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EPIDEMIOLOGICAL CHARACTERIZATION OF BOVINE PARATUBERCULOSIS IN THE STATE OF PARAÍBA

PATOS, 2016

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Tese apresentada ao programa de pós-graduação em Medicina Veterinária da Universidade Federal de Campina Grande – UFCG em cumprimento do requisito para obtenção do título de Doutor em Medicina Veterinária.

Prof. Dr. Sérgio Santos de Azevedo
Orientador

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Dedico

A meu pai Lindonôr Tôrres Vilar

(*in memorian)*

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ABSTRACT

This study focused on estimating the herd-level and animal-level prevalences, and identifying herd-level spatial clustering and risk factors associated with herd-level prevalence for bovine paratuberculosis in the State of Paraíba, Northeastern Brazil. The state was divided into three sampling groups: sampling stratum 1 (mesoregion of Sertão), sampling stratum 2 (mesoregion of Borborema), and sampling stratum 3 (mesoregions of Zona da Mata and Agreste). For each sampling stratum, herd-level and animal-level prevalences were estimated by a two-stage sampling survey. In the first stage, a pre-established number of herds (primary sampling units) were randomly selected; in the second stage, a pre-established number of cows aged ≥ 24 months were randomly selected (secondary sampling units). In total, 2504 animals were sampled from 480 herds. Serological diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection was performed by the indirect ELISA kits. A herd was deemed positive if it included at least one positive animal in herds of up to 24 females, and two positive animals in herds with more than 24 females. The herd-level prevalence in the State of Paraíba was 34.5% (95% CI = 30.2–39.1%), 41.4% (95% CI = 34.0–49.1%) in Sertão, 26.6% (95% CI = 20.2–34.2%) in the region of Borborema, and 30.5% (95% CI = 23.9–38.0%) in Agreste/Mata. The animal-level prevalence was 10.7% (95% CI = 7.3–15.4%) in the State of Paraíba, 9.4% (95% CI = 7.3–12.1%) in Sertão, 7.9% (95% CI = 5.2–11.7%) in the region of Borborema and 13.9% (95% CI = 6.2—28.3%) in Agreste/Mata. The frequency of seropositive animals per herd ranged from 6.7% to 100% (median of 20%).The risk factors identified were as follows: Sertão region (OR = 1.9), more than 12 adult animals in the herd (OR = 1.9), and not using maternity pens (OR = 1.7). Two significant clustering of positive herds were detected in Northern part of Borborema mesoregion. Our findings suggest that MAP herd-level seroprevalence in the State of Paraíba, Northeastern Brazil, is high, and support the idea that the use of maternity pens will be important for preventing transmission of MAP in the herds. As serological tests for MAP diagnosis are not widely available and are very expensive, as well as replacement or maintenance of livestock by animal purchasing is common in the region, it is concluded that prevention measures should be applied at herd level.

Key words: Paratuberculosis; Cattle; Epidemiology; Spatial clustering; Control; Northeastern Brazil.

RESUMO

Os objetivos destes trabalhos foram estimar as prevalências da paratuberculose bovina em nível de rebanho e animal bem como identificar os fatores de risco associados com a ocorrência da paratuberculose e identificar agrupamentos espaciais em nível de rebanho para paratuberculose bovina no Estado da Paraíba, Nordeste do Brasil. O Estado foi dividido em três grupos amostrais: estrato amostral 1 (mesorregião do Sertão), estrato amostral 2 (mesorregião da Borborema) e estrato amostral 3 (mesorregiões da Zona da Mata e Agreste). Para cada estrato amostral, as prevalências de rebanhos e de animais soropositivos foram estimadas por amostragem em dois estágios. No primeiro estágio, um número pré-estabelecido de rebanhos (unidades primárias de amostragem) foi selecionado aleatoriamente; no segundo estágio, um número pré-estabelecido de vacas com idade ≥ 24 meses (unidades secundárias de amostragem) foi selecionado aleatoriamente. No total, 2.504 animais foram amostrados de 480 propriedades. Para a detecção e anticorpos anti-*Mycobacterium avium* subsp. *paratuberculosis* (MAP) foram usados kits de ELISA indireto. Um rebanho foi considerado positivo se incluiu pelo menos um animal positivo em rebanhos de até 24 fêmeas, e dois animais positivos em rebanhos com mais de 24 fêmeas. A prevalência de rebanhos positivos no Estado da Paraíba foi de 34,5% (IC 95% = 30,2%–39,1%), 41,4% (IC 95% = 34,0%–49,1%) no Sertão, 26,6% (IC 95% = 20,2%–34,2%) na Borborema e 30,5% (IC 95% = 23,9%–38,0%) no Agreste/Mata. A prevalência de animais soropositivos foi de 10,7% (IC 95% = 7,3%–15,4%) no Estado da Paraíba, 9,4% (IC 95% = 7,3%–12,1%) no Sertão, 7,9% (IC 95% = 5,2%–11,7%) na Borborema e 13,9% (IC 95% = 6,2%–28,3%) no Agreste/Mata. A frequência de animais soropositivos por rebanho variou de 6,7% a 100% (mediana de 20%). Os fatores de risco identificados foram os seguintes: propriedade ser localizada no Sertão (OR = 1,9), mais de 12 animais adultos no rebanho (OR = 1,9), e não usar piquetes de parição (OR = 1,7). Foram detectados dois agrupamentos significativos de rebanhos positivos na parte norte da mesorregião da Borborema. Os resultados sugerem que a soroprevalência de paratuberculose bovina em nível de rebanho no Estado da Paraíba, Nordeste do Brasil, é alta, bem como que a utilização de piquetes de parição será importante para a prevenção da transmissão de MAP nos rebanhos. Tendo em vista que os testes sorológicos para diagnóstico de MAP não são amplamente disponíveis e são muito caros, bem como que é comum na região a reposição e manutenção dos rebanhos por compra de animais e conclui-se que medidas de prevenção devem ser aplicadas em nível de rebanho.

Palavras-chave: Paratuberculose, Bovino; Epidemiologia; Análise de aglomerados espaciais, Controle, Nordeste do Brasil.

SUMMARY

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LIST OF ABBREVIATIONS AND ACRONYMS

MAP = *Mycobacterium avium* subsp. *paratuberculosis*

PTB = Paratuberculosis

JD = Disease Johne

PCR = Polymerase chain reaction assays

ZN = *Ziehl–Neelsen*

ELISA Enzyme-Linked Immunosorbent Assay

CI = Confidence interval

CD = Disease Crohn

DNA = Deoxyribonucleic acid

OR = *Ods Ratios*

SEDAP = Agricultural and Livestock Defense Service of the Paraiba

PC= Positive control

NC = Negative control

PNCBT = National Program for Control and Eradication of Brucellosis and Tuberculosis

Se = Sensitivity

Sp = Specificity

ICP= Individual calving pens

GCP = Group calving pens

GENERAL INTRODUCTION

Paratuberculosis, also known as Johne's disease is a chronic infectious disease with a long incubation period, caused by *Mycobacterium avium subsp. paratuberculosis* (MAP), mainly affecting domestic ruminants and less often wild ruminants, horses, pigs, hares, foxes and rodents(RIET-CORREA et al., 2001; RAIZMAN et al.,2005;YAMASAKI et al., 2013). The typical symptoms of paratuberculosis in cattle and buffalos occurs in animals after two years of age and is characterized by progressive weight loss, although the animals had normal appetite or even exacerbated, dehydration, intermittent diarrhea, profuse, homogeneous semifluid or liquid, non-responsive to treatments which gradually becomes continuous and the elimination of feces is in the form of spray (CLARKE, 1997; BEHR e COLLINS, 2010; YAMASAKI et al., 2013).

The importance of the disease in domestic ruminants has been reported because of the economic losses due to the paratuberculosis presence in cattle and buffalo herds. The main losses include reduced feed efficiency, reduced fat and milk protein, reduced slaughter weight, decreased fertility and increased incidence of mastitis, early animals sale, decrease in milk production, reproductive changes as increased calving interval , reducing the animal value and restrictions on their marketing, reducing the milk value; in addition to other costs such as veterinary care, tests, medications. (NIELSEN, 2011; YAMASAKI et al., 2013, RADOSTITS et al., 2007).

The zoonotic potential of the disease is not fully established. It is known that 10% of animals with subclinical phase eliminate agent in milk. The bacteria can withstand the pasteurization and the milk intake may be an important route of transmission to humans. The MAP agent appears was related to the Crohn's disease etiology, as its causative agent, or a factor of a multi-etiologic syndrome. The agent was isolated in 70% of patients with Crohn's disease, which could indicate a relevant role in infection (COUSSENS, 2004; SHIN et al., 2004). Furthermore, in 33.3% of patients with Crohn's disease was the agent's DNA detection (JUAREZ et al., 2001).

In Paraíba, between the years 2009 and 2012, five paratuberculosis outbreaks were diagnosed at the Veterinary Hospital of the Federal University of Campina Grande (UFCG) in Patos, PB, in dairy cattle herds of the State (personal information - Riet - Correa) . These data suggest that paratuberculosis is an important disease of ruminants in the semiarid region of Paraíba and it is necessary to know its epidemiology to determine appropriate control measures for the region.

This thesis consists of three chapters. Chapter I includes a literature review on the current status of bovine paratuberculosis in Brazil, which was submitted to the journal *Semina: Agricultural Sciences.* In Chapter II was carried out an epidemiological study on 480 serum farms in State of Paraiba in order to determine the prevalence of positive herds and animals, and to identify risk factors associated with prevalence of positive herds. The article related to this chapter was

published in the journal *Preventive Veterinary Medicine* (v. 121, p. 49-55, 2015). Chapter III contains an analysis of spatial clusters of positive properties in order to identify risk area of transmission of the disease in the state of Paraiba, and article derivative was submitted to the journal P*esquisa Veterinária Brasileira.*

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Bovine paratuberculosis: epidemiological situation in Brazil

Paratuberculose bovina: situação epidemiológica no Brasil

Ana Letícia Torres Vilar[1]; Sérgio Santos de Azevedo[2*]

Abstract

Bovine paratuberculosis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is an infectious disease characterized by chronic granulomatous enterocolitis, incurable and difficult to control. That presents ecomonic importance for ruminants in several countries and may poses a threat to the development of brazilian cattle production as it can cause economic losses related to decreasing in productivity, premature disposal of animals and increased health costs. This review covers the current epidemiological situation of bovine paratuberculosis in Brazil related to serological and MAP isolation and identification surveys, as well as issues related to economic importance and public health.

Key words: MAP. Paratuberculosis.Bovine.Epidemiology. Brazil.

