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Genetic Analysis Method for *Staphylococcus chromogenes* Associated with Goat Mastitis

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Abstract

Mastitis in goats is mainly caused by coagulase-negative *Staphylococcus* (CNS). The identification methods for this group are based on evaluating the expression of phenotypic characteristics such as the ability to metabolize various substrates; however, this is disadvantageous as these methods are dependent on gene expression. In recent years, genotyping methods such as the Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) and gene identification have been useful for epidemiological study of several bacterial species. To develop a genotyping method, the genome sequence of *Staphylococcus chromogenes* MU970 was analysed. The analysis showed nine virulence genes described in *Staphylococcus aureus*. The MLVA was developed using four loci identified in the genome of *S. chromogenes* MU970. This genotyping method was examined in 23 strains of CNS isolated from goat mastitis. The rate of discrimination for MLVA was 0.8893, and the highest rates of discrimination per the index of Simpson and Hunter-Gaston were 0.926 and 0.968 for the locus 346_06, respectively. The virulence genes were present in all strains of *S. chromogenes* but not in other CNS. The genotyping method presented in this paper is a viable and easy method for typifying CNS isolates from mastitis cases in different regions and is an ideal mean of tracking this disease.

Key words: genotyping, mastitis in goats, MLVA, *Staphylococcus chromogenes,* VNTR

Introduction

Goat mastitis is primarily caused by CNS. CNS are classified into 47 species and 23 subspecies (Becker *et al.*, 2014), and in mastitis cases, more than 10 CNS species have been isolated (Taponen and Pyörälä, 2009; Zadocks and Watts, 2009). The CNS represent a heterogeneous group within the *Staphylococcus* genus, and the identification methods for this group evaluate the expression of genetically encoded characteristics, such as enzyme production (Stepanovick *et al.*, 2006; Becker *et al.*, 2014; Vanderhaeghem *et al.*, 2015). One disadvantage of the phenotypic identification method is the expression variability of phenotypic traits between isolates of the same species, and because of this, genotypic identification methods have been developed (Monir *et al.*, 2007). These methods are based on the genetic material analysis of the organism; therefore, they are independent of changes in the gene expression pattern. Molecular methods represent a more stable and reproducible alternative and provide useful information about the genetic connections between isolates from different sources, allowing epidemiological monitoring of disease outbreaks (Hollender *et al.*, 2013; Wang *et al.*, 2016). Genotyping bacteria by typing their loci containing a variable number of tandem repeats (VNTR) may become the gold standard for many pathogens (Ramisee *et al.*, 2004; Vergnaud and

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Pourcel, 2009). MLVA is a DNA-based molecular typing method frequently used in the study of prokaryotes. It records size polymorphisms in several VNTR loci amplified by stringent PCR protocols (Le Fleche *et al.*, 2001). MLVA is useful in epidemiology because it replaces older, slower and dangerous methodologies (phenotypic identification methods require handling live pathogenic bacteria) for typing microorganisms. VNTRs are a powerful tool for determining evolutionary relationships and population genetics of bacteria (Hardy *et al.*, 2004). The development of genome sequencing has shown that VNTR sequences are present in many bacterial species and that polymorphism exists in most of them. These repetitions have become a source for locating markers to identify pathogenic bacteria (Vergnaud and Pourcel, 2009). In *S. aureus*, MLVA is useful for strain typing. An MLVA analysis of 130 strains isolated from raw-milk dairy products (122 isolates) and human samples (eight isolates) revealed marked genomic variability among the samples. In this study, the MLVA technique correctly assigned isolates from outbreaks and discriminated isolates that were not from outbreaks (Ikawaty *et al.*, 2008). In this study, we developed the MLVA for *S. chromogenes* using the *S. chromogenes* MU970 genome sequence (NCBI Reference Sequence: NZ_JMJF00000000.1). This genotyping method included nine virulence genes previously described in other *Staphylococcus* spp.

