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Virulence factors, antimicrobial resistance and phylogeny of bovine mastitis-associated *Streptococcus dysgalactiae*

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Research Article

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Abstract

We carried out a thorough genetic evaluation of *Streptococcus dysgalactiae* isolated from clinical bovine mastitis cases and performed a phylogenetic analysis to represent the evolutionary relationship between *S. dysgalactiae* sequences. A total of 35 *S. dysgalactiae* strains were isolated from cases of clinical mastitis identified at a large commercial dairy farm located near Ithaca, New York. Whole-genome sequencing identified twenty-six antibiotic resistance genes, four of which were acquired genes, in addition to fifty virulence genes. Multi-locus sequence typing detected three new sequence types (STs). We conclude that a high proportion of this microorganism carries multiple virulence determinants and resistance genes, and that this indicates its potential to cause mastitis. Eight different STs were identified, of which ST453 ($n = 17$) was the most prevalent and ST714, ST715, ST716 were novel STs.

Bovine mastitis is a disease that compromises milk quality and causes economic losses in the dairy industry. Direct and indirect losses in the milk production chain, due to mastitis, are related to the following: the costs associated with treating affected animals; decrease in the milk volume during lactation and the culling of diseased animals or animals with a shorter productive life (Bettanin *et al*., 2019). Several bacterial pathogens can cause mastitis, however, the majority of species responsible for causing infections are streptococci, staphylococci and enterobacteria (Gomes and Henriques, 2016). The use of antimicrobials is the main form of treatment for mastitis, and the first-line treatments are penicillin (alone or in combination with aminoglycosides), macrolides and lincosamides, fluoroquinolones and tetracyclines (Haenni *et al*., 2018). It is important to recognize that recurrent and uncontrolled use of antibiotics may promote the occurrence of antimicrobial-resistant bacteria (Abd *et al*., 2020).

Mastitis caused by *Streptococcus dysgalactiae* is usually identified in the clinical form of the disease and has been reported in many different countries (Vélez *et al*., 2017; Tian *et al*., 2019). Clinical mastitis causes an increase in the somatic cells of the mammary gland, and has greater incidence during the postpartum period and the rainy season (Ulsenheimer *et al*., 2020). Cows infected with mastitis-causing *S. dysgalactiae* can infect healthy cows, likewise, *S. dysgalactiae* present in the environment can also infect the herd. Thus, this microorganism has the characteristics of both a contagious and environmental pathogen (Vélez *et al*., 2017). The level of *Streptococcus* pathogenicity depends on its ability to produce a variety of virulence factors (Tian *et al*., 2019). *S. dysgalactiae* isolated from cattle possess several potential cell-associated and extracellular virulence factors (Calvinho *et al*., 1998). The success of bacterial host infection and proliferation requires the acquisition of virulence factors, to nullify the host's defense mechanisms. These factors may include enzymes that effectively inhibit the defense responses of the host, structural components and toxins (Calvinho *et al*., 1998).

The objective of this study was to characterize the virulence and antibiotic resistance profiles of *S. dysgalactiae* isolates obtained from the milk of cows affected with clinical mastitis using whole genome sequences; and to perform a phylogenetic analysis, to represent the evolutionary relationship between *S. dysgalactiae* sequences according to the severity of the cases studied.

Material and methods

Streptococcus dysgalactiae was isolated from 35 cows with clinical mastitis. These strains are accessioned and stored in the bacteria collection of the Department of Population Medicine

and Diagnostic Sciences College of Veterinary Medicine, Cornell University Ithaca (Tomazi *et al.*, 2021). Clinical mastitis isolates were defined as those from cows with clinical signs of intramammary infection (alteration of milk and/or normal udder appearance).

DNA was extracted from each bacterial isolate using the DNAasy Power food Microbial Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions.

Then, PCR amplification of the 16S ribosomal DNA gene, purification of product and analyses of FASTA sequences was performed according to Silva *et al.*, 2021.