Resumo

A paratuberculose bovina causada por *Mycobacterium avium* subsp. *Paratuberculosis* (MAP) é uma doença infecciosa caracterizada por enterocolite granulomatosa crônica, incurável e de difícil controle. A doença apresenta importância econômica para ruminantes em vários países e pode representar uma ameaça ao desenvolvimento da pecuária brasileira, pois pode causar perdas econômicas na produção de bovinos relacionadas à redução na produtividade, descarte prematuro de animais e aumento dos custos sanitários. Esta revisão aborda a situação epidemiológica atual da paratuberculose bovina no Brasil no que diz respeito aos estudos sorológicos e de isolamento e identificação de MAP, bem como os aspectos relacionados à importância econômica e Saúde Pública.

Palavras-chave: MAP. Paratuberculose. Bovino. Epidemiologia.Brasil.

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Introduction

Paratuberculosis (PTB), also known as Johne's disease (JD), is an infectious disease caused by Mycobacterium avium subsp. paratuberculosis (MAP), characterized by granulomatous enterocolitis chronic, incurable and difficult to control, and that has economic importance for ruminants in many countries and can pose a threat to the development of brazilian cattle (MOTA et al., 2010), it can cause economic losses in the production of cattle related to reduced productivity, premature disposal of animals and increased health costs (KUDAHL; NIELSEN, 2009).

Mycobacterium avium subsp. *paratuberculosis* (MAP) is classified as a member of the order *Actinomicetales*, family *Mycobacteriaceae*, and *Mycobacterium avium-intracellulare* complex. This complex comprises four species: *M. avium avium*, *M.avium paratuberculosis* (MAP), *M. avium silvaticum* and M. *avium intracellulare*.(LILEMBAUM, MARASSI e OELEMANN, 2007). It is an aerobic bacillus, immobile, non-sporulated Gram-positive, with approximate dimensions of 2 mm by 0.5 mm, with a high content of mycolic acids in their cell wall that was first described in Germany in the year 1895.Because the presence of the waxy material on its surface, this is an alcohol-acid resistant cells (GRANT, 2005).

In cattle, clinical signs more suggestive of infection by MAP are progressive weight loss, intermittent or chronic diarrhea (CLARKE, 1997). Clinical disease can be facilitated by childbirth, lactation or other stress conditions (CHIODINI, VANKRUININGEN and MERKAL, 1984). The diagnosis of cattle PTB is divided into two parts - the clinical disease diagnosis and subclinical infection detection - and this latter is essential to control the disease in the flock level, national or international. In cattle, clinical cases can be diagnosed without difficulty because chronic diarrhea in adult animals is indicative of the disease. Laboratory diagnosis can be performed by agent isolation in stool or autopsy material, histopathological examination of the lesions and the Polymerase Chain Reaction (PCR). In the staining method Ziehl-Neelsen stain, bacteria acid-alcohol are found in stool smears and samples taken from the end small intestine portions, however, the ELISA test has been referred to as higher than the Ziehl-Neelsen the presumptive diagnosis confirmation of clinical PTB in cattle (WEBER et al., 2009).

Subclinical cases can be diagnosed by bacterial isolation in stool, serum or allergy tests (LILENBAUM, MARASSI and OELEMANN, 2007). Hendrick et al. (2005), observed that the indirect ELISA applied to serum and milk can be efficient and convenient in PTB subclinical detection in dairy cows, with sensitivity of 73.6% (95% CI = 61.9 to 83.3%), 61.1% (95% CI = 48.9 to 72.4%), and specificity of 87.5% (95% CI = 84.7 to 90.0%), and 94.7% (95% CI = 92.6 to 96.3%) respectively compared to the stool culture.

In Brazil, the PTB bovine has been described in several Federative Units, as shown in Table 1, which shows that the disease is present in the country. The carrying out epidemiological surveys

and conducting studies aimed at isolating / agent identification are important to guide the effective control measures implementation. Thus, this review has been prepared in order to describe the bovine PTB in Brazil epidemiological situation.

Economic importance

Economic losses in ruminant herds vary considerably from country to country. Overall, the disease can cause losses in the production of cattle related to the reduction in milk production and premature disposal of animals (RAIZMAN et al., 2009), decreased slaughter value (KUDAHL; NIELSEN, 2009), and eventual death (KUDAHL et al., 2007). Other economic losses due to the presence of PTB in herds include reduced feed efficiency, reduced fat and milk protein, reduced fertility and increased incidence of mastitis (NIELSEN; TOFT, 2011), herd replacement, conducting diagnostic tests and veterinary care for the treatment of chronic diarrhea (HASONOVA; PAVLIK, 2006).

In Brazil, there is still no quantitative data of actual production losses of affected herds by PTB, however, estimates in other countries. In the United States of America (USA), Johne's-positive herds experience an economic loss of almost US$ 100 per cow when compared to Johne's-negative herds due to reduced milk production and increased cow-replacement costs. For Johne's-positive herds that reported at least 10% of their cull cows as having clinical signs consistent with Johne's disease, economic losses were over US$ 200 per cow. These high-prevalence herds experienced reduced milk production of over 700 kg per cow, culled more cows but had lower cull-cow revenues, and had greater cow mortality than Johne's-negative herds. (OTT et al., 1999). Averaged across all herds, Johne's disease costs the US dairy industry, in reduced productivity, US$ 22 to US$ 27 per cow or US$ 200 to US$ 250 million annually. (OTT et al., 1999). Also in the US, Meyer and Hall (1994) found that economic losses for the milk and beef cattle industry revolved around six million dollars per year in the state of Kentucky, and the impact is much more serious for the dairy industry than for the beef industry.

In the province of Buenos Aires, Argentina, the estimated economic losses for the gross value of livestock production and marketing and transportation during the year 2001 was approximately 22 million dollars (Paolicchi et al., 2001).

In New Zealand, in an infected herd, the loss of one cow per year from Johne’s disease in an infected herd is estimated to cost an average of $1,616 per year. Clinical disease is responsible for the majority of the economic loss, comprising losses from lower milk production (52% of the total cost) and from lower carcass values (26% of the total cost). Overall, the disease may account for losses in the dairy industry up to 3.8 million dollars (BRETT 1998).

Public health importance

The DJ has some histopathologic similarities with Crohn's disease (CD), a chronic inflammatory bowel disease of humans controversial etiology, however, MAP can possibly be related to its etiology as the causal agent or as one of the syndrome multi-aetiologicalfactors (ATREYA et al., 2014). This association has awakened great scientific interest since the 1980, when Chiodini, Vankruiningen and Merkal (1984) isolated MAP and detected their DNA in patients with CD tissues. Based on these observations, the link between MAP and DC were investigated by the direct and indirect diagnostic methods application for the MAP detection. (CHIODINI et al., 2012).

Humans are exposed to MAP during direct contact with infected animals, exposure to faecal material or through contaminated meat or dairy products. The latter point is of major concern since particularly young children with an immature antibacterial host defense may be at risk to acquire infection by the ingestion of milk (Atreya et al., 2014).

MAP has been detected in milk and dairy products on several occasions and in several countries, including Brazil. Carvalho et al. (2009) used 222 samples of raw cow's milk, with 206 individual samples 16 and cooling tank in Viçosa, MG, and detected DNA in eight MAP (3.6%) samples. Carvalho et al. (2012), also in Viçosa, MG, used 37 samples of pasteurized milk sold in supermarkets and, based on colony morphology after culture in medium HEYN to mycobactin J, observed growing colonies suspected of MAP in a sample, which was confirmed by PCR IS900. Faria et al. (2014) detected by PCR and culture, the presence of MAP in curd cheese sold at retail in the Parnaíba region, in the Piaui state, Northeast Brazil. Of the30 rennet cheese samples collected in the formal and informal market, there was a specific DNA detection of MAP in three (10%) samples and one (3.3%) sample was achieved isolation of MAP.

Seroepidemiological surveys conducted in Brazil

In Brazil, seroepidemiological studies were conducted in cattle in various conditions (Table 1), however, methodologies relating to selecting properties / animal and serological testing was used distinct. A total of eight studies were carried out to determine the prevalence of animal level (FONSECA et al., 2000; FERREIRA et al., 2001; ACYPRESTE et al., 2005; SILVA, 205; COSTA et al., 2010; MEDEIROS et al, 2012;. SÁ et al, 2013;. VILAR et al, 2015), whereas seven studies were determined the prevalence at herd level (FONSECA et al., 2000; FERREIRA et al., 2001; ACYPRESTE et al., 2005; COSTA et al, 2010;. MEDEIROS et al., 2012;. SÁ et al., 2013; VILAR et al., 2015). In only three studies were used random sampling to select properties / animals (ACYPRESTE et al., 2005; SILVA, 2005; VILAR et al, 2015).

The prevalence of positive herds (at least one positive animal in the herd) were 95% in São Paulo (FONSECA et al., 2000), 82% in Rio de Janeiro (FERREIRA et al., 2001), 100% in Goiás (ACYPRESTE et al., 2005), 87% in the Espirito Santo (COSTA et al., 2010), 58.33% in Paraíba (MEDEIROS et al., 2012), 47.4% in Pernambuco (SÁ et al., 2013) and 34.5% also in the state of Paraiba (VILAR et al., 2015), and animal level, the prevalence was 37.9% in São Paulo (FONSECA et al., 2000), 18% in Rio Janeiro (FERRERIA et al., 2001), 35.4% in Pará (SILVA, 2005), 60.24% in Goiás (ACYPRESTE et al., 2005), 11.4% in the Espírito Santo (COSTA et al. , 2010), 10.1% (MEDEIROS et al., 2012) and 10.7% (VILAR et al., 2015) Paraiba, and 2.7% in Pernambuco (SÁ et al., 2013).

MAP isolation and identification in cattle in Brazil

In Brazil, several studies were conducted in order to isolate / detect MAP in cattle. In the state of Rio Grande do Sul, Gomes et al. (2002), using 229 samples of tissues (intestine and lymph nodes) eight cows with clinical signs suggestive of PTB in dairy herd of Rio Grande do Sul, isolated MAP eight (3.5%) samples and Fiss et al. (2015) detected DNA of MAP in five (14.7%) of the 34 stool samples of the rectum of beef cattle from a farm.

In the Rio de Janeiro state, Ristow et al. (2008), studying three cattle from two herds with PTB history in Rio de Janeiro MAP recovered from three (42.8%) of the seven samples with minor injuries, five (55.5%) of the nine samples with injuries moderate, and all eight samples with severe injuries. In Resende, Rio de Janeiro, where had description of PTB clinical cases in cattle, Yamasaki et al. (2010) 203 stool specimens submitted for bacterial isolation means Herold with mycobactin, and 14 (7.0%) samples grew colonies compatible with MAP, as well as in 14 fecal samples, and the milk was detected DNA MAP IS900 PCR.

In the Pernambuco state, Mota et al. (2007) isolated four MAP (50.0%) of the eight faecal samples inoculated in HEYM to mycobactin J, shaved and a sample of the animal mucosa with the clinical form of PTB, and in three samples was detected DNA MAP IS900 PCR.

In the Paraiba state, Mota et al. (2009) examined 160 stool samples from a dairy Gyr herd and observed the growth of bacterial colonies compatible with MAP in two (1.3%) samples.

