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COMMUNICATION

Isolation, identification, and antimicrobial susceptibility of *Brucella* spp. cultured from cows and goats manure in Mexico[#]

Aislamiento, identificación y susceptibilidad antimicrobiana de *Brucella* spp. cultivadas de materia fecal de vacas y cabras en México

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ABSTRACT. Brucellosis is a zoonosis that affects many animal species worldwide. Humans are often infected through direct animal contact, through contact with animal excretions, or through ingestion of unpasteurized dairy products. Livestock manure is extensively used as a pasture fertilizer in many production systems, especially in non-developed countries. Some microbiological studies warn of the risk of manure being a disseminator vector of pathogenic microorganisms. The present study aimed to isolate *Brucella* in manure from cows and goats that were serologically positive for brucellosis in an endemic region of Mexico. We then used E-test methods to evaluate the isolated microorganisms' susceptibility to antimicrobial agents *in vitro*. *Brucella* isolation was performed via a pre-enriched selective culture, and subcultures using selective agar plates. Isolates were identified using microbiological tests as well as BCSP31 PCR and Bruce-Ladder multiplex PCR. We isolated 10 *Brucella* spp. strains, all of which amplified the genus-specific gene that encodes the BCSP31 protein. The species were identified using multiplex PCR. Interestingly, *B. melitensis*, *B. abortus,* and *B. suis* were isolated from cow manure, while *B. abortus* was just isolated from goat manure. The isolates did not include any strains that are routinely vaccinated against. Some isolates were resistant to ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, and rifampicin. Manure from infected animals could represent a vehicle for human exposure to *Brucella.* More detailed studies are needed to evaluate *Brucella*-contaminated manure as a risk factor in endemic regions.

Key words: brucellosis, *Brucella*, manure, zoonosis.

RESUMEN. La brucelosis es una zoonosis distribuida mundialmente que afecta varias especies animales. Los humanos se infectan por el contacto con animales infectados, sus excreciones o por la ingesta de derivados lácteos no pasteurizados. Las heces excretadas por el ganado se usan como fertilizante en países no desarrollados. Estudios microbiológicos han alertado sobre el riesgo de las heces o estiércol como vector de diseminación de microorganismos patógenos. El objetivo de este trabajo fue aislar *Brucella* de heces de vacas y cabras y evaluar la sensibilidad contra antimicrobianos mediante el método de E-test. Se analizaron heces de vacas y cabras serológicamente positivas a brucelosis de una región endémica de México. El aislamiento de *Brucella* se llevó a cabo en un medio de preenriquecimiento y posteriormente se aisló en medio selectivo. La identificación se realizó mediante pruebas microbiológicas y por PCR. Se obtuvieron 10 aislados de *Brucella* spp., que fueron positivos a la amplificación del gen específico de género que codifica para la proteína BCSP31 y mediante multiplex Bruce-Ladder se identificó la especie. Se demostró la presencia de *B. melitensis*, *B. abortus* y *B. suis* en las heces de las vacas, mientras que *B. abortus* solo se aisló de las cabras. Ninguna cepa vacunal se encontró en el estudio. Algunos aislados fueron resistentes a ciprofloxacina, levofloxacina, trimetoprim-sulfametoxazol, y rifampicina. Las heces de animales infectados representan un riesgo de exposición a *Brucella*. Se requieren estudios más detallados para evaluar el factor de riesgo asociado a la materia fecal contaminada con *Brucella* en regiones endémicas.

Palabras clave: brucelosis, *Brucella*, heces, zoonosis.

INTRODUCTION

Animal manure, a fecal waste generated by livestock, has a long tradition of use in agriculture as a valuable source of available plant nutrients and organic matter, improving soil quality and fertility. However, the manure of wild and domestic animals can contain a wide variety of pathogenic viruses, bacteria, and parasites, and thus manure use carries a risk of spreading animal diseases and zoonotic agents. Several studies have investigated the prevalence and levels of pathogenic bacteria in manure, including *Salmonella*, *Campylobacter*, *Escherichia coli* O157:H7, and *Listeria* (Hutchison *et al* 2005). *Brucella* can survive in manure experimentally, but little is known about the risk for humans in contact with naturally infected manure in the field. Wallach *et al* (2008) reported an outbreak of human brucellosis among farm workers in Mendoza, Argentina, which was attributed to contact with manure of infected goats. In this outbreak, *Brucella melitensis* was isolated from milk of goats and in blood cultures from farm workers.