Whole genome sequencing identification was performed using the Illumina platform. The library was generated using the Nextera XT DNA Sample Prep Kit (Illumina Inc. San Diego, CA), and the run was performed using the Illumina v2. Quality control of the reads was carried out using FASTQC. The sequencing reads were submitted to the comprehensive genome analysis service using Pathosystems Resource Integration Center (PATRIC 3296), and the reads were assembled using SPAdes. The genomes were annotated using the Rast tool kit, available in the PATRIC system, and the multilocus sequence typing (MLST) was determined (https://cgecbsdtudk/services/MLST/) (Silva *et al.*, 2021).

Acquired genes were identified using ABRicate version 05 (https://githubcom/tseemann/abricate), by aligning genome sequences to the ResFinder database. The virulence genes were identified using the VFDB database. Plasmid replicon types were detected using PlasmidFinder v13. Finally, the phylogenetic tree was constructed, as described by Silva *et al.*, 2021.

The gene mutations were analyzed with Geneious Prime 2021 v. 2.2 (Biomatters, Auckland, New Zealand) (Kearse *et al.*, 2012). *EF-G*, *gid*B, *lia*R, *lia*S, *pgs*A, *rpo*B, *rpo*C and *S10p* reference sequences were obtained from the genomes of *S. agalactiae* (NGBS128, Genbank accession number: CP012480.1), *S. dysgalactie* (FDA-ARGOS 1157, GenBank accession number: CP068057.1) and *S. pyogenes* (MGAS23530, Genbank accession number: CP013839.1), from the National Centre for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/).

Multiple alignments were performed using ClustalW; the reading frames were adjusted according to the genome annotations and synonymous/non-synonymous mutations were analyzed (Kearse *et al.*, 2012).

Results and discussion

Twenty-six genes associated with antibiotic resistance have been identified. Concerning acquired resistance genes, the genes that were present in our analysis were *lsa*(C) (22.8%), *mef*E and *tet* (M) (17.1%) and *lnu*(C) (5.7%) (online Supplementary Table S1).

Fifty virulence genes were identified in the analyzed strains (Table 1). All strains presented the *per*R gene; 94.2% had the *leu*S gene and 91.4% had the *gid*A, *gld*A, *has*C, *pur*H, *SP_0251* and *SpyM3_0013* genes. Thirty-five other genes were present in most isolates, with their presence ranging from 88.5% to 48.5% in frequency. The least frequent genes identified were: *sda* (22.8%), *emm*, *emm*1 and *SpyM3_0386* (17.1%), *spe*K (11.4%), *SP_0338* and *SP_1399* (5.7%).

Resistance genes

Some antibiotics are used to treat mastitis and we identified resistance associated genes that are related to these antibiotics in our genetic analysis. Mutations in the *lnu*(C), *lsa*(C) genes are associated with lincosamide resistance. The resistance to lincosamide by *S. dysgalactiae* and other *Streptococcus* species isolated from infected cattle has previously been reported (Haenni *et al.*, 2018; Silva *et al.*, 2021) and should be a concern, both for mastitis treatment and for other cattle infections. In our study, 2 (5.7%) and 8 (22.8%) strains presented *lnu*(C) and *lsa*(C) respectively.

With expanding bacterial resistance to conventional antibiotics, antimicrobial peptides have become increasingly important as a last resort for combating multi-resistant bacteria; and some of these have already been used in veterinary medicine (Davis and Janssen, 2020). Antimicrobial peptides have certain advantages, such as adverse effects against biofilm formation and immune response stimulators, thereby enhancing immunity and protection against infections (Saeed *et al.*, 2022).

