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Genetic diversity of *Streptococcus agalactiae* from dairy cattle with mastitis in Argentina

Gabriela Gerez[1†], Luciana Belén Hernandez[1†], Jimena Cadona[1], Andrea Mariel Sanso[1] and Ana Victoria Bustamante[1*]

Abstract

Background Bovine mastitis is an important health problem in dairy cattle which affects the quality and yield of milk and causes significant economic losses in the dairy industry. *Streptococcus agalactiae* is a Gram-positive and zoonotic bacterium that causes clinical and subclinical contagious bovine mastitis. The main strategy for the control of this pathogen in dairy herds is the antimicrobial therapy. The aim of this study was to determine the genetic diversity of *S. agalactiae* using Multiple Locus Variable number tandem repeat -VNTR- Analysis (MLVA), serotypes, virulence factors (VF) and antimicrobial resistance (AMR) profiles and to compare the discrimination power of these different methods in strains isolated from cattle with mastitis in Argentinian dairy farms.

Results Eighty-seven *S. agalactiae* isolates obtained from dairy cattle with mastitis in Argentina were analyzed. The detected serotypes were III, II and Ia. The most frequent virulence and AMR detected genes were *cpsA, hylB*, *PI-2b*, *cylE*, *rib, spb1*, and *tetO* and *ermB* respectively. A total of 36 VF + AMR profiles were detected with a discriminatory power of the method of Ds = 0.96. The MLVA based on six VNTRs showed 29 profiles with a Ds = 0.90. The analysis of VF + AMR + MLVA data together showed 59 profiles with an increased discriminatory power (Ds = 0.98).

Conclusion This study highlights that the MLVA is recommended to add to other methodologies in order to study epidemiological relationships in this species Although within each dairy farm there was a predominance of certain serotypes/virulence profiles, the characteristics did not show total homogeneity, as expected due to the contagious nature of the pathogen. This suggests the incorporation of animals from other herds at some point, a practice not uncommon among dairy farms in Argentina. By other hand, the detection of a same clone in the same farm in different periods confirms that *S. agalactiae* strains can persist on dairy farms for a long time.

Keywords *Streptococcus agalactiae*, Mastitis, Dairy cattle, Genetic diversity, MLVA

Background

Bovine mastitis is an important health problem in dairy cattle which affects the quality and yield of milk [1]. Some of the major etiological species belong to the genus *Streptococcus*, where *S. agalactiae* and *S. uberis*, can be distinguished as contagious and environmental, respectively. Another pathogen related to mastitis of environmental origin is *Escherichia coli* [2].

S. agalactiae, a Gram-positive bacterium, causes clinical and subclinical contagious bovine mastitis. It can survive inside the mammary gland for a long time and, at present, it has been demonstrated that the bacteria can

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survive in extramammary sources [3]. Also, there is evidence that suggests interspecific transmission from cattle to humans and vice-versa and, therefore, it is considered a zoonotic pathogen [4].

The main strategy for the control of *S. agalactiae* in dairy herds infections is the antimicrobial therapy. Penicillin is the first option used for the prevention and treatment of *S. agalactiae* infections, however reports of reduced susceptibility to penicillin have been published [5, 6]. Alternative antibiotics such as macrolides and lincosamides are used, but increased resistance to them has been documented worldwide [7]. Also, isolates with reduced penicillin susceptibility, tend to be resistant to other antibiotics such as fluoroquinolones and macrolides, and exhibit multidrug-resistance [8]. Antimicrobial resistance is an area of concern in both human and veterinary medicine [9]. Strain characterization and surveillance are important to obtain information that allows evaluating the level and evolution of antimicrobial resistance [10]. Some of the antimicrobial resistance genes detected in *S. agalactiae* are *erm, lnuB, tet, and aphA3/aad6,* involved in resistance to macrolides, lincosamides, tetracyclines and aminoglycosides [11, 12].

Virulence factors contribute to the pathogenesis infection of *S. agalactiae*, allowing the colonization and invasion of epithelial barriers, immune system evasion and persistence in host tissues [13]. Among the virulence factors that have been detected in bovine isolates are the capsular polysaccharide, encoded by c*psA*, cell surface proteins mediating adherence and invasion such as C5a peptidase, encoded by *scpB*, α-C protein (*bca*), β-C protein (*bac)*, Rib (*rib*) and laminin binding protein (*lmb)* [14]. Extracellular toxins and enzymes capable of forming pores have been described, such as that encoded by the *cylE* gene [15–18]. The product of gene *hylB*, a secreted hyaluronate lyase, can hydrolyze hyaluronan polymers, suggesting that this enzyme can facilitate the spread of bacteria during infection [15]. Other surface structures, such as *pili,* are also crucial virulence factors that promote the adherence and attachment of *S. agalactiae* to host cells. *Pili* are encoded by two loci in different regions of the genome designated *PI-1* and *PI-2*, with the latter presenting two distinct variants, *PI-2a* and *PI-2b* [19].