Experimental

Materials and Methods

Bacterial isolates. Fifty-three *Staphylococcus* strains were isolated from the milk of both healthy goats and goats with subclinical mastitis from farms in Queretaro and Guanajuato, Mexico. To isolate the strains, the milk was plated on trypticase soy agar and incubated at 37°C for 24 hours. The microorganisms were Gram-stained and catalase tested to identify the *Staphylococcus* isolates. Coagulase tests were performed to identify coagulase-negative *Staphylococcus*, using the API Staph® (V4.1) Biomerieux laboratory system per the manufacturer's instructions. A chemotherapeutic susceptibility test was conducted on all isolates identified as *S. chromogenes* using the Kirby-Bauer technique (Bernal and Guzman 1984) with antimicrobial susceptibility disks (polymyxin B, ampicillin, tobramycin, gentamicin and tetracyclines) (BD Becton-Dickinson and Company).

DNA extraction and polymerase chain reaction (PCR). DNA extraction from the 53 bacterial isolates was made as per the protocol described by Cremonesi *et al.* (2006). DNA concentration was measured by spectrophotometry (BIORAD SmartSpec-Plus Spectrophotometer). To identify the *Staphylococcus* genus, primers designed by Mason *et al.* (2001) were used. To identify the isolates within the CNS group, we used a pair of primers corresponding to the *coa* gene of *S. aureus*. This primer amplifies a 1200-bp fragment from these genes (Ruiz *et al.*, 2013). DNA from *S. chromogenes* ATCC® 43764 TM and *S. aureus* ATCC® 29737 TM were used to validate the PCR test (PCR Master Mix, Fermentas). The thermocycler conditions were specific for each primer. The PCR products were analysed by electrophoresis on 1% agarose gel using TAE 1X as the running solution. To identify the virulence genes in *S. chromogenes*, nine pairs of primers were designed to amplify different proteins defined as virulence factors in *S. aureus*. Primer design was performed by obtaining the amino acid sequence of the aim genes, using the BLAST database (Basic Local Alignment Search Tool) from the NCBI (National Center of Biotechnology Information; http://blast.ncbi.nlm.nih.gov/Blast.cgi) for verifying the sequence homology. We then located the amino acid sequence within the genome, and the primers were designed using the DNAman program (Lynnon Corporate) version 7.02 for Windows (Table I).

MLVA. The *S. chromogenes* MU970 strain genome was analysed to locate the tandem repetitions using the Tandem Repeats Finder Program (Benson, 1999). The primers of the loci flanking regions from the tandem repetitions were designed using the DNAman v. 7.02 program. Six loci of 15- to 96-bp were selected from the tandem repetitions, with the number of copies ranging from 7 to 23, to observe the amplifications on an agarose gel. Tandem repetitions were named according to the genome where they were found and the size of the expected product in the *S. chromogenes* MU970 strain (Table II). MLVA analysis was performed with all *S. chromogenes* isolates. The PCR products of the repeated sequences were analysed with the fragment QIAxcel DNA High Resolution Kit (QIAGEN México S. DE R.L. DE C.V. CP 01090, México City. Catalogue number 929002) analyser, following the manufacturer's instructions. The size of each VNTR was determined using a molecular weight marker of 100- to 3000-bp.

Data Analysis. The size of each amplicon was determined using the molecular weight marker of 3000-bp, and the repetition number of each allele was derived from the size of the obtained amplicon. For each VNTR locus, we calculated the diversity indexes of Simpson and Hunter-Gaston (1988) with confidence intervals of 95%, using the VNTR Diversity and Confidences Extraction Software program (V-DICE), from the Health Protection Agency website (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). The MLVA discrimination power was calculated using the Discriminator Power Calculator program (http://insilico.

Table I
Primers used to identify *S. chromogenes.*

Primers	Sequence (5'-3')	Conditions	Size [bp]
16S rRNA	F'- CCTATAAGACTGGGATAACTTCGGG R'- CTTTGAGTTTCAACCTTGCGGTCG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	791
coa	F'- CAAAGCAGATGCGATAG R'- CCTGTACCAGCATCTCTAT	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	1200
Aureolysin	F'- GCATTAAACGAAASCTTYTCWG R'- GTATGCAGCTTTATTTGGWATACC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	247
Hemolysin	F'- ATGAATATTGGAATAACTTTAGTCAG R'- TTAGGAAGCATACAATTGATGT	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	600
Extracelular protein	F'- ACGTGAGCAATATATGAACGC R'- TTATAAATACCTGTTAATGCGCC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	133
Zin Metaloprotease	F'- ATTTGCAATCGGGATGG R'- ATGTTGATCATCTAAAATAATATGCG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	250
Surface protein SasH	F'- GGCWAAAGCRATGAATGC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m,	201
	R'- ACCGATAATKCCRTAACG	72°C/1 m/ 1 cycle 72°C/5 m.	
TRAP (Transduction signal protein)	F'-AACTCTATTCACTTATGGAACATATGG R'- CTGTTCAACATTTTGCTGTTG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	529
B antigen	F'- CAAAAACACTATTAGCGACTGG R'- CTTTTATGACGATGGAGTTTCC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	488
Virulence B factor	F'- ATGTCTTTTAAAGAAAATGAAATCG R'- TATGCATGATTTTCACTGTGC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	905
FemA protein	F'- GTGTGCTTRTACCWYTAGC R'- CCAGCATAATAAACWASTTC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 50°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	250

