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Brucellosis Prevalence in Livestock of Tavush and Kotayk Marzes of Armenia and Assessment of Diagnostic Algorithm

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ABSTRACT

Brucellosis is a common bacterial zoonosis caused by *Brucella spp.*, which are facultative intracellular gram-negative cocco-bacilli, causing disease in cattle, small ruminants, and humans. The occurrence of brucellosis is widespread across a wide geographical area as well as endemic throughout the Caucasus region. The Ministry of Agriculture of Armenia has adopted a diagnostic algorithm consisting of screening samples with the Rose Bengal Test (RBT) followed by confirmation with the complement fixation test (CFT). This study determined the feasibility of the algorithm and diagnostics in Armenia. Sampling and testing were conducted in two marzes of Armenia with disparate brucellosis prevalence. The screening was performed in 2020 at regional laboratories, followed by retesting at the reference laboratory with RBT and confirmation by CFT. Results indicated that RBT testing was reproducible between laboratories, although positive rates in CFT testing were slightly higher. Brucellosis incidence in cattle was not geographically dependent, while sheep incidence was affected by location. The addition of CFT to the diagnostic algorithm improves the results.

Introduction

Brucellosis is a common bacterial zoonosis caused by *Brucella spp.*, which are facultative intracellular gram-negative cocco-bacilli. It is most commonly a disease of cattle, small ruminants, and humans, with animal cases typically recognized by abortion (Acha, 2003; Głowacka, et al., 2018). The main species are *B. abortus, B. suis, B. melitensis, B. neotame, B. ovis*, and *B. canis* (Theron, 2014). Brucellosis is transmitted to humans through contact with infected animals and animal products, especially milk products (Khurana et al., 2021). The Food and Agriculture Organization (FAO), World Health Organization (WHO), and World Organization for Animal Health (WOAH) consider brucellosis one of the most pervasive zoonoses in the world (Corbel, 2005). Brucellosis also poses a hazard to laboratory staff handling specimens containing Brucella species, as the pathogen is

readily aerosolized and has a low infective dose. Therefore, brucellosis is becoming one of the most prevalent laboratory infections (Yagupsky and Baron, 2005; Sayin-Kutlu, et al., 2012). The most effective prevention strategy is infection elimination in animals (Corbel, et al., 2006). Many countries at risk of brucellosis lack diagnostic capacity to identify disease cases, which threatens affected countries and their neighbors (Kisman, 2010).

Brucellosis is widespread geographically, with cases reported in North Africa, the Mediterranean, the Middle East, and Central Asia. The disease is endemic in the Caucasus region (Porphyre, et al., 2010; Yumuk and O'Callaghan, 2012; Akhvlediani, et al., 2017). In Armenia, brucellosis is one of the most widespread zoonotic diseases, with more than 300 new cases annually (2019). Disease incidence is depends on geographical and demographic factors. Geographically, the Kotayk marz is dominated by pastures located centrally in the country, where it is a crossroads for seasonal animal movement. Farmers from multiple regions use pastures. The Tavush marz is dominated by forest zones. A primary demographic factor is the presence of a national minority group, the Yezidis in the Kotayk marz. This group maintains small ruminants in large numbers. In Armenia, previous risk mapping indicated a significant prevalence of 29 % and 21 % for cattle and small ruminants, respectively, in 858 communities surveyed. However, the distribution was uneven, which prevents accurate disease predictions risk, and suggests a possible problem with testing methods (Porphyre, et al., 2010).

Diagnosis of brucellosis is primarily accomplished through serological methods, but bacterial culture methods can also be used, although, culturing requires additional safety precautions (Dal, et al., 2019). Primary binding assays, such as enzyme-linked immunosorbent assay (ELISA), in serological diagnosis, determine the interaction of antibodies and antigens. Conventional tests measure secondary phenomena such as agglutination in the Rose Bengal Test (RBT), or the activation of complement in the complement fixation test (CFT). Each diagnostic has performance and cost considerations, such as low-cost screening tests versus high-cost and highly specific binding assays (Gall and Nielsen, 2004). One potentially confounding factor in brucellosis testing programs is vaccinating animals with strains such as S19 in cattle and Rev-1 in sheep and goats. These vaccine strains cause false positives in RBT. This issue is not important for Armenia. A State vaccination program has not been initiated to date. Importation and use of vaccines are restricted by the Armenia government and private individuals can not access them.

In 2019, the Ministry of Agriculture of Armenia made changes to its state brucellosis testing algorithm due to past limitations on the information supplied by the brucellosis testing regime in Armenia. Currently, in addition to screening livestock within each marz by RBT, positive samples must be confirmed by the Armenia Reference Laboratory for Especially Dangerous Pathogens (RLEDP) followed by an additional confirmatory test by complement fixation test (CFT). In 2020 following the implementation of the CFT, a pilot study was conducted in two marzes of Armenia. This study determined the feasibility of the algorithm and diagnostic tests in two marzes of Armenia with disparate brucellosis prevalence.

Materials and methods

The investigation was performed in 2020. The tests were conducted in the Reference Laboratory for Especially Dangerous Pathogens of the "Republican Veterinary-Sanitary and Phytosanitary Center of Laboratory Services" SNCO, under the Food Safety Inspection Body of the Republic of Armenia.

Sample collection

The two marzes selected for this study were Tavush and Kotayk, with a historically low and high prevalence of brucellosis, respectively. All regions of the Tavush marz were sampled, while two of three regions in Kotayk were sampled due to geographical barriers of the mountainous region. A total of 1.298 samples were collected with 535 blood samples from Tavush marz (cattle=257, sheep=278) and 763 samples from Kotayk marzes (cattle=215, sheep=548). From each animal, 5-10 ml of blood was collected in vacutainer tubes and transported to a regional laboratory. Blood was processed by centrifugation and serum was removed and stored at +4 ^{0}C until initial analysis.