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Brucellosis is caused by multiple species of the *Brucella* genus. *B. melitensis* is the most pathogenic species, followed by *B. abortus* and *B. suis*. These bacteria can infect a wide variety of animals, including cattle, pigs, sheep, goats, horses, and dogs. *Brucella* can be excreted in vaginal fluids, including with aborted fetuses, and thus may contaminate feces and bedding (Corbel 1997). *Brucella* can be transmitted from infected animals to humans through direct contact with aborted fetuses and related materials, as well as through consumption of raw milk or unpasteurized dairy products (Corbel 1997). This study reports *Brucella* isolation and identification from the manure of cows and goats that were serologically positive for brucellosis. It also evaluated the antimicrobial susceptibility of these *Brucella* isolates.

MATERIAL AND METHODS

ANIMAL SAMPLES

The investigated animals were from 3 farms located in a brucellosis endemic region of Mexico. We serologically analyzed 5 dairy herds with approximately 60 cows, and 3 herds with 50 goats. Sampling was performed during August and September of 2012. Animal blood was taken from the jugular vein for use in serological tests. Cow serum was analysed using the card agglutination test, and positive results were confirmed by rivanol tests (agglutination titers ≥ 1:50 were considered positive for brucellosis) (Norma Oficial Mexicana NOM-041). Goat serum was also analysed using the card agglutination test, and positive results were confirmed by complement fixation test (titers ≥ 1:8 were considered positive for brucellosis) (Norma Oficial Mexicana NOM-041). Testing revealed that 20 cows and 10 goats were serologically positive for brucellosis, corresponding to around 60% of the population of these farms. Manure samples were collected in sterilised plastic bottles directly from the anus of each animal, and were transported to the laboratory at 4 °C. Samples were analysed in the laboratory within 24 h after collection.

BRUCELLA ISOLATION AND IDENTIFICATION

The pre-enriched broth medium was prepared with tryptic soy broth (Becton Dickinson); 0.5% yeast extract; 5% horse serum; 1% glucose; and commercially available *Brucella* selective supplement (Oxoid catalog number SR0209E), which contains polymyxin B, bacitracin, natamycin, nalidixic acid, nystatin, and vancomycin and was prepared according to the manufacturer's recommendations. For agar plates, the formulation was the same with addition of 2% agar.

We added 15 g of each manure sample to bottles with 200 mL of pre-enriched broth medium, and these bottles were incubated at 37 °C for 48 h in a shaking incubator. The contents of each bottle were then filtered by syringe with a sterile cotton ball to remove solid debris, and the liquid was centrifuged at 3,000 × *g* for 30 min. After centrifugation, 0.5 mL of the resulting pellet was spread on selective agar plates containing antibiotics, which were incubated at 37 °C with 5% CO_2 for 5 to 15 days. Colonies showing a *Brucella* phenotype were selected and microbiologically typed based on H_2S and urease production, fuchsin and thionin sensitivity, lysis by *Brucella*-specific bacteriophages (Tbilisi, Weybridge and Berkeley), and agglutination with monospecific A and M antisera (Alton *et al* 1988).

To estimate the number of *Brucella* in each manure sample, the pellet obtained by centrifugation (as described above), was dissolved in 0.5 mL sterile saline. We then performed a ten-fold serial dilution in sterile saline. Aliquots of 0.5 mL were inoculated on selective antibiotic-containing agar plates, and the colonies on the plates were counted to obtain the CFU of *Brucella*/g.

