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Comparison of commercial enzyme-linked immunosorbent assays for diagnosis of contagious agalactia caused by *Mycoplasma agalactiae*

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Abstract

Introduction: Contagious agalactia (CA) is a disease affecting small ruminants with worldwide distribution and caused by several mycoplasmas, especially *M. agalactiae*. The main option for systematic diagnosis under monitoring control programmes is the enzyme-linked immunosorbent assay (ELISA) test. **Material and Methods:** This study was designed to appraise the performance of two commercial indirect ELISA tests using *M. agalactiae* p48 protein and one using total protein, for antibody detection in small ruminants after natural infection with different *M. agalactiae* strains. We carried out the test evaluation using sera of confirmed *M. agalactiae*-positive goats with clinical signs. In addition, test agreement was assessed by kappa between the three commercial ELISA tests. **Results:** All three ELISA tests showed high validity scores (Youden's J: 72.9–84%). The sensitivity values for the P48 protein-based tests were 76.9% and 84.6%, and was 79% for the total protein-based test. The specificity of all tests was 100%. In addition, between the total protein-based ELISA test and the other two ELISA tests based on the P48 protein, the agreement was substantial (kappa: 0.762–0.763) and the agreement between the latter two tests was almost perfect (kappa: 0.93). **Conclusion:** The validity parameters for all tests allowed their application for diagnostic purposes in lactating goats excreting *M. agalactiae* in milk and presenting clinical signs. The agreements show that any of these ELISA tests could be equally well used for diagnosis in programmes against CA.

Keywords: contagious agalactia, *Mycoplasma agalactiae*, enzyme-linked immunosorbent assays, small ruminants, goat.

Introduction

While contagious agalactia (CA) was first described more than two centuries ago in 1816 (20), it remains a neglected disease in many countries around the world. In countries with small ruminant production, its control has been complicated because of diagnostic limitations and aetiological, clinical and epidemiological variability (17). The economic impact of CA (14) and its consequences for animal welfare were sufficient reasons for the World Organisation for Animal Health (OIE) to list it as a notifiable disease. Despite its notifiable status, underestimation of CA for its effect on milk production has been suggested for many regions (17). On the other hand, the Mediterranean basin can be considered an endemic CA area due to continual official notifications and descriptions of outbreaks of the disease (5, 17).

Mycoplasma agalactiae is the main agent responsible for CA syndrome both in sheep and goat herds. In addition, *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. putrefaciens* have been associated with the disease in goat herds (5, 9). Besides aetiological variability, different clinical signs associate with CA, from acute outbreaks to asymptomatic chronically infected herds in endemic areas. The main CA related symptoms are mastitis, keratoconjunctivitis and arthritis, but respiratory and reproductive consequences including abortion have also been described (14). It should be pointed out that the symptoms and the *Mycoplasma* species involved are not

directly associated and, in addition, several grades of their frequency and intensity or clinical sign subsets occur at individual or herd level (5, 14).

Direct diagnosis of *Mycoplasma* spp. infections is based on culture or PCR methods as appropriate for different types of samples, which may be bulk tank milk (2, 3, 31), milk secretion from clinical mastitis (2), caprine ear swabs (1), ovine nasal swabs (26) and caprine or ovine semen samples (10, 27). These different samples have been useful for CA monitoring and have allowed the improvement of the design of CA control programmes at local (2) or at country level, as in France and Spain (22, 24). Alternatively, indirect diagnosis using serological strategies has been implemented for *M. agalactiae* in unvaccinated herds. Among serological methods, the enzyme-linked immunosorbent assay (ELISA) is the main option because it is an economical and simple procedure which provides a means of diagnostic screening (6). The combination of direct and indirect CA diagnosis has enabled the control of CA in different regions and the determination of the factors affecting the efficiency of that control (23, 25).

