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ARTÍCULO DE INVESTIGACIÓN

Antimicrobial resistance of *Streptococcus* spp. isolated from bovine clinical mastitis in Argentinean Mar y Sierras Region Dairy Farms

Resistencia antimicrobiana de *Streptococcus* spp. aislado de mastitis clínica en tambos de la Región Mar y Sierras, Argentina

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SUMMARY

Streptococcus dysgalactiae, and *S. uberis* are the most prevalent *Streptococcus* species isolated from clinical mastitis. Most of all antimicrobials administered on dairy farms are for treating mastitis. Considering that antimicrobial resistance can vary between regions, it is crucial to monitor the susceptibility of microorganisms to antimicrobials used. Particularly, it is known that clinical mastitis caused by *S. uberis* frequently do not respond to antimicrobial therapy and that this pathogen causes recurrent infections. The general aim was to evaluate the antimicrobial susceptibility of *S. uberis* and *S. dysgalactiae* isolates from 23 dairy farms located in the *Cuenca Mar y Sierras*, Argentina, for the main antimicrobials used in this region, and characterize the dairy farms from which the pathogens come. A total of 39 *S. uberis* and *S. dysgalactiae* strains were isolated and tested for susceptibility to 5 antibiotics using a disc diffusion method. Results showed frequencies of AMR to tetracycline of 26 %, pirlimycin 18 %, rifaximin 15 %, penicillin 8 %, and kanamycin 5 %, the detection of resistance to all the antibiotics in *S. uberis*, and that the multi-resistant isolates belonged to dairy farms that carry out antimicrobial treatments in an empirical manner.

Key words: (*Streptococcus uberis)*, (*S. dysgalactiae)*, (mastitis), (antimicrobial resistance), (dairy farms)

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RESUMEN

Streptococcus dysgalactiae y *S. uberis* son las especies de *Streptococcus* más frecuentemente aisladas de mastitis clínica. La mayoría de los antimicrobianos administrados en los tambos se usan para tratar infecciones intramamarias. Teniendo en cuenta que la resistencia a los antimicrobianos puede variar entre regiones, es crucial monitorear la susceptibilidad de los microorganismos a los antimicrobianos utilizados. En particular, se sabe que la mastitis clínica causada por *S. uberis,* con frecuencia, no responde fácilmente a la terapia antimicrobiana y, que este patógeno tiende a causar infecciones recurrentes. El objetivo fue evaluar la susceptibilidad antimicrobiana de aislamientos de *S. uberis* y de *S. dysgalactiae* obtenidos en 23 tambos de la Cuenca Mar y Sierras, Argentina, y caracterizar los tambos de donde provienen. Se aislaron 39 cepas de *S. uberis* y *S. dysgalactiae* y se analizó la susceptibilidad a 5 antibióticos mayormente utilizados en la región mediante difusión en disco. Los resultados mostraron frecuencias de resistencia a tetraciclina de 26 %, pirlimicina 18 %, rifaximina 15 %, penicilina 8 % y kanamicina 5 %, la detección de resistencia a todos los antibióticos en *S. uberis* y que los aislamientos multirresistentes pertenecieron a tambos que realizan tratamientos antimicrobianos de manera empírica.

Palabras clave: (*Streptococcus uberis)*, (*S. dysgalactiae)*, (mastitis), (resistencia antimicrobiana), (tambos)

INTRODUCTION

Bovine mastitis is the most common disease in the dairy industry. In addition to negatively impacting production efficiency and quality, it is an important animal health and welfare issue[12]. Mastitis is a clear example of a multifactorial disease, where many factors are involved in its presentation. It is controlled using diverse management and intervention practices. Its occurrence is predisposed by an interaction among the causative agent, the host, and the environment[23].