MOLECULAR IDENTIFICATION OF BRUCELLA ISOLATES

Genomic DNA was obtained using the previously described cetyltrimethylammonium bromide (CTAB) method (Wilson 1987). All isolates were tested by amplification of the *bcsp*31 genus-specific gene, and multiplex Bruce-Ladder PCR to identify *Brucella* species as reported previously (Morales-Estrada *et al* 2012). The reference strains *B. melitensis* 16M, *B. abortus* 2308, and *B. suis* 1330 were used as positive controls, and *Ochrobactrum anthropi* was used as a negative control.

Antimicrobial susceptibility testing

We used the E-test method (Biomerieux, Sweden) to determine the minimal inhibitory concentration (MIC) values of tetracycline, rifampicin, ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazole (TMP-SMX). Briefly, for each *Brucella* strain, we created a bacterial suspension adjusted to a turbidity equivalent of 0.5 McFarland standard. These bacterial suspensions were spread onto Mueller-Hinton (Oxoid catalog number CM337) agar plates supplemented with 5% sheep's blood, and one E-test strip was placed on the plate. The plates were incubated at 37 °C for 48 h. The MIC was considered the value at which the inhibition zone intercepted the scale on the E-test strip. *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* 1330 were used as positive controls, and *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains for susceptibility testing (Hashim *et al* 2014). The MIC values of tetracycline, ciprofloxacin, and TMP-SMX for *Brucella* spp. were interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI). For rifampicin and levofloxacin, we used the *Haemophilus* spp. breakpoint recommended for slow-growing bacteria because no breakpoints were defined for *Brucella*. Based on these criteria, the tested strains were classified as resistant or sensitive to these antimicrobials.

RESULTS AND DISCUSSION

Dilution plating revealed 2-100 CFU of *Brucella*/g from each sample. Based on monospecific antisera, bacteriophages, and microbiological results, we identified three isolates from cow manure as *B. abortus* biovar 1, three isolates from cow manure as *B. melitensis* biovar 1, three isolates from goat manure as *B. abortus* biovar 1, and one isolate from cow manure as *B. suis* biovar 1.

Brucella species are generally host-specific but cross-species infections commonly occur when different species of animals share the same stables, pasture, or facilities. Cases of *B. melitensis* cross-infection have been described in mixed herds of sheep and goats in southern Europe (Verger *et al* 1989) and are regularly reported in the Middle East (Samaha *et al* 2008). Our present results show cross-infections with *B. abortus*, *B. melitensis*, and *B. suis* in a brucellosis endemic region of Mexico. Within this region, the main farm animals are cattle, followed by goats, sheep, and pigs. Herds of these animals co-exist closely, which likely contributes to the difficulty of eradicating brucellosis in this area.

Usually, brucellosis in cattle and goats is diagnosed based on serological studies, and it is not routine practice to isolate *Brucella* from animals in endemic regions of Mexico. Thus, little information is available regarding the prevalence of *Brucella* species in herds and flocks. Vaccines against brucellosis, *B. abortus* RB51 and B19, and *B. melitensis* Rev. 1, are administered to bovines and goats, respectively, in this region. Our present study included the use of the Bruce-Ladder, a multiplex PCR that can differentiate *Brucella* species and vaccine strains: *B. abortus* S19 and RB51 and *B. melitensis* Rev 1 (Morales-Estada *et al* 2012). Results of Bruce-Ladder PCR showed no vaccine strains among the *Brucella* isolates from manure (figure 1).

Brucella spp. are fastidious and relatively slow-growing microorganisms. Thus, their isolation from organs, tissue, or fluids of infected animals requires the use of selective media to reduce or eliminate the associated microbiota. Based on our experience and the reports of other authors, *Brucella* recovery in milk from *B. abortus*-infected cows ranges from 10 to 30% (Langoni *et al* 2000). Our present results showed approximately 33% recovery of *Brucella* spp. from the manure of serologically positive cows and goats using a pre-enriched selective medium.