Risk factors

The bovine paratuberculosis has a worldwide distribution and many countries have implemented control programs to prevent transmission among and within herds. For these programs to be efficient, knowledge of the risk factors involved in transmission is essential in a particular geographical area (DORÉ et al., 2012), since, due to the complexity of the causal network for

transmission of infection different risk conditions may be important in different geographical areas (ANSARI-LARI et al., 2009).

Rangel et al. (2015), in a systematic review about the risk factors associated with infection by MAP, selected scientific articles published from 1996 to 2011 and found that the purchase / introduction of animals in herds was an important risk factor associated with paratuberculosis. In fact, animals entering the herd may have subclinical infection (WELLS; WAGNER, 2000; SIBLEY; ORPIN; PEARSE, 2012), eliminate the bacteria and contaminate the environment for months to years before the clinical signs onset. Similarly, Hirst et al. (2004) also reported the introduction of animals in the herd as an important risk factor for PTB in dairy cattle in Colorado, USA. Already Doré et al. (2012) also entered systematic review and found that the contact calves adult animal feces was the most important risk factor in the MAP transmission.

Nielsen et al. (2008), Denmark, reported that calves fed colostrum from multiple cows had higher risk of being positive compared to animals fed only mother's colostrum. Ansari-Lari et al. (2009) in cross-sectional study in southern Iran observed that contamination of cows udders during the periparturient with manure and history have suspected cases of PTB in the herd were significantly associated with MAP infection in the herd.

In Brazil, risk factors analysis were performed in studies conducted in the Pernambuco and Paraíba states, and according to the results, the most important variables were: annual rate of higher birth to 51 calves per year (SA et al, 2013) the Pernambuco state; herd over 12 adult animals and the non-use of farrowing paddocks (VILAR et al., 2015) in the Paraiba state. In fact, high annual birth rates and high numbers of adult animals are related to herd size and generally the higher the larger herd size is the animals density, which can facilitate the spread of the agent in the herd. The use of paddocks parturition is a very important management strategy for the various infectious diseases prevention in animals, and in particular, the PTB case, the proper management of the birthing area with the cleaning and disinfection of the environment, reduces the chances of MAP infection (NIELSEN and TOFF, 2008).

The high percentage of reactive animals to the ELISA test, presents one of the most important categories in the disease control, for not presenting clinical signs intermittently eliminate the agent in the environment and keeps the infection in the herd (CHIODINI, CHAMBERLIN AND MERKAL, 1984). The implementation of control measures such as changes in the handling and feeding of animals, such as animals elimination with diarrhea, selective slaughter of cows that had high antibody concentrations in the ELISA test, which had recurrent mastitis, reproductive and problems or advanced age, separation of calves from cows shortly after birth and use of colostrum bank, can bear fruit some positive effect in reducing the number of clinical casesoccurrence. (YAMASAKI et al, 2010).

Final considerations

Despite controversies with the MAP involvement in the CD etiology in humans, there are strong indications of possible zoonotic paratuberculosis. This observation raises concerns from a public health point of view in the case of milk and milk products consumption from infected animals because in Brazil there are several reports of isolation and / or MAP DNA identification in milk and milk products. It is noteworthy that MAP has been isolated from tissues and cattle feces on several occasions in Brazil.

Analyzing the seroepidemiological studies for MAP in Brazil, concluded that such studies are scarce and mostly without planned sampling use, which can generate an epidemiological bias in determining indicators. On the other hand, these studies indicate a high incidence of herds with seropositive animals, so it is important to concentrate efforts on driving more work with sampling planned in all country regions in order to determine the real epidemiological situation disease, seeking to standardize the methodologies used for decision-making and implementation of appropriate control measures for each region.

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Table 1. Sero-epidemiological studies in Brazil to determine the prevalence of paratuberculosis in animal level and herd level.

State	Study period	Nº of herds/animals	Selection of herds / animals	Diagnostic test	Prevalence of herds / prevalence of animals	References
São Paulo	N.R.	20 herds/403 animals	N.R. / N.R.	ELISA (Idexx)	95% / 37,9%	Fonseca et al., 2000
Espírito Santo	N.R.	24 herdes/1450 animals	N.R./ nonrandom sampling	ELISA (Pourquier)	87% / 11,4%	Costa et al., 2010
Pará	N.R.	514 animals	random sampling / random sampling	ELISA (Svanova)	35,4 % in animal level	Silva 2005
Paraíba	N.R.	36 herds/486 animals	N.R./ N.R.	ELISA (ID Screen)	58,33% / 10,1 %	Medeiros et al., 2012
Paraíba	September 2012 to january 2013	480 herds/ 2504 animals	random sampling / random sampling	ELISA (Pourquier – IDEXX)	34,5% / 10,7 %	Vilar et al., 2015
Goiás	January - july 1998	17 herds/166 animals	random sampling / random sampling	ELISA (Bratexlaboratories	100% / 60,24 %	Acyprest et al., 2005
Pernambuco	N.R.	19 herds/408 animals	N.R./ N.R.	ELISA (Pourquier – IDEXX)	47,4% / 2,7 %	Sá et al., 2013
Rio de Janeiro	N.R.	45 herds/1004 animals	N.R./ N.R.	ELISA (Parachek)	82% / 18 %	Ferreira et al., 2001

N.R .: not reported

Source: Adapted from Fernandes-Silva et al. (2014)

CHAPTER II

Herd-level prevalence and associated risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in cattle in the State of Paraíba, Northeastern Brazil

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Herd-level prevalence and associated risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in cattle in the State of Paraíba, Northeastern Brazil

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ABSTRACT. A cross-sectional study based on a planned sampling was carried out to determine herd-level and animal-level prevalences, and to identify risk factors associated with herd-level prevalence for bovine paratuberculosis in the State of Paraíba, Northeastern Brazil. The state was divided into three sampling groups: sampling stratum 1 (mesoregion of Sertão), sampling stratum 2 (mesoregion of Borborema), and sampling stratum 3 (mesoregions of Zona da Mata and Agreste). For each sampling stratum, herd-level and animal-level prevalences were estimated by a two-stage sampling survey. In the first stage, a pre-established number of herds (primary sampling units) were randomly selected; in the second stage, a pre-established number of cows aged ≥ 24 months were randomly selected (secondary sampling units). Ten animals were sampled in herds with up to 99 cows aged over 24 months; 15 animals were sampled in herds with 100 or more cows aged over 24 months; and all animals were sampled in those with up to 10 cows aged over 24 months. In total, 2,504 animals were sampled from 480 herds. Enzyme-linked immunosorbant assay (ELISA) test kits were used for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) antibody detection. A herd was deemed positive for the presence of MAP if it included at least one positive animal in herds of up to 24 females, and two positive animals in herds with more than 24 females. The herd-level prevalence in the State of Paraíba was 34.5% (95% CI = 30.2%–39.1%), 26.6% (95% CI = 20.2%–34.2%) in the region of Borborema, 30.5% (95% CI = 23.9%–38.0%) in Agreste/Mata, and 41.4% (95% CI = 34.0%–49.1%) in Sertão. The animal-level prevalence was 10.7% (95% CI = 7.3%–15.4%) in the State of Paraíba, 7.9%

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(95% CI = 5.2%–11.7%) in the region of Borborema, 9.4% (95% CI = 7.3%–12.1%) in Sertão, and 13.9% (95% CI = 6.2%–28.3%) in Agreste/Mata. The frequency of seropositive animals per herd ranged from 6.7% to 100% (median of 20%). The risk factors identified were as follows: Sertão region (OR = 1.9), more than 12 adult animals in the herd (OR = 1.9), and not using maternity pens (OR = 1.7). Our findings suggest that MAP herd-level seroprevalence in the State of Paraíba, Northeastern Brazil, is high, and support the idea that the use of maternity pens will be important for preventing transmission of MAP in the herds.

Keywords: Paratuberculosis; Bovine; Epidemiology; Control; Northeastern Brazil

RESUMO: Foi realizado um estudo transversal com base em uma amostragem planejada para determinar a prevalência de propriedades e animais soropositivos para MAP e identificar os fatores de risco associados à prevalência da paratuberculose a nível de rebanho de bovinos no Estado da Paraíba, Nordeste do Brasil. O estado foi dividido em três estratos amostrais: estrato amostral 1 (Mesorregião do Sertão), estrato amostral 2 (Mesorregião da Borborema), e estrato amostral 3 (Mesorregiões do Zona da Mata e Agreste). Para cada estrato amostral, a prevalência a nível de animais e a nível de rebanho foi estimada por uma pesquisa de amostragem de duas fases. Na primeira etapa, um número pré-estabelecido de rebanhos (unidades primárias da amostra) foram selecionados aleatoriamente; na segunda fase, um número pré-estabelecido de fêmeas com idade ≥24 meses foram selecionados aleatoriamente (unidades secundárias da amostra). Dez animais foram amostrados em rebanhos com até 99 fêmeas com idade ≥ 24 meses; 15 animais foram amostrados nos rebanhos com 100 ou mais fêmeas com idade ≥ 24 meses; e todas as fêmeas foram amostradas em rebanhos com até 10 fêmeas com idade ≥ 24 meses. No total, 2504 animais foram amostrados em 480 rebanhos. Para a detecção de anticorpos para *Mycobacterium avium subsp. paratuberculosis* (MAP) foram utilizados kits de teste imunoenzimático (ELISA). Um rebanho foi considerado positivo para a presença de MAP se apresentasse pelo menos um animal positivo em rebanhos de até 24 fêmeas e dois animais positivos em rebanhos com mais de 24 fêmeas. A prevalência a nível de rebanho no Estado da Paraíba foi de 34,5% (IC 95% = 30,2-39,1%), 41,4%(IC 95% = 34,0-49,1%) no Sertão, 26,6% (IC 95% = 20,2-34,2%) na região da Borborema e 30,5% (95% IC = 23,9-38,0%) na região Agreste / Zona da Mata. A prevalência em nível animal foi de 10,7% (IC 95% = 7,3-15,4%) no Estado da Paraíba, 9,4% (IC 95% = 7,3-12,1%) no Sertão, de 7,9% (IC 95% = 5,2-11,7%) na região da Borborema e 13,9% (IC 95% = 6.2--28.3%) no

Agreste / Mata. A frequência de animais soropositivos por rebanho variou de 6,7% a 100% (média de 20%). Os fatores de risco identificados foram os seguintes: região Sertão (OR = 1,9), rebanhos com mais de 12 animais adultos (OR = 1,9), e o não uso de currais de maternidade (OR = 1,7). Nossos resultados sugerem que a soroprevalência de MAP a nível rebanho no Estado da Paraíba, Nordeste do Brasil, é alta, e apoia a ideéa de que o uso de currais de maternidade será importante para a prevenção da transmissão do MAP nos rebanhos.