Vélez *et al.* (2017) determined the occurrence of antimicrobial resistance genes in *S. dysgalactiae* isolated from Canadian dairy herds. The study demonstrated that mutations in the beta subunit of RNA polymerase (*rpo*B) produced resistance to rifamycin in other bacteria species. The authors continue to raise awareness regarding this significant public health risk, as rifamycins are used to treat human tuberculosis (Vélez *et al.*, 2017). Several antibiotics, such as rifampicin, myxopyronine, and tagetitoxin specifically target the genes *rpo*B and *rpo*C, which encode for subunits of RNA polymerase (RNAP) (Wang *et al.*, 2018). In our study, 20 nucleotide mutations were found in the *rpo*B gene, 18 of which were synonymous (same amino acid) and 2 were non-synonymous – indicated in the alignment (positions 295 and 1003). Considering the reference strains, in both positions the amino acid valine changed to isoleucine in 8 strains (online Supplementary Fig. S1). In the *rpo*C gene, 19 nucleotide mutations were found, 16 of which were synonymous and 3 were non-synonymous (positions 1799, 2598 and 2600). Considering the reference strains, at position 1799 the amino acid cysteine changed to arginine in 10 strains, at position 2598 the amino acid histidine changed to arginine also in 10 strains, and at position 2600 the amino acid glutamic acid changed to lysine in 13 strains (online Supplementary Fig. S2).

The *pgs*A gene encodes for CDP-diacylglycerolglycerol-3-phosphate 3-phosphatidyltransferase, which is involved in the production of phosphatidylglycerol. This in turn facilitates daptomycin binding and the permeabilization of membranes, aside from also being the biosynthetic precursor of cardiolipin and lysyl-phosphatidylglycerol. This conversion into cardiolipin or lysyl-phosphatidylglycerol, as well as mutations suffered by the bacteria, can cause changes in daptomycin susceptibility (Taylor and Palmer, 2016). In the *pgs*A gene, 2 nucleotide mutations were identified, and were non-synonymous mutations (positions: 109 and 284). Considering the reference strains, in position 109 the amino acid phenylalanine changed to isoleucine in 2 strains, and in position 284 the amino acid valine changed to glycine also in 2 strains (online Supplementary Fig. S3).

In Gram-positive bacteria, the LiaFSR system is well conserved. It encodes for a three-component regulatory system that controls the integrity of the cell envelope in order to neutralize membrane damage caused by external agents, such as antibiotics. The *lia*F gene encodes a protein that acts as a specific inhibitor of *lia*S and *lia*R which are, respectively, the histidine kinases and response regulators of the LiaFSR system (Ota *et al.*, 2021). In this study, 43 nucleotide mutations were found in the *lia*R gene, 35 of which were synonymous and 7 were non-synonymous (positions 311, 845, 957, 986, 1397, 1400, 1442). Considering

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Table 1. Distribution of virulence factors genes of 35 *Streptococcus dysgalactiae* strains isolated from cows with mastitis