The use of animal manure contaminated with zoonotic pathogens may promote the transfer of these pathogens. Our present finding of *Brucella* species in manure from domestic animals suggests a means of *Brucella* dissemination, and may represent a potential risk for animal and human health. Moreover, *Brucella* species can reportedly persist for several days or weeks in various environmental conditions depending on temperature and presence of organic materials. Data show that *B. melitensis* can survive in moist faeces for less than 75 days, while *B. suis* can survive for 120 days in cattle feces at room temperature, and for 4–37 days in soil (Burton and Turner 2003). Another report indicates that *B. abortus* can survive for 250 days in manure at 12 °C (Hagan *et al* 1988). In wolves experimentally infected with *B. abortus* biovar

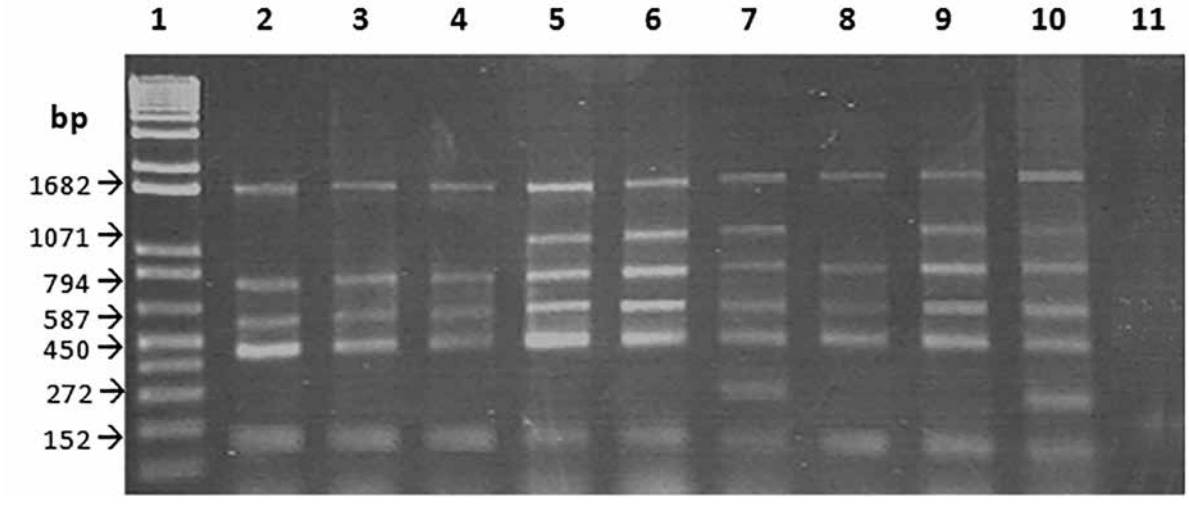

Figure 1. Multiplex PCR assay for the identification of *Brucella* strains isolated from samples of cow and goat faeces. Lane 1, 1-kb ladder; Lanes 2–4, *B. abortus* strains from goats; Lanes 5 and 6, *B. melitensis* strains from cows; Lane 7, *B. suis* strain from cow; Lanes 8, 9, and 10, positive control DNA from *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* 1330, respectively; Lane 11, negative control DNA from *Ochrobactrum anthropi*.

Ensayo de PCR multiplex para la identificación de cepas de *Brucella* aisladas de la materia fecal de vacas y cabras. Pozo 1, Marcador de peso molecular 1Kb; pozo 2-4, cepas de *B. abortus* aisladas de cabras; pozos 5 y 6, cepas de *B. melitensis* aisladas de vaca; pozo 7, cepa de *B. suis* aislada de vaca; pozos 8-10, testigo positivo (DNA de *B. abortus* 2308, *B. melitensis* 16M y *B. suis* 1330, respectivamente); pozo 11, testigo negativo (DNA de *Ochrobactrum anthropi*).

1, bacteria were cultured from manure for up to 50 days post-infection (Tessaro and Forbes 2004). One report describes a case of *B. melitensis* isolated from an abscess of the left foot in 3-year-old child who lived on a ranch and was in contact with cattle. The girl suffered a trauma from an unidentified object that caused the foot lesion, and it likely became contaminated with manure or other animal material present in the soil (Resendiz-Sanchez *et al* 2009). Recently, Jahne and coworkers (2015) reported that bioaerosol emitted from manure application sites following manure application may present significant public health risks to downwind receptors. The Center for Diseases Control and Prevention (CDC) states that *Brucella* has a low infectious dose and shows ease of aerosolization; however, the health risk of *Brucella*-containing manure bioaerosols has not been documented.

