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Characterization of *Mannheimia haemolytica* and *Pasteurella multocida* isolated from kids with respiratory problems

Caracterización de aislamientos de *Mannheimia haemolytica* y *Pasteurella multocida* obtenidos de cabritos con problemas respiratorios

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ABSTRACT

The objective of this study was to characterize and determine the presence of *Mannheimia haemolytica* and *Pasteurella multocida* isolated from kids with respiratory problems in Mexico. Convenience samplings were carried out in seven states of Mexico, obtaining 1.498 samples of nasal exudate from kids, the samples were inoculated on blood agar plates, and the isolates underwent biochemical tests to determine gender and species, finding 535 isolates, of which 491 were identified as *M. haemolytica* and 44 as *P. multocida.* Fifty isolates *of M. haemolytica* were randomly selected to be characterized with real-time PCR and endpoint PCR, confirming molecular identification in 46 of the 50 isolates. The capsular type and biotype of the *P. multocida* isolates were determined, finding that 36 isolates corresponded to type A and eight isolates to type D.

Keywords: *Mannheimia haemolytica, Pasteurella multocida*, kids, Mexico.

RESUMEN

El objetivo del presente estudio fue caracterizar y determinar la presencia de *Mannheimia haemolytica* y *Pasteurella multocida* aisladas de cabritos con problemas respiratorios en México. Se realizaron muestreos de conveniencia en siete estados de México, obteniéndose 1, 498 muestras de exudado nasal de cabritos, las muestras fueron inoculadas en placas de agar sangre, a los aislamientos se les realizaron pruebas bioquímicas para determinar género y especie, encontrando 535 aislamientos, de los cuales 491 fueron identificados como *M. haemolytica* y 44 como *P. multocida*. Se seleccionaron aleatoriamente 50 aislamientos de *M. haemolytica* para caracterizarlos con PCR tiempo real y PCR punto final, confirmándose la identificación molecular con ambas pruebas en 46 de los 50 aislamientos. A los 44 aislamientos de *P. multocida* se les determinó el tipo capsular y biotipo, encontrándose que, 36 correspondieron al tipo A y 8 al tipo D.

Palabras clave: *Mannheimia haemolytica, Pasteurella multocida*, cabritos, México.

INTRODUCTION

The goat population in Mexico exceeds 8.7 million head and has a carcass meat production of more than 77 thousand tons, and milk production of more than 160 thousand liters. The profitability of goat farming in Mexico lies in the production of meat for sale and consumption, as well as in the production of goat milk for human consumption and for making cheese and sweets (SADER, 2023).

Goat production is concentrated mainly in the arid and semi-arid zones, which account for 60 % of the country. Goat production (80 %) takes place in the extensive system, due to its low production costs, with the use of large arid areas of shrubs and grasses. The limitations it faces are large technological lags and serious sanitary problems (Cuéllar *et al*., 2012). According to the final product, there are three production models: suckling goat of 8 to 10 kg, fattened goat that have 40 to 45 kg, and for milk production (Andrade-Montemayor, 2017).

Although there is little information on aspects referring to goat health, it is known that respiratory diseases are important in Mexico, but diagnosis is rarely performed and prevention programs are established (Cuéllar *et al*., 2012). These problems are temporarily solved by applying broad-spectrum antibiotic treatments, which can later lead to bacterial resistance. Several factors are involved in the presentation of Ruminant Respiratory Infectious Respiratory Complex (RIRC), such as the environment, the conditions of the animal and the presence of infectious agents such as viruses and bacteria (Laishevtsev, 2020; Rahal *et al.*, 2014).
Mannheimia haemolytica and *Pasteurella multocida* are the bacteria frequently involved in respiratory ailments and they are considered the most important bacteria, affecting goats of all ages, with a wide distribution, appearing in temperate, subtropical and tropical climates (Rawat *et al.*, 2019; Amin, 2020). *P. multocida* and *M. haemolytica* have been observed to be responsible for outbreaks with septicemic or pneumonic presentation, causing death of lactating kids exposed to fatigue and chilling (Hakim *et al.*, 2014). Both microorganisms are part of the normal microbiota of the respiratory tract and oropharynx of ruminants, maintaining a symbiotic relationship with their host (Hakim *et al.*, 2014; Laishevtsev, 2020).

