+	–	–	–	–	–	+	–
A1		V263	S	S		R	R	–	–	–	–	–	–	+	–
A2		V250.1	S	S		S	S	–	–	–	–	–	–	–	–
A3		F103am	S	S		S	S	–	–	–	–	–	–	–	–
B1		MBV212	R	S		R	S	+	–	–	–	–	–	–	–
B1		MBV253	S	S		S	S	–	–	–	–	–	–	–	–
B2	705	MBV123	R	S		R	S	+	–	–	–	–	–	–	–
B2		MBV257.1	S	S		S	S	+	–	–	–	–	–	–	–
C		2072	S	S		S	S	–	–	–	–	–	–	–	–
D		MBE3	S	R	S	S	S	+	–	–	–	–	–	–	–
E		MBV25	S	S		S	S	–	–	–	–	–	–	–	–
F1		2015B	S	S		S	S	+	–	–	–	–	–	–	–
F2		MB038	S	S		S	S	–	–	–	–	–	–	–	–
G1		127Nbca	S	S		S	S	–	–	–	–	–	–	–	–
G2		71N	S	S		S	S	–	–	–	–	–	–	–	–
H		MBV163	R	S		S	S	+	–	–	–	–	–	–	–
I1		K46/8	R	R	S	S	S	+	–	–	–	–	–	–	–
I2		MBV133	R	S		R	S	+	–	–	–	–	–	–	–
J1		K43.1	R	S		S	S	+	–	–	–	–	–	–	–
J2		MBV204	R	S		S	S	+	–	–	–	–	–	–	–
K1		MBV78	R	S		S	S	+	–	–	–	–	–	–	–
K2		MBV80	R	S		S	S	+	–	–	–	–	–	–	–
K3		A11	R	S		S	S	+	–	–	–	–	–	–	–
L		V265	S	S		S	S	–	–	–	–	–	–	–	–
M1		MBV167.3	R	S		S	S	+	–	–	–	–	–	–	–
M2		MBV30	S	S		S	S	–	–	–	–	–	–	–	–
N1		K48/1	R	S		S	S	+	–	–	–	–	–	–	–
N2		MBV296.1	R	S		R	R	+	–	–	+	+	+	–	–
N3	97	F161.3	R	S		S	S	+	–	–	–	–	–	–	–
N4		D20	R	S		S	S	+	–	–	–	–	–	–	–
N5		MBV147	S	S		S	S	–	–	–	–	–	–	–	–
O		MBV264	R	S		S	S	+	–	–	–	–	–	–	–
P1		F85.2	S	S		S	S	–	–	–	–	–	–	–	–
P2		T4	R	S		S	R	+	–	–	–	–	–	+	–
P3		F109bca	S	S		S	S	–	–	–	–	–	–	–	–
P4		MB034	S	S		S	S	–	–	–	–	–	–	–	–
P5		MBV036	S	S		S	S	–	–	–	–	–	–	–	–
P6	746	F67.1	S	S		S	S	–	–	–	–	–	–	–	–
P7		MBV046	S	S		S	S	–	–	–	–	–	–	–	–
P8		F100.1	S	S		S	S	–	–	–	–	–	–	–	–
P9		MB044	S	S		S	S	–	–	–	–	–	–	–	–
Q		V1	S	S		S	S	–	–	–	–	–	–	–	–

Table 1. (*Cont.*)

			Antibiotic susceptibility pattern					Resistance gene profile							
PFGE type	MLST ST	Isolate	PEN	OXA	FOX†	ERY	CLI	*blaZ*	*mecA/C*	*ermA*	*ermB*	*ermC*	*lnuA*	*mefA*	*msrA*
R		F100	S	S		S	S	–	–	–	–	–	–	–	–
S1		MBV131	S	S		S	S	–	–	–	–	–	–	–	–
S2		MBV5	S	S		R	R	–	–	–	–	–	–	+	–
S3	2187	119N	S	S		S	S	–	–	–	–	–	–	–	–
S3		179N	S	S		S	S	–	–	–	–	–	–	–	–
S4		125N	S	S		S	S	–	–	–	–	–	–	–	–
T1		F151.2	R	S		R	R	+	–	–	–	–	–	+	–
T2		MBV043	S	S		S	S	–	–	–	–	–	–	–	–
T3		K44	R	S		S	S	+	–	–	–	–	–	–	–
T3	746	K47	R	S		S	R	+	–	–	–	–	–	–	–
T3		K48	R	S		S	S	+	–	–	–	–	–	–	–
T4	746	A10	R	R		S	S	+	–	–	–	–	–	–	–
T4		A12	R	S		S	S	+	–	–	–	–	–	–	–
T4		A15	R	S		S	S	+	–	–	–	–	–	–	–
U1		266N	S	S		S	S	–	–	–	–	–	–	–	–
U1		278N	S	S		S	S	–	–	–	–	–	–	–	–
U1		285N	S	S		S	S	–	–	–	–	–	–	–	–
U1		92N	S	S		S	S	–	–	–	–	–	–	–	–
U2		MBV114	S	S		S	S	–	–	–	–	–	–	–	–
U3		126Nbca	S	S		S	R	–	–	–	–	–	–	–	–
U4		108N	S	S		R	R	–	–	+	–	–	–	–	–
U4		82Ncrema	S	S		R	R	–	–	+	+	+	–	+	–
U4		89N	S	S		S	S	–	–	–	–	–	–	–	–
U5		102N	S	S		S	S	–	–	–	–	–	–	–	–
U5		112N	S	S		S	S	–	–	–	–	–	–	–	–
U5		117N	S	S		S	S	–	–	–	–	–	–	–	–
U5		121N	S	S		S	S	–	–	–	–	–	–	–	–
U5		122N	S	S		S	R	–	–	–	+	–	+	+	+
U5		124N	S	S		S	S	–	–	–	–	–	–	–	–
U5		137N	S	S		S	S	–	–	–	–	–	–	–	–
U5		148N	S	S		S	S	–	–	–	–	–	–	–	–
U5	97	26N	S	S		R	R	–	–	–	+	–	–	+	–
U5		60Ncrema	S	S		R	R	–	–	–	+	+	–	+	–
U5		94N	S	S		S	S	–	–	–	–	–	–	–	–
V1	2102	MBV118	R	S		S	S	+	–	–	–	–	–	–	–
V1		MBV119	R	S		S	S	+	–	–	–	–	–	–	–
V1		MBV136	R	S		S	S	+	–	–	–	–	–	–	–
W		MB037	S	S		S	S	–	–	–	–	–	–	–	–

