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RESEARCH ARTICLE

Serological Evidence of *Mycoplasma mycoides* Subspecies *mycoides* in the Central Area of Veracruz, Mexico

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ABSTRACT

A high morbidity and mortality of goats has been observed in the central area of Veracruz, Mexico, where goat production is practiced. The clinical signs showed by these goats may be related to mycoplasmosis, a highly contagious disease that causes serious economic losses. Thus, our objective was to evaluate the seroprevalence of *Mycoplasma mycoides* subspecies *mycoides* (Mmm) using a competitive ELISA (c-ELISA) and to assess risk factors associated with seropositivity. The seroprevalence of 556 goats sampled in random was 11.2%, the frequency by local municipality was 64.3%, and 25.3% per animal small production farm (SPF). The identified risk factors were associated with breeding in the municipalities of Coacoatzintla (OR=14.2; 95%CI: 6.9-29.5), Villa Aldama (OR=4.9; 95%CI:2.5–9.5) and Chiconquiaco (OR=7.71; 95%CI:3.6–16.47); the crossbreeding phenotype (OR=1.78; 95%CI:1.05–3.04), stage of suckling goat kids (OR=2.87; 95%CI:1.64–5.03), mobilization of animals (OR=4; 95%CI:2.33–6.88), semi intensive production system (OR=2.51; 95%CI:1.24–5.07) and coexistence with cattle (OR=3.28; 95%CI:1.53–7.02). Possible protective factors were associated with drink water from stream (OR=0.54; 95%CI: 0.29–0.98), well water (OR=0.11; 95% CI:0.01–0.82), Saanen breed (OR=0.39; 95%CI:0.19–0.8), purchased animals (OR=0.47; 95%CI:0.24–0.93) and the extensive system of production (OR=0.13; 95%CI:0.03–0.55). In conclusion, this study showed that the overall seroprevalence of Mmm in goats has a low general distribution, moderated by animal small production farms and high by municipality, in the central region of Veracruz, Mexico. Considering these results is required to perform further studies by use of molecular techniques for the precise identification of the etiological agent.

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INTRODUCTION

Mycoplasma is the smallest bacteria that cause disease of the respiratory tract of goats in worldwide, being the most frequent pathogens: *Mycoplasma agalactiae* (Ma), *Mycoplasma mycoides* subspecies *capri* (Mmc), *Mycoplasma capricolum* subspecies *capricolum* (Mcc), and *Mycoplasma putrefaciens* (Mp) (DaMassa *et al*., 1992; Sadique *et al*., 2012). These bacteria can be simultaneously present in a same herd or in an individual and this may result in mixed infections, along with the presence of other mycoplasma spp. considered as part of the normal flora; such a situation complicates the diagnostic and control measures of the disease (Gómez-Martín *et al*., 2012a). Among several *Mycoplasma* species that cause severe disease in goats, Mmc and Mcc are associated with the syndrome called MAKePS (mastitis, arthritis, keratoconjunctivitis, pneumonia and septicemia), which is characterized by high fever, respiratory failure with cough and nasal discharge (Thiaucourt and Bölske, 1996; Hussain *et al*., 2012). Another infectious agent, *Mycoplasma mycoides* subspecies *mycoides* Small Colony

type (MmmSC) could also produce severe respiratory distress, fever, and mucopurulent nasal discharge in goats. In an outbreak of contagious caprine pleuropneumonia in Eastern Tanzania, MmmSC and Mccp were isolated from sick goats (Kusiluka *et al*., 2000a). The small ruminant infection by MmmSC is not rare, since MmmSC has been also isolated in milk samples from ewes with mastitis and from samples of goats with interstitial pneumonia (Brandão, 1995). A microbiological examination in the respiratory tracts of goats and cattle identified as MmmSC in all isolates from both animal species (Kusiluka *et al*., 2000b). Regarding to the mycoplasma transmission, one significant route is the close contact between an infected goat and healthy goat. This route involves inhaling infective droplets from sick-goat breathing (D´Angelo *et al*., 2010). The lactation and milking are also considered the main routes of transmission where goat kids become infected (Bergonier *et al*., 1997). Other routes although of lesser importance, include excretion of mycoplasmas in feces, semen as well as other body secretions from infected goats at flock level. Specifically, the excretion of Ma and Mmc has been demonstrated in semen samples from buck goats (Gómez-Martín *et al*., 2012b), which may affects the sperm quality.