Palavras-chave: Paratuberculose, Bovino, Epidemiologia, Controle, Nordeste do Brasil

1. Introduction

Paratuberculosis, also known as Johne's disease, is a chronic intestinal infection of global importance in mainly domestic and wild ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). This infection can cause significant economic losses in cattle primarily related to reduced milk production and premature culling (Raizman et al., 2009), decreased value at slaughter (Kudahl and Nielsen, 2009), and eventual death (Kudahl et al., 2007). Other economic losses resulting from the presence of paratuberculosis in cattle herds include reduced feed efficiency, decreased fat and protein content in the milk, decreased fertility, and increased incidence of mastitis (Nielsen and Toft, 2011).

Johne's disease shows some histopathological analogies with Crohn's disease (CD), a human chronic inflammatory bowel disease of still unresolved etiology, and MAP appears to be related to the etiology of CD as either a causative agent or one of the factors of a multi-etiological syndrome (Atreya et al., 2014). This association has aroused great scientific interest since the 1980s, when Chiodini et al. (1984) isolated MAP and its DNA from biological tissues in CD patients. Based on these observations, the link between MAP and CD has been widely investigated through direct and indirect methods for MAP detection (Chiodini et al., 2012). In Brazil, the presence of MAP DNA and the viability of MAP in raw and retail pasteurized milk (Carvalho et al., 2009, 2012) and artisanal Coalho cheese (Faria et al., 2014) has been proven, suggesting that live organisms might be transmitted to humans by ingestion of contaminated products.

The diagnosis of paratuberculosis is divided into two parts - the diagnosis of clinical disease and the detection of subclinical infection – and the latter is essential for control of the disease at the farm, national or international level. In cattle, clinical cases can be diagnosed

without difficulty because chronic diarrhea in adult animals is indicative of the disease. In the laboratory, a diagnosis can be made by isolating the agent from feces or necropsy material, by histological study of the lesions, and polymerase chain reaction (PCR) assays. By using the Ziehl-Neelsen staining method (ZN-test), acid-alcohol resistant bacteria are observed in feces smears and at the end portions of the small intestine, however, serum-ELISA has been referred as superior to the ZN-test to confirm the presumptive diagnosis of clinical paratuberculosis in cattle (Weber at al., 2009). Subclinical cases can be diagnosed by isolating the bacteria from the feces, serological tests, or allergy tests (Lilenbaum et al., 2007). Hendrick et al. (2005) found that serum- and milk-ELISAs may be potentially useful and convenient methods in detecting subclinical paratuberculosis in lactating dairy cattle, with sensitivities of 73.6% (95% CI = 61.9% - 83.3%) and 61.1% (95% CI = 48.9% - 72.4%), and specificities of 87.5% (95% CI = 84.7% - 90.0%) and 94.7% (95% CI = 92.6% - 96.3%), respectively, compared to fecal culture.

During the last few decades, dairy cattle have become significantly important within animal husbandry in the State of Paraíba, Northeastern Brazil. Except for the Zona da Mata region (where sugarcane crops prevail), small cattle-raising farms are widespread in the Agreste, Borborema and Sertão regions. Whereas cultivated grasses (mostly *Brachiaria* spp.) are the basis for Agreste livestock, cattle are usually reared extensively on native Caatinga in most of the Borborema and Sertão farms. Following the Brazilian scenario of milk production, in the State of Paraíba around 69% of milk was produced in small cattle-raising farms (IBGE, 2006). In this context, the performance of epidemiological studies to investigate infectious agents as well as infection by MAP is important. MAP prevalence estimation is a key element to assess the disease impact, and for the design of control programmes (Verdugo et al., 2014), therefore, the objective of this study was to determine the herd-level and animal-level prevalence of MAP infection using serology in cattle from the State of Paraíba, Northeastern Brazil, as well as to identify risk factors associated with herd-level prevalence.

2. Material and methods

2.1. Characterization of the study area

The State of Paraíba, located in the Northeastern region of Brazil, is characterized by warm weather throughout the year. The state is geographically subdivided into the following

four major regions, based mostly on vegetation type and rainfall: (i) Zona da Mata (Atlantic forest), (ii) Agreste, (iii) Borborema, and (iv) Sertão. The Zona da Mata and Agreste have relatively higher rainfall regimes (Cabrera and Willink, 1973). Both Borborema and Sertão (the semiarid region) are typically within the Caatinga biome, which encompasses an area of 900,000 km² (11% of Brazilian territory) and is the only major biome that occurs exclusively in Brazil. Caatinga is xeric shrubland and thorn forest, which consists primarily of small, thorny trees that shed their leaves seasonally. Cacti, thick-stemmed plants, thorny brush and arid-adapted grasses make up the ground layer; however, during the dry periods there is no ground foliage or undergrowth (Andrade-Lima, 1981). The weather is characterized by a hot and semiarid climate, with temperatures averaging 27 °C, and the mean annual rainfall is typically ≈500 mm. There are typically two seasons: a rainy season from February to May, and a long drought period from June to January. However, occurrences of droughts sometimes lasting for longer than one year is also a characteristic of the region (Batista et al., 2007).

2.2. Division of the State of Paraíba into stratified sampling groups

The State of Paraíba was divided into three sampling groups: sampling stratum 1 (mesoregion of Sertão), sampling stratum 2 (mesoregion of Borborema), and sampling stratum 3 (mesoregions of Zona da Mata and Agreste) (Fig. 1). When creating this stratification scheme, the operational capacity of the Agricultural and Livestock Defense Service of the State of Paraíba (SEDAP) was considered based on the areas of action of its regional units in order to ensure that the agency could conduct the fieldwork.

2.3. Sampling, target condition and herd-level case definition

For each sampling stratum, the prevalence of herds infected with bovine paratuberculosis and the prevalence of seropositive animals were estimated by a two-stage sampling survey. In the first stage, a pre-established number of herds (primary sampling units) were randomly selected; in the second stage, a pre-established number of cows aged ≥ 24 months were randomly selected (secondary sampling units).

In farms with more than one herd, the cattle herd of greater economic importance was chosen as the target of the study; the animals in the selected cattle herd were subjected to the same type of management system as the other herds, i.e., had the same risk factors as the

202 other herds. The selection of the primary sampling units was random (random drawing), and
203 was based on the records of farms of the SEDAP. If a herd that was selected could not be
204 visited, the herd was replaced by another one in the vicinity with the same production
205 characteristics. The number of selected herds per sampling stratum was determined by using
206 the formula for simple random samples proposed by Thrusfield (2007). The parameters
207 adopted for the calculation were as follows: 95% confidence level, 47.4% estimated
208 prevalence (Sá et al., 2013), and 8% error. Further, the operational and financial capacity of
209 the SEDAP was taken into consideration when determining the sample size of the sampling
210 stratum.

211 For the secondary units, the minimum number of animals to be examined within each
212 herd was estimated in order to allow its classification as positive herd. For this purpose, the
213 concept of aggregate sensitivity and specificity was used (Dohoo et al., 2003). For the
214 calculations, the following values were adopted: 73.6% (Hendrick et al., 2005) and 98%
215 (Sweeney et al., 1995) for the sensitivity and specificity, respectively, of the test protocol and
216 37.9% (Fonseca et al., 2000) for the intra-herd estimated prevalence. Herdacc version 3
217 software (Jordan, 1995) was used during this process, and the sample size was selected so that
218 the herd sensitivity and specificity values would be ≥ 90%. Therefore, 10 animals were
219 sampled in herds with up to 99 cows aged over 24 months; 15 animals were sampled in herds
220 with 100 or more cows aged over 24 months; and all animals were sampled in those with up
221 to 10 cows aged over 24 months. The selection of the cows within the herds was systematic.

222 The target condition (Gardner et al., 2011) was a seropositive animal within an
223 infected herd. The herd-level case definition was based on the size of the population (cows
224 aged ≥ 24 months), number of females sampled, an intra-herd apparent prevalence of 37.9%
225 (Fonseca et al., 2000), and the sensitivity and specificity of the diagnostic test used (indirect
226 ELISA), with the goal of obtaining a herd sensitivity and specificity of ≥ 90%. After new
227 simulations using Herdacc software, a herd was deemed positive for the presence of MAP if it
228 included at least one positive animal in herds of up to 24 females, and two positive animals in
229 herds with more than 24 females.

230 The field activities included blood collection, provision of an epidemiological
231 questionnaire, and sending the samples to the laboratory. The veterinarians and agricultural
232 and livestock technicians of the SEDAP were involved in the fieldwork. Blood samples (10-
233 mL volume) were collected from September 2012 to January 2013, from cows aged ≥ 24
234 months by jugular vein puncture with a disposable needle and a 15-mL capacity vacuum tube
235 (without anticoagulant). An 11-digit code was used for identification of the tubes, of which

the first nine digits referred to the herd code and the final two digitsto the number sequence of the sampled cow. After draining, the serum was transferred to microtubes and was frozen.

2.4. Data collection

A structured questionnaire including closed-ended questions was designed to obtain information concerning (a) the identification and location of the herd; (b) management practices; (c) structure and composition of the herd; and (d) presence of other domestic and wildlife species in the farm. Questionnaires were administered to the owner or person in charge of the herd either by the primary author or by a veterinarian from the SEDAP at the same time of the visit to blood collection. The description of the questions included in the questionnaire can be found in the supplementary material.

2.5. Serological diagnosis

The serological examination was performed according to the protocol recommended by the kit for detection of antibodies to MAP (Pourquier-IDEXX ELISA Paratuberculosis Screening Ab Test). To calculate the results, the reactions were considered valid when the average of the positive control (PCx) had a minimum OD450 mean value of 0.350 and the coefficient between the average of the PCx and the negative control (NC OD450) was ≥ 3.00. Samples with percentages ≥ 70% were considered positive for the presence of antibodies against MAP.

2.6. Prevalence calculations

A herd was deemed positive for the presence of MAP if it included at least one positive animal in herds of up to 24 females, and two positive animals in herds with more than 24 females. EpiInfo 6.04 software was used to calculate the apparent prevalences and respective confidence intervals (Dean et al., 1996). Stratified random sampling was utilized to calculate the herd-level prevalence in the State of Paraíba (Thrusfield, 2007). The required parameters were as follows: (a) condition of the herd (positive or negative), (b) sampling stratum to which the herd belonged, and (c) statistical weight. The statistical weight was determined by applying the following formula (Dean et al., 1996):

$$Weight = \frac{\text{number of herds in the stratum}}{\text{number of herds sampled in the stratum}}$$

The calculation of the herd-level prevalence per sampling stratum employed the sampling design of a simple random sample by using the following parameters: (a) number of positive herds and (b) number of herds sampled in the stratum.

The sampling design for calculating the animal-level prevalence in the State of Paraíba employed a two-stage stratified cluster sampling, and a two-stage cluster sampling in each stratum (Thrusfield, 2007), where each herd was considered a cluster. The following parameters were used: (a) animal condition (seropositive or seronegative), (b) sampling stratum to which the animal belonged, (c) herd code (to identify each cluster), and (d) statistical weight. The statistical weight was calculated with the following formula (Dean et al., 1996):

$$Weight = \frac{\text{cows} \geq 24 \text{ months in the stratum}}{\text{cows} \geq 24 \text{ months in the sampled herds}} \times \frac{\text{cows} \geq 24 \text{ months in the herd}}{\text{cows} \geq 24 \text{ months sampled in the herd}}$$

In the above expression, the first term refers to the weight of each animal in the calculation of the animal-level prevalence within the stratum.