CPS is the primary virulence determinant which confers anti-phagocytic properties and plays a pivotal role in evading host defense mechanisms [20]. Differences in CPS allow to distinguish *S. agalactiae* into 10 different serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX) [21–23], but this classification does not have the enough differentiating power to discriminate between isolates [24]. Genetic diversity and clonal relatedness of *S. agalactiae* strains from human and animal infections have been previously demonstrated by pulsed-field gel electrophoresis (PFGE) [25]. Other efficient subtyping methods like Multiple Locus Variable number tandem repeat-VNTR- Analysis (MLVA) are faster and with greater resolution power [24, 26, 27], and it has been extensively used for genotyping isolates of various bacterial species [28]. The MLVA identifies a variable number of tandem repeat (VNTR) in several loci. The VNTRs loci are dispersed throughout the bacterial genomes and they comprise short nucleotide sequences, called Repeat Units (RU) which can differ in the number of copies inside the tandem at each locus.

The objective of this study was to determine the genetic diversity of *S. agalactiae* strains isolated from dairy cattle with mastitis in Argentina using different methods: MLVA, data on serotype, virulence and antimicrobial resistance profile, in combination or separately to compare their discrimination power.

Results

Eighty-seven *S. agalactiae* isolates obtained from dairy farms located in one of the largest milk-producing regions of Argentina, were molecularly characterized. The isolates belonged to serotypes III (63.2%), II (26.4%), and Ia (5.8%), while the remaining 4.6% were not typeable (NT). The most frequently detected virulence genes were *cpsA* and *hylB* (100% of the isolates), followed for the *pilus* gene *PI-2b* (93%) and *cylE* (89%). *PI-2b* was absent only in six isolates (B69, B81; B86, B89; B95; B97) meanwhile the genes *bac*, *lmb, scpB*, *hvgA* and the *pili PI-1 / PI-2a* were not detected in any isolates. In relation to antimicrobial resistance genes*, tetO* and *ermB* were the most frequently detected genes (64% and 54%, respectively) meanwhile *tetM* and *lnuB*, the less frequent ones (Table 1).

Taking into account the virulence factors (VF) and antimicrobial resistance (AMR) genes, 36 genetic profiles were identified, with 18 clusters grouping two to eleven isolates and 18 singletons (Fig. 1). The Simpson´s diversity indices for the VF and VF + AMR analyses were $D_S = 0.85$ and $D_S = 0.96$, respectively.

The MLVA genotype was expressed as an allelic string profile: SAG2, SAG3, SAG4, SAG7, SAG21, SAG22. The number of alleles detected per locus varied between 2 (SAG21) and 5 (SAG4), with the SAG4 and SAG22 markers showing the highest Nei´s diversity indices ($D_N = 0.74$ and 0.73, respectively) (Table 2; Fig. 2). The UPGMA clustering MLVA analysis revealed 29 different genotypes, which were distributed in 14 clusters with two to 22 isolates and 15 singletons (Fig. 3). The Simpson´s diversity index for the MLVA was $D_S = 0.90$. Dairy farm A, represented with 28 isolates, collected between 2017 and 2018, showed the greatest diversity of MLVA profiles (11) followed by farm B, with 7 MLVA profiles which

Table 1 Virulence and antimicrobial resistance genes frequencies in Argentinian bovine *S. agalactiae* isolates. N: number of isolates

Dairy farms (N)	Virulence factor genes (N)							Antimicrobial resistance genes (N)					
	bca	*rib*	*spb1*	*hylB*	*cylE*	*PI-2b*	*cpsA*	*ermB*	*tetM*	*tetO*	*lnuB*	*aad6*	*aphA3*
Farm A (*N* = 28)	0,071 (2)	0,89 (25)	1 (28)	1 (28)	1 (28)	1 (28)	1 (28)	0,25 (7)	0	0,5 (14)	0,036 (1)	0	0,25 (7)
Farm B (*N* = 11)	0,09 (1)	0,27 (3)	1 (11)	1 (11)	1 (11)	1 (11)	1 (11)	1 (11)	0	1 (11)	0,18 (2)	0	0,27 (3)
Farm C (*N* = 2)	0,5 (1)	0	0,5 (1)	1 (2)	1 (2)	0,5 (1)	1 (2)	0	0,5 (1)	0,5 (1)	0	0	0,5 (1)
Farm D (*N* = 7)	1 (7)	0	1 (7)	1 (7)	1 (7)	1 (7)	1 (7)	0	0,143 (1)	0,143 (1)	0	0	0,143 (1)
Farm E (*N* = 3)	1 (3)	0	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)	0	0	0	0	0	0
Farm G (*N* = 5)	1 (5)	1 (5)	1 (5)	1 (5)	1 (5)	1 (5)	1 (5)	0	0,4 (2)	0	0	0	1 (5)
Farm H (*N* = 1)	1 (1)	0	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	0	0	0	0	0	0
Farm I (*N* = 2)	0,5 (1)	1 (2)	0,5 (2)	1 (2)	1 (2)	1 (2)	1 (2)	1 (2)	0	1 (2)	0	0	0,5 (1)
Farm J (*N* = 28)	0,18 (5)	0,96 (27)	0,036 (1)	1 (28)	0,64 (18)	0,82 (23)	1 (28)	0,96 (27)	0,11 (3)	0,96 (27)	0	0,75 (21)	0,43 (12)
Total (*N* = 87)	0,3 (26)	0,71 (62)	0,67 (58)	1 (87)	0,88 (77)	0,93 (81)	1 (87)	0,54 (47)	0,08 (7)	0,64 (56)	0,03 (3)	0,24 (21)	0,34 (30)

grouped 11 isolates collected during 2016–2018. Seven MLVA profiles were detected in isolates from different dairy farms. On the other hand, two profiles grouped isolates from the same farm (J) with different virulence profiles (Fig. 3).