The accurate diagnosis for respiratory mycoplasma infection is difficult due to multiple variations such as genetic, age, and different clinical manifestations that are strain specific (Chakraborty *et al*., 2014). Moreover, as a result of deficiency of diagnosis services in several countries, some species of mycoplasmas may be almost unnoticed. Mmc is the main agent that causes contagious agalactia in goats, but sometimes, it is isolated from sheep or cattle (Chazel *et al*., 2010). Furthermore, Mmc has been also isolated from goat-mastitis milk (Kumar *et al*., 2013).

In Mexico, few outbreaks caused by Mmc have occurred in the years 1963, 1965 and 2003; the first of them affected 2,000 goats in the State of Oaxaca, the second 1,000 goats in the State of Guerrero, and a third involved 2,000 goats in the State of Durango (Hernández *et al*., 2007). In the latter case, 200 animals were killed by the disease, and infection affected animals of all ages. Goat kids died after a week of appearance of clinical signs, which were characterized by abundant nasal discharge, fever, difficulty for breathing with extended neck, prostration, drooping ears, and low milk production. Fourteen goats were sacrificed and Mmc was identified in the pulmonary lesion samples by the PCR (Hernández *et al*., 2007). In Veracruz, professionals in Veterinary Medicine have declared their concern about the increase in the number of animals with respiratory diseases, which show clinical signs that suggest respiratory mycoplasma infection possibly caused by Mmm. Thus, our goal was to evaluate the presence of serum antibodies against Mmm by means of c-ELISA and the possible risk factors associated with seropositivity in goats of the central area of Veracruz, Mexico.

MATERIALS AND METHODS

Study area: Sampling was conducted in the Rural Development District 004 Coatepec (RDD-004), a division of the Agriculture Secretariat in Veracruz. This area has an extension of 402,057 ha including 31 municipalities, 14 of which concentrate 90% goat production of the region (Fig. 1).

Study design: The study was cross-sectional, multistage and stratified. Herds were randomly selected. In RDD-004 Coatepec, there are 536 small production farms (SPF), with herds ranging from 6 to 50 goats and a population of 36,660 goats. Number of farms was calculated by Cannon and Roe (1982) equation, producing 81 SPF. Thus, 556 goats were analyzed, considering 6-7 animals for each one of the 81 SPF. All bucks and female 3 months or older were included.

Implementation of survey: A general survey for each sampled SPF included variables such as production system, grazing animals, milking system, reproduction method, source of water, mobilization. An individual survey for each sampled animal included variables such as intake of colostrum, provenance, productive stage, age, sex, and breed.

Blood sample collection: Ten ml of blood were obtained from each animal by puncture of the jugular vein. Samples were transported at 4°C to the Microbiology Laboratory, of the Faculty of Veterinary Medicine, and then centrifuged for 15 minutes at 1,000 *g*. Sera were stored at -20°C until processing.