2.7. Risk factor analysis

Data obtained with the epidemiological questionnaires were used in the analysis of risk factors associated with the herd-level prevalence. The analyzed variables and respective categories were as follows: sampling stratum (Sertão/Borborema/Agreste and Zona da Mata), type of production (beef/milk/mixed), management system (intensive/semi-intensive/extensive), number of lactating cows (cut-off point: median), daily milk production (cut-off point: median, 3rd quartile), use of artificial insemination (no/yes), predominant breed (Zebu/crossbreed/European dairy and beef breeds), number of cattle young stock (cut-off point: 3rd quartile), number of cattle adult animals (cut-off point: 3rd quartile), presence of sheep/goats, horses, pigs, poultry, wild canids, cervids, and capybaras (no/yes), animal purchasing (no/yes), rental of pastures (no, yes), sharing of pastures (no, yes), presence of

301 flooded pastures (no, yes), use of maternity pens (no, yes), milk cooling (no/yes), and
302 veterinary assistance (no/yes).

303 The variables were organized for presentation in ascending or descending order
304 regarding scale of risk. When necessary, these variables were re-categorized. The lower-risk
305 category was considered the basis for comparison for the other categories. An initial
306 exploratory analysis of the data (univariable) was conducted for selection of variables with P
307 ≤ 0.2 by the chi-square test or Fisher's exact test; subsequently, the variables that passed this
308 cut-off were utilized for logistic regression (Hosmer and Lemeshow, 2000). The fit of the
309 final model was verified with the Hosmer and Lemeshow test, and collinearity between
310 independent variables was verified by a correlation analysis; for those variables with a strong
311 collinearity (correlation coefficient > 0.9), one of the two variables was excluded from the
312 multiple analysis according to the biological plausibility (Dohoo et al., 1996). Confounding
313 was assessed by monitoring the changes in the model parameters when adding new variables.
314 If substantial changes (i.e., higher than 20%) were observed in the regression coefficients, this
315 was considered as indicative of confusion. The calculations were performed by using SPSS
316 software version 20.0.
317

318 **3. Results**
319

320 The census data and the sample studied in each sampling stratum are shown in Table
321 1. In total, 2,504 animals were sampled from 480 herds. Herd-level and animal-level
322 prevalences are presented in Tables 2 and 3, respectively; further, the geographical
323 distribution of positive and negative herds are shown in Fig. 1. The herd-level prevalence in
324 the State of Paraíba was 34.5% (95% CI = 30.2–39.1), 26.6% (95% CI = 20.2%–34.2%) in
325 the region of Borborema, 30.5% (95% CI = 23.9%–38.0%) in Agreste/Zona da Mata, and
326 41.4% (95% CI = 34.0%–49.1%) in Sertão. The animal-level prevalence was 10.7% (95% CI
327 = 7.3%–15.4%) in the State of Paraíba, 7.9% (95% CI = 5.2%–11.7%) in the region of
328 Borborema, 9.4% (95% CI = 7.3%–12.1%) in Sertão, and 13.9% (95% CI = 6.2%–28.3%) in
329 Agreste/Zona da Mata. The frequency of seropositive animals per herd ranged from 6.7% to
330 100% (median of 20%).

331 The results of the univariable analysis for the risk factors are presented in Table 4. The
332 variables selected ($P \leq 0.2$) for the multiple analysis were as follows: sampling stratum, daily
333 milk production, predominant breed, number of cattle adult animals, presence of pigs, and use
334 of maternity pens. In the final logistic regression model (Table 5), the risk factors identified

were as follows: Sertão region (OR = 1.9), more than 12 adult animals in the herd (OR = 1.9), and not using maternity pens (OR = 1.7). Final model had a good fit (Hosmer and Lemeshow test: chi-square = 6.146; $P = 0.631$).

4. Discussion

High herd-level (34.5%; 95% CI = 30.2–39.1) and animal-level (10.7%; 95% CI = 7.3%–15.4%) prevalences were found in the State of Paraíba, especially in the Sertão mesoregion, where the herd-level prevalence was 41.4% (95% CI = 34.0%–49.1%), indicating that the infection is widely spread in Paraíba. It is believed that the animal-level prevalence might even be higher, once the National Program for the Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT) in Brazil, which has been conducted in the State of Paraíba since 2001, recommended that animals should be tested by tuberculin test and that positive animals should be culled. This recommendation may have helped to decrease the animal-level prevalence for paratuberculosis because *M. avium* infection can produce false-positive results in the diagnosis of infection by *M. bovis* in the tuberculin test (simple intradermic test) (Balseiro et al., 2003) since MAP and *M. bovis* belong to the same genus. However, it is important to emphasize that paratuberculosis has a relatively long incubation period, and the antibody levels against MAP only become detectable by the ELISA test at the end of the incubation period, making this test ineffective for detecting infected animals in the early infection period (Collins, 2003; Collins, 2011). Thus, the animals that are infected at a young age (i.e., less than one year) will remain in the herd, shedding MAP through feces and contaminating water and food, and will only present clinical signs in the adult phase. On the other hand, it should be pointed out that the intra-herd prevalence ranged from 6.7% to 100% (median of 20%).

Sertão mesoregion was identified as risk factor associated with herd-level prevalence, and this mesoregion is the largest milk producer in the State of Paraíba. According to Nielsen and Toft (2008), dairy herds have a high likelihood of being affected because confinement produces conditions that contribute to the spread of the infection among the animals. Furthermore, the Sertão mesoregion borders the States of Rio Grande do Norte, Ceará and Pernambuco (Fig. 1), where there is an intense animal trade among these states without knowing the sanitary condition of the animals. In general, paratuberculosis is an unknown disease for most farmers in Paraíba State, and although the high herd-level and animal-level prevalences found in this state, most farmers are not aware of the impact of the infection and

the economic losses that it can cause. Notably, most farms in the State of Paraíba are family farming systems, and the largest amount of milk produced in the state comes from such properties.

In the present survey, the existence of more than 12 adult animals in the herd was identified as risk factor for MAP infection. MAP infections are thought to occur predominantly early in life, with cattle up to 1 year of age thought to be most susceptible (Windsor and Whittington, 2010). In fact, in larger herds, closer contact among animals exists and paddocks that separate animals by age group are absent; thus, calves eventually have contact with the facilities contaminated with feces from infected adult animals as well as with udders contaminated with feces. Marcé et al. (2011) referred that a reduction in the exposure of susceptible calves to any environment contaminated by adults led to a sharp decrease in MAP prevalence and could lead to the fadeout of infection if exposure was decreased by >90%, and that preventing the exposure of calves to adult feces was essential for controlling MAP transmission within a dairy herd.

Not using maternity pens was also identified as risk factor for MAP infection. Transmission of MAP is primarily thought to occur via the fecal-oral route, although infections in uteru and via ingestion of contaminated colostrum or milk are likely routes of transmission (Sweeney, 1996), so that the use of maternity pens is a practice that help to decrease calf exposure to feces, mainly if cleaning of the maternity pen is performed after each use (Johnson-Ifearulundu and Kaneene, 1998). Pithua et al. (2013) found that the instantaneous hazard of a MAP test positive outcome was significantly lower for cows born in individual calving pens (ICP) compared with herd mates born in group calving pens (GCP), and that this protective effect was expected because removal of manure and other wastes from the ICP after successive calving minimized early intake of infectious manure, curtailing the level and duration of MAP transmission in this group of calves. It was found that the use of maternity pens was referred in only 123 of the 480 herds sampled (Table 4), and it suggests that efforts should be concentrated to encouraging the use of this management practice for preventing transmission of MAP in the herds.

A matter of concern is the possible zoonotic link between CD and MAP, although this association remains controversial and is the target of continuous debates in the scientific community. However, MAP has been detected in raw and pasteurized milk (Grant et al., 2002; O'Reilly et al., 2004; Ayele et al., 2005; Slana et al., 2009; Okura et al., 2012), and the habit of consuming raw cow milk is common in the State of Paraíba, predominantly in family farms. Therefore, the confirmation of a zoonotic link between MAP and CD could have

serious consequences for the dairy industry of the State of Paraíba and Brazil, the latter of which assuming a prominent position in world milk production, with an estimated milk production of around 37 billion liters.

According to Fernández-Silva et al. (2014), who conducted a systematic review of the prevalence of paratuberculosis in cattle, goats, and sheep in Latin America and the Caribbean, to date, six reports on herd-level prevalence for paratuberculosis in cattle in Brazil have been published. However, because random sampling was not used in any of these studies, the present study is the first one in Brazil to determine the prevalence of bovine paratuberculosis at herd-level by using random sampling of herds and animals. In a review of prevalences of paratuberculosis in farmed animals in Europe, Nielsen and Toft (2009) referred that many studies have been conducted to estimate both the herd-level and the animal-level prevalences, but corrections for sensitivity (Se) and specificity (Sp) prior to classification of the herd were not included in the study design and data analyses, which could be a weakness in the present study, however, herd-level case definition was based on the size of the population (cows aged ≥ 24 months), number of females sampled, intra-herd apparent prevalence and the Se and Sp of the diagnostic test used, which was important to minimize misclassification bias.

5. Conclusion

The results presented here suggest that MAP herd-level seroprevalence in the State of Paraíba, Northeastern Brazil, is high, especially in the Sertão mesoregion, the largest milk producer region in the state, and in larger herds (adult cattle). Our findings further support the idea that the use of maternity pens will be important for preventing transmission of MAP in the herds.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Figure caption

Fig. 1. Division of the State of Paraíba into three sampling groups, and geographical distribution of positive and negative herds. Detail shows the State of Paraíba within Brazil.

663 **Table1**

664 Census data of the cattle population in the State of Paraíba, Northeastern Brazil, according to
665 sampling stratum.

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Sampling stratum	No. of herds		No. of cows ≥ 24 months of age	
	Total	Sampled	Total	Sampled
Sertão	24,356	162	288,764	967
Borborema	11,603	154	83,428	754
Agreste/Zona da Mata	18,398	164	192,320	783
State of Paraíba	54,357	480	564,512	2,504

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689 **Table 2**

690 Herd-level prevalence for bovine paratuberculosis in the State of Paraíba, Northeastern Brazil,
691 according to sampling stratum.

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Sampling stratum	No. of herds		Prevalence (%)	95% CI
	Tested	Positive		
Sertão	162	67	41.4	[34.0 – 49.1]
Borborema	154	41	26.6	[20.2 – 34.2]
Agreste/Zona da Mata	164	50	30.5	[23.9 – 38.0]
State of Paraíba	480	158	34.5	[30.2 – 39.1]

693 CI: Confidence interval

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Table 3

Animal-level prevalence for bovine paratuberculosis in the State of Paraíba, Northeastern Brazil, according to sampling stratum.