Several microorganisms are implicated in mastitis infection, some are environmental pathogens, whereas others are contagious ones[24]. *Streptococcus* species frequently reported to cause mastitis are *S. agalactiae*, *S. dysgalactiae*, and *S. uberis*. Although both *S. dysgalactiae* and *S. uberis* can have contagious transmission[31], they are ubiquitous in dairy farms and can survive for long periods in the environment; hence, these organisms are difficult to eradicate. These two pathogens are the most prevalent *Streptococcus* species isolated from cases of clinical mastitis and are also commonly found in subclinical infections. Furthermore, *S. uberis* accounts for more than 15 percent of all mastitis cases mainly occurring during the dry period and early lactation[28].

At present, intramammary antibiotics are the first-line treatment for bovine mastitis; approximately 60–70 % of all antimicrobials administered on dairy farms are for treating mastitis[5,27]. In Argentina, antimicrobial intramammary administration in lactating dairy cows accounted for the 85 % of total drug usage[9]. In recent years, the ability of pathogens to resist antimicrobial agents has become a serious problem. A study demonstrated that approximately 62 % of isolated mastitis-causing agents are resistant to at least one antimicrobial agent[11]. In Argentina, information about veterinary antimicrobial drug usage (DU) at herd level is still limited, and there are no veterinary medical products sales statistics or official surveillance programs of veterinary antimicrobial consumption. Previous studies had demonstrated that beta-lactams, macrolides and aminoglycosides are the most frequently used intramammary compounds in dairy farms of Argentina, while other antibacterial drugs such as sulfonamides, quinolones and oxytetracyclines preparations are mostly administered to treat other diseases[9,20]. The World Organization for Animal Health (OIE, 2016)[30] informed that, between 2010 and 2015, tetracyclines and macrolides were the two classes of antibiotics most commonly used in animals worldwide.

Considering that antimicrobial resistance can vary between regions and even within the same region, it is crucial to monitor the susceptibility of microorganisms to common antimicrobials used. Particularly, it is known that clinical mastitis caused by *S. uberis* frequently do not respond easily to antimicrobial therapy and that this pathogen causes recurrent infections, being a permanent barrier to control mastitis[16].

The general aim of this study was to evaluate the antimicrobial susceptibility (AMS) of *Streptococcus uberis* and *S. dysgalactiae* isolated from dairy farms located in the *Cuenca Mar y Sierras*, Argentina, for the main antimicrobials used in this region, and characterize the dairy farms from which the pathogens were isolated.

MATERIALS AND METHODS

Dairy farms management and characteristics

Isolates were recovered from cows with clinical mastitis (CM) that belonged to 23 dairy farms located, primarily, in the *Cuenca Mar y Sierras*, except four (farms 2, 4, 10, and 23), between 2017 and 2022. The management and dairy farms characteristics such as location, dairy cattle housing, dairy cattle breed, number of milking cows, dairy milk yield, intramammary treatment application, bulk somatic cell count (BSCC), use of antimicrobials drugs, milking equipment, use of dairy herd improvement programs (DHI), and milking routine type were recorded (Table 1).

Isolation and biochemical identification of *Streptococcus uberis* and *Streptococcus dysgalactiae*

Milk samples were collected from quarters with abnormal milk by trained farm personnel using the following procedures: (1) affected teat were dipped in a pre-milking disinfectant solution; (2) after 30 seconds, the teat was wiped with a disposable paper towel; (3) the first 3–4 milk streams were discarded and the teat end was scrubbed using a gauze soaked in alcohol 70 %; (4) milk samples were collected into a sterile 15-mL tube and transported to the laboratory in isothermal boxes with ice.

At the laboratory, a loop of milk sample was streaked on blood agar and incubated at 37 °C for 48 hs in aerobiosis. Colonies suspected to be *Streptococcus* spp. (small, pinpoint white or grey translucent, haemolytic or not), were Gram stained and streaked into a slant agar for catalase test, NaCl and bile esculin test, Christie-Atkins-Munch-Peterson (CAMP) test, esculin, hippurate, inulin and sorbitol tests as described by the NMC (National Mastitis Council, 2017)[19]. Isolates identified as *S. uberis* and *S. dysgalactiae* were stored at -20 ºC.