Table II
Primers used to amplify the VNTR.

Primer	Sequence (5'-3')	Conditions	Size [bp]
266_07	F'- ATTCTGGATTTTGCACAGC R'- ATCTGCTAAAATGACAGAATTACAAC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	266
346_06	F'- CAAAACAACGATATCTGTATCTGA R'- TGTCGGTTTATTAGGTTGAGAAG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	346
360_06	F'- CAAGTGCATAACCGTTATTCC R'- TGTCTGATGTCGGTTTATTAGG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	360
854_08	F'- GAAGCACTTACACTTTCGGAG R'- GACTTCACTAAGTGAGTCAACAAGTAC	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	854
638_07	F'- TGAGGTCTCCGCTGTAGG R'- GCAGACGTCCCTGTTGAG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	638
613_12	F'- AGTAGCTAACCATTTTGTTAATTGC R'- GTTAAAGAAAATTCTTCACAGTCG	1 cycle 95°C/5 m, 30 cycles 95°C/1 m, 56°C/1 m, 72°C/1 m/ 1 cycle 72°C/5 m.	613

ehu.es/mini_tools/discriminatory_power/index.php). The 23 *S. chromogenes* isolates were grouped by their phenotypic characteristics through a cluster analysis, using the Euclidean distance and the Ward minimum variance method with the DELL STATISTICA (Data Analysis Software System) program. The cluster analysis of the *S. chromogenes* genogroups was performed with the BioNumerics software version 7.6.1 (Applied Maths, St-Martens-Latern, Belgium) using the Pearson correlation coefficient and the pair grouping method with an unweighted arithmetic mean (UPGMA).

Results

Bacterial Identification. The 53 bacterial isolates were Gram-positive cocci and were grouped in clusters of catalase positive and coagulase negative using a macroscopic morphology characteristic of the genus. From the results of the API system Staph®, 23 isolates corresponded to *S. chromogenes*, 18 to *Staphylococcus simulans*, nine to *Staphylococcus xylosus*, two to *S. sciuri*, and one to *Staphylococcus warneri*. These results, as well as the results of the chemotherapeutic susceptibility

test for the isolates identified as *S. chromogenes*, are shown in Table III.

Molecular identification. PCR was standardized for the detection of the 16s *Staphylococcus* region and for the *coa* gene from *S. aureus* to confirm the expected size of each product using DNA from the ATCC strains at concentrations of 100 ng/µl. The 53 isolates were positive for the 16s gene and negative for the *coa* gene. The *S. chromogenes* isolates were positive for all virulence genes, except for the gene encoding the *virulence B factor*, in which only 95.65% of the isolates were positive. All *S. simulans* isolates were positive for the genes encoding the extracellular protein, zinc metalloprotease, SasH and FemA, and 27.78% were positive for the aureolysin gene, 16.67% for the *TRAP* gene and 11.11% for haemolysin and B antigen. In addition, all of these were negative for the virulence B factor. All *S. xylosus* isolates were positive for the extracellular protein genes, zinc metalloprotease and FemA. Of these, 66.67% were positive for SasH, 44.44% for aureolysin, and 33.33% for SasH and virulence B factor; however, none of these isolates were positive for the B antigen. All *S. sciuri* isolates were positive for all virulence genes except haemolysin and the B antigen, for which none of the isolates were positive. Finally, the *S. warneri* isolate was positive for the extracellular protein, zinc metalloprotease and the virulence B factor and was negative for the rest of the virulence genes (Table IV).