Diagnostics

The diagnostic algorithm used for the processing of samples through the final result was as follows: Screening was conducted by RBT at the regional laboratory level. All sera were then transferred on ice to the RLEDP for retesting by RBT, followed by confirmation with CFT. Final results were determined at RLEDP and reported through government-access.

Screening for antibodies against Brucella was first conducted by the RBT (ANTIGEN, LTD) at regional laboratories. Then samples were transferred to RLEDP where RBT was repeated in duplicate. Positive samples were confirmed using CFT. In the case of split results between RBT and CFT, CFT was used as the final diagnosis. Briefly, serum samples and controls were aliquoted in 96 well plates and diluted 1:4 (25 µl) in veronal buffer. Equal volumes of diluted antigen (IDEXX) and complement (Rockland, USA) were added and incubated for 16-20 hours at +2-8 ^{0}C. Plates were incubated for 10 minutes at +37 ^{0}C followed by the addition of 50 µl of hemolysis serum consisting of

equal volumes of 2.5 % sheep red blood cells and optimized hemolysin (Rockland, USA). Plates were incubated at +37 ^{0}C for 30 minutes followed by 60 minutes at +2-8 ^{0}C. The percentage of hemolysis was determined by comparison to prepared standards, consisting of a series of dilutions of complement control from 0-100 % hemolysis. Samples with less than 50 % hemolysis were considered positive.

Statistical analysis

The relation of marz to disease status in cattle and sheep was calculated by the Pearson chi-square test for independence, computation of odds ratio, and likelihood test ratio. Analysis was performed utilizing SAS software.

Results and discussions

Among tested cattle from Tavush, 11.3 % tested positive (29/257) for brucellosis as indicated by the confirmatory CFT results. Incidence among Kotayk cattle was higher, with 18.6 % positive (40/215). Analysis indicates that there was a significant interaction between marzes (environment) and disease status in cattle, with Kotayk cattle 1.8 times more likely to be positive. An even stronger association between marz and disease status was indicated in sheep, with Kotayk sheep 2.4 times more likely to be positive for brucellosis. Disease incidence was higher in sheep than in the cattle in both marzes. This was 18.3 % sheep positive (51/278) in Tavush marz and 35.8 % positive (196/548) in Kotayk marz. The higher density of sheep kept in the Kotayk marz coupled with shared-farming husbandry practices should account for the higher prevalence.

When the results were examined by region within each marz (Table 1), there was no significant difference in the incidence of brucellosis among cattle. However, in sheep, the incidence of disease was significantly dependent on the region (Table 2). In the Kotayk marz, the odds of having a brucellosis-positive sheep are 2.0 times greater in the Nairi region than in the Abovyan region. The Tavush marz also revealed significant differences in brucellosis incidence by region. The odds of detecting a positive sheep in the Dilijan region were 3.5x higher than in Ijevan, 5.1x higher than in Noyemberyan, and 11.0x higher than in the Berd regions. The difference in odds of positive sheep was 3.1x higher in the Ijevan region than in Tavush. Brucellosis testing results by marz followed the historical trend of higher incidence in Kotayk marz than in Tavush. The current diagnostic algorithm for brucellosis was an initial screening test by RBT in each region's laboratory. This was followed by confirmatory testing at RLEDP by RBT and CFT. For this pilot study, all samples were tested by RBT and CFT for comparison purposes. Under normal testing parameters, only RBT positives and 10 % of negative samples would be forwarded to RLEDP for confirmation.

Test results were nearly identical between regional laboratory RBT results (59/472 positive cattle, 231/826 positive sheep) and Reference Laboratory results (63/472 cattle, 238/826 sheep). There were no statistical differences in the odds of testing positive by RBT the regional versus reference in laboratories. CFT produced slightly more brucellosis positives than RBT, especially in Tavush marz cattle (29 by CFT versus 18 by RBT), where additional positives were detected in samples that tested negative by RBT in both laboratories. Differences by test and region may be due to the skill level of technical staff or the condition of testing reagents. It has been shown that RBT antigens can deteriorate when used frequently due to repeated cold-to-room temperature cycles.

Table 1. Brucellosis incidence in cattle by marzes and region communities*

Marz	Region	Brucellosis positive animals quantity/ animals total quantity	Positive frequency within the region, %
Tavush	Ijevan	11/69	15.9
	Dilijan	5/55	9.1
	Noyemberyan	4/57	7.0
	Berd	9/76	11.8
Kotayk	Abovyan	40/206	19.4
	Nairi	0/9	0.0

Table 2. Brucellosis incidence in sheep by marzes and region*

Marz	Region	Brucellosis positive animals quantity/ animals total quantity	Positive frequency within the region, %
Tavush	Ijevan	15/64	23.4
	Dilijan	12/27	44.4
	Noyemberyan	15/86	17.4
	Berd	9/101	8.9
Kotayk	Abovyan	154/463	33.26
	Nairi	42/85	49.4

*Composed by the authors.

Conclusion

A summary of the pilot study results indicates that adding a confirmatory test (CFT) to the state brucellosis testing algorithm is an important step to the improvement of the

results before reporting. Additionally, baseline data on the prevalence of brucellosis in Tavush and Kotayk marzes has increased since 2010. These results also indicate that while geographic region does not impact the incidence of disease in cattle, there is a significant impact of location on the incidence of brucellosis in sheep. This pilot study should be expanded in the future to all marzes of Armenia. Further expansion of the data available would better inform public health policy as well as allow the development of improved testing and slaughter management plans. This would reduce the incidence of brucellosis in Armenia.

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Declarations of interest

The authors declare no conflict of interest concerning the research, authorship, and/or publication of this article.

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