Different ELISA tests based on total antigens or recombinant proteins have been evaluated. The sensitivity of total antigen ELISAs elaborated from strains of different origins ranged from 48% (23) up to 89% (7), whereas the sensitivity of the ELISA test using total proteins from the reference strain PG2 ranged from 72% (23) through 74% (13) and 76% (19) to 84% (24). The use of local strains of *M. agalactiae* for antigen elaboration showed an improvement in the sensitivity to 99% (4). With to specificity, the ELISA test based on total antigens ranged from 94% (23) to 99% (19, 23), showing significant differences between the values yielded when testing ovine (95.7%) and caprine (99.3%) samples (25). Using recombinant protein P48 as the antigen (28), the sensitivity ranged from 56% (19) up to 82% (23), with a specificity of nearly 100% in all cases (19, 23, 25). An ELISA test with P80 and P55 proteins developed for non-commercial use yielded 94% sensitivity and 97% specificity (13).

The above differences in ELISA test validity parameters could be explained by the genetic and protein differences of the circulating *Mycoplasma* strains, the assay designs, or the infection phase of the sampled animals. Therefore, since the efficacy of an ELISA test is affected by the circulating *M. agalactiae* strains in the place of the test's use, an evaluation of the relevant tests should be performed prior to generalising their use in control programmes (25), especially knowing the antigenic variability of *M. agalactiae* (11).

Because some CA control programmes have been implemented in Spain by serological diagnosis, this work was designed to compare the three commercial ELISAs available for testing small ruminants naturally infected with *M. agalactiae*. The assays were compared by means of evaluation and agreement tests. The evaluation test was carried out using true-negative and true-positive herds with previous records of their sanitary status. Infection cases in ruminants enrolled from herds known to be positive for *M. agalactiae* were confirmed by means of bacterial culture and molecular identification of samples from clinically affected animals.

Material and Methods

Herds, animals and sera studied. After blood collection by jugular venipuncture, a total of 761 sera samples were collected and processed during 2018 from nine herds (400 sera obtained from five dairy sheep herds and 361 from four dairy goat herds) located in three Spanish regions: Castilla-León, Murcia and Andalucía. The number of animals in the herds studied ranged from 500 to 3500. Most of the samples came from lactating females and all came from animals not vaccinated against CA in at least the two years prior to sampling. The sanitary status of each herd was obtained from the records of the monitoring programs for CA, which screened bulk tank milk and mastitis samples for asymptomatic carrier detection, and as previously described (1, 2, 26). The herds were classified according to the criterion defined by Pépin *et al.* (23). The herds and samples obtained were profiled as follows:

True-negative herds (TN) (n = 4): A total of 367 sera samples from herds without a history of CA in the two years prior to sampling. The sampling strategy included bulk tank milk, mastitis samples and ear swabs, and all samples for isolation or molecular detection of *M. agalactiae* were negative.

True-positive herds (TP) (n = 5): A total of 394 sera samples from herds with a previous history of clinical cases of CA. The clinical cases included both acute and chronic incidences. In all herds, *M. agalactiae* was detected from bulk tank milk, mastitis samples or ear swabs.

ELISA test procedures. Three commercial ELISA tests designed to detect the presence of specific antibodies against *M. agalactiae* were used. The first was the CIVTEST OVIS *M. agalactiae* (Laboratorios HIPRA, S.A., Amer Girona, Spain). It uses an inactivated *M. agalactiae* total antigen and protein G/horseradish peroxidase (HRP) conjugate. Ratio values (Rz) based on optical density (OD) are given by the formula: Rz = sample OD/2× mean negative control OD. The results are interpreted as follows: negative when $Rz < 1.0$, doubtful when $1 < Rz \leq 1.5$ and positive when $Rz > 1.5$.

The second assay was the *M. agalactiae* Screening Ab Test (IDEXX Institut Pourquier, Montpellier, France). It uses a fusion protein equivalent to *M. agalactiae* P48 protein and an anti-ruminant IgG conjugate. Normalised values (NV) based on OD are given by the formula: NV = (sample OD − negative control OD) × 100/(average positive control OD − average negative control OD). The results are interpreted as follows: negative when $NV \leq 50\%$, doubtful when $50\% < NV \leq 60\%$ and positive when $NV \geq 60\%$.