Table 3 shows the discrimination power of the different methods applied to study genetic diversity in *S. agalactiae* isolates. The greatest power of discrimination is achieved by analyzing all the characters together ($D_S = 0.98$).

Table 4 summarizes the genetic diversity of each dairy farm evidenced by their VF, AMR and MLVA profiles. Taking into account genetic information of VF, AMR and MLVA data, 59 genetic profiles were detected. The dendrogram presented two principal branches, one of which was integrated only by strains from dairy farm J, of which all, with the exception of one, harbored the aminoglycoside resistance gene *aad6* (Fig. 4).

For the correlation analysis between MLVA profiles and virulence/ resistance genes, the correlations between the MLVA A (2, 2, 0, 2, 3, 4), MLVA B (2, 1, 1, 0, 2, 3), and MLVA C (1, 2, 2, 2, 2, 2) and the presence/ absence of *bca, rib, spb1, cylE, PI-2b, ermB, tetM, tetO, lnuB, aad6, aphA3* were analyzed. MLVA A exhibited statistically significant correlations with *aad6* ($r = 0.95$, $p < 0.0001$), a moderate positive correlation with *ermB* ($r = 0.56$, $p = 0.00012$), *rib* ($r = 0.47$, $p = 0.0017$), and *tet O* ($r = 0.39$, $p = 0.011$) and a strong negative correlation with *spb1* ($r = -1.00$, $p < 0.0001$). MLVA B was significantly correlated with *spb1* ($r = 0.66$, $p = 0.0000017$) and *aad6* ($r = -0.63$, $p = 0.000007$), meanwhile a negative correlation was observed with *ermB* ($r = -0.88$, $p < 0.0001$) and *tetO* ($r = -0.63$, $p = 0.0000069$). MLVA C showed positive significant correlations with *spb1* ($r = 0.51$, $p = 0.00058$), and *ermB* ($r = 0.31$, $p = 0.048$) and a strong negative correlation with *rib* ($r = -0.92$, $p < 0.0001$).

Also, a correlation analysis was conducted between dairy farms A, B, D, G, J and genetic markers of virulence and resistance. Dairy farm A exhibited a positive correlation with *spb1* ($r = 0.52$, $p < 0.0001$) and a negative correlation with *ermB* ($r = -0.51$, $p < 0.0001$). Additionally, significant negative correlations were found with *bca, tetO,* and *aad6*, and positive correlation, with *cylE*. In relation to dairy farm B, a negative correlation was found with *rib* ($r = -0.42$, $p < 0.0001$). In addition, weak positive correlations were observed with *lnuB, tetO, ermB* and *spb1.* Dairy farm D showed a negative correlation with *rib* ($r = -0.55$, $p < 0.0001$), and a positive correlation with *bca* ($r = 0.54$, $p < 0.0001$), meanwhile, dairy farm G showed moderate positive correlation with *bca* ($r = 0.45$, $p < 0.0001$), *tetM* ($r = 0.32$, $p < 0.01$), and *aphA3* ($r = 0.35$, $p < 0.01$). Finally, dairy farm J was the one that showed outstanding correlations. It was strongly and negatively correlated with *spb1* ($r = -0.97$, $p < 0.0001$) and positively, with *aad6* ($r = 0{,}81$, $p < 0.0001$). In addition, significant negative correlation was detected with *cylE* ($r = -0.51$, $p < 0.0001$) and positive, with *ermB* ($r = 0.59$) and, *tetO* ($r = 0.46$).

Discussion

In this study, we described the genetic diversity in relation to virulence, antimicrobial resistance and multi locus VNTR analysis (MLVA) of *S. agalactiae* recovered from cows with mastitis in Argentina. MLVA, a typing method based on tandem repeat polymorphisms at multiple loci, has been successfully applied to many other bacterial species and, recently, to study human isolates of this species [7, 30]. We investigated the relevance of this tool for genotyping bovine isolates of *S. agalactiae* and compared it with methods that use other genetic characters.

An important genetic diversity among the bovine isolates of *S. agalactiae* obtained from different dairy farms