Sampling stratum	Animals		Prevalence (%)	95% CI
	Tested	Positive		
Sertão	967	103	9.4	[7.3 – 12.1]
Borborema	754	63	7.9	[5.2 – 11.7]
Agreste/Zona da Mata	783	86	13.9	[6.2 – 28.3]
State of Paraíba	2,504	252	10.7	[7.3 – 15.4]

CI: Confidence interval

733 **Table 4**

734 Univariable analysis for risk factors associated with the herd-level prevalence of bovine
735 paratuberculosis in the State of Paraíba, Northeastern Brazil.

Variables	Categories	No. of herds sampled	No. of positive herds (%)	*P*
Sampling stratum	Borborema	154	41 (26.6)	
	Agreste/Zona da Mata	164	50 (30.5)	
	Sertão	162	67 (41.4)	0.015*
Type of production	Beef	62	19 (30.6)	
	Mixed	284	101 (35.6)	
	Milk	134	38 (28.4)	0.316
Management system	Intensive	28	7 (25.0)	
	Semi-intensive	283	91 (32.2)	
	Extensive	169	60 (35.5)	0.501
No. of lactating cows	0 – 9	434	140 (32.3)	
	> 9	46	18 (39.1)	0.436
Daily milk production (litres)	0 - 5	242	68 (28.1)	
	6 - 15	132	48 (36.4)	
	> 15	106	42 (39.6)	0.067*
Use of artificial insemination	No	478	158 (33.1)	
	Yes	2	0 (0)	1.000
Predominant breed	Zebu	27	16 (59.3)	
	Crosbreed	408	129 (31.6)	
	European (dairy and beef)	45	13 (28.9)	0.010*
No. of cattle young stock	1 – 11 animals	365	120 (32.9)	
	> 11 animais	115	38 (33.0)	1.000
No. of cattle adult animals	1 – 12 animals	370	110 (29.7)	
	> 12 animais	110	48 (43.6)	0.009*
Presence of sheep/goats	No	295	99 (33.6)	
	Yes	185	59 (31.9)	0.781
Presence of horses	No	228	73 (32.0)	
	Yes	252	85 (33.7)	0.763
Presence of pigs	No	336	103 (30.7)	
	Yes	144	55 (38.2)	0.132*
Presence of poultry	No	176	60 (34.1)	
	Yes	304	98 (32.2)	0.752

Presence of wild canids	No	299	96 (32.1)	
	Yes	181	62 (34.3)	0.700
Presence of cervids	No	474	155 (32.7)	
	Yes	6	3 (50.0)	0.400
Presence of capybaras	No	477	158 (33.1)	
	Yes	3	0 (0)	0.554
Animal purchasing	No	315	97 (30.8)	
	Yes	165	61 (37.0)	0.206
Rental of pastures	No	373	118 (31.6)	
	Yes	107	40 (37.4)	0.318
Sharing of pastures	No	409	138 (33.7)	
	Yes	71	20 (28.2)	0.432
Presence of flooded pastures	No	319	100 (31.3)	
	Yes	161	58 (36.0)	0.354
Use of maternity pens	No	357	124 (34.7)	
	Yes	123	34 (27.6)	0.183*
Milk cooling	No	470	155 (33.0)	
	Yes	10	3 (30.0)	1.000
Veterinary assistance	No	401	128 (31.9)	
	Yes	79	30 (38.0)	0.360

736 * Variables selected and used in the multiple analysis ($P \leq 0.2$)

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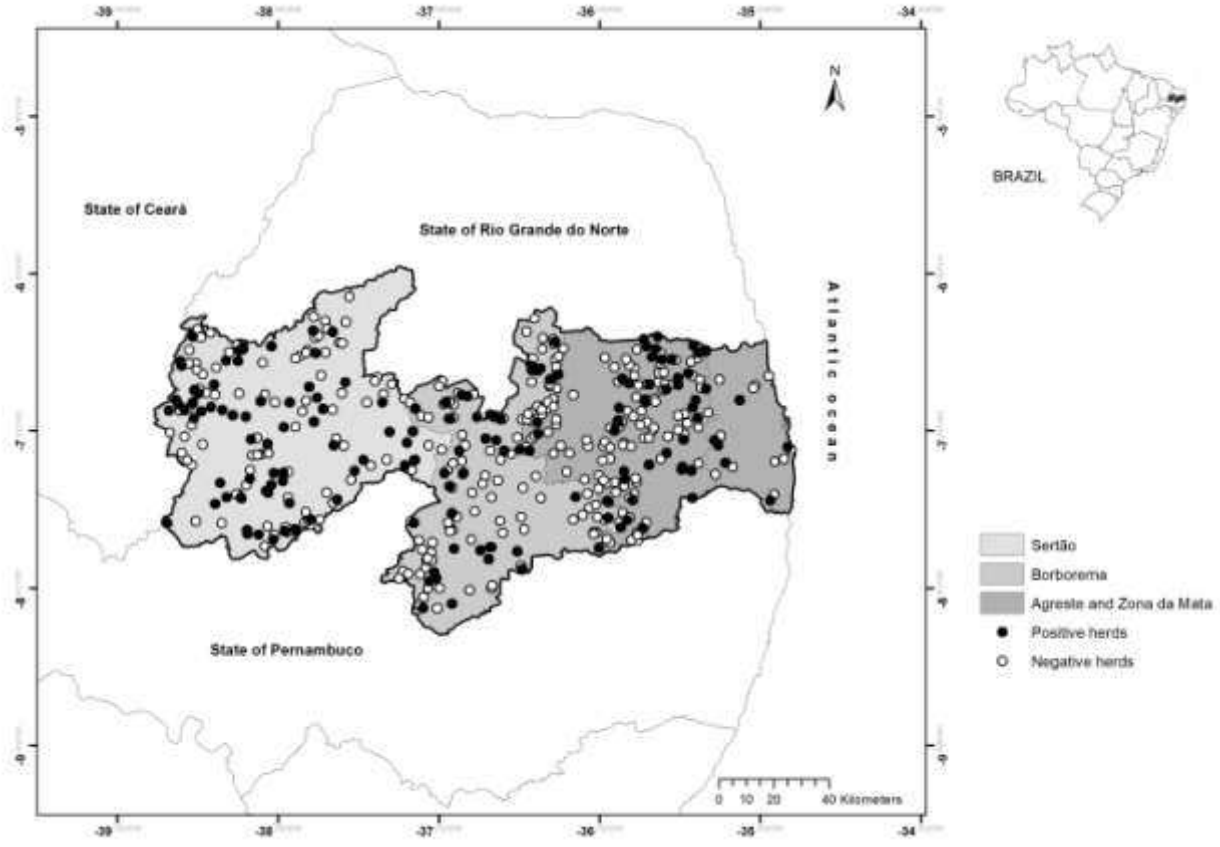
Table 5

Risk factors associated with herd-level prevalence of bovine paratuberculosis in the State of Paraíba, Northeastern Brazil.

Risk factors	Logistic regression coefficient	Standard error	Wald	Degrees of freedom	Odds ratio	95% CI	*P*
Sertão region	0.623	0.249	6.235	1	1.9	1.1 – 3.0	0.013
More than 12 adult animals	0.624	0.230	7.382	1	1.9	1.2 – 2.9	0.007
Not using maternity pens	0.540	0.251	4.620	1	1.7	1.0 – 2.8	0.032
Intercept	-1.843	0.301	37.411	1	0.2	...	< 0.001

Hosmer and Lemeshow chi-square = 6.146; degrees of freedom = 8; *P* = 0.631


State of Ceará
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BRAZIL
Sertão
Borborema
Agreste and Zona da Mata
Positive herds
Negative herds
0 10 20 40 Kilometers

Fig. 1

CHAPTER III

Spatial cluster analysis for bovine paratuberculosis in Paraiba State, Northeastern Brazil

Scientific paper submitted to the journal PESQUISA VETERINÁRIA BRASILEIRA (Qualis A2)

Spatial cluster analysis for bovine paratuberculosisinParaiba State, Northeastern Brazil[3]

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ABSTRACT.- Vilar A.L.T., Santos C.S.A.B., Clementino I.J., Fernandes L.G., Alves C.J. & Azevedo S.S. 2016. **Spatial cluster analysis for bovine paratuberculosis in Paraiba State, Northeastern Brazil**. *Pesquisa Veterinária Brasileira 00(0):000-000*. Unidade Acadêmica de Medicina Veterinária, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Av. Universitária s/n, Caixa Postal 61, Santa Cecília, Patos, PB 58700-970, Brasil. E-mail: sergio@vps.fmvz.usp.br

The aim of this survey was to identify spatial clustering of bovine paratuberculosis positive herds in the State of Paraíba, Northeastern Brazil. The state was divided into three sampling groups: sampling stratum 1 (mesoregion of Sertão), sampling stratum 2 (mesoregion of Borborema), and sampling stratum 3 (mesoregions of Zona da Mata and Agreste). Ten animals were sampled in herds with up to 99 cows aged over 24 months; 15 animals were sampled in herds with 100 or more cows aged over 24 months; and all animals were sampled in those with up to 10 cows aged over 24 months.In total, 2504 cows aged ≥ 24 months were sampled from 480 herds. Indirect enzyme-linked immunosorbant assay (ELISA) test kits were used for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) antibody detection.A herd was deemed positive for paratuberculosis if it included at least one positive animal in herds of up to 24 females, and two positive animals in herds with more than 24 females. Spatial clustering was assessed using the Cuzick-Edwards' *k*-nearestneighbormethod and spatial scanstatistics. Two significant clustering of positive herds were detected in Northernpartof Borborema mesoregion, a border region with the State of Rio Grande do Norte, in which there is a largeanimal movement from different locations without knowing the sanitary condition of animals. As serological tests for MAP diagnosis are

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not widely available and are very expensive, as well as replacement or maintenance of livestock by animal purchasing is common in the region, it isconcludedthat prevention measures should be applied at herd level.

INDEX TERMS: Paratuberculosis, cattle, epidemiology, cluster analysis, control.