Molecular Identification

Biochemical identification of *S. uberis* isolates was confirmed by PCR-based amplification of the species-specific *pau* gene and, of *S. dysgalactiae*, by amplification of *16S rRNA* gene[22]. The DNA template was obtained by boiling frozen bacteria suspended in sterile water for 10 min. The PCR products were separated in 2 % agarose gel stained by ethidium bromide and visualized in a UV transilluminator.

Antimicrobial susceptibility testing

All the isolates were tested for susceptibility to 5 antibiotics used extensively in dairy farms using a disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI)[3] instructions. A bacterial suspension in sterile saline solution from an overnight pure culture, adjusted to a turbidity of 0.5 on the McFarland scale, was inoculated on a Muller-Hinton agar (Britania, CABA, Argentina) plate, supplemented with 5 % sheep blood. Antibiotic discs were placed on the agar surface and plates were incubated overnight (16-18 h) at 37 °C in atmosphere with 5 % CO_2. The diameters of the zones of inhibition were then measured and data were categorized according to the animal (or human-derived if not available) interpretive criteria of CLSI (2018)[3] supplements VET08 and CLSI M100 (2019)[4], or manufacturer' instructions (Rifaximin). Since no kanamycin official standards are available, only isolates presenting no zones of inhibition were considered as resistant (Table 2). The following discs were used: pirlimycin (2 µg), penicillin (10 units), tetracycline (30 µg), kanamycin (120 µg) and rifaximin (30 µg). Isolates that showed resistance to three or more antibiotic classes were considered multidrug resistant (MDR)[25].

Table 1. Management characteristics of *Cuenca Mar y Sierras* dairy farms from which *Streptococcus uberis* and *S. dysgalactiae* isolates come.

REFERENCE	LOCATION	DAIRY CATTLE HOUSING	DAIRY CATTLE BREED	MILKING CATTLE (N)	AVERAGE MILK YIELD l/cow/day	INTRAMMARY TREATMENT APPLICATION	BULK SSC	ANTIMICROBIAL USE (*)	MILKING EQUIPMENT	DHI	MILKING ROUTINE
TBO1	GARDEY - TANDIL	GRAZING LAND	CROSSBREED	400	19	EMPIRICAL	300000	YES	HERRINGBONE	NO	COMPLETE
TBO2	PUAN	GRAZING LAND	CROSSBREED	350	19	EMPIRICAL	260000	YES	HERRINGBONE	NO	COMPLETE
TBO3	GARDEY - TANDIL	DRY LOT	AMERICAN HOLSTEIN	550	38	OFC	220000	YES	ROTARY	NO	COMPLETE
TBO4	LAMADRID	GRAZING LAND	CROSSBREED	600	19	OFC	250000	YES	HERRINGBONE	NO	INCOMPLETE
TBO5	BASE AEREA TANDIL	GRAZING LAND	AMERICAN HOLSTEIN	350	28	EMPIRICAL	320000	YES	HERRINGBONE	YES	COMPLETE
TBO6	BASE AEREA TANDIL	GRAZING LAND	CROSSBREED	400	19	EMPIRICAL	450000	YES	HERRINGBONE	NO	INCOMPLETE
TBO7	NAPALEUFU – BALCARCE	GRAZING LAND	CROSSBREED	700	20	EMPIRICAL	400000	YES	HERRINGBONE	YES	COMPLETE
TBO8	DE LA GARMA - A. G. CHAVES	DRY LOT	AMERICAN HOLSTEIN	700	32	OFC	180000	YES	HERRINGBONE	NO	COMPLETE
TBO9	BALCARCE	GRAZING LAND	AMERICAN HOLSTEIN	240	25	EMPIRICAL	250000	YES	HERRINGBONE	YES	COMPLETE
TBO10	JUNIN	GRAZING LAND	AMERICAN HOLSTEIN	600	27	OFC	350000	YES	HERRINGBONE	NO	COMPLETE
TBO11	BASE AEREA TANDIL	GRAZING LAND	CROSSBREED	700	19	OFC	300000	YES	HERRINGBONE	YES	COMPLETE
TBO12	BARKER - B. JUAREZ	GRAZING LAND	AMERICAN HOLSTEIN	700	28	OFC	230000	YES	HERRINGBONE	YES	COMPLETE
TBO13	NAPALEOFU - BALCARCE	GRAZING LAND	CROSSBREED	400	17	OFC	240000	YES	HERRINGBONE	NO	INCOMPLETE
TBO14	NAPALEOFU - BALCARCE	GRAZING LAND	CROSSBREED	400	17	OFC	300000	YES	HERRINGBONE	NO	INCOMPLETE
TBO15	AZUCENA - TANDIL	GRAZING LAND	AMERICAN HOLSTEIN	400	25	EMPIRICAL	250000	YES	HERRINGBONE	NO	COMPLETE
TBO16	RAUCH	GRAZING LAND	CROSSBREED	800	18	EMPIRICAL	200000	YES	HERRINGBONE	NO	INCOMPLETE
TBO17	PASTORA - TANDIL	GRAZING LAND	AMERICAN HOLSTEIN	500	23	OFC	250000	YES	HERRINGBONE	NO	COMPLETE
TBO18	NAPALEOFU - BALCARCE	GRAZING LAND	CROSSBREED	800	19	OFC	230000	YES	HERRINGBONE	NO	INCOMPLETE
TBO19	VELA - TANDIL	CBPB	AMERICAN HOLSTEIN	240	40	OFC	220000	YES	HERRINGBONE	NO	COMPLETE
TBO20	OLAVARRIA	GRAZING LAND	CROSSBREED	400	18	EMPIRICAL	400000	YES	ROBOT	NO	INCOMPLETE
TBO21	CHILLAR - AZUL	GRAZING LAND	AMERICAN HOLSTEIN	380	24	EMPIRICAL	400000	YES	HERRINGBONE	NO	COMPLETE
TBO22	NAPALEOFU - TANDIL	GRAZING LAND	AMERICAN HOLSTEIN	550	26	OFC	400000	YES	HERRINGBONE	NO	COMPLETE
TBO23	BELGRANO	GRAZING LAND	AMERICAN HOLSTEIN	800	24	EMPIRICAL	230000	YES	HERRINGBONE	YES	COMPLETE