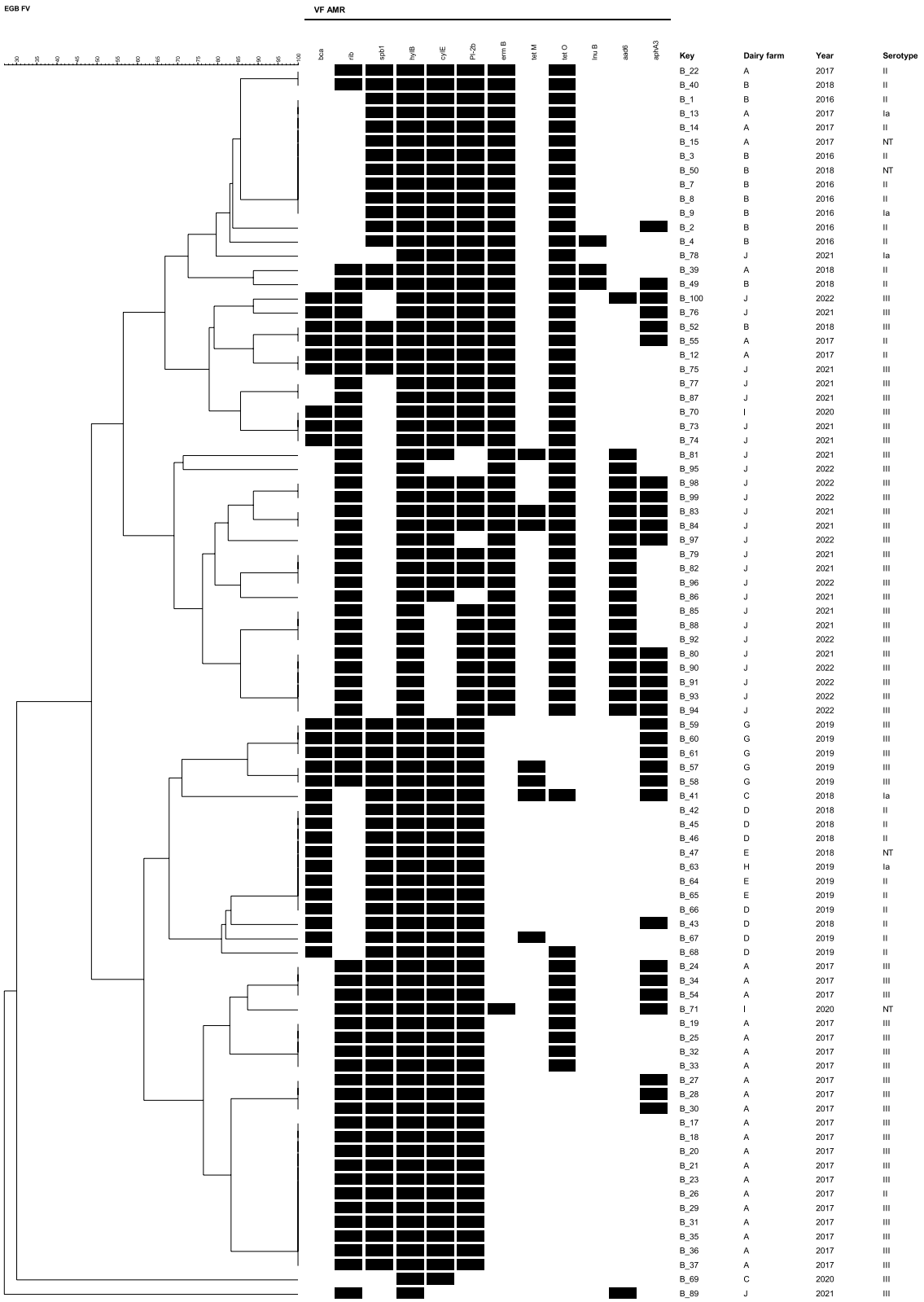

Fig. 1 Cluster analysis of *S. agalactiae* isolated from dairy cattle with mastitis in Argentina based on virulence and AMR profiles. The presence (black) or absence (white) of genes, the isolate name, dairy farm, isolation year, and serotype of the isolates are shown. NT: non-typeable. Genes not found in any of the studied isolates: *bac, lmb, hvgA, PI-1, PI-2a*, and *scpB*

Table 2 VNTR *loci* characterization

VNTR loci	Repeat Unit (RU) size (bp)	Number of observed alleles[a]	D_N[b]
SAG2	32	3	0.29
SAG3	24	3	0.53
SAG4	60	5	0.74
SAG7	18	3	0.49
SAG21	48	2	0.46
SAG22	159	5	0.73

[a] null alleles were taken into account

[b] Nei´s diversity index, calculated by $D_N = 1-\Sigma(fra)^2$, where fra is the allelic frequency)

in Argentina, as well as the presence of different clones within one of the dairy farms included in this study, were detected by MLVA. We could identify 29 distinct MLVA profiles with a Simpson´s diversity index (D_S) of 0.90 among the 87 bovine mastitis isolates analysed. Other authors, who applied MLVA to study human *S. agalactiae* strains, obtained D_S values from 0.84 to 0.88 [13, 27, 30]. Interestingly, Haguenoer et al. [24] calculated a D_S value of 0.96 in an analysis that included human and bovine strains. These data could be indicating that bovine isolates are more diverse in relation to VNTR loci than human ones. On the other hand, several virulence and antimicrobial susceptibility profiles associated with *S. agalactiae* intramammary infections were detected. According to our results and comparing them with previous works in which MLVA was applied [7, 27, 30], it can state that this methodology shows a satisfactory discriminatory power in order to genotype *S. agalactiae* isolates. Most shared MLVA profiles were presented by isolates from the same dairy farm, with the exception of isolates from farms A and B which shared profiles and one isolates from farm I which was included among farm J isolates. In the first case, it is known that there was an exchange of cattle between both dairy farms (A and B).

The complement of the MLVA data with data from genetic profiles of virulence and AMR increased the discrimination power (D_S: 0.98 versus 0.90). Our results show that, therefore, MLVA, is recommended to add to other methodologies in order to study epidemiological relationships between *S. agalactiae* strains. On the other hand, correlation analysis suggests that the presence or absence of specific genes could be related to different MLVA profiles and/or sources (dairy farms).