RESUMO.-[Análise de aglomerados espaciais para paratuberculose bovina no Estado da Paraíba, Nordeste do Brasil.]O objetivo deste estudo foi identificar agrupamentos espaciais de rebanhos positivos para paratuberculose bovina no Estado da Paraíba, Nordeste do Brasil. O estado foi dividido em três grupos amostrais: estrato amostral 1 (mesorregião do Sertão), estrato amostral 2 (mesorregião da Borborema), e estrato amostral 3 (mesorregiões da Zona da Mata e Agreste). Dez animais foram amostrados em rebanhos com até 99 vacas com idade maior ou igual a 24 meses; 15 animais foram amostrados em rebanhos com 100 ou mais vacas com idade maior ou igual a 24 meses; e todos os animais foram amostrados naqueles rebanhos com até 10 vacas. No total, foram amostradas 2504 vacas com idade ≥ 24 meses de 480 rebanhos. Para a detecção de anticorpos anti-*Mycobacterium avium* subsp. *paratuberculosis* (MAP) foram utilizados kits do teste imunoenzimático indireto (ELISA). Um rebanho foi considerado positivo para paratuberculose se apresentasse pelo menos um animal positivo em rebanhos de até 24 fêmeas, e dois animais positivos em rebanhos com mais de 24 fêmeas. Os agrupamentos espaciais foram avaliados com o uso da metodologia *k*-vizinhos mais próximos de Cuzick-Edwards e estatística espacial de varredura.Dois agrupamentos significativos de rebanhos positivos foram detectados na parte norteda mesorregião da Borborema, uma região de fronteira com o Estado do Rio Grande do Norte onde há intenso movimento de animais de diferentes locais sem o conhecimento do estado sanitário desses animais. Tendo em vista que os testes sorológicos para diagnóstico de MAP não são amplamente disponíveis e muito caros, bem como é comum na região a reposição e manutenção dos rebanhos por compra de animais, conclui-se que medidas de prevenção devem ser aplicadas em nível de rebanho.

TERMOS DE INDEXAÇÃO: Paratuberculose, bovinos, epidemiologia, análise de cluster, controle.

INTRODUCTION

Paratuberculosis is a chronic intestinal infection of global importance in mainly domestic and wild ruminantscaused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Paratuberculosis, also known as Johne's disease, can cause significant economic losses in cattle primarily related to reduced milk production and premature culling (Raizman et al. 2009), decreased value at slaughter (Kudahl & Nielsen 2009), and eventual death (Kudahl et al. 2007), reduced feed efficiency, decreased fat and protein content in the milk, decreased fertility, and increased incidence of mastitis (Nielsen et al. 2008).

MAP can be transmitted via milk and colostrum from infectious animals and intrauterine route (Streeter et al., 1995). Infected animals shed MAP in faeces and can lead to widespread contamination of environment, including the presence of viable MAP in settled dust particles suggesting potential transmission of MAP infection through bio-aerosols (Eisenberg et al., 2010). In cattle, clinical cases can be diagnosed without difficulty because chronic diarrhea in adult animals is indicative of the disease. In the laboratory, a diagnosis can be made by isolating the agent from feces or necropsy material, by histological study of the lesions, and polymerase chain reaction (PCR) assays. Subclinical cases can be diagnosed by isolating the bacteria from the feces, serological tests, or allergy tests (Lilenbaum et al. 2007). However, the assessment of MAP infection status is subject to misclassification, especially low sensitivity of the diagnostic test used in the control programme (Nielsen & Toft 2011).

In the State of Paraíba, a cross-sectional study based on a planned sampling was carried out to determine the epidemiological situation of the disease (Vilar et al. 2015).The herd-level prevalence in the State of Paraíba was 34.5% (95% CI = 30.2%–39.1%), 26.6% (95% CI = 20.2%–34.2%) in the region of Borborema, 30.5% (95% CI = 23.9%–38.0%) in Agreste/Mata, and 41.4% (95% CI = 34.0%–49.1%) in Sertão (Table 1). In understanding risk and controlling disease it is important to know the spatial distribution of the disease in the environment. To date, there is no survey on spatial clustering analysis for bovine paratuberculosis in Brazil. Spatial clustering analysis is a useful tool to study the spread of infectious diseases in animal populations, and the identification of clusters might yield important information about the transmission and/or control of such diseases (Carpenter 2001). Thus, in the present study a spatial cluster analysis was performed aiming to determine the spatial distribution of the disease in the State of Paraíba.

MATERIALS AND METHODS

Data used in the present study were originated from the epidemiological survey for bovine paratuberculosis in the State of Paraíba (Vilar et al. 2015). The statewas divided into three sampling groups: sampling stratum 1 (mesoregion of Sertão), sampling stratum 2 (mesoregion of Borborema), and sampling stratum 3 (mesoregions of Zona da Mata and Agreste) (Fig. 1). For each sampling stratum, a two-stage sampling survey. In the first stage, a pre-established number of herds (primary sampling units) were randomly selected; in the second stage, a pre-established number of cows aged ≥ 24 months were randomly selected (secondary sampling units).

The number of selected herds per sampling stratum was determined by using the formula for simple random samples proposed by Thrusfield (2007). The parameters adopted for the calculation were as follows: 95% confidence level, 47.4% estimated prevalence (Sá et al. 2013), and 8% error. Further, the operational and financial capacity of the SEDAP was taken into consideration when determining the sample size of the sampling stratum.

For the secondary units, the minimum number of animals to be examined within each herd was estimated in order to allow its classification as positive herd. For this purpose, the concept of aggregate sensitivity and specificity was used (Dohoo et al. 2003). For the calculations, the following values were adopted: 73.6% (Hendrick et al. 2005) and 98% (Sweeney et al. 1995) for the sensitivity and specificity, respectively, of the test protocol and 37.9% (Fonseca et al. 2000) for the intra-herd estimated prevalence. Herdacc version 3 software (Jordan 1995) was used during this process, and the sample size was selected so that the herd sensitivity and specificity values would be ≥ 90%. Therefore, 10 animals were sampled in herds with up to 99 cows aged over 24 months; 15 animals were sampled in herds with 100 or more cows aged over 24 months; and all animals were sampled in those with up to 10 cows aged over 24 months. The selection of the cows within the herds was systematic. In total, 2504 animals were sampled from 480 cattle herds.

The target condition (Gardner et al. 2011) was a seropositive animal within an infected herd. The herd-level case definition was based on the size of the population (cows aged ≥ 24 months), number of females sampled, an intra-herd apparent prevalence of 37.9% (Fonseca et al., 2000), and the sensitivity and specificity of the diagnostic test used (indirect ELISA), with the goal of obtaining a herd sensitivity and specificity of ≥ 90%. After new simulations using Herdacc software, a herd was deemed

positive for the presence of MAP if it included at least one positive animal in herds of up to 24 females, and two positive animals in herds with more than 24 females.

The serological examination was performed according to the protocol recommended by the kit for detection of antibodies to MAP (Pourquier-IDEXX ELISA Paratuberculosis Screening Ab Test). To calculate the results, the reactions were considered valid when the average of the positive control (PCx) had a minimum OD450 mean value of 0.350 and the coefficient between the average of the PCx and the negative control (NC OD450) was ≥ 3.00. Samples with percentages ≥ 70% were considered positive for the presence of antibodies against MAP.

Spatial clustering of bovine paratuberculosis positive herds was assessed using two methods (Ward & Carpenter 2000). First, the Cuzick-Edwards'*k*-nearestneighbor method (Cuzick & Edwards 1990) was used to detect the possibility of global spatial clustering at herd level using the Cluster Seer 2.5.1 software (Bio Med ware, Ann Arbor, MI, United States). Existence of potential spatial clustering was analyzed at each of the first 10 neighborhood levels, and the overall p-value was adjusted for multiple comparisons with the Simes approach. Further, scan statistics by the SatScan software version 9.0 (Kulldorff & Nagarwalla 1995) was used to identify local clusters of positive herds. A Bernoulli model was applied, the scanning window was circular, and the spatial size of scan window was limited to 25% ofthe total population. Because of the large proportion of positive herds (Table 1), analysis was not run on herd-level, and then considering within-herd prevalence.

RESULTS AND DISCUSSION

Significant clusters were not identified (Simes $p > 0.05$) by the Cuzick and Edwards' method for the entire Paraiba State. However, when considering the state division into separate strata a significant global clustering (Simes $p < 0.05$) of positive herds was detected by the Cuzick and Edwards' method at k=3 neighborhood level in Borborema mesoregion. The resultsoftheSatScan cluster analysisisshown in Table 2 and Fig. 1. Usingthe Bernoulli model, two spatial clusters of positive herds with high within-herd prevalence were detected in Northern part of Borborema mesoregion. In the primary cluster, the number of herds was 4, the radius of the cluster was 14.57 km, and the number of observed and expected cases (positive animals) were 11 and 2.60, respectively, where the risk for infection was 4.91 (relative risk = 4.91; p = 0.014)times

higher in herds located inside cluster than in those located else where. In the secondary cluster, the number of herds was 4, the radius of the cluster was 19.20 km, and the number of observed and expected cases (positive animals) were 7 and 1.17, respectively, and the risk for infection was 6.58 (relative risk = 6.58; p = 0.022).

In asurvey to describe the spatial pattern of MAP prevalence throughout Denmark it was found a number of significant clusters, identifying geographical areas with higher apparent within-herdprevalence (Bihrmann et al. 2012). This study found consistency between kriging and scan statistics results with respect to location of areas with high apparent within-herd prevalence of MAP. However, these authors did not take any covariate information into account. Recently, Bihrmann et al. (2016) identified the spatial pattern in infection prevalence in Denmark and found a significant spatial component, suggesting that the estimated range of influence and the overall location of areas with increased prevalence are not very sensitive to diagnostic misclassification.

In the present study there was a lack of spatial cluster of bovine paratuberculosispositive herds throughout the Paraíba State, but spatial clusters were identified when considering the separate mesoregions. However, it can be inferred that these clusters cannot be explained by spatially structured factors as referred by Ávila et al. (2013), which detected cluster for bovine tuberculosis in Bahia State only when analyzed regions separately. The geographic division (Sertão, Borborema, Agreste/Zona da Mata) created in this study for analysis purposes is not subject to real parameters occurrence of paratuberculosis, and does not respect geographical boundaries. Thus, the clusters found in the Borborema region can be explained by being a border region with the State of Rio Grande do Norte, in which there is a large movement of animals from different locations without knowing the sanitary condition of the animals, which may result in a greater number of traded animals subclinically infected with MAP.

The detection of spatial clustering is a complex methodology and has limitations, however, the obtainment of more accurate results and security for decision-making lead to a greater efficiency of sanitary defense actions (Ávila et al. 2013). In this context, it is not plausible to suggest measures based on animal testing prior to purchasing because serological tests for MAP diagnosis are not widely available and are very expensive, as well as replacement or maintenanceof livestock by animal purchasing is common in the region. Furthermore, in general, the design quality, implementation, and reporting of test results for paratuberculosis have been generally poor (Nielsen & Toft 2008). Therefore, measures should be based on the prevention of

the disease at herd level, such as keep calvesin areas free of manure and raised separate from adults until at least one year old, reducing fecal contamination in animal housing areas by elevating food and water sources, and using colostrum only from the dam of the calf (Nielsen et al. 2008, OIE 2016).

CONCLUSION

We detected two spatial clusters of cattle herds with a high within-herd seroprevalence of paratuberculosis in the State of Paraíba, in a border region with the State of Rio Grande do Norte, which suggests a between-states trade of infected animals. It is also suggested that paratuberculosis prevention measures should be applied at herd level.

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Figure caption

Fig. 1. Significant clusters of cattle herds with a high within-herd prevalence of paratuberculosis in Paraíba State. Primary cluster: circular red line; secondary cluster: circular dark line. Detail shows Paraíba State within Brazil.