Phenotypic characteristics. To determine the variability of the 23 *S. chromogenes* isolates from the different biochemical tests and the chemotherapy susceptibility test, a dendrogram was constructed showing three clusters of the 23 *S. chromogenes* isolates. The first cluster contains six isolates, divided into two subgroups. In the first subgroup, there are two isolates from farm number 2, which has an intensive production system, and four isolates from farm number 1, which has an extensive production system. This group is characterized for being maltose-negative, having a variable N-acetyl-glucose response, and being sensitive to the five antibiotics that were used. The second cluster has six isolates from farm number 3, which are divided into two subgroups. In this cluster, the isolates are maltose-positive, N-acetyl-glucose-negative, and polymyxin B and ampicillin-resistant. The third cluster has one subgroup with two isolates from farm number 1, two isolates from farm number 2 and five isolates from farm number 3, which has an intensive production system. This cluster is characterized as being maltose-positive, N-acetyl-glucosamine-positive and polymyxin B and ampicillin-resistant. In the principal component analysis, the *S. chromogenes* isolates are further grouped according to cluster analysis (Fig. 1).

MLVA. The *S. chromogenes* isolates were used to perform the MLVA, and 63 VNTR loci were identified in the genome of *S. chromogenes* MU970. Six were used for the *S. chromogenes* isolates, and the amplification of four loci was achieved in the 23 isolates (Table V). From the VNTR, the 23 isolates were grouped using the Unweighted Pair Group Method with Arithmetic

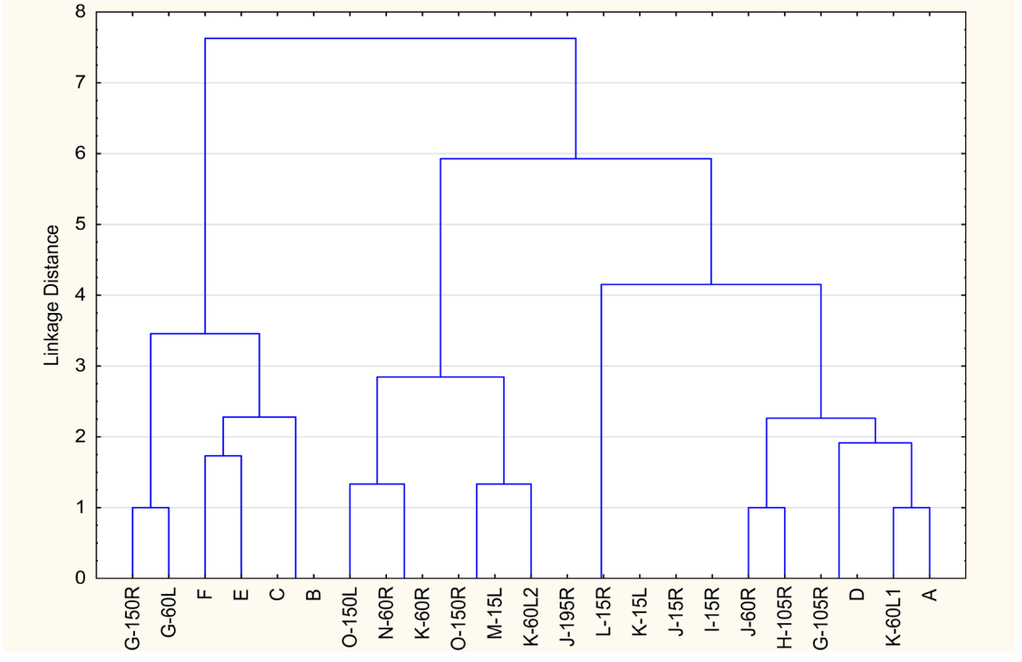

Fig. 1. Cluster analysis according to the phenotypic characteristics and chemotherapeutic susceptibility of 23 *S. chromogenes* strains according to Ward's minimum variance method.

Table III
Biochemical tests results with Api Staph System and antibiograms.

