Short communication

Serological technique for detecting tuberculosis prevalence in sheep in Atlantic Spain

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Abstract

Recent studies show that sheep could be considered to be a maintenance host for the causative agents of animal tuberculosis (TB). The performance of diagnostic tests is not well established, and new tests need to be developed for this species. In addition, information about TB prevalence in sheep is scarce. Our objectives were to evaluate a new P22 ELISA for detection of specific antibodies against *Mycobacterium tuberculosis* Complex (MTC), and to assess the seropositivity in 3,998 sheep from herds sampled in TB hotspot areas of northern Atlantic Spain with a low TB prevalence in cattle. Results based on 80 sheep of known infection status suggest excellent sensitivity and specificity (100% and 98%, respectively) even in a *M. avium* susbsp. *paratuberculosis* infected flock. The observed TB seroprevalence was 17.96% (698/3,998; CI95% 16.31- 18.67). Our results indicate that the P22 ELISA may constitute a good option for TB screening at the herd level in sheep, and that sheep are an important host and control programs should be implemented at least in hotspots or when cohabiting with other TB-infected species, i.e. cattle and goats.

Keywords: Animal tuberculosis; P22 ELISA; Serum antibodies; Sheep

Animal tuberculosis (TB) is one of the most important diseases worldwide in animal and public health. It is caused by members of the *Mycobacterium tuberculosis* Complex (MTC), mainly *M. bovis* and *M. caprae* (Rodríguez-Campos et al., 2014). MTC infects a wide range of hosts, including domestic and wild animals, resulting in a multi-host system where the relationship and contact between species poses an important risk of transmission and, therefore, hinders the control of the disease (Gortázar et al., 2015). Although sheep have been regarded as less susceptible to MTC, they have recently been described as a relevant host for TB when cohabiting with infected cattle or goats in specific epidemiological situations (Muñoz-Mendoza et al., 2016). The importance of sheep as a host may vary between regions depending on sheep density, production model, interspecies relationships with other ruminants and cattle and goat TB prevalence in the area (Muñoz-Mendoza et al., 2016).

There is little information on TB tests in sheep; ELISA and interferon-gamma (IFN-γ) provided good sensitivity (Se), while the single intradermal tuberculin test (SIT) provided low Se (Muñoz-Mendoza et al., 2016). In addition, SIT and ELISA showed low specificity (Sp), explained by potential cross-reactivity with *M. avium* subsps. *paratuberculosis* (MAP) (Muñoz-Mendoza et al., 2016). Due to the increasing number of TB reports in sheep (López et al., 2016; Muñoz-Mendoza et al., 2016; Vallejo et al., 2018; Gelalcha et al., 2019), new tests need to be developed. Sheep are not routinely subjected to TB testing within the European eradication scheme; therefore serological tests appear to be an attractive option. In this regard, an ELISA for specific antibody detection against MTC based on P22, a multiprotein complex purified from bovine purified protein derivative (bPPD) was developed (Infantes-Lorenzo et al., 2017). The ELISA showed good Se in high prevalence settings in cattle and goats

(Casal et al., 2017; Bezos et al., 2018) and excellent Sp in cattle, pigs and a *Corynebacterium pseudotuberculosis*-infected sheep herd (Infantes-Lorenzo et al., 2019).

Since the diagnostic tests available are limited and the epidemiological information of MTC in sheep is poor, the aims of this study were i) to evaluate the Se and Sp of the P22 ELISA in sheep and ii) to estimate the seroprevalence in sheep from herds sampled in TB hotspot areas in northern Atlantic Spain with low cattle TB prevalence.

The previously developed multispecies ELISA based on the P22 protein complex (Infantes-Lorenzo et al., 2019) was evaluated in order to calculate Se and Sp, using three sets of samples. The Se was evaluated using serum samples from 24 natural infected sheep with gross and microscopic TB compatible lesions, positive by *M. bovis* or *M. caprae* isolation (Muñoz-Mendoza et al., 2016). The Sp was evaluated in two different TB-free sheep herds from Castilla y León (north of Spain). Twenty-two (herd 1) and 34 animals (herd 2) were tested, respectively. Both herds were considered TB-free based on the absence of TB compatible lesions for more than 5 years at slaughter. Moreover, herds did not cohabit with other species and TB was absent in nearby herds. In addition, the 22 animals from herd 1 were negative for both *M. bovis* and MAP by culture and herd 2 was confirmed as a MAP-infected herd by culture. None of the herds studied were vaccinated against paratuberculosis (Johne´s disease).

Between 2012 and 2016, a cross sectional study was carried out to approach the prevalence of antibodies against MTC in sheep in TB hotspots from Atlantic Spain