Not all farms in this study had a specific MLVA profile, in agreement with other authors, who also found the presence of profiles unique to some dairy farms, different profiles within the same farm, and profiles shared by isolates from farms located in different regions, obtained at different times [27]. Dairy farms in Argentina tend, at times, to produce movements with the cattle due to the sale/trade of milking cows. Transmission of *S. agalactiae* is therefore likely between farms. On farms with multiple samples (A, B, J), strains with identical or single locus variant profiles were found over a period of several years, indicating subclinical infection. On the other hand, several profiles were detected intra-farm supporting the occurrence of different clones (farms A, B, D, J).

The most prevalent serotypes detected among bovine *S. agalactiae* were III (63%) and II (26%), followed by Ia (6%). The most frequently detected virulence genes were

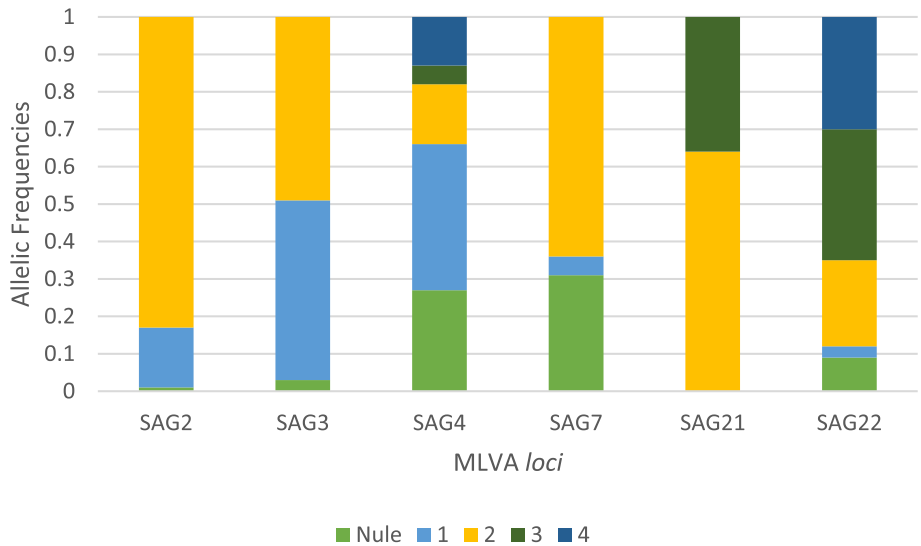

Fig. 2 VNTR allelic frequencies distibution *per locus* in the analysed Argentinian bovine *S. agalactiae* isolates. Alleles are indicated in colours: nule: green, 1: light blue, 2: yellow, 3: dark green, and 4: blue