Virulence factor genes	Total of positive strains (%)	Product
*per*R	35/35 (100)	Peroxide stress regulator PerR, FUR family
*leu*S	33/35 (94.2)	Leucyl-tRNA synthetase
*gid*A	32/35 (91.4)	tRNA-5-carboxymethylaminomethyl-2-thiouridine(34) synthesis protein MnmG
*gld*A	32/35 (91.4)	Glycerol dehydrogenase
*has*C	32/35 (91.4)	UTP--glucose-1-phosphate uridylyltransferase
*pur*H	32/35 (91.4)	IMP cyclohydrolase (EC 3.5.4.10)/Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3)
SP_0251	32/35 (91.4)	Pyruvate formate-lyase
SpyM3_0013	32/35 (91.4)	Cationic amino acid transporter – APC Superfamily
*cyd*A	32/35 (91.4)	Cytochrome d ubiquinol oxidase subunit I
mf/spd	31/35 (88.5)	Streptodornase B; Mitogenic factor 1
*rpo*E	31/35 (88.5)	DNA-directed RNA polymerase delta subunit
SP_0121	31/35 (88.5)	Ribonuclease J1 (endonuclease and 5′ exonuclease)
SP_0494	31/35 (88.5)	CTP synthase
*opp*A	31/35 (88.5)	Oligopeptide ABC transporter, substrate-binding protein OppA
*gln*A	30/35 (85.7)	Glutamine synthetase type I
fba	30/35 (85.7)	Fructose-bisphosphate aldolase class II
*lux*S	30/35 (85.7)	S-ribosylhomocysteine lyase (EC 4.4.1.21) @ Autoinducer-2 production protein LuxS
*cov*S	30/35 (85.7)	Transmembrane histidine kinase CsrS
*atm*B	30/35 (85.7)	Methionine ABC transporter substrate-binding protein
*clp*P	29/35 (82.8)	ATP-dependent Clp protease proteolytic subunit ClpP
*lux*S	29/35 (82.8)	S-ribosylhomocysteine lyase (EC 4.4.1.21) @ Autoinducer-2 production protein LuxS
*pur*B	29/35 (82.8)	Adenylosuccinate lyase
SPy_1718	29/35 (82.8)	Esterase
*vic*K	29/35 (82.8)	Two-component sensor kinase SA14-24
*ccp*A	29/35 (82.8)	Two-component sensor kinase SA14-24
SPy_1633	28/35 (80.0)	Ribonuclease Y
fbp54	28/35 (80.0)	Fibronectin/fibrinogen-binding protein
SP_2086	28/35 (80.0)	Phosphate ABC transporter, permease protein PstA
SP_1970	27/35 (77.1)	Aspartate--ammonia ligase
SP_0095	27/35 (77.1)	Rhodanese domain protein UPF0176, Firmicutes subgroup
*dlt*A	27/35 (77.1)	D-alanine--poly(phosphoribitol) ligase subunit 1
lgt	26/35 (74.2)	Prolipoprotein diacylglyceryl transferase
lmb	25/35 (71.4)	Laminin-binding surface protein
lsp	25/35 (71.4)	Lipoprotein signal peptidase
SP_0320	25/35 (71.4)	2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase (EC 1.1.1.127) @ 2-deoxy-D-gluconate 3-dehydrogenase (EC 1.1.1.125)
SP_1396	25/35 (71.4)	Phosphate ABC transporter, ATP-binding protein PstB
SP_1398	25/35 (71.4)	Phosphate ABC transporter, permease protein PstA
*cps*Y	24/35 (68.5)	Methionine biosynthesis and transport regulator MtaR, LysR family
*lep*A	24/35 (68.5)	Translation elongation factor LepA
SP_0829	24/35 (68.5)	Phosphopentomutase
SP_0856	24/35 (68.5)	Branched-chain amino acid aminotransferase

(*Continued*)

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Table 1. (*Continued.*)

Virulence factor genes	Total of positive strains (%)	Product
EF1623	23/35 (65.7)	Ethanolamine utilization polyhedral-body-like protein EutM
*gua*A	22/35 (62.8)	GMP synthase [glutamine-hydrolyzing], amidotransferase subunit (EC 6.3.5.2)/GMP synthase [glutamine-hydrolyzing], ATP pyrophosphatase subunit (EC 6.3.5.2)
mf3	17/35 (48.5)	Streptococcal extracellular nuclease 3; Mitogenic factor 3
sda	8/35 (22.8)	Streptodornase D
emm	6/35 (17.1)	Antiphagocytic M protein
*emm*1	6/35 (17.1)	Antiphagocytic M protein
SpyM3_0386	6/35 (17.1)	Uncharacterized MFS-type transporter
*spe*K	4/35 (11.4)	Streptococcal pyrogenic exotoxin K (SpeK)
SP_0338	2/35 (5.7)	ClpE-like protein
SP_1399	2/35 (5.7)	Phosphate ABC transporter, permease protein PstC (TC 3.A.1.7.1)

the reference strains, at position 311 the amino acid aspartic acid changed to alanine in 1 strain, at position 845 the amino acid lysine changed to arginine in 5 strains, at position 957 the amino acid serine changed to arginine in 5 strains, at position 986 the amino acid threonine changed to methionine in 5 strains, at position 1397 the amino acid serine changed to leucine in 5 strains, at position 1400 the amino acid lysine changed to methionine in 5 strains and at position 1442 the amino acid asparagine changed to serine in 5 strains (online Supplementary Fig. S4).