(MAPA, 2016), located from the universal trade marcator (UTM) 42/43ºN 8ºW to 42ºN 1ºW (SIGPAC, 2019). Serum samples were obtained by jugular venipuncture into serum separation tubes (Vacutainer®, BD Diagnostics, Plymouth, UK) and stored at -20ºC. A total of 3,998 samples were analyzed; 1,840 sera were obtained from herds with a medium size of 10 sheep, and 2,158 sera from seven herds with a medium size of 308.3 animals. Serum samples were analysed by the indirect P22 ELISA (Infantes-Lorenzo et al., 2019). Briefly, plates were coated with P22 and blocked with 5% skimmed milk solution. After washes, sera were added in duplicated at 1:100 dilution in skimmed milk and secondary antibody (Rabbit anti sheep IGg(H/L)-HRP) at 1:2000 in PBS. Colour was developed by adding 100 µl of o-phenylenediamine dihydrochloride substrate (FAST OPD, Sigma–Aldrich, St Louis, USA). The reaction was stopped with 50 µl of H_2SO_4 (3 N) and the optical density (OD) was measured at 492 nm using a microplate reader Model 680 (Bio-Rad, Hercules, CA, USA). Sample results were expressed as an ELISA percentage (E %), calculated by the following formula: [sample E% = (mean sample OD/ 2 x mean of negative control OD) x 100]. The selected cut-off point was E%=150%. Data were analysed using SPSS 20.0 (IBM, Somers, NY, USA). A Wilson 95% confidence interval (95%CI) was calculated for each percentage. The true prevalence was calculated using the Rogan-Gladen estimator. Statistically significant differences were evaluated using Pearson Chi-Square test.

The overall Sp achieved with the P22 indirect ELISA was 98.21% (CI95% 90.55-99.68). Only one animal from the MAP-infected herd tested positive by the ELISA while no positive animals were observed in the MAP non-infected herd (Table 1). However, no significant difference was observed between groups (p= 0.286). Regarding Se, all animals were positive by the P22 ELISA, yielding a Se of 100%

(CI95% 86.20-100) (Table 1). The individual MTC seroprevalence obtained was 17.96% (698/3,998; CI95% 16.31- 18.67), and the estimated true prevalence using the Rogan-Gladen correction 16.28%. When results were expressed by herd type, the TB prevalence in sheep was 20.7% (95%CI 18.92-22.62) in small herds and 14.69% (95%CI 13.26-16-25) in large herds. The estimated true prevalence was 19.02 and 12.94% in small and large herds, respectively.

We found that the ELISA showed great Se and Sp even in a MAP-infected herd. In addition, TB prevalence was high as expected in sheep from TB hotspot areas (Muñoz-Mendoza et al., 2016).

Sheep were not regarded as an important MTC host until recently (Muñoz-Mendoza et al., 2016), thus, there are few reports of diagnostic tests in this species. Only a few studies have reported the accuracy of SIT, IFN-γ test or ELISA (Muñoz-Mendoza et al., 2016). Regarding ELISA, the Se reported was 100% and the Sp varied between 37 and 50% depending on the gold standard technique used (Muñoz-Mendoza et al., 2016). The Se shown by the previously reported ELISA (using bPPD as antigen) was similar to the Se of our ELISA. However, this result could be biased since samples were obtained from animals with TB-like lesions and culture positive that are known to be animals with high levels of antibodies. The analysis of a greater number of samples from MTC-infected animals, including cases without visible lesions, are needed for better estimation of Se. Regarding Sp, the previous ELISA yielded a very low Sp (37-50%), possibly due to MAP infection (Muñoz-Mendoza et al., 2016). In contrast, the P22 ELISA showed excellent Sp in the MAP-infected herd (98%), MAP-free herd (100%) and also in a *C. pseudotuberculosis* infected herd (98%) (Infantes-Lorenzo et

al., 2019). Further studies are necessary to evaluate the correlation between cellular based immunity and antibody tests; in goats, for example, the combination of humoral and cellular based tests showed the highest Se (Bezos et al., 2018). High Sp is particularly important in areas of low TB prevalence. Due to the excellent Sp obtained in previous and present studies in MAP vaccinated (Infantes-Lorenzo et al., 2019) or MAP infected herds, respectively, the P22 ELISA may constitute a good option for TB screening at the herd level in sheep, as we have demonstrated in this study.

The high TB seroprevalence obtained here is consistent with previous study where up to 59.42% of examined animals were positive by ELISA and 83% showed TB-like lesions by histopathology, albeit this was biased because those animals showed gross TB-like lesions at slaughterhouse (Muñoz-Mendoza et al., 2016). In that study, the high seroprevalence observed was mostly explained by cohabitation with TB-infected cattle and goats. We used the P22 ELISA to estimate seroprevalence in TB hotspots, and our results showed lower prevalence (17.96%) likely because animals were analysed without that bias.

The TB prevalence in cattle herds is low in Atlantic Spain (from 0.05 to 0.54 in 2018) (MAPA, 2019) and, although it has been decreasing in recent years, TB still remains an issue. The role of other domestic hosts such as sheep has been neglected in multi-host systems. Northern Spain has the largest number of sheep and cattle herds but the smallest average herd size (10.7 animal per herd *vs* national average of 158) (MAPA, 2019). In addition, herds are not under intensive management and sheep can cohabit with other species such as goats and cattle, which may facilitate the transmission of mycobacteria among animals. Furthermore, the weather in these regions

enables the mycobacteria to better survive in the environment (Fine et al., 2011). Since sheep are susceptible to MTC infection (Muñoz-Mendoza et al., 2016; Balseiro et al., 2017; Vallejo et al., 2018; Vidal et al., 2018) and the seroprevalence could be high as shown in this study, this species should be considered as a potential host and therefore as a potential source of infection for cattle and other species. Nevertheless, the immunohistochemical study of TB granulomas in sheep has demonstrated a particular slow development of lesions with a small number of mycobacteria present in granulomas (Vallejo et al., 2018), which would suggest less risk of shedding and thus of transmission. In any case, testing sheep for TB is recommended as well as the sacrifice of positive animals at least in hotspots or when cohabiting with other infected species such as cattle or goats (Vidal et al., 2018).

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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