The assortment was completed by the ID Screen *M. agalactiae* Indirect ELISA (IDvet, Grabels, France). It uses a purified *M. agalactiae* P48 recombinant antigen and an anti-ruminant IgG HRP conjugate. For each sample, the S/P percentage (S/P%) is calculated as (sample OD − negative control OD) ×100/(positive control OD – negative control OD). Results are interpreted as follows: negative when S/P% ≤ 50%, doubtful when 50% < S/P% ≤ 60% and positive when S/P% ≥ 60%.

Mycoplasma cultures. Milk samples were inoculated into solid and liquid mycoplasma media and incubated at 37°C in a 5% CO_2 humid atmosphere for 15 days before being considered negative; the ear swabs were twirled and left in 1 mL of liquid mycoplasma medium for 30 min at room temperature. Subsequently, after discarding the swabs, aliquots of the remaining fluid were cultured under the same conditions as previously described (1, 2). With positive cultures, isolates from previously cloned single colonies were used for final identification performed by PCR.

DNA extraction and PCR. DNA was extracted from 200 µL of *Mycoplasma* spp.-positive cultures and culture aliquots from milk and ear swabs using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. Subsequently, *M. agalactiae* was detected using a specific PCR protocol (21).

Evaluation of ELISA commercial tests. A total of 71 sera from lactating goats were processed. The samples came from three herds from Murcia (n = 2) and Andalucía (n = 1). According to the CA status of the herd of origin and the bacteriological analyses performed as gold standard on the selected animals, the goats were classified as follows:

Non-infected goats (n = 45): Animals from herds classified as TN. Their samples of milk (n = 90) and ear swabs (n = 90) were negative for *M. agalactiae* and the other mycoplasmas associated with CA.

Infected goats (n = 26): Animals from two herds classified as TP. All were suffering acute or subacute mastitis and *M. agalactiae* was isolated in at least one of the two udder half milk samples processed (n = 52) by culture procedures.

The evaluation of the three ELISA commercial tests was performed using their sensitivities and specificities and following Thrusfield's recommendations (32). Data were processed with the WinEpi program (http://www.winepi.net/), and other parameters such as predictive values, true prevalence, apparent prevalence and Youden's J were also obtained at a 95% confidence interval.

Agreement test of commercial ELISAs. A total of 761 sera from lactating goats were processed. The agreement test between the three ELISA commercial tests was carried out by estimating the Cohen's kappa coefficient at a 95% confidence interval, using the WinEpi program. The criteria for result interpretation were based on Fleiss *et al.* (12) and Thrusfield (32).

Results

Table 1 shows the global results of the three studied ELISA commercial tests. The IDEXX test yielded the highest proportion of positive results (47.17%), followed by the ID.Vet test (46.52%) and HIPRA test (42.52%). A higher proportion of doubtful results was obtained by the HIPRA test (9.33%) and this contrasted sharply with the substantially lower proportions of doubtful results for the IDEXX (0.79%) and ID.Vet (0.66%) tests.

Table 1. Positive, negative and doubtful results yielded by the ELISA tests studied at a 95% confidence interval (CI)

Test	Results	n (%)	CI, 95%
HIPRA[1]	Negative	366 (48.09%)	44.56%, 51.65%
	Positive	324 (42.58%)	39.11%, 46.12%
	Doubtful	71 (9.33%)	7.46%, 11.61%
IDEXX[2]	Negative	396 (52.04%)	48.49%, 55.57%
	Positive	359 (47.17%)	43.65%, 50.73%
	Doubtful	6 (0.79%)	0.36%, 1.71%
ID.Vet[3]	Negative	402 (52.82%)	49.27%, 56.35%
	Positive	354 (46.52%)	43.00%, 50.07%
	Doubtful	5 (0.66%)	0.28%, 1.53%