ELISA: The serological analysis was performed by c-ELISA version P05410/02 kit for contagious bovine pleuropneumonia, which was acquired from CIRAD/Institut Pourquier (Montpellier, France). The assay was performed following manufacturer´s instructions. Briefly, each serum was diluted and mixed with the anti-MmmSC antibody called Mab 117/5. This mixture was incubated in an MmmSC lysate-coated plate followed by incubation with peroxidase substrate solution after incubation with the peroxidase-labelled anti-mouse IgG antibody. The reaction was stopped when the intensity of color was developed. Reading was performed in a spectrophotometer BioTek model 680 (BioTek Instruments Inc., Winooski, VT) at 450 nm wavelength using the IDEXX xChekPlus™ software (IDEXX Laboratories, Inc., Westbrook, ME). The percentage of inhibition (PI) for each serum was calculated as follows:

$$PI = 100 \times [(OD\ Cm - OD\ Test)/ (OD\ Cm - OD\ Cc)]$$

Where OD = optical density, Cm = monoclonal control, Test = test serum, and Cc = conjugate control. The validity criteria were an OD between 0.5 and 2.0 for Cm; an OD below 0.3 for Cc; a PI of negative control lower than 35%; a PI of positive control between 50 and 80%; and a PI of strong positive control between 60 and 90%. Thus, a test serum with a PI equal to or greater than 50% was considered positive.

Statistical analysis: Data were analyzed by SPSS 17 software. Prevalence was determined and confidence intervals (CI) were calculated using VassarStats online software. To determine the association between variables, odds ratio (OR) was calculated using the formula proposed by Thrusfield (2005). In OR, when a CI includes

the value of 1, the odds of the measured outcome are the same for all treatment groups, even without a significance test, so all those variables with an OR>1, and a value of P<0.10 were analyzed by logistic regression (Thrusfield, 2005) to know interactions among the variables tested, where the independent variable was goat seropositivity to the c-ELISA test and explanatory variables those with P<0.10.

RESULTS

The OD values obtained from controls used in the c-ELISA were as follows: Cm showed OD between 1.33 and 1.51, whereas Cc displayed OD between 0.095 and 0.206. With regard to PI, negative control sera showed proportions ranging from 21.3 to 32.8%, whereas those positive control sera were from 72.5 to 79.0%. The strong positive control sera showed PI that reached 83.5% on average. The test sera considered as positive displayed PI ranging from 50.4 to 91.2%. Serological evidence of *Mycoplasma mycoides* subspecies *mycoides* in 8 out of 14 sampled municipalities was found with the highest prevalence at Chiconquiaco, Coacoatzintla, and Villa Aldama, as shown in table 1. As risk factors were found the breeding in the municipalities of Coacoatzintla, Villa Aldama, and Chiconquiaco; the crossbreeding phenotype (OR=1.78; 95% CI:1.05–3.04), stage of suckling goat kids (OR=2.8; 95%CI:1.64–5.03), semi intensive production system (OR=2.5; 95%CI:1.24–5.07) and animal born in the SPF (OR=4.3; 95%CI:2.2–8.1). As animal protective factor were found the Saanen breed and the extensive system.

Analysis of risk factors associated with *Mycoplasma mycoides* subspecies *mycoides* by logistic regression: According to the inclusion criteria for logistic regression (LR) model, 14 variables were analyzed, of which seven were significant. However, coexistence of goats with cattle, dead animals without appropriate elimination, and the semi intensive production system were the ones with greater significance (Table 2). In addition, in order to identify possible protective factors, the interaction of those variables with statistical significance (P<0.05) but with OR<1 was performed; the most important variable was animals come out to pasture (OR=0.37; 95%CI:0.19–0.72). Other important variables were the purchased animals (OR=0.35; 95%CI: 0.17–0.73), the Saanen breed (OR=0.4; 95%CI:0.19–0.84), the extensive system (OR= 0.17; 95%CI:0.03–0.81) and animals that drink water from a well (OR=0.11;95%CI:0.16–0.93) as shown in Table 3.

DISCUSSION

The results showed that seroprevalence to *Mycoplasma mycoides* subspecies *mycoides* in herds from small production farmers, was 25.3% by a c-ELISA kit for CBPP. However, the obtained data must be interpreted at the herd level and never at the individual level (Thiaucourt *et al*., 1994). In our study, in municipalities belonging to the RDD004 Coatepec, showed a total prevalence of 11.5% (OR=8.72–14.14), which represents the first report of goat infection by Mmm in Mexico. The CBPP in Mexico is considered an exotic disease; therefore, a bacteriological confirmation must be done. Additionally, an experimental evaluation of Mycoplasma-infected goats coexisting with healthy cattle or vice versa must also be carried out as suggested by Ruhnke *et al*. (1983).