CBPB: compost bedded pack barns; OFC: on farm culture; SCC: somatic cell count: (*) betalactamics, macrolides, rifaximin, ubrolexin (kanamycin + cephalexin) use; DHI: Dairy Herd Improvement

Table 2. Antimicrobial susceptibility tested in studied *Streptococcus uberis* and *S. dysgalactiae* isolates and the used breakpoints.

Antibiotic	Concentration disc	Zone diameter (mm)	
		S	R
Aminoglycosides – Kanamycin (Britania)	120 µg	Without reference	
Beta -lactams – Penicillin (Britania)*	10 U	≥24	
Lincosamides – Pirlimicyn (Oxoid)**	2 µg	≥13	≤12
Pyridoimidazols – Rifaximin (Sigma -Aldrich)***	30 µg	≥17	≤13
Tetracyclines – Tetracycline (Britania)*	30 µg	≥23	≤18

*CLSI (2019); ** CLSI (2018); *** Antimicrobial manufacturer' instructions; S: susceptible; R: resistant.

Data analysis

A cluster analysis based on antimicrobial resistance profiles was carried out using the UPGMA clustering method. The dendrogram was generated using the BioNumerics v.6.6 software.

RESULTS AND DISCUSSION

A total of 39 *S. uberis* and *S. dysgalactiae* strains were isolated and identified by biochemical methods and further confirmed by PCR, 79 % (31) were *S. uberis*, and 21 % (8) to *S. dysgalactiae*.