[1]CIVTEST OVIS *M. agalactiae*; [2]IDEXX *M. agalactiae* Screening Ab Test; [3]ID Screen *Mycoplasma agalactiae* Indirect ELISA

Table 2. Distribution of the qualitative results for each ELISA test studied according to the sanitary classification of the herds

ELISA kit	Result	True-negative herds	True-positive herds
HIPRA[1]	Negative	317	49
	Positive	9	315
	Doubtful	41	30
IDEXX[2]	Negative	351	45
	Positive	12	347
	Doubtful	4	2
ID.Vet[3]	Negative	359	43
	Positive	4	350
	Doubtful	4	1

[1]CIVTEST OVIS *M. agalactiae*; [2]IDEXX *M. agalactiae* Screening Ab Test; [3]ID Screen *Mycoplasma agalactiae* Indirect ELISA

Table 3. Distribution of the evaluation results in lactating goat sera (n = 71) for the three ELISA commercial tests studied

ELISA test	Results	Gold standard	
		Negative (confirmed previously as n = 45)	Positive (confirmed previously as n = 26)
HIPRA[1]	Negative	44	5
	Positive	0	19
	Doubtful	1	2
IDEXX[2]	Negative	45	6
	Positive	0	20
	Doubtful	0	0
ID.Vet[3]	Negative	45	4
	Positive	0	22
	Doubtful	0	0

[1]CIVTESTOVIS *M. agalactiae*; [2]IDEXX *M. agalactiae* Screening Ab Test; [3]ID Screen *Mycoplasma agalactiae* Indirect ELISA

Table 4. Validity parameters obtained for each ELISA commercial test evaluated at a 95% confidence interval (CI)

Test	Parameter	Value (CI, 95%)
HIPRA[1]	Sensitivity	79.2% (62.9%, 95.4%)
	Specificity	100% (100%, 100%)
	True prevalence	35.3% (23.9%, 46.7%)
	Apparent prevalence	27.9% (17.3%, 38.6%)
	Youden's J	79.2% (62.9%, 95.4%)
IDEXX[2]	Sensitivity	76.9% (60.7%, 93.1%)
	Specificity	100% (100%, 100%)
	True prevalence	36.6% (25.4%, 47.8%)
	Apparent prevalence	28.2% (17.7%, 38.6%)
	Youden's J	76.9% (60.7%, 93.1%)
ID.Vet[3]	Sensitivity	84.6% (70.7%, 98.5%)
	Specificity	100% (100%, 100%)
	True prevalence	36.6% (25.4%, 47.8%)
	Apparent prevalence	31.0% (20.2%, 41.7%)
	Youden's J	84.6% (70.7%, 98.5%)

[1]CIVTESTOVIS *M. agalactiae*; [2]IDEXX *M. agalactiae Screening* Ab Test; [3]ID Screen *Mycoplasma agalactiae* Indirect ELISA

Table 5. Agreement test between the three commercial ELISA tests studied at a 95% confidence interval (CI)

	HIPRA[1]/IDEXX[2]	HIPRA[1]/ID.Vet[3]	IDEXX[2]/ID.Vet[3]
Kappa coefficient	0.763	0.762	0.930
CI for kappa se(0)	0.701, 0.824	0.701, 0.824	0.860, 0.999
CI for kappa se(1)	0.723, 0.803	0.722, 0.802	0.904, 0.955
Observed agreement	87.0%	87.0%	96.5%
Expected agreement	45.2%	45.3%	49.4%
Observed agreement minus hazard	41.8%	41.7%	47.0%
Maximum agreement not due to hazard	54.8%	54.7%	50.6%
Concordant values			
Negatives	346	348	389
Positives	313	313	345
Doubtful	3	1	0
Discordant values	99	99	27
Total studied sera	761	761	761

[1]CIVTEST OVIS *M. agalactiae*; [2]IDEXX *M. agalactiae* Screening Ab Test; [3]ID Screen *Mycoplasma agalactiae* Indirect ELISA

Table 2 shows the distribution of the results yielded by each test according to the previous herd qualifications. Most of the sera positive in the three studied tests came from herds qualified as TP. Nevertheless, herds with previous TN qualification also yielded several positive sera (false positives). More false positive results were given by the IDEXX test and 12 such results were observed, while the HIPRA and ID.Vet tests yielded nine and four false positive results, respectively. Doubtful results were produced for both TP- and TN-qualified herds by all three ELISA tests.