We used a c-ELISA kit, which has been developed and validated by IDEXX-Montpellier/CIRAD (Peyraud *et al*., 2014). In this method, serum antibodies from sick goats are mixed with the mAb 117/5 specific for MmmSC. This mixture is brought to react with the MmmSC antigen, so any serum specific antibody will compete with the mAb 117/5 for the specific epitope. Nonetheless, the sick goat-antibodies are polyclonal and they could recognize diverse MmmSC-epitopes preventing the binding between the mAb 117/5 and its specific epitope. This likelihood is low since polyclonal antibodies to different MmmSC-epitopes should have very high affinity for nearby epitope to that recognized by mAb 117/5. Regardless, the binding of serum polyclonal antibodies to a close epitope indicates recognition of MmmSC antigen because the used c-ELISA kit has specificity at 99.9% for MmmSC (Peyraud *et al*., 2014). Likewise, goat serum polyclonal antibodies bound to a distant MmmSC-epitopes cannot be recognized by secondary anti-IgG mouse antibody and consequently they could not compete with the mAb 117/5. Therefore, the possibility that goat antibodies recognize MmmSC is very high. Another possibility is the serological cross-reaction between MmmSC and Mmc that are most likely because the two species show genetic similarity and both are also very similar in antigenicity (Thiaucourt *et al*., 2011). However, the cross-reaction polyclonal antibodies should have the same or higher affinity than the mAb 117/5 for an epitope that is not their specificity. Furthermore, an important risk factor was the coexistence of goats with cattle (OR=3.289, P<0.002), which strongly suggests exchange of MmmSC between the two species.

Table 1: General Seroprevalence of *Mycoplasma mycoides mycoides*

Municipality	No. of animals	Positive by c-ELISA	% Prevalence	*95%CI	OR
Emiliano Zapata	34	1	2.94	0.15–17.05	OR=0.22; 95%CI:0.03-1.7
Coacoatzintla	36	20	55.56	38.29–71.67	OR=14.23;95%CI:6.86-29.5
Villa Aldama	49	16	32.65	20.36–47.65	OR=4.85; 95%CI:2.48-9.49
Yecuatla	34	1	2.94	0.15–17.05	OR=0.25; 95%CI:0.03-1.88
Las Vigas	40	7	17.5	7.89–33.36	OR=1.77; 95%CI:0.75-4.21
Jalacingo	33	0	0	0	
Perote	71	1	1.41	0.07–8.65	OR=0.09; 95%CI:0.01-0.72
Tatatila	39	0	0	0	
Tlacolulan	39	0	0	0	
Chiconquiaco	32	14	43.75	26.84–62.12	OR=7.71;95%CI:3.61-16.47
Coatepec	41	0	0	0	
Ixhuacán	26	1	3.85	0.2–21.59	OR=0.3; 95%CI:0.04-2.31
Las Minas	33	1	3.03	0.16–17.51	OR=0.23; 95%CI:0.03-1.76
Xico	49	0	0	0	
Total	556	62	11.15	8.72-14.14	