American Holstein/ Jersey crossbreds (N=11) and American Holstein (N=12) cows were the breeds present in the dairy farms. One dairy farm housed their lactating cows in compost-bedded pack barns (CBPB), two in dry-lots and the remaining in grazing land or pasture. The CBPB is a housing system characterized by an open resting area (free of stalls or partitions) and bedded with organic materials (e.g., straw), which must be mechanically stirred on a regular basis. In pasture-based dairy farm systems, the pasture itself is a primary reservoir of *S. uberis,* and the original infection usually occurs between milking. So, improving environment conditions such as alleys, penning pens, water drinkers rounding, renewing of bedding material, rotating heavily used pastures, and manure removal will help control this type of infection[5,17]. *S. dysgalactiae* has been isolated not only from the udder but also from many sites of the cow, including the tonsils, genital tract, rumen, rectum and coat, showing that it can almost live anywhere in the cow, in addition to farm bedding[13]. Also, *S. uberis* and *S. dysgalactiae* have been also isolated from the cattle fly *Hidrotea irritans* which appears to play a significant role in the establishment and maintenance of bacterial contamination of teats of healthy cattle, in countries where this head fly is present[2,18]. On the other hand, *S. uberis* was also found in the fly *Musca domestica*, from milking parlors[8]. Both species have characteristics of both contagious and environmental pathogens[21].

Seventy-eight percent (78 %) of the farms had 400 or more milking cows. The predominant milking management of the dairy farms was a complete milking routine by pre-dipping and forestripping, use of individual paper or cloth towel to dry udders, wore gloves and use of post-dipping disinfectant. All herds milked their cows in herringbone milking parlors, except for a dairy farm that had a rotary milking system and a dairy farm which had a robotic milking system. Milk yield was between 17 and 40 litters per cow and the bulk somatic cell counts were between 180000 and 400000 cel/ml.

Fifty-two percent (12/23) of the dairy herds routinely use on farm culture (OFC) system (for rational antimicrobial use) and all but one (farm 19) of them had more 400 or more milking cows. The remaining farms (44 %) carry out empirical antimicrobial treatments. Upon diagnosis of a clinical case of mastitis, the cow is rapidly assigned a severity score and a milk sample is obtained. After scoring and collection of the milk sample, the eligible cows (severity scores 1 and 2) are sent to a hospital pen for monitoring and to ensure that abnormal milk is discarded. The milk sample is used to set up an OFC and no antibiotic treatment is given until results of the OFC are known. Generally, after 24 hours, the culture plate is read and based on the results, a treatment protocol is assigned. Typical decisions that are made as a result of the OFC results include the decision to use an intramammary antibiotic, use a drug that has greater activity against Gram negative bacteria, extend the duration of treatment, or to withhold antibiotic treatment and discard milk while the immune system remove the infection. In the farms which did not use OFC, every cow detected with clinical mastitis received immediately intramammary antibiotics. The majority of the herds (74 %) were not enrolled in Dairy Herd Improvement (DHI) control programs to work with subclinical mastitis. Only 36 % of the dairy herds used DHI and monitored individual somatic cell counts. In the cases of clinical mastitis severity 1 and 2 is recommended to delay the antimicrobial treatment until laboratory results are obtained, in relation with gram-positive growth. On the contrary, for clinical mastitis severity 3, the recommendation is to be treated with antibiotics immediately[14, 15].