The diagnostic results obtained with the three ELISA commercial tests studied for antibody detection of *M. agalactiae* (Table 3) were processed to estimate the validity parameters at a 95% confidence interval (Table 4). The three doubtful results, which were yielded by the HIPRA test, were not considered for this evaluation. Generally, the three studied ELISA tests

showed high validity parameters because the Youden's J ranged from 72.9% to 84.0%, with the ID.Vet test being the one with the highest global scores. With to sensitivity, the highest value was obtained for the ID.Vet test (84.6%), the middle-ranked value was for the HIPRA test (79.2%) and the lowest was for the IDEXX test (76.9%). For all, the specificity value reached 100%.

Table 5 shows the agreement test at a 95% confidence interval. According to the interpretation of Cohen's kappa coefficient (31), the agreement between the IDEXX and ID.Vet tests was almost perfect, whereas it was adequate between the HIPRA and IDEXX tests and between the HIPRA and ID.Vet tests. On the other hand, since the three kappa coefficients obtained were higher than 0.75, the concordance between them can be considered excellent according to the criteria of Fleiss *et al.* (12).

Discussion

Due to the diversity of the circulating strains of *M. agalactiae*, its genetic variability and other factors affecting the diagnostic results, the use of ELISA tests for serological diagnosis of *M. agalactiae* requires a previous evaluation of those tests (3, 11, 25). The results of the present study show that the three commercial tests are valid for use in CA control programmes, since their quantitative results for positive and negative sera were similar.

Most of the positive results (97.6%) were obtained in sera from herds qualified as TP, even if a few positive sera (2.4%) were detected in herds qualified as TN (Table 2), in agreement with previous findings (25). Herds in this situation, from samples of which the isolation of *M. agalactiae* was not achieved, without clinical signs of CA and without a history of CA in recent years, have been proposed as qualifying as false-positive herds (23). One explanation for the presence of these false-positive herds could be the occurrence of cross-reactions between different mycoplasmas, as some authors have pointed out (18, 29). These cross-reactions could be related to some similar epitopes in different *Mycoplasma* species, in this sense two of the studied tests use as an antigen one recombinant protein only present in *M. agalactiae* and in the bovine pathogen *M. bovis*. Appositely, the assessment of these kinds of sera by an immunoblotting technique has shown that the profiles of the antigens detected differ from those usually seen in sera from negative animals, suggesting that these sera could be false positive for the pathogen most commonly causing CA (25).

Similarly to the present work, the diagnostic evaluation of ELISA tests for antibody detection against *M. agalactiae* has been carried out in other countries such as New Zealand (19), Brazil (7), Italy (13) and France (23, 25). In addition to methodological differences, the geographic location has been identified as one of the factors affecting the efficacy of the different ELISA tests studied, because of the variation in the serological results obtained for different strains of *M. agalactiae* (25). In this context, ELISAs in Spain are investigated for the first time in this study with the evaluation of three commercial tests in small ruminant herds under field conditions. The assays showed sensitivity values between 76.9% and 84.6% (Table 4). Our sensitivity results agree with those reported for total antigen (48–89%) or protein P48 (56–82%) ELISAs (7, 13, 19, 23, 25) which means that we could not categorically define the antigen type detecting the higher percentage of infected animals. Because of the high antigenic variability of the circulating strains of *M. agalactiae* (11), the detection of specific antibodies in infected animals is affected by the degree of similarity between the antigen used to design the test and the circulating strains in the herds from which the tested sera originate (25). In addition, the sensitivity of ELISA tests for serological detection of *M. agalactiae* is compromised for the gold standard used and due to the antibodies' kinetics. A disassociation between the excretion of *M. agalactiae* in milk and the serological response has been reported, with 16% and 31% animals excreting *M. agalactiae* in milk and nevertheless being detected as seronegative by ELISA tests based on total antigens and P48 protein, respectively (25). A related findingin *M. agalactiae* experimental infections in goats was a decline in detectable antibody titres at 37 days post-infection (8). Despite its limitations, the isolation of *M. agalactiae* is the criterion recommended by the OIE to define infected animals (16) and was the criterion selected in the evaluation carried out in the present study. However, other non-infectious factors are related to a decline in antibody titres against *M. agalactiae*, such as the time until parturition (5), or the age of the animal, this being because of the limited serological response in young sheep (30).