M. haemolytica capsule gives it the property of adherence (Laishevtsev, 2020; Wilson & Ho, 2013). In addition to the capsule, there are other virulence factors in M. haemolytica, one of them is leukotoxin, which is a pore-forming cytolysin that destroys ruminant leukocytes, and it is a 104-kDa exotoxin that is produced during the logarithmic phase of bacterial growth. The synthesis, activation and production of leukotoxin is regulated through a polycistronic operon, which contains the *lktC*, *lktA*, *lktB* and *lktD* genes (Oppermann *et al.*, 2017; Laishevtsev, 2020).

Sequencing of the *sodA* gene has been used for the identification of *Mannheimia* spp (Aulik *et al.*, 2010; Nefedchenkoa *et al.*, 2016). Regarding *P. multocida*, it has five capsular serogroups named: A, B, D, E and F (Wilson & Ho, 2013); few studies have been conducted in Mexico in this animal species; however, the presence of serogroups A and D has been reported (Blanco *et al.*, 1993). The objective of the present study was to characterize and determine the presence of *M. haemolytica* and *P. multocida* isolated from kids with respiratory problems in Mexico.

MATERIAL AND METHODS

Obtaining the samples

A non-probabilistic convenience sampling (Hernández, 2021) was performed during one year, in kids with respiratory problems presenting nasal and ocular secretion, cough, rales in thoracic auscultation, depression and fever. Samples (1,498) were obtained in San Luis Potosí (707), Baja California Sur (363), Coahuila (211), Puebla (148), Durango (35), Guerrero (20), Guanajuato (9) and Querétaro (5) states. Nasal exudates were collected using swabs with Ames transport medium with activated charcoal. The swabs were kept refrigerated at 4 ºC until the bacteriological study was performed in the laboratory.

Bacteriological isolation and bacterial identification

The bacteriological study of the nasal exudates obtained was carried out on blood agar and Mc Conkey agar plates, incubated at 37 ºC for 24 hours. Genus and species identification of isolates was performed according to colonial and microscopic morphology, Gram staining, sugar fermentation, H_2S, indole, urease and oxidase production (Legesse *et al.*, 2018).

DNA extraction

DNA extraction from bacterial isolates was performed using the technique with guanidine thiocyanate (Pitcher *et al.*, 1989). DNA concentration was quantified by spectrophotometry (SmartSpec-Plus spectrophotometer BIORAD Laboratories, USA) at an absorbance of 260/280. For isolates identified as *Mannheimia*, the species was determined using real-time PCR, identifying the *sodA* gene, using the primer pair designed by Guenther *et al.*, (2008). Strains identified as *M. haemolytica* had the presence of the *lktA* gene determined by PCR (Table 1).

Multiplex PCR for the detection of virulence genes associated with *P. multocida*

Isolates identified by bacteriology as *P. multocida* underwent multiplex PCR for genus using the *KMT*1 gene sequence, for the biotype of the strains, the presence of the *hyaD-hyaC* genes coding for capsular type A and the *dcbF* gene to identify type D was identified (Rawat *et al.*, 2019).

Determination of *P. multocida* somatic serotypes

The somatic serotype of *P. multocida* was determined by gel precipitation with specific antisera (Chengappa *et al.*, 1982).

Table 1. Oligonucleotide sequences used for *P. multocida* and *M. haemolytica*

	Gen	Primers	Sequence 5' to 3'	Amplicon size (bp)	Reference
M. haemolytica	*sodA*	SODA-FWD SADA-REV	AGCAGCGACTACTCGTGTTGGTTCAG AAGACTAAAATCGGATAGCCTGAAACGCCTG	143	Guenther 2008
	lktA	LKTA-FWD LKTA-REV	GGTGAAGGTTACGACCGAGTT CTTCACGGTTGCCCACTAATG	172	This study
P. multocida	*KMT1*	KMT1T7 KMT1SP6	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460	Rawat *et al.*, 2009
	hyaD-hyaC	CAPA-FWD CAPA-REV	TGCCAAAATCGCAGTCAG TTGCCATCATTGTCAGTG	1044	Townsend *et al.*, 2001
	dcbF	CAPD-FWD CAPD-REV	TTACAAAAGAAAGACTAGGAGCCC CATCTACCCACTCAACCATATCAG	657	Townsend *et al.*, 2001

RESULTS

Bacterial isolates from nasal swabs

From the 1.498 swabs of nasal exudate, which underwent bacteriological study, 535 isolates of bacteria of the *Pasteurellaceae* family were obtained, with a recovery rate of 36 %; of which 9 2 % (491/535) were identified as *M. haemolytica* and 8 % (44/535) as *P. multocida*. The distribution of the isolates obtained in each of Mexican Republic states where sampling was carried out is shown in Table 2.