Strain	Origin	Goat id	Farm	Production system	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	Guanajuato	A	1	Extensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	R	S	S	S	S
2	Guanajuato	B	1	Extensive	+	+	+	–	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	S	S	S	S
3	Guanajuato	C	1	Extensive	+	+	+	–	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	S	S	S	S
4	Guanajuato	D	1	Extensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	R	S	S	S
5	Guanajuato	E	1	Extensive	+	+	+	–	–	+	+	–	–	+	+	–	–	–	+	–	–	+	+	S	S	S	S	S
6	Guanajuato	F	1	Extensive	+	+	+	–	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	S	R	S	S	S
7	Querétaro	G-105R	2	Intensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	S	R	S	S	S
8	Querétaro	H-105R	2	Intensive	+	–	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	S	R	S	S	S
9	Querétaro	G-60L	2	Intensive	+	+	–	–	+	+	+	–	–	+	+	–	–	–	+	–	+	+	–	S	S	S	S	S
10	Querétaro	G-150R	2	Intensive	+	+	–	–	+	+	+	–	–	+	+	–	+	–	+	–	+	+	–	S	S	S	S	S
11	Querétaro	I-15R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	R	R	S	S	S
12	Querétaro	J-15R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	R	R	S	S	S
13	Querétaro	K-15L	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	R	R	S	S	S
14	Querétaro	J-60R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	S	R	S	S	S
15	Querétaro	K-15L1	3	Intensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	+	+	R	R	S	S	S
16	Querétaro	K-15L2	3	Intensive	+	+	–	+	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	R	R	S	S	S
17	Querétaro	K-15R3	3	Intensive	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	S	R	S	S	S
18	Querétaro	L-15R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	I	R	S	S	S
19	Querétaro	J-195R	3	Intensive	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	+	+	I	R	S	S	S
20	Querétaro	M-15L	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	R	R	S	S	S
21	Querétaro	N-60R	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	S	R	S	S	S
22	Querétaro	O-150L	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	–	–	+	+	I	R	S	S	S
23	Querétaro	O-150R	3	Semiintensive	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	–	+	+	R	R	S	S	S

The physiological tests used with *S. chromogenes* were:
1. Glucose; 2. Fructose; 3. Manose; 4. Maltose; 5. Lactose; 6. Trehalose; 7. Manitol; 8. Xilitol; 9. Mellobiose; 10. Nitrates; 11. Alkaline Phosphatase; 12. Voges-Proskauer; 13. Rafifnose; 14. Xylose; 15. Saccharose; 16. Metil-Gluco-Pyranosidase; 17. N-Acetyl-Glucose; 18. Arginin Dihydrolase; 19. Urea; 20. Polymyxin B; 21. Ampicillin; 22. Gentamicine; 23. Tobramycine and 24. Tetracycline.

Table IV
The genes identified in the isolated CNS.

The gen coding for	*S. chromogenes*		*S. simulans*		*S. xylosus*		*S. sciuri*		*S. warneri*	
	+/total	%	+/total	%	+/total	%	+/total	%	+/total	%
Aureolysin	23/23	100	5/18	27.78	4/9	44.44	2/2	100	0/1	0
Hemolysin	23/23	100	2/18	11.11	0/9	0	0/2	0	0/1	0
Extracellular protein	23/23	100	18/18	100	9/9	100	2/2	100	1/1	100
Zin metaloprotease	23/23	100	18/18	100	9/9	100	2/2	100	1/1	100
Surface protein SasH	23/23	100	18/18	100	6/9	66.67	2/2	100	0/1	0
TRAP	23/23	100	3/18	16.67	3/9	33.33	2/2	100	0/1	0
B Antigen	23/23	100	2/18	11.11	0/9	0	0/2	0	0/1	0
Virulence B factor	22/23	95.65	0/18	0	3/9	33.33	2/2	100	1/1	100
FemA protein	23/23	100	18/18	100	9/9	100	2/2	100	0/1	0

Table V
Number of VNTR detected by capillary electrophoresis.