In Argentina, there are few data available on antimicrobial resistance level in *Streptococcus s*pecies that produce mastitis in dairy cattle, especially for *S. uberis* and *S. dysgalactiae,* since recently Hernandez *et al.*[10] published data on AMS of *S. agalactiae* isolates. Denamiel *et al*.[7], analysed resistance to penicillin, erythromycin, and clindamycin in both species, but in a low number of isolates (*S dysgalactiae*, 8; *S. uberis*, 3). We recorded that all dairy farms used beta-lactams, macrolides/lincosamides, rifaximin, and beta-lactam-aminoglycoside combinations, in agreement in part with González Pereyra *et al.*[9]. Particularly, in Argentina is a common practice the use of a product containing kanamycin in association with beta-lactam drug[10]. In order to have a regional view of antimicrobial resistance occurrence, all isolates were tested against five antimicrobials, which were selected taking into account their use for mastitis treatment in cattle in this region. Regarding to the aminoglycoside kanamycin, since not reference breakpoint values were available, only isolates presenting no inhibition area were considered resistant. Eight AMR (antimicrobial resistance) profiles were detected (Table 3), being more than 50 % of the isolates susceptible to all tested antimicrobial agents. The global frequencies of AMR observed in the present study to tetracycline were 26 %, pirlimycin 18 %, rifaximin 15 %, penicillin 8 %, and kanamycin 5 %. Regarding to *S. dysgalactiae*, 38 % of strains presented tetracycline resistance, and 12 %, rifaximin resistance. *S. uberis* isolates presented pirlimycin, tetracycline, rifaximin, penicillin and kanamycin resistance, 23 %, 23 %, 16 %, 10 % and 6 % respectively. Particularly, three *S. uberis* isolates showed penicillin resistance. Two of these three isolates plus another one showed AMR to more than 3 antibiotic groups, therefore they can be considered as multi drug resistance (MDR) isolates (Figure 1). All the MDR isolates were *Streptococcus uberis,* obtained from dairy farms which manage mastitis treatments in an empiric manner. Recently, Crespi *et al*.[6] detected penicillin resistance in *Streptococcus* but they did not report the species.

The resistance (black box) or susceptibility (white box) to penicillin, pirlimycin, tetracycline, rifaximin, and kanamycin, isolate name, species and dairy farm are shown.

Regarding to the oxytetracycline resistance, we detected it in 38 % of *S. dysgalactiae* and 23 % of *S. uberis.* Our results are in agreement with previous reports[20,30]. For lincosamides, the prevalence of resistance phenotypes previously reported was between 5.5 % and 56 %[1,26,29] meanwhile our data were of 26 % pirlimycin resistance. Kanamycin, pirlimycin, and tetracycline, added to erythromycin (macrolide) and clindamycin (lincosamide) resistance in *Streptococcus* has been reported previously in Argentina for *S. agalactiae*[10]. The pirlimicyn-resistance detected by us reinforces the premise about the careful use of this group of antibiotics.

The results emphasize the need to identify the pathogen that causes mastitis and carry out antimicrobial susceptibility tests in an established frequency before applying an antibiotic therapy in dairy farms. An accurate diagnosis will help improve antimicrobial usage, obtain better treatment response and greater bacteriological cure rates by strengthening the awareness about trends within the field.

Table 3. Distribution of antimicrobial resistance patterns among *Streptococcus uberis* and *S. dysgalactiae* isolates from dairy cattle with clinical mastitis in *Cuenca Mar y Sierras*, Argentina.

Antimicrobial resistance pattern	Total resistant isolates N (%)	Resistant *S. uberis* N (%)	Resistant *S. dysgalactiae* N (%)
S	22 (56)	18 (58)	4 (50)
TET	6 (15)	3 (10)	3 (38)
PYR	3 (8)	3 (10)	0
RIX	3 (8)	2 (6)	1 (1 2)
PEN-PYR-TET-RIX	2 (5)	2 (6)	0
PEN-KAN	1 (3)	1 (3)	0
PYR-TET	1 (3)	1 (3)	0
PYR-TET-RIX-KAN	1 (3)	1 (3)	0

S: susceptible to all tested antimicrobials; KAN: kanamycin; PEN: penicillin; PYR: pirlimycin; RIX: rifaximin; TET: tetracycline.