*CI= 95 % confidence interval; OR=Odds Ratio

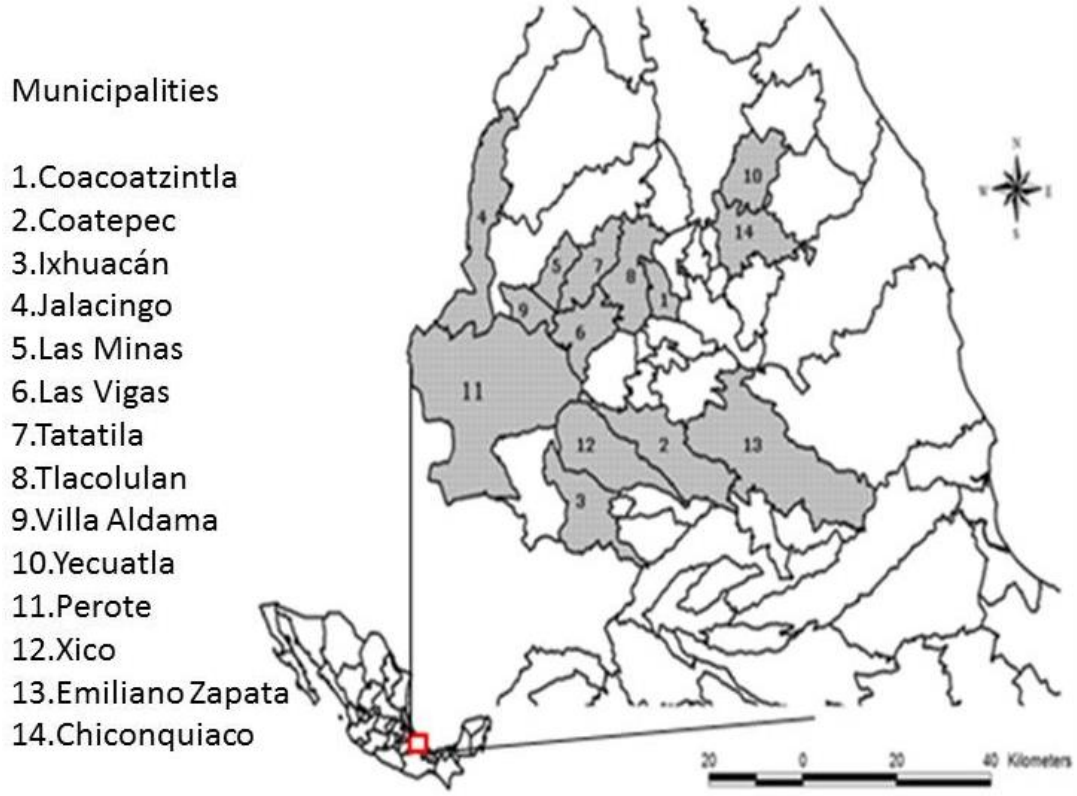

Fig. 1: Municipalities belonging to RDD 004 Coatepec, Veracruz, Mexico. Taken from the Encyclopedia of the Municipalities of Mexico, INAFED 2010 (www.inafed.gob.mx/work/enciclopedia).

Table 2: Interaction of variables that are considered as risk factors for *Mycoplasma mycoides* species *mycoides* in the central area of Veracruz, Mexico

Variable	OR	Lower limit	Upper limit	P<0.05
Wooden feeder	2.4044	0.9354	6.1805	0.0686
Wildlife armadillo	1.2878	0.5756	2.8813	0.5381
Drinking water from stream	0.2464	0.0731	0.8302	0.0238
Coexistence with cattle	3.289	1.539	7.0288	0.0021
Crossbreds	1.4592	0.7624	2.7927	0.2539
corpses whitewashed	5.6422	1.8242	17.4512	0.0027
Bury dead animal	0.8191	0.3742	1.7927	0.6175
Incinerated animals	11.3927	1.0791	120.2827	0.043
Nursing stage	2.2027	1.1234	4.319	0.0215
Mobilization	3.7377	1.3747	10.1624	0.0098
Drinking water from elsewhere (not steam or well)	2.3342	0.823	6.62	0.111
Semi -intensive system	4.4068	1.6697	11.6303	0.0027
Food supplement	1.3985	0.6263	3.1226	0.4132
Presence of vultures	1.5994	0.6588	3.8833	0.2994

Table 3: Interaction of variables that are considered as protective factor for *Mycoplama mycoides* species *mycoides* in the central area of Veracruz, Mexico