Strain	Origin	Farm	Production system	266_07	VNTR	346_06	VNTR	360_06	VNTR	854_08	VNTR
1	Guanajuato	1	Extensive	955	27	1092	73	1187	40	1101	11
2	Guanajuato	1	Extensive	955	27	1166	78	1156	39	301	3
3	Guanajuato	1	Extensive	963	27	1153	77	1183	39	1101	11
4	Guanajuato	1	Extensive	953	26	1161	77	1181	39	1077	11
5	Guanajuato	1	Extensive	952	26	1159	77	1181	39	1210	13
6	Guanajuato	1	Extensive	936	26	1164	78	1175	39	1201	13
7	Querétaro	2	Intensive	453	13	1181	79	1175	39	329	3
8	Querétaro	2	Intensive	453	13	1177	78	1177	39	329	3
9	Querétaro	2	Intensive	1075	30	1176	78	1176	39	1221	13
10	Querétaro	2	Intensive	1080	30	1178	79	1182	39	1229	13
11	Querétaro	3	Intensive	384	11	1155	77	1176	39	329	3
12	Querétaro	3	Intensive	383	11	1157	77	1178	39	329	3
13	Querétaro	3	Intensive	385	11	1156	77	1172	39	329	3
14	Querétaro	3	Intensive	382	11	1164	78	1181	39	204	2
15	Querétaro	3	Intensive	382	11	1150	77	1181	39	329	3
16	Querétaro	3	Intensive	382	11	1155	77	1183	39	330	3
17	Querétaro	3	Intensive	385	11	1178	79	1181	39	329	3
18	Querétaro	3	Intensive	989	27	1158	77	1181	39	329	3
19	Querétaro	3	Intensive	384	11	1156	77	1178	39	329	3
20	Querétaro	3	Semi-intensive	383	11	1168	78	1186	40	328	3
21	Querétaro	3	Semi-intensive	386	11	1163	78	1186	40	205	2
22	Querétaro	3	Semi-intensive	872	24	1192	79	1185	40	329	3
23	Querétaro	3	Semi-intensive	0	0	1187	79	1186	40	329	3

Mean (UPGMA) and the Pearson correlation coefficient. An 85.7% similarity was obtained for the 23 isolates, which were then grouped in ten genogroups and divided into two larger groups. Per the MLVA results, the six Guanajuato isolates are grouped with four isolates from Queretaro with a 93.2% similarity, while the 13 remaining isolates from Queretaro are grouped with a 90.4% similarity (Fig. 2). The discrimination ability of the MLVA was determined using the discrimination index (*D*) for the 23 isolates, which showed discrimination level with a D value of 0.8893. The discrimination power of each VNTR was estimated from the number of alleles detected and their diversity. The highest diversity rates using the Simpson and Hunter-Gaston index were 0.926 and 0.968, respectively, for the 346_06 locus, whereas the lowest diversity indexes were from the 854_08 locus with rates of 0.654 and 0.684, respectively (Table VI).