In relation to the detection of seronegative animals, the three ELISA tests studied had perfect specificity (100%) (Table 4) and in this characteristic agreed with previously reported ELISA data ranging from 94% to 100% (19, 23, 25).

The percentage of doubtful results yielded by the three ELISA tests studied was 3.7%. The HIPRA test returned most (86.6%) of the 82 such results and these constituted 9.33% of its data (Table 1). The high number of doubtful results obtained with the HIPRA test, regardless of the sanitary status of the herd, could compromise its use in low prevalence conditions, because of how it limits the decision-making process (23).

Overall, the agreement test performed showed good agreement between the three commercial ELISAs studied (Table 5). Since the kappa coefficient was higher than 0.75 in all cases, an adequate agreement can be stated between all tests according to Thrusfield's (32) criteria, and this agreement could even be acclaimed excellent according to the methodology of Fleiss *et al.* (12). Notwithstanding this good agreement between the three tests, the best agreement (kappa value of 0.93) was

shown when comparing the tests based on the P48 protein, IDEXX and ID.Vet, which gave almost perfect agreement when measured according to Thrusfield (32). The concordance of the three commercial tests studied agrees with those reported between other commercial tests in previous studies (23) and suggests the usefulness of any one of them in CA control programmes. However, at the time of writing this paper, the IDEXX *M. agalactiae* Screening Ab Test is no longer commercially available, so only the other two commercial tests examined can be used for the detection of antibodies against *M. agalactiae*.

Regarding the study's limitations, we should point out the low number of positive sera (n = 26) *versus* negative sera (n = 45) selected for evaluation purposes (Table 3). Using specimens with a known health status or 'convenience samples' that do not come from observational studies, and which are not representative of the population, limits the external validity of the study (15), but it is useful to refine the case definition. In this sense, the results of this evaluation are valid for CA diagnosis of non-vaccinated lactating goats with clinical or subclinical mastitis and shedding of *M. agalactiae* by the galactogenic route. For this use case, and considering the validity parameters obtained, combining the serological ELISA tests with a direct diagnosis of *M. agalactiae* should be considered and determined appropriate or not according to previous CA records, prevalence levels and programme objectives. In addition, the epidemiological perseverance of *Mycoplasma* spp. infections in small ruminants from endemic areas is associated with the chronic infections of herds with a low frequency of clinical signs (14). In these situations, asymptomatic carriers have no detectable serological response (14, 26). The only study offering validity parameters of ELISA tests in asymptomatic goats was carried out with five animals and achieved sensitivities of 20% and 40% (19). Therefore, further observational studies are necessary to increase the knowledge of the effectivity of serological ELISA tests in detection of *M. agalactiae* in chronically infected herds.

The good validity parameters obtained for the three commercial ELISA tests studied for detection of antibodies against *M. agalactiae* recommend their use in CA control programmes for serological diagnosis of lactating goats excreting *M. agalactiae* in milk and with clinical signs. The adequate concordance demonstrated between the HIPRA test and the other two tests studied, and the almost perfect concordance achieved between the IDEXX test and the ID.Vet test make replacing one with another entirely feasible in CA control programmes.

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