Variable	OR	Lower limit	Upper limit	P<0.05
Dead animal outdoors	0.9013	0.447	1.8176	0.7716
Purchased animal	0.3555	0.1726	0.732	0.005
Cockroaches	0.6105	0.325	1.1468	0.125
Extensive system	0.1779	0.039	0.8113	0.0257
Grazing (consumption of grass)	0.3708	0.1902	0.7229	0.0036
Drinking water well	0.1194	0.0153	0.9342	0.0429
Saanen breed	0.4038	0.1935	0.8425	0.0157
No drinking fountain	0.8556	0.2769	2.644	0.7865
There are rats in the place	0.8988	0.3671	2.2006	0.8154
Mice	0.7207	0.2704	1.921	0.5126
Eat on the floor	0.6528	0.1995	2.1353	0.4806
Drinking water from steam	1.2816	0.6887	2.3849	0.4336
CONSTANT	*	*	*	0.9924

The etiologic agent of CBPP has been isolated from goats in different countries including disease-free areas (Tardy *et al*., 2011). Thus, the goats can be reservoirs of MmmSC and should be considered a risk factor as pointed out by Tardy *et al*. (2011). Our results agree with this possibility, cattle in coexistence with goats constitute a risk factor for both animals.

As additional risk factors, it was found that covering corpses with lime and incineration are common practices in the area of study. Possibly the deficient corpses removal techniques, and the low hygiene used by producers is due to the fact that belong to a poor and unprepared social population. This observation coincides with the opinion published by Al-Momani *et al*. (2011) indicating that the lack of cleaning in general, and utensils used for milking in particular (OR= 4.7), are considered an important risk factor. The study performed in the North of Jordan by Al-Momani *et al*., (2011) pointed out that the purchase of new healthy animals for replacement (OR= 0.3) is considered as a protector factor. Similar evidences were found in our study in the RDD004 Coatepec where the buying of animals was confirmed by the logistic regression as a protection factor. It is important to establish relevant biosafety measures with regards to the new animals to be introduced into the herd, in order to reduce the possibility of the introduction of asymptomatic carriers of infectious diseases. These measures will promote the presence of healthy animals for reproduction and as a consequence more benefits for the producers. Other relevant information obtained, is related to the intake of water well or stream since it was also proved to be a protective factor. This could be due to the fact that while the water flows diminish environmental conditions

for possible infection, contrary of what occurs stagnant water. Another protective factor was race, since the Saanen breed animals gave less seropositivity than animals of other races or even the Crusaders, perhaps from the genetic point of view; it retains the characteristics of resistance to certain diseases or microorganisms as Mmm, while being crossbreeds weakens the possibility of conserving these resistance genes.

It is required to carry out studies of this type in main regions of goat's production in Mexico. Additionally, the use of molecular techniques and bacteriological studies should be performed in order to have a more accurate identification of agents involved in the respiratory problems of goats. At the present time these diseases are causing severe damages and losses in the goat herds, due to diminish in the goat production. The respiratory problems affect the economic producer's stability, since the goat production is their economic livelihood.

Conclusions: The serum analysis of goats with symptoms suggestive of mycoplasmosis pointed out the presence of antibodies against MmmSC by a competitive ELISA kit for CBPP. The logistic regression analysis indicated as important risk factors in descending order: incinerated animals, corpses whitewashed, semi-intensive system, mobilization, coexistence with cattle, and nursing stage. Whereas the protective factors were Saanen breed, drinking water well, consumption of grass, extensive system, and acquisition of new animals. We emphasize that there are not previous records of Mmm infection in goat herds belonging to the RDD004 Coatepec, in the central area of Veracruz.

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Authors contribution: DM conceived the idea of study, RL and SL analyzed samples of sera, SL, DM, JM, FA, FM, RF, RL analyzed data and results. All the authors interpreted the data, analyzed information, reviewed the manuscript and approved the final version.

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