Molecular characterization of *M. haemolytica*

From 491 isolates biochemically identified as *M. haemolytica*, 50 were randomly selected for characterization by real time PCR and end point PCR techniques, and they were classified by real time PCR to locate the *sodA* gene, obtaining amplification in 46 isolates, which were confirmed as *M. haemolytica*.

The 46 isolates confirmed by PCR as *M. haemolytica* were subjected to the endpoint PCR test to amplify a 172 pb segment of the *lktA* gene; 39 isolates were amplification positive.

Table 2. Isolates obtained from kids with respiratory problems in Mexican Republic states where samples were collected

State	Number of samples	Positive isolations *Mannheimia haemolytica*	Positive isolations *Pasteurella multocida*
San Luis Potosí	707	231 (47.1%)	4 (9.1%)
Baja California Sur	363	113 (23%)	9 (20.4%)
Coahuila	211	86 (17.5%)	16 (36.4%)
Puebla	148	34 (6.9%)	11 (25%)
Durango	35	10 (2%)	1 (2.3%)
Guerrero	20	16 (3.3%)	1 (2.3%)
Guanajuato	9	0	2 (22%)
Querétaro	5	1 (0.2%)	0
Total	1498	491 (12.8)	44 (2.9)

Determination of the capsular type of *P. multocida*

The 44 isolates identified by biochemical tests as *P. multocida*, when tested simultaneously for the detection of the *kmt* gene to confirm the genus and the *hyaD-hyaC* genes to determine the capsular type A, amplified a 460 pb segment, which indicated and confirmed that all belong to the genus *Pasteurella*. With respect to the capsular type, 36 isolates amplified a segment of 1044 pb, being thus characterized as belonging to capsular type A. The remaining eight isolates when tested for type D, amplified a band of 657 pb corresponding to the presence of the *dcbF* gene that determines their belonging to capsular biotype D (Figure 2).

Somatic serotypes of *P. multocida*

The eight isolates confirmed as *P. multocida* type D, upon IDD, showed a precipitation band for serotype 3, confirming that these isolates obtained from nasal swabs of kids belonged to the D:3 type. Reference strains of *P. multocida* serotypes D:3, A:3 and A:1 were used in this test.

DISCUSSION

Information related to respiratory problems in goats is scarce in Mexico and even in the American continent, perhaps because this animal species is not considered important from an economic point of view. Literature on this subject is usually generated in countries of other continents.

In Mexico, there is the antecedent of a study conducted by Blanco *et al.*, (1993), in which the capsular and somatic types of *P. multocida* and the serotypes of *P. haemolytica* (currently *M. haemolytica*), isolated from 148 lungs with inflammatory lesions of sheep and goats collected at the slaughterhouse were determined. The isolates (79) were obtained of bacteria of the *Pasteurellaceae* family, 30 corresponding to *M. haemolytica* and 49 to *P. multocida*. When typing was carried out, specifically two isolates of M. haemolytica from goats corresponded to A serotype and of the three isolates of *P. multocida*, one corresponded to A type and two to D type. It is important to mention that in this study molecular methodologies were not used for bacterial identification, nor for the determination of capsular types, using only conventional tests such as indirect hemagglutination, decapsulation by hyaluronidase and flocculation by acriflavine. An important difference is the proportion of isolates, since Blanco *et al.*, (1993), obtained a greater number of isolates of *P. multocida* than *M. haemolytica*, in contrast to the present study.