In the *lia*S gene, 5 nucleotide mutations were found, all of which were synonymous (online Supplementary Fig. S5). The elongation factor G (EF-G) protein is involved in the resistance mechanism against the antibiotic fusidic acid. Due to its heterologous expression, the fusB protein family is the main source of clinical resistance to fusidic acid (Tomlinson *et al.*, 2020). By binding to EF-G in the ribosome, fusidic acid inhibits bacterial protein synthesis, prevents disassembly of the post-translocation complex (as well as the steric occlusion resulting from site A by EF-G), blocks the delivery of aminoacyl-tRNA species from entry into the ribosome and, finally, causes protein synthesis interruption (Wilson, 2020). In our study, 9 nucleotide mutations were found in the *EF-G* gene, 8 of which were synonymous and 1 non-synonymous; (position 699). Considering the reference strains, at position 699 the amino acid aspartic acid changed to glutamic acid in 12 strains (online Supplementary Fig. S6).

Streptomycin is an antibiotic widely used in veterinary medicine, and it belongs to the class of aminoglycosides, which is an important group of natural or semi-synthetic antibiotics. By binding to the highly conserved 16S region of the rRNA and 30S subunit, aminoglycosides exhibit a bactericidal action. This interaction can lead to translation errors in the synthesis of amino acids and, consequently, the interruption of protein synthesis of the target bacterium (Samanta and Bandyopadhyay, 2020). In this study, the whole genome sequencing of *S. dysgalactiae* showed the presence of the *gid*B resistance gene and the ribosomal protein S12p, which are associated with resistance to the aminoglycoside class of antibiotics. This gene (*gid*B) showed 4 nucleotide mutations, 3 synonymous and 1 non-synonymous (position 364). Considering the reference strains, at position 364 the amino acid valine changed to leucine in 5 strains (online Supplementary Fig. S7).

Tetracyclines are antibiotics that target a wide range of Gram-positive and Gram-negative bacteria. They have bacteriostatic activity through the inhibition of ribosomal protein synthesis (Samanta and Bandyopadhyay, 2020). So far, the possible mechanisms of resistance to tetracycline explored are efflux pumps, ribosomal protection, enzymatic degradation of drug molecules and mutations in the rRNA, resulting in reduced affinity for binding to the drug (Grossman, 2016; Samanta and Bandyopadhyay, 2020). In this study, the presence of tetracycline resistance appears to be associated with the *tet*(M) gene and also with the ribosomal protein S10p. The *tet*(M) gene is a tetracycline ribosomal protection protein, and in streptococci from clinical isolates, it is considered the most prevalent determinant of tetracycline resistance (Wilson, 2020). However, we found no mutations in ribosomal protein S10p (online Supplementary Fig. S8).

We showed that the *mef*E gene was present in 6 of the 35 *S. dysgalactiae* isolates analyzed (17.1%), and this gene is associated with resistance to macrolide antibiotics. Zhang *et al.* (2018) analyzed the presence of the *mef*E gene in 88 *S. dysgalactiae* isolates from milk samples of clinical bovine mastitis, and this gene was present in 2 of the 88 *S. dysgalactiae* analyzed (2.3%). This resistance to macrolides is related to the presence of the efflux pump and is encoded by the *mef*E gene (Latini *et al.*, 1999).

Virulence genes

In this study, *S. dysgalactiae* showed a wide variety of virulence genes, and the distribution of genes and their respective products can be seen in Table 1. Some virulence genes found in our study are associated with pathogenic mechanisms, such as *has*C (antiphagocytosis, adherence and tissue invasion), *mf/spd* (exoenzyme and propagation factor), *fbp54* (adherence and cell invasion), *mf3* (exoenzyme and propagation factor), *sda* (exoenzyme and propagation factor), *emm* (antiphagocytosis, adherence and cell invasion) and *spe*K (toxin, membrane-acting and superantigen). The *spe*K gene was present in 11.4% of the analyzed strains. The *spe*K, *spe*L and *spe*M genes are virulence genes associated with bacteriophages, and this suggests that bacteriophages may also play a role in genetic plasticity and virulence of bovine mastitis (Rato *et al.*, 2011). The *lmb, emm* and *emm1* genes found in this study are related to adherence (Rato *et al.*, 2011). The virulence of *emm1* and *emm* strains can be attributed to their ability to adapt to different host environments and by diversification through phage mobilization (Matsue *et al.*, 2020).