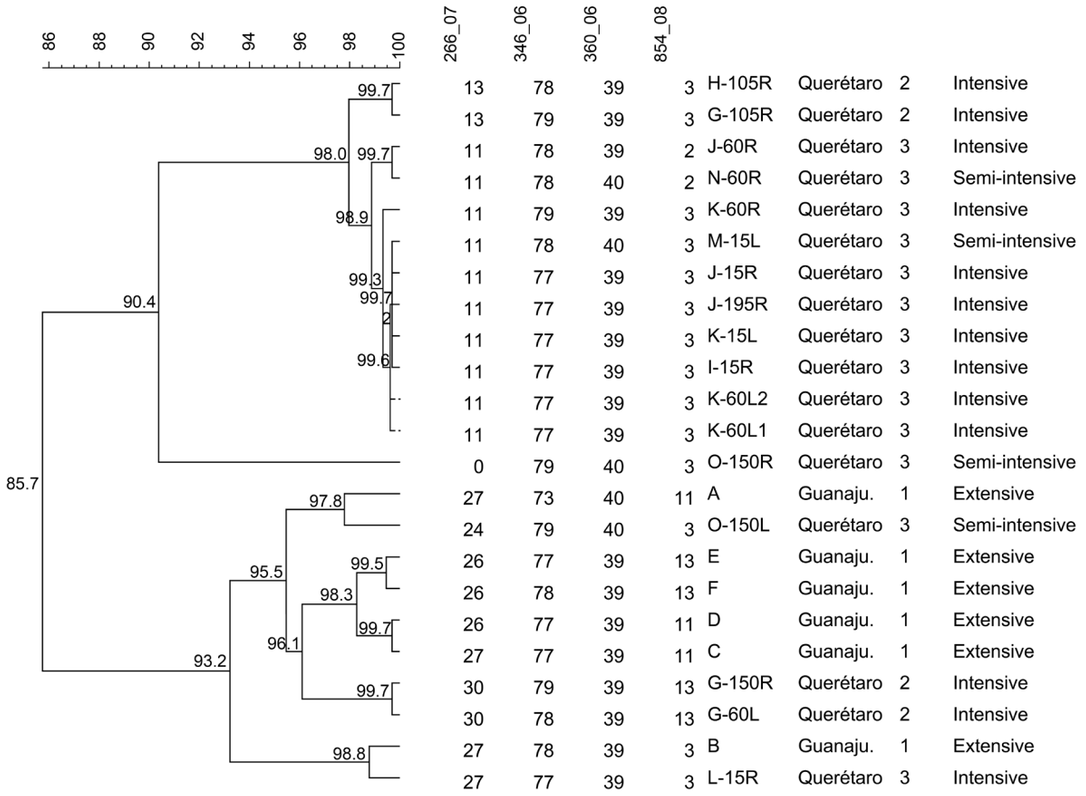

Fig. 2. Cluster analysis according to the VNTR of 23 strains of *S. chromogenes* using Pearson's correlation coefficient and the UPGMA algorithm.

Discussion

The *Staphylococcus* genus is commonly isolated from mastitis cases in ruminants. Subclinical mastitis in goats is primarily caused by coagulase-negative *Staphylococcus*, which is considered a minor and opportunistic pathogen (Bergonier *et al.*, 2003; Vliegher *et al.,* 2004; Schukken *et al.,* 2009; Taponen and Pyorala, 2009). This group can cause mastitis due to its many virulence factors, both in human and animal isolates. Some of these virulence genes are shared with *S. aureus*, such as haemolysins, leucocidins, toxins, biofilm formation and adhesins (Pyörälä and Taponen, 2009; Taponen and Pyorala, 2009; Vergnaud and Pourcel, 2009; Park *et al.*, 2011; Supré *et al.*, 2011; Vanderhaeghem *et al.*, 2015). In this study, some of the virulence factors that are shared with *S. aureus* corresponded to aureolysin, extracellular metalloproteinase, and SasH protein. Although CNS species are less virulent than *S. aureus*, the different virulence factors in this group could influence their clinical features and the persistence of intramammary infections, which should be regarded as pathogenic and are not part of the normal microbiota (Haveri *et al.*, 2007; Schukken *et al.*, 2009; Taponen and Pyorala, 2009). We identified the isolates of *S. chromogenes* with *S. chromogenes* MU970 genes, which is presently the only reported sequence and was isolated from a bovine mastitis case in the United States.

Table VI
Diversity Index (Simpson and Hunter-Gaston) and confidence intervals for each VNTR loci for *S. chromogenes.*

Locus	Size [bp]	Simpson index	Confidence intervals 95%	Hunter-gaston index	Confidence intervals 95%
266_07	36	0.87	0.803–0.936	0.909	0.843–0.975
346_06	15	0.926	0.896–0.957	0.968	0.938–0.999
360_06	30	0.756	0.650–0.862	0.791	0.684–0.897
854_08	96	0.654	0.449–0.859	0.684	0.479–0.888