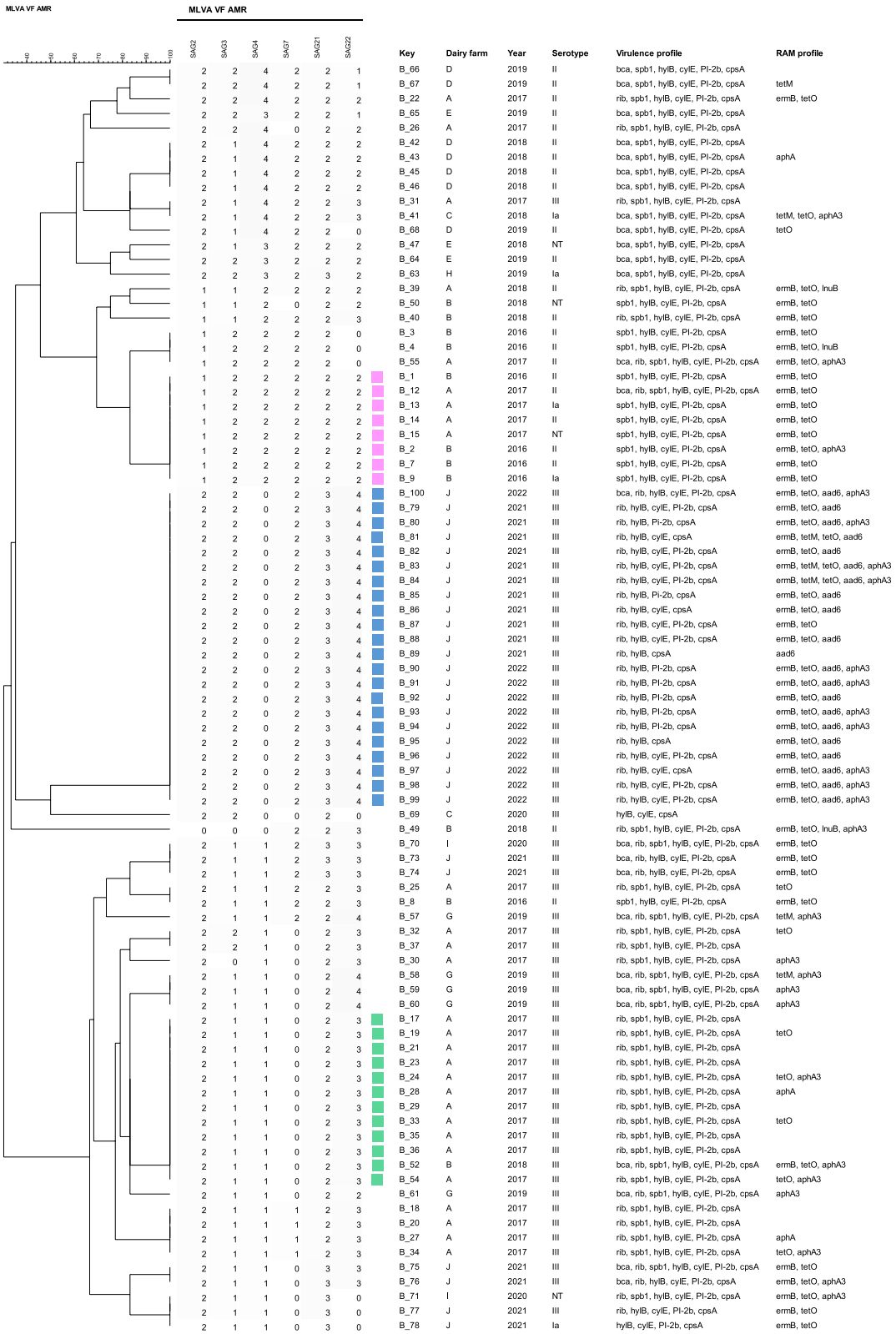

Fig. 3 Cluster analysis of *S. agalactiae* isolated from dairy cattle with mastitis in Argentina based on MLVA profiles. MLVA profiles which were included in correlation analysis are colored: MLVA A: blue, MLVA B: green, and MLVA C: purple

Table 3 Comparison of the discrimination power of the different methods applied to study Argentinian bovine *S. agalactiae* isolates

Method	Number of profiles	D_S[a]
VF profiles analysis	11	0.85
VF and AMR profiles analysis	36	0.96
MLVA	29	0.90
VF and AMR profiles analysis + MLVA	59	0.98

[a] Simpson´s diversity index, calculated according to Hunter and Gaston [29]

cpsA and *hlyB* (100% of the isolates), followed for the *pilus* gene *PI-2b* (93%) and *cylE* (89%). The *PI-2b* pilus variant is associated with bovine isolates [31] and, in this study was absent only in six isolates (B69, B81; B86, B89; B95; B97) meanwhile *cylE* which encodes a β-hemolysin which causes tissue damage and the systemic spread of the bacteria and is involved in the recruitment of cytotoxic and pro-inflammatory cytokines [17] was absent in ten isolates. Particularly, all the negative- *PI-2b- cylE* isolates were from the same dairy farm (J). On the other hand, genes *bac*, *lmb, scpB*, *hvgA,* and the *pili PI-1/ PI-2a* were not detected in any isolates. These results agree with those of other authors, who described some of these genes as typical of human isolates [32, 33]. However, in relation to *scpB*, more recently Parasana et al. [34] detected it in *S. agalactiae* milk isolates.

In Argentinian dairy farms, to treat mastitis via the intramammary route, the groups of antibiotics most used are beta-lactams, macrolides and aminoglycosides, while tetracyclines (together with sulphonamides and quinolones) are administered, mainly, systemically, to other infections [35, 36]. The presence of genetic determinants of AMR was heterogeneous among the nine dairy farms. Isolates from D and E farms present only one AMR gene of the six analyzed and, on the contrary, the isolates from farm J, up to five AMR genes were detected. Regarding the *ermB* gene, encoding cross-resistance between macrolides and lincosamides (erythromycin and clindamycin-pirlimycin), it was detected in more than 50% of the bovine isolates meanwhile *lnuB*, which confers resistance exclusively to lincosamides, only in three isolates (farms A and B). The high level of kanamycin resistance detected by our group in previous studies [37, 38] can be explained by the *aphA3* presence. This gene encodes a phosphotransferase that confers resistance to kanamycin and mediates synergism with beta-lactams [39]. On the other hand, a group of isolates from farm J harbored *aad6,* an aminoglycoside acetyltransferase encoding-gene, which also eliminates the synergism between cell wall–active antimicrobials and aminoglycosides [39]. Studies carried out in Brazil did not detect *aphA3* or *aad6* [40]. However, other ones carried out in France [41] described the presence of *aphA3* as responsible for high levels of resistance to kanamycin and, of *aad6*, as responsible for across resistance to kanamycin and streptomycin and, in a study from China [42], this gene was associated with gentamicin and amikacin resistance.

Tetracycline resistance is explained mostly by the presence of ribosome protection gene *tetO* and *tetM*. The gene *tetO* is the most common gene in bovine *S. agalactiae* strains [43, 44]. Only in few isolates both tetracycline resistance determinants were detected in combination (B41, B83, B84, B81). The majority of *tet* genes are associated with mobile genetic elements (MGE) [45, 46]. On the other hand, MGE in which tetracycline resistance genes are present, also contain AMR determinants of aminoglycosides, macrolides and lincosamides [44, 47–49]. In our study, the presence of *ermB/tetO*, macrolide/tetracycline resistance genes among isolates considered unrelated (assessed by MLVA) suggests a possible horizontal transfer of these genes.