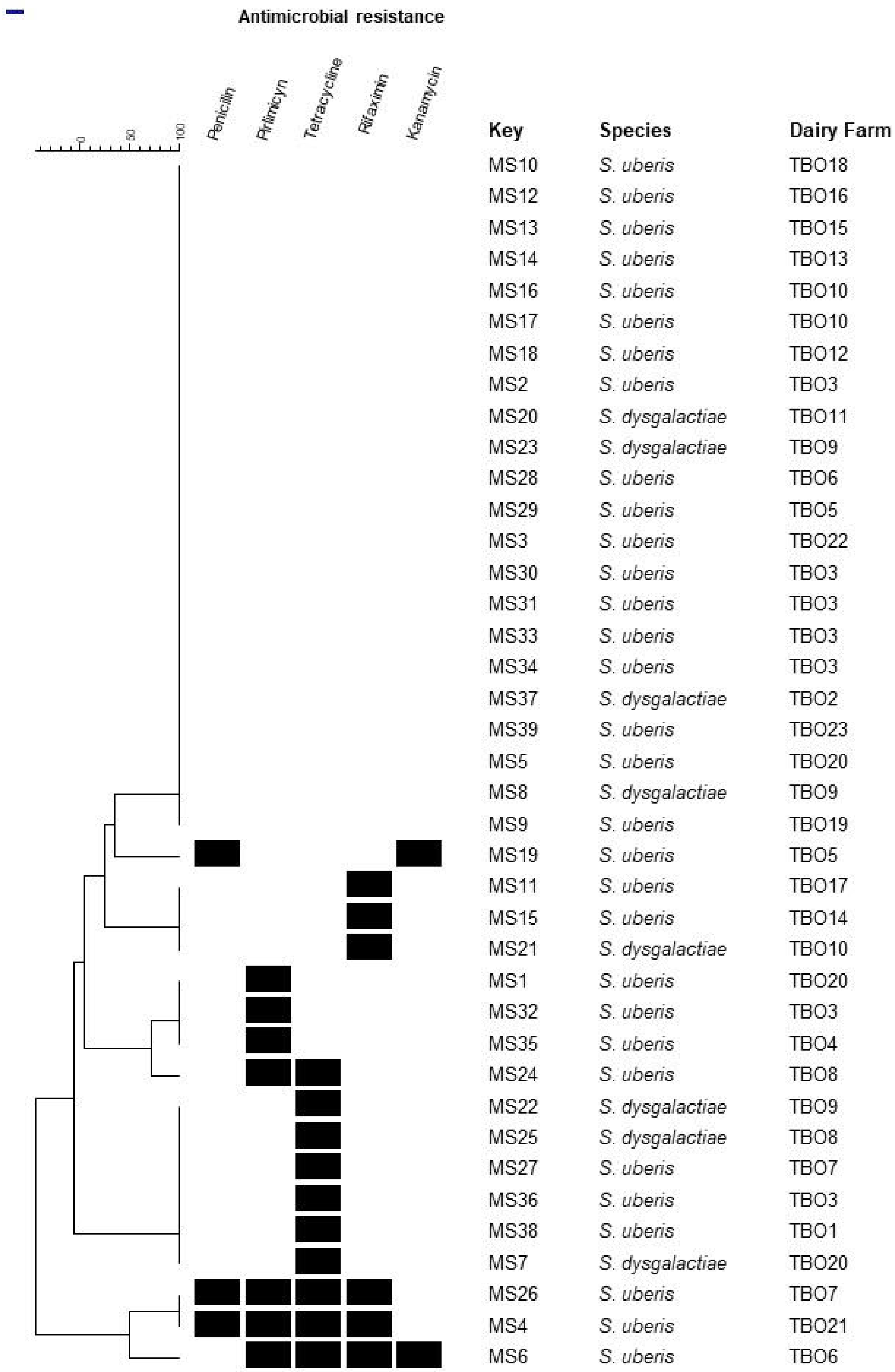

Figure 1. Cluster analysis of *Streptococcus* spp. isolates from *Cuenca Mar y Sierras* region, Argentina, based on antimicrobial resistance profiles.

CONCLUSIONS

These results provide the information on antimicrobial sensibility of *Streptococcus uberis* and *S. dysgalactiae* strains isolated from clinical mastitis in *Mar y Sierras Cuenca*, Argentina, and on management practices and structures of dairy farms in this region. Some of our main findings were the detection of resistance to all the antimicrobials agents tested *in S. uberis*, kanamycin, penicillin, pirlimicyn, rifaximin, and tetracycline, and that the multi-resistant isolates belonged to dairy farms that carry out antimicrobial treatments in an empirical manner.

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