This type of CNS is the primary cause of subclinical mastitis in goats worldwide (Ruiz *et al.*, 2013). In this work, we detected adhesins, antigens associated with the cell wall and haemolysins, which are shared with other species of CNS. From these results and those in the literature, the presentation of the mammary gland disease and the resultant damage is influenced by the virulence factors that are present in the bacteria. Because there are no studies that focus on a single species in this group, it is important to know the virulence factors of CNS mastitis in ruminants to develop control measures and disease prevention (Schukken *et al.*, 2009; Sampaio *et al.*, 2015; Vanderhaeghem *et al.*, 2015). From the identification of these virulence genes in *S. chromogenes*, it is clear that the mastitis caused by this bacterium is significant; therefore, we developed the first specific MLVA for *S. chromogenes* isolates. Other studies used different genotyping methods rarely focus on a particular species; for example, authors have standardized genotyping methods for *S. epidermidis* isolates from bovine milk and human skin using PFGE, where five patterns were obtained. One of these skin isolates showed a pattern common to animal milk, indicating that it may be zoonotic (transmitted from humans to animals) (Thordberg *et al.*, 2006). For *S. chromogenes*, Shimizu *et al*. (1997) reported genotyping by PFGE of 138 *Staphylococcus hyicus* isolates from pigs, chickens, cows and goats, and 21 isolates of *S. chromogenes* obtained from pigs and dairy cows with mastitis. The patterns obtained for *S. hyicus* were different according to the animal's country of origin, whereas the *S. chromogenes* patterns were more conservative; however, the authors managed to differentiate them from the *S. hyicus* isolates (Shimizu *et al*., 1997). The MLVA presented here, could be used for molecular epidemiology studies on *S. chromogenes*, as well as other CNS species, since other species, identified in this work, were also isolated from subclinical mastitis cases in goats. Therefore, this analysis would be useful to examine CNS isolates from both animals and humans, in different geographic locations, in order to compare the genetic diversity within this group and standardize genotyping techniques. It is worth noting the importance of genotyping methods, as per the results of our cluster analysis that revealed three large clusters. Two of them comprised isolates from Queretaro and Guanajuato, whereas the third cluster contained only isolates from Queretaro. The main disadvantage of these phenotypic identification methods is that they determine biochemical and/or physiological traits. These phenotypic methods represent the first tool for comparing microorganisms and are an important tool for characterizing many pathogens; however, phenotypic traits are susceptible to environmental influence, which can cause variation in gene expression. Therefore, these results would be less stable, less reproducible and less discriminatory, making it difficult to determine whether the isolates have the same genetic pattern (Vilchez and Alonso, 2009). In this study, the MLVA classified the six isolates from Guanajuato within a cluster with four isolates from Queretaro, whereas the rest of the isolates from the two farms of Queretaro are grouped in a second cluster. In addition, isolates from the same goat were obtained on different lactation days, which indicates that the bacteria can survive for long periods of time in the mammary gland. In addition, isolates from different animals on the same farm belonged to the same genogroup, indicating that the isolates were from the same strain, and transmission was likely due to improper hygiene and/or management practices during milking. Furthermore, these results indicate that the genogroups of *S. chromogenes* differ based on geographical location since the genotypic identification methods developed in this work can be used to classify closely related or divergent microorganisms. Thus, the knowledge derived from epidemiological studies can determine the natural history of a disease, its aetiology, occurrence frequency, distribution, pathways and spreading patterns, reservoirs and factors that increase the risk of contracting the disease (Vilchez and Alonso, 2009). The discrimination index of the *S. chromogenes* MLVA was 0.8893, indicating that this method is capable of distinguishing between different isolates, because a D value of 1 indicates that the genotyping method is able to distinguish each member of the population from any other member of the same population, while an index of 0 indicates that all members of the population are identical, and a 0.50 index indicates that if an isolate is chosen randomly, there is a 50% chance that the next isolate chosen will be indistinguishable from the first (Hunter and Gaston, 1988; Zaluga *et al*., 2013). The highest discrimination rate pertained to the 346_06 locus, representing a viable option for genotyping the *S. chromogenes* isolates, since this locus present more variability. The MLVA has several advantages, such as the use of common and inexpensive laboratory equipment. One of the most important features of the MLVA is that analysis of a limited number of loci can provide information about the diversity that exists within the species, which is crucial for typifying different bacteria (Vergnaud and Pourcel, 2009). Tandem repetitions are useful as molecular markers because they represent one of the most diverse genomic loci in bacterial populations. They are made of smaller sequences known as repetition units or motifs, which are repeated in tandem and vary in their number of repeated copies (Van Belkum *et al.*, 1998; Vogler *et al.*, 2006,). These repeated copies result from insertion mutations and/or deletions, so they can gain or lose several of these repetition units and create many alleles. It has been pro-

posed that these mutations occur primarily due to a mismatch by strain landslides, but recombination events also occur (Taylor *et al.*, 2000; Vogler *et al.*, 2006). Most VNTR do not have any phenotypic effects and generate neutral genetic variations, so they have not been associated with biological effects (Vogler *et al.*, 2006). The speed of these repetition sequences remains unclear, so studies to determine the mutation rate would facilitate molecular epidemiologic investigations. In conclusion, the MLVA presented in this paper is an easy and viable method of typying CNS isolates from mastitis cases from different regions and is an ideal option for tracking this disease.

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