The difference could be due to several factors such as geographical origin and type of samples, since Blanco *et al.*, (1993) took samples of lung tissue with lesions from adult animals from the central part of the country, mainly from Mexico, Queretaro, Jalisco, Guanajuato and Aguascalientes states unlike the present work in which nasal exudate was collected mainly from sick kids in San Luis Potosi, Baja California Sur, Coahuila and Puebla. A review of the published literature showed variable results. For example, in Ethiopia, a bacteriological study was carried out on 112 goat lungs with pneumonic lesions, from which they obtained 21 isolates of M. haemolytica and six of *P. multocida*, in which they did not use molecular techniques for identification, nor did they perform typing of the isolates (Demissie *et al.*, 2014).). Another similar study carried out in Sudan where from 200 goat lungs with pneumonic lesions. They obtained 102 isolates of different bacteria, predominantly *M. haemolytica* with 85 isolates, followed by *Corynebacterium pseudotuberculosis* with seven isolates, *Streptococcus* spp. α hemolytic with five and *P. multocida* with one isolate, thus concluding that *M. haemolytica* serotype A is the main cause of respiratory problems in goats in this region of Sudan (Elsheikh *et al.*, 2012).). In another investigation, carried out in India, they sampled goats affected by respiratory problems, obtaining 20 nasal swabs, from which 15 isolates of *M. haemolytica*, two of *Staphylococcus* spp, two of *Proteus* spp, and one of *E. coli*. They also conducted a bacteriological study of five goat tracheal washings, from which four isolates of *M. haemolytica* and one of *E. coli* were obtained, so the authors conclude that in this region of India the main etiological agent involved in outbreaks of respiratory disease in goats is *M. haemolytica* (Ponnusamy *et al.*, 2017).

A different situation is presented in the results found in a study conducted in Iran, where sheep and goats were sampled with swabs from nostrils and tonsils (Sahragard *et al*., 2012), finding 26 isolates of *P. multocida*. When they were characterized with multiplex PCR, they found that 24 of them were type A and two belonged to type D, although they do not specifically refer how many isolates correspond to sheep and how many to goats (Tahamtan *et al.*, 2016). It is important to note that in this study they tested for some virulence genes related to the production of toxins and outer membrane proteins. In another study carried out in Egypt (Amin, 2020), with 20 goats sick with pneumonia, when they performed PCR of lung tissue they found a positivity of 95 % to *Pasteurella multocida* as the only bacterial agent participating in the respiratory problem.

From 50 isolates selected for definitive identification by PCR, 46 showed the presence of the *sodA* gene, confirming their definitive identification as *M. haemolytica*; the remaining 4 isolates were negative and were not considered for the present study. The initial bacterial identification was performed using phenotypic and metabolic characteristics that are not necessarily highly specific, which can lead to the collection of information that does not coincide when compared with genotypic information obtained with molecular tools.

With respect to the study to determine the presence of *lktA* gene in the 46 isolates of *M. haemolytica*, it was found that 39 (84.7 %) amplified a segment of the gene and the remaining 7 (15.3 %) did not amplify. When consulting the information published on the subject, variable results were found, for example Vougidou *et al.,* (2013) studied 11 isolates of goat origin and 70 of sheep; 100 % of strains evidenced the presence of the *lktA* gene. In other studies Fisher *et al*., (1999) reported that when studying 147 strains of *Mannheimia haemolytica* and 101 of *Pasteurella trehalosi*, the *lktA* gene was detected in 108 (43.5 %) isolates and 140 (56.5 %) were negative; the authors do not specify the percentage by bacterial genus. In another study, Kelley *et al.,* (2007), analyzed 48 strains obtained from wild sheep and found that 20 (41.6 %) isolates had the gene *lkt*A and 28 (58 %) lacked it.

M. haemolytica isolates have the l*kt*A gene and others do not Davies *et al.,* (2001) because of some genes directly involved in pathogenesis are transferred between bacterial species by bacteriophages and the genes of the leukotoxin operon seem to be transferred horizontally between strains of *M. haemolytica* and even between *M. glucosida* and *P. trehalosi*.

It can be observed that in general, there is little information related to studies of respiratory problems in goats, since most of the studies describe them as “carried out in sheep and goats” or simply “carried out in small ruminants” and when presenting the results they do not clearly mention the separation by animal species. It contributes to the fact that there is little specific information on the subject in goats.

CONCLUSION

What can be highlighted from the studies carried out on respiratory problems in goats, as well as in ruminants in general is that the frequency of the main etiological agents such as *M. haemolytica* and *P. multocida* differs according to the geographic region. It is necessary to carry out this type of studies since they contribute with information that has practical application in the diagnosis, virulence factors and the formulation of specific biologicals for the region. An aspect that should not be omitted in future studies is related to bacterial resistance to chemotherapeutics, since it currently has an important impact on both animal and public health.

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Errata Erratum

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