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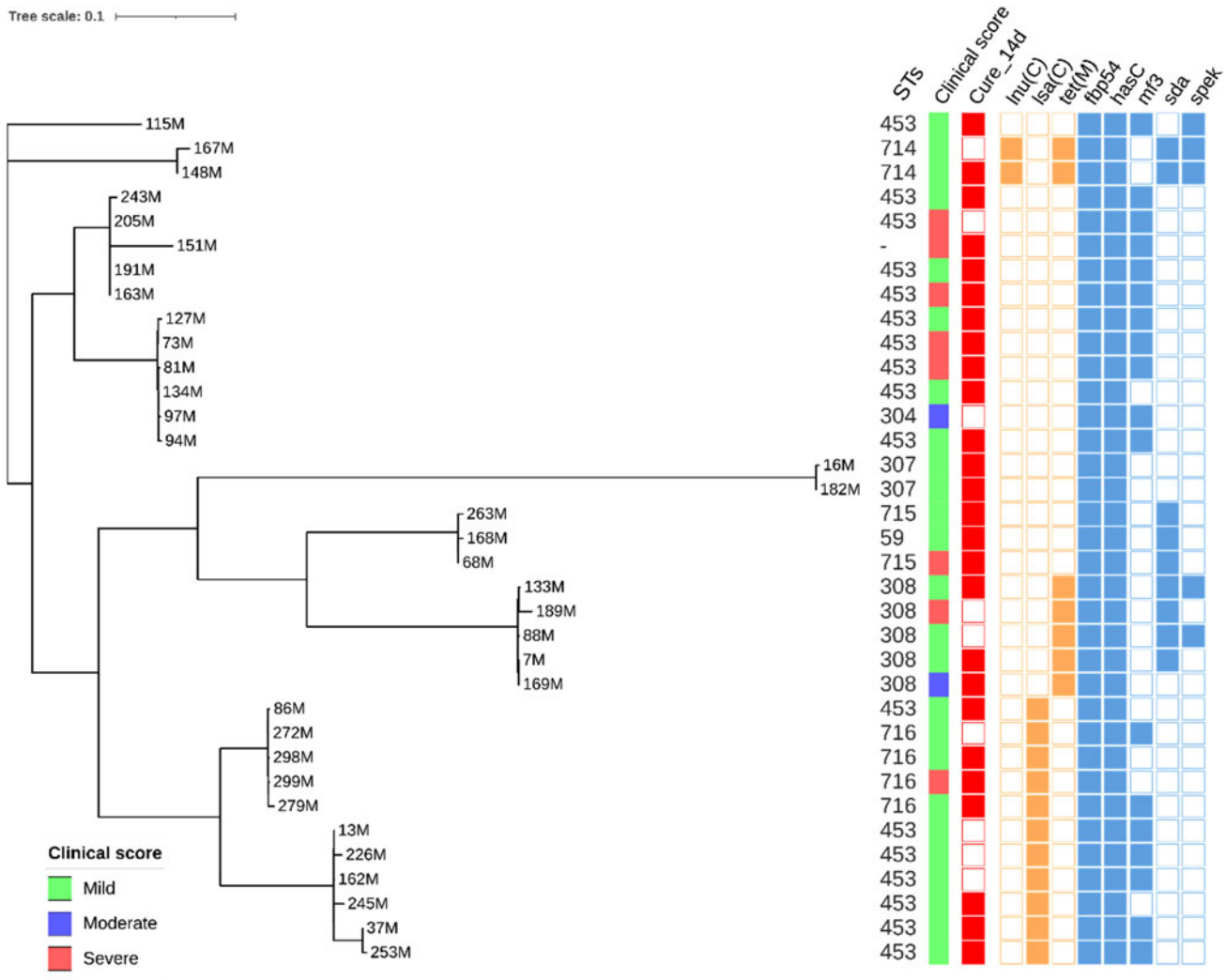

Fig. 1. Maximum likelihood phylogeny of *Streptococcus dysgalactiae* based on core genomic SNPs. An unassigned ST was indicated by a dash under the STs. The clinical scores were indicated as mild, moderate, and severe. The red squares indicate whether the cow was cured (solid) or not (hollow). Genes for acquired antimicrobial resistance and virulence are denoted by the yellow and blue squares, respectively. *Clinical score: Severity of mastitis.*ST: Sequence type.