Brucella is an intracellular facultative bacterium that can potentially evade the immune system and produce a relapsing or chronic disease presenting with a broad spectrum of clinical manifestation and complications (Corbel 1997). Thus, the treatment for brucellosis in humans is long and based on the use of two antibiotics. Farm animals receive antibiotics during breeding and *Brucella* is transmitted from animals to humans; therefore, it was important to determine the sensitivity of *Brucella* isolates to antibiotics. For this purpose, we used the E-test method, an antibiotic susceptibility test that determines the MIC and that is recommended for fastidious and slow-growing microorganisms. The E-test uses strips that contain an antibiotic concentration gradient, enabling simple MIC determination. Based on breakpoints outlined by the CLSI in 2013 and 2014, we found that one *B. abortus* isolated from goats was resistant to rifampicin and TMP-SMX, and showed a MIC of 6 µg/mL for tetracycline. One *B. melitensis* isolate from cow manure was resistant to ciprofloxacin, levofloxacin, and TMP-SMX, and showed a MIC of 32 µg/mL for tetracycline. Finally, a *B. suis* isolate from cow manure was resistant to ciprofloxacin. The rest of the isolates were sensitive to all antimicrobial agents (table 1).

Hashim *et al* (2014) recently reported the isolation of rifampicin-resistant *B. melitensis* from human patients. A previous study also tested *Brucella* isolates from Mexico against different antimicrobial agents, and found that all isolates (97 strains) were sensitive to quinolones, tetracycline, doxycycline, TMP-SMX, and rifampicin, but 6 strains of *B. abortus* were resistant to streptomycin (Lopez-Merino *et al* 2004). The results in this study showed that 3 isolated *Brucella* strains were resistant to quinolones. Furthermore, 2 isolated strains showed a high MIC for tetracycline (above 6 µg/mL), which is one of the best options for brucellosis treatment. Unfortunately, we did not test streptomycin in the present work. The E-test method had previously been used by Maves *et al* (2011) with *Brucella* strains from Peru, and by Kasymbekov *et al* (2013) to investigate *Brucella* strains from Kyrgyzstan. Both of these prior reports showed that all isolates were sensitive to the tested antimicrobials. Due to the potential existence of resistant isolates, evaluating the sensitivity of *Brucella* strains to the antimicrobials used in therapy should be established as a routine method in clinical laboratories.

In summary, our present results showed the presence of different *Brucella* species in manure from cows and goats in an endemic brucellosis region. Some of the isolates were resistant to quinolones, TMP-SMX, and rifampicin. Manure from infected animals could be a vehicle for *Brucella* dissemination; therefore, we recommend careful management of this material. Further studies are needed concerning the environmental impact of *Brucella*-contaminated manure in endemic regions.

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Table 1. Antibiotic susceptibility of *Brucella* strains isolated from manure.
Susceptibilidad antimicrobiana de cepas de *Brucella* aisladas de materia fecal.

Antibiotics	*B. abortus* (6)				*B. melitensis* (3)		*B. suis* (1)		Breakpoint for susceptibility (µg/mL)
	goat		cow		cow		cow		
	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)	
Tetracycline	2	1	3	0	2	1	1	0	≤ 1[a]
Trimethoprim/ Sulfamethoxazole	2	1	3	0	2	1	1	0	≤ 2/38[a]
Ciprofloxacin	3	0	3	0	2	1	0	1	≤ 1[a]
Levofloxacin	3	0	3	0	2	1	1	0	≤ 2[b]
Rifampicin	2	1	3	0	3	0	1	0	≤ 1[b]

[a] Standard breakpoints for *Brucella* spp. from Clinical and Laboratory Standards Institute guidelines.
[b] Standard breakpoints for slowly growing bacteria (*Haemophilus* spp.) from Clinical and Laboratory Standards Institute guidelines.

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