†Used only on oxacillin resistant isolates to screen for MRSA PEN, penincillin; OXA, oxacillin; FOX, cefoxitin; ERY, erythromycin; CLI, clindamycin; R, resistant; S, susceptible; +, presence of the gene; –, absence of the gene.

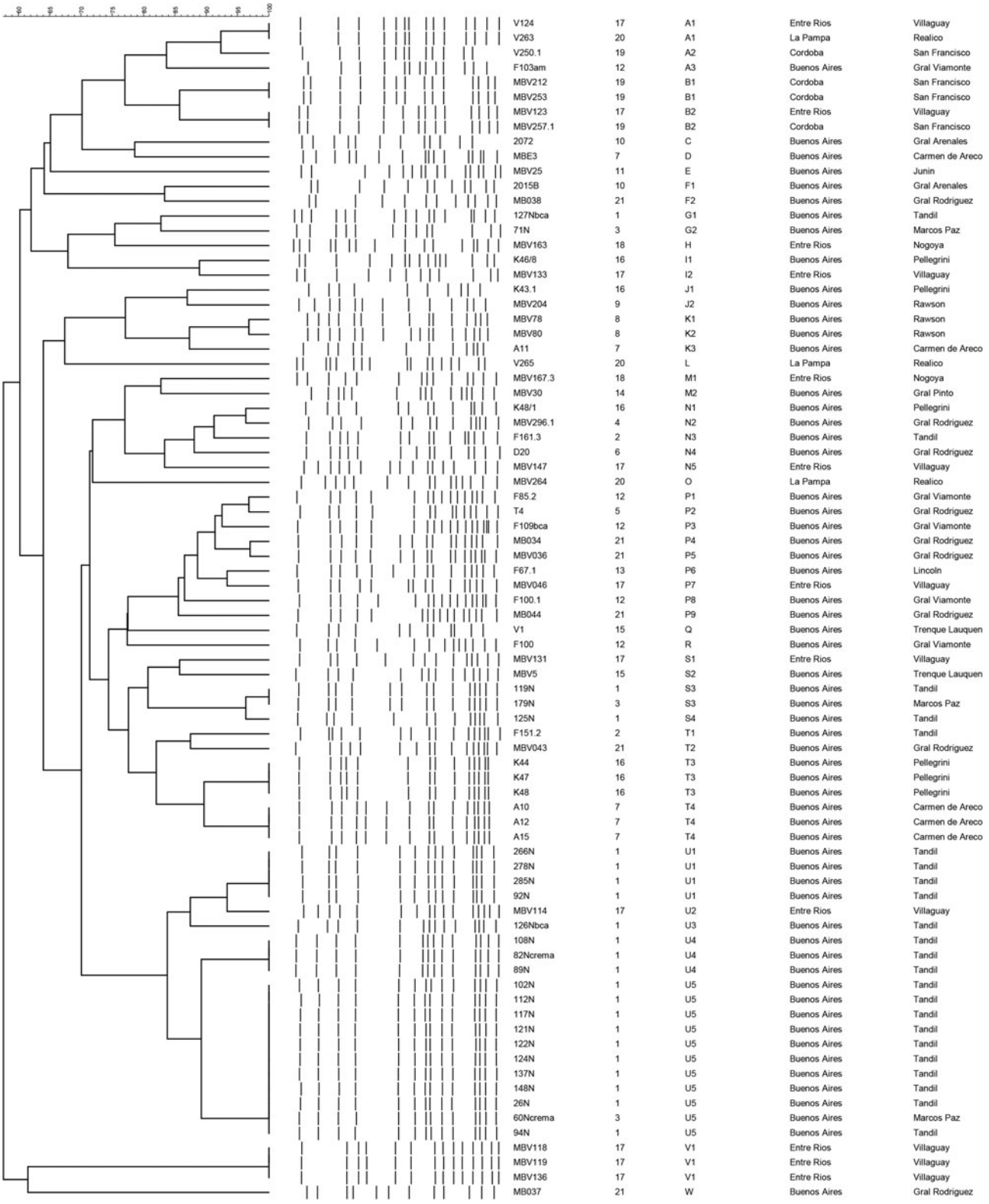

Fig. 1. Dendogram of PFGE patterns showing the relatedness of the 80 *S. aureus* isolates. The cluster cutoff was set at 80% similarity.

with findings from China (Li et al. 2009). Most of the resistance isolates were associated with pulsotypes present on more than one farm. However, our results contrast with the findings of Schmidt et al. (2017) who reported a low genetic diversity of *S. aureus* isolates among herds. None of the isolates in this study were methicillin resistance.

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Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029917000851.

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