The hyaluronic acid capsule gene (*has*C) was present in 91.4% of the *S. dysgalactiae* strains analyzed in this study. This gene, in addition to playing an important role in pathogenicity, also helps make the microorganism contagious (Calonzi *et al.*, 2020). The fibronectin-binding gene *fbp54* was present in 80% of the *S. dysgalactiae* isolates we analyzed. Fibronectin is a glycoprotein and acts as a substrate for bacteria to attach to the host cell surface. *Streptococci* express several fibronectin-binding adhesins, and this binding to epithelial cells *via* fibronectin facilitates their entry into cells (Miller-Torbert *et al.*, 2008).

The mf3 protein is related to *Streptococcal* extracellular nuclease 3 and mitogenic factor 3. This gene was present in 48.5% of the studied strains. MF has DNase activity and extracellular streptococcal DNases are considered to be potential virulence factors. DNase B, in particular, participates in the induction of anti-DNase antibody production after skin and pharyngeal infection (Hasegawa *et al.*, 2002; Sharma *et al.*, 2019).

The virulence factor associated with the prophage streptodornase (*sda*) was present in 22.8% of the *S. dysgalactiae* analyzed in this study. Streptodornase has been related to diseases caused by other *Streptococci*, and in addition there are reports that the coding of the *sda* gene occurred due to inducible bacteriophages (Smeesters *et al.*, 2010).

Phylogenetic analysis and multi-locus sequence type (MLST)

The results revealed deep branching and a scattered population structure that was broadly classified into distinct phylogenetic lineages. The phylogenetic tree (Fig. 1) showed the diversity of strains associated with clinical mastitis in the analyzed samples. Isolates with observed sequence types (ST) are: ST59 ($n = 1$), ST304 ($1n = 1$), ST307 ($n = 2$), ST308 ($n = 5$), ST453 ($n = 17$), ST714 ($n = 2$), ST715 ($n = 2$) and ST716 ($n = 4$). Novel STs observed were ST714, ST715 and ST716. Only one ST was not identified. Strains with high similarity were observed, suggesting cow-to-cow transmission, for example, the strains 16 and 182 M. However, the great diversity suggests environmental contamination, since the strains were isolated from just one herd. It appears that both types of transmission can occur in *S. dysgalactiae* mastitis, with the most common being environmental transmission.

It should be noted that strains with ST453 appear in different clades of the presented tree, calling attention to the use of next-

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generation sequencing for phylogenetic analysis. This means that MLST could be used to type the strains, but the whole genome sequence is more specific and displays more diversity, which could be more relevant to epidemiological studies.

Considering resistance genes, we observed that the strains which carry these genes belong to the same clade, for example, 167 and 148 M carry three acquired resistance genes (*mef*E, *tet*M and *lnu*C). Also, the strains which carry the resistance gene *lsa*C are clustered together in the phylogenetic tree, suggesting a close relationship among them. However, there was no relation between the severity of mastitis, bacteriological cure, and the strains, as it is possible to observe severe and mild mastitis cases in the same clade.

In conclusion, this study showed a great diversity of virulence and resistance genes in the studied *S. dysgalactiae* isolates. This microorganism has genes associated with resistance to the main antibiotics used for the treatment of mastitis and other diseases. This demonstrates its potential as a cause of mastitis, as well as increasing the understanding regarding the difficulties of treating this disease. Furthermore, three new STs were documented in this study and the results presented here provide important information on the genomic characteristics, and on the genetic profile of *S. dysgalactiae* causing mastitis.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000195.

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