Regarding the complementation of MLVA with virulence and AMR genetic profiles data, correlation analysis showed different associations between specific genes and

Table 4 Genetic diversity of *S. agalactiae* population of each dairy farm assessed on number of serotypes and genotypes detected

Dairy farm	Number of isolates	Serotypes	VF profiles	AMR profiles	MLVA profiles
A	28	**III** (20), II (6), Ia (1), NT (1)	3	7	11
B	11	**II** (8), Ia (1), III (1), NT (1)	3	4	7
C	2	Ia, III	2	2	2
D	7	**II**	1	4	3
E	3	II, NT (1)	1	1	3
G	5	**III**	1	2	3
H	1	Ia	1	1	1
I	2	III, NT	2	2	2
J	28	**III**, Ia (1)	7	6	4

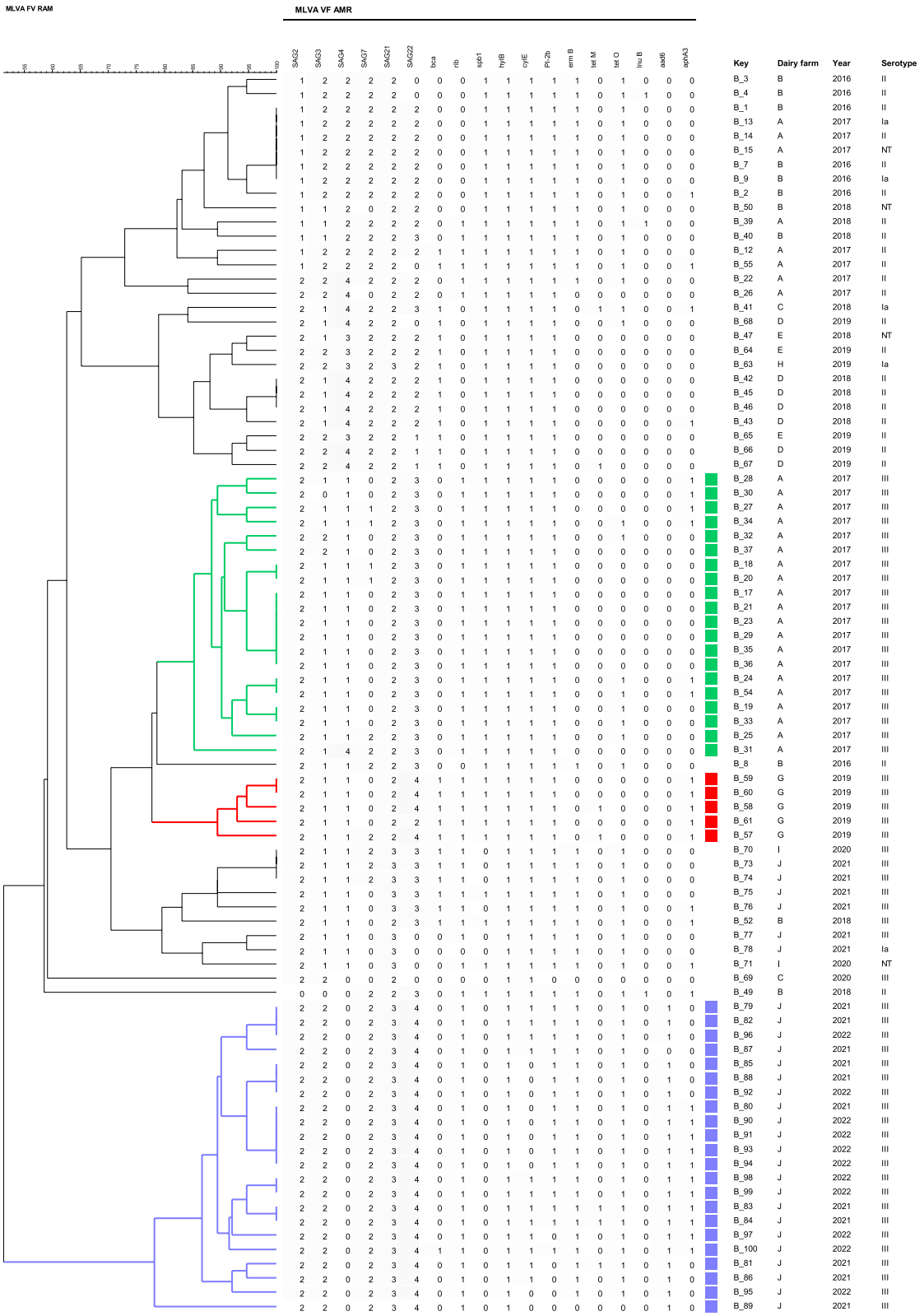

Fig. 4 Cluster analysis of *S. agalactiae* isolated from dairy cattle with mastitis in Argentina based on virulence factors, AMR and MLVA genotypes. Some clades made up of isolates from the same dairy farm are colored

dairy farms. In particular, dairy farm A was correlated with *spb1* presence, farm D, with the presence of *bca* and the absence of *rib,* and farm J, very noticeably, with the presence of *aad6* and the absence of *spb1,* added to the presence of *ermB* and *tetO,* and the absence of *cylE*.

Conclusions

This study highlights that MLVA is recommended to add to other methodologies in order to study epidemiological relationships in *S. agalactiae*. Bovine mastitis caused by this pathogen is responsible for one of the main types of contagious mastitis in dairy farms. The infected cow is the primary source of infection within the herd and the infection typically spreads from cow to cow during milking. Although in this study within each dairy farm there was a predominance of certain serotypes/virulence profiles, the characteristics did not show total homogeneity, as expected due to the contagious nature of the pathogen. This suggests the incorporation of animals from other herds at some point, a practice not uncommon among dairy farms in Argentina. By other hand, the detection of a same clone in different samples carried out in the same farm in different periods confirms that *S. agalactiae* strains can persist on dairy farms for a long time, more than a year in this study, 2–12 months according to Wataradee et al. [50]. On the other hand, correlation analyses suggest that the presence or absence of specific genes could be related to different MLVA profiles and/or dairy farms. This information could lead to better control and prevention strategies in the dairy sector in Argentina.

Methods

S. agalactiae isolates

A total of 87 *S. agalactiae* isolates collected between 2016 and 2022, from nine dairy farms (A-G, I, J) located in the Cuenca Mar y Sierras, Argentina, were studied. They were obtained from milk of cows presenting clinical or subclinical mastitis. Isolates were stored at − 80 °C.

Molecular confirmation of species and serotype, virulence factors (VF) and resistance antimicrobial (AMR) genes identification

Fifty-six isolates had been previously analyzed by Polymerase Chain Reaction (PCR) for molecular confirmation of species and serotype, virulence factors and antibiotic resistance genes identification [37] and, using the same PCR conditions, 31 isolates (B69-B100) were analyzed in this study. Briefly, the DNA were obtained by boiling bacterial colonies suspended in sterile water for 10 min. The species was confirmed by amplifying the *dltR* gene [51] and the serotype assigned by a multiplex PCR (capsular types Ia, Ib, II-IX) according to Imperi et al. [52]. A total of ten virulence genes: *bac, bca, rib*, *spb1* [53], *cpsA*, *scpB* [54], *cylE, hylB* [13], *hvgA* [51], *lmb* [44], plus three *pili* genes *PI-1, PI 2a*, and *PI-2b* [55] were identified according to the reference conditions. The antibiotic resistance genotype was performed amplifying some genes representatives of important groups in which phenotypic antimicrobial resistance was detected: the macrolide resistance gene *ermB* [56], tetracycline resistance genes *tetM* and, *tetO* [57], lincosamide resistance gene *lnuB* [12] and, aminoglycosides resistance genes *aphA3* and *aad6* [41]. PCR products were plated on 2% agarose gels containing 1 µg/ml ethidium bromide, run by electrophoresis for 30 min at 100 V and, visualized using a UV transilluminator.

Multiple locus VNTR analysis (MLVA)

Six *loci* VNTR, specific for *S. agalactiae*, were amplified with the primers described by Haguenouer et al. [24] for the 87 isolates. The PCR were carried out in two multiplex reactions, RI: SAG2, SAG4 and SAG21, and RII: SAG3, SAG7 and SAG22. Each PCR was performed in a final volume of 25 µl containing: 10 ng of DNA, 2 mM $MgCl_2$ (InBio Highway, Argentina), 200 µM of each dNTP (InBio Highway, Argentina), 1 × *Taq* DNA polymerase Buffer (InBio Highway, Argentina), 1 U *Taq* DNA polymerase and 5 pmol of each primer (Genbiotech SRL, Argentina). Amplification was performed under the following conditions: initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C and elongation for 60 s at 72 °C plus a final elongation step for 10 min at 72 °C. PCR products were plated on 2% agarose gels containing 1 µg/ml ethidium bromide, run by electrophoresis for 30 min at 100 V and, visualized using a UV transilluminator. Allelic number names corresponded to different amplimer sizes (not to the exact number of repetitions). Absence of amplification product was considered null allele and it was designed with the number 0. Allelic variants identified for each VNTR were sequenced (Macrogen, Inc., Korea) and used as a reference size in the electrophoresis runs. The MLVA genotype of each strain were expressed as an allelic profile string: SAG2, SAG3, SAG4, SAG7, SAG21, SAG22. Each allelic profile was classified as a distinctive MLVA type (MT).

Diversity analysis

Nei´s diversity index (D_N) was calculated for each locus using the formula $D_N = 1 - \sum(fr_a)^2$, where fr_a is the allelic frequency [58]. Clustering analysis (UPGMA -unweighted pair-group method with arithmetic mean-based on categorical coefficient and binary data for MLVA and virulence/RAM profiles, respectively), were constructed using BioNumerics, vs 6.6 (Applied Maths, Belgium). The discrimination power of each subtyping

method separately and combined was assessed using the Simpson diversity index (D_S) [29].

Correlation analysis

Data analysis to calculate the Pearson correlation coefficients and the corresponding p-values using Python packages Scipy (1.9.3). A *p-value* of less than 0.05 was considered statistically significant, indicating a meaningful association between the variables. Only those MLVA profiles (A, B, C) and dairy farms (A, B, D, G, J) that presented a frequency greater than 5 were considered for the analysis.

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Authors' contributions
MS and AVB contributed to conception and design of the study. GG and LBH performed the molecular characterization. JSC and AVB performed the statistical analysis. MS and AVB wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The work was carried out with the approval of the Ethics and Animal Welfare Committee of the Faculty of Veterinary Sciences -UNCPBA (Resolution No 087/02). Access to the dairy farms had the consent of the dairy owners.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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