Table 1. Census data of the cattle population in the State of Paraíba, Northeastern Brazil, according to sampling stratum, and herd-level prevalence for bovine paratuberculosis.

Sampling stratum	Total no. of herds	No. of herds		Prevalence (%)	95% CI
		Tested	Positive		
Sertão	24,356	162	67	41.4	[34.0 – 49.1]
Borborema	11,603	154	41	26.6	[20.2 – 34.2]
Agreste/Zona da Mata	18,398	164	50	30.5	[23.9 – 38.0]
State of Paraíba	54,357	480	158	34.5	[30.2 – 39.1]

Source: Vilar et al. (2015)

Table 2. Statistically significant clusters of herds with a high within-herd prevalence of paratuberculosis in the State of Paraíba.

Radius (km)	No. of herds in cluster	No. of cases in cluster		RR[a]	p-value
		Observed	Expected		
14.57[b]	4	11	2.60	4.91	0.014
19.20	4	7	1.17	6.58	0.022

[a] Relative risk

[b] Primary cluster

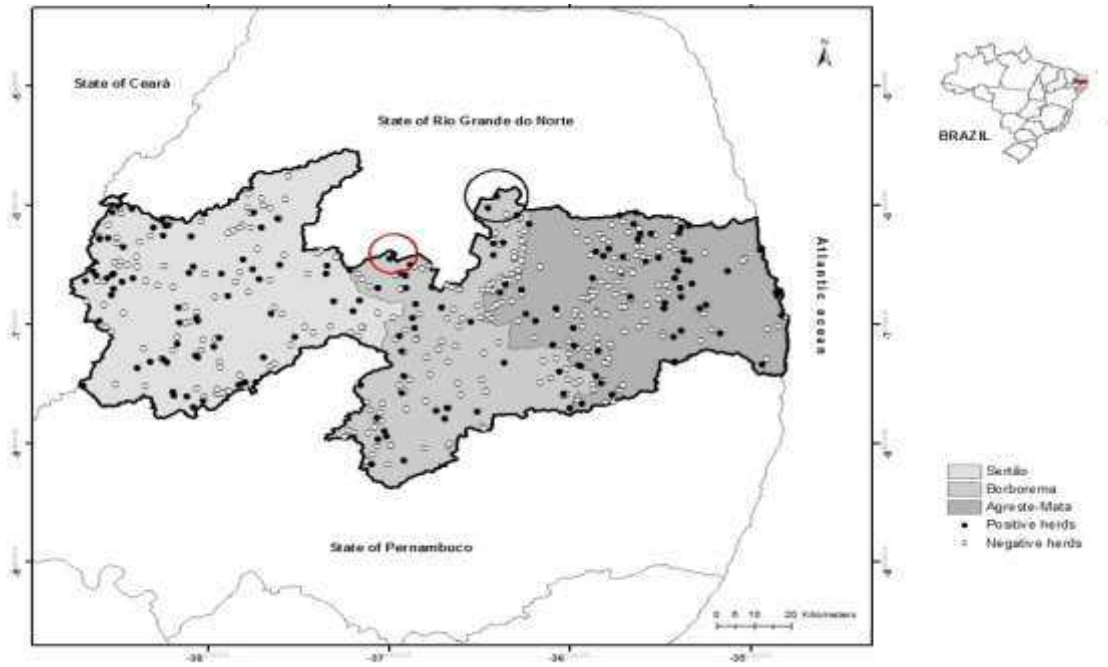
State of Ceará
State of Rio Grande do Norte
State of Pernambuco
Atlantic ocean
BRAZIL
Sertão
Borborema
Agreste-Mata
Positive herds
Negative herds

Fig. 1

FINAL CONSIDERATIONS

The present study has important as regards the absence of epidemiological studies of paratuberculosis in cattle conducted at state level based on planned sample and the collection of epidemiological important indicator was possible. According to the results it can be seen that there is a high prevalence of positive herds in the state of Paraiba, and that the knowledge of this reality is important for decision-making and future control measures. It was also possible to identify risk factors associated with the presence status (positive property), and the determination of areas with greater spread of risk of infection, which should also be taken into account in planning future actions.

On the other hand, it is important to concentrate efforts with regard to the conduction of studies aimed at identifying infected animals with clinical symptoms, as well as carrying out insulation work and identification of the agent from infected animals and dairy products, once the disease, and the recognized economic impact, can be related to zoonotic occurrence of Crohn's disease in humans.

ATTACHMENTS

ATTACHMENT I

Normas do periódico *SEMINA: CIÊNCIAS AGRÁRIAS*

Taxa de Submissão de novos artigos: RS 80,00. Em caso de rejeição do artigo, esta taxa não será devolvida.
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Em caso de aceite do artigo para publicação, o valor pago de R$ 80,00 referente à taxa de submissão, não será deduzido da taxa de publicação.
O comprovante de depósito deverá ser digitalizado e anexado no sistema como documento suplementar
Depósito em nome do Instituto de Tecnologia e Desenvolvimento Econômico e Social (ITEDES), CNPJ: 00.413.717/0001-65, em uma das três contas abaixo:
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Normas editoriais para publicação na Semina: Ciências Agrárias, UEL.
A partir de 01 de abril de 2014, os artigos poderão ser submetidos em português ou inglês, mas somente serão publicados em inglês. Os artigos submetidos em português, após o aceite, deverão ser obrigatoriamente traduzidos para o inglês.
Os artigos enviados para a revista até esta data e que estão em tramitação poderão ser publicados em português, entretanto, se traduzidos para o inglês terão prioridade na publicação.
Todos os artigos, após o aceite deverão estar acompanhados (como documento suplementar) do comprovante de tradução ou correção de um dos seguintes tradutores:
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O autor principal deverá anexar no sistema o documento comprobatório dessa correção na página de submissão em "Docs. Sup."

OBSERVAÇÕES:
1) Os manuscritos originais submetidos à avaliação são inicialmente apreciados pelo Comitê Editorial da Semina: Ciências Agrárias. Nessa análise, são avaliados os requisitos de qualidade para publicação na revista, como: escopo; adequação às normas da revista; qualidade da redação; fundamentação teórica; atualização da revisão da literatura; coerência e precisão da metodologia; contribuição dos resultados; discussão dos dados observados; apresentação das tabelas e figuras; originalidade e consistência

das conclusões. Se o número de trabalhos com manuscrito ultrapassar a capacidade de análise e de publicação da Semina: Ciências Agrárias é feita uma comparação entre as submissões, e são encaminhados para assessoria Ad hoc, os trabalhos considerados com maior potencial de contribuição para o avanço do conhecimento cientifico. Os trabalhos não aprovados nesses critérios são arquivados e os demais são submetidos a análise de pelo menos dois assessores científicos, especialistas da área técnica do artigo, sem a identificação do(s) autor(es). Os autores cujos artigos forem arquivados, não terão direito à devolução da taxa de submissão.

2) Quando for o caso, deve ser informado que o projeto de pesquisa que originou o artigo foi executado obedecendo às normas técnicas de biosegurança e ética sob a aprovação da comissão de ética envolvendo seres humanos e/ou comissão de ética no uso de animais (nome da Comissão, Instituição e nº do Processo).

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a) O arquivo do artigo anexado do trabalho contenha os nomes dos autores e respectiva afiliação; b) Não tenha sido realizado o cadastro completo de todos os autores nos metadados de submissão; Exemplo: Nome completo; Instituição/Afiliação; País; Resumo da Biografia/Titulação/função c) Não tenha sido incluído no campo COMENTÁRIOS PARA O EDITOR, um texto que aponte a relevância do trabalho (importância e diferencial em relação a trabalhos já existentes), em até 10 linhas; d) Não estejam acompanhados de documento comprobatório da taxa de submissão, em documento suplementar "Docs. Sup." no ato da submissão; e) Não estejam acompanhados dos seguintes documentos suplementares: gráficos, figuras, fotos e outros, EM VERSÃO ORIGINAL. (Formato JPEG; TIFF; EXCEL)

f) Não constem no artigo original: título, resumo e palavras-chave em português e inglês, tabelas e figuras.

RESTRIÇÃO POR ÁREA: PARA A ÁREA DE AGRONOMIA NÃO SERÃO ACEITOS MANUSCRITOS EM QUE:

a) Os experimentos com cultura in vitro sejam limitados ao melhoramento dos protocolos já padronizados ou que não forneçam novas informações na área;

b) Os experimentos de campo não incluam dados de pelo menos dois anos ou de várias localidades dentro do mesmo ano;

c) Os experimentos se refiram apenas a testes sobre a eficiência de produtos comerciais contra agentes bióticos, abióticos ou estresses fisiológicos;

d) Envolvam apenas bioensaios (screening) de eficácia de métodos de controle de insetos, ácaros ou doenças de plantas, exceto se contiverem contribuição importante sobre mecanismos de ação numa perspectiva de fronteira do conhecimento;

e) O objetivo seja limitado a registrar a ocorrência de espécies de pragas ou patógenos ou associações entre hospedeiros em novas localidades dentro de regiões geográficas onde eles já sejam conhecidos. Registros de espécies ou associações conhecidas só serão considerados em novas zonas ecológicas. Os registros de distribuição devem se basear em ecossistemas, e não em fronteiras políticas.

PARA A ÁREA DE VETERINÁRIA

a) A publicação de relatos de casos é restrita e somente serão selecionados para tramitação àqueles de grande relevância ou ineditismo, com real contribuição ao avanço do conhecimento para a área relacionada.

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a) Artigos científicos: no máximo 20 páginas incluindo figuras, tabelas e referências bibliográficas;
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c) Artigos de revisão: no máximo 25 páginas incluindo figuras, tabelas e referências bibliográficas.

Apresentação dos Trabalhos

Os originais completos dos artigos, comunicações, relatos de casos e revisões podem ser escritos em português ou inglês no editor de texto Word for Windows, em papel A4, com numeração de linhas por página, espaçamento 1,5, fonte Times New Roman, tamanho 11 normal, com margens esquerda e direita de 2 cm e superior e inferior de 2 cm, respeitando-se o número de páginas, devidamente numeradas no canto superior direito, de acordo com a categoria do trabalho.

Figuras (desenhos, gráficos e fotografias) e Tabelas serão numeradas em algarismos arábicos e devem ser incluídas no final do trabalho, imediatamente após as referências bibliográficas, com suas respectivas chamadas no texto. Alem disso, as figuras devem apresentar boa qualidade e deverão ser anexadas nos seus formatos originais (JPEG, TIF, etc) em "Docs Supl." na página de submissão. Não serão aceitas figuras e tabelas fora das seguintes especificações: Figuras e tabelas deverão ser apresentadas nas larguras de 8 ou 16 cm com altura máxima de 22 cm, lembrando que se houver a necessidade de dimensões maiores, no processo de editoração haverá redução para as referidas dimensões.

Observação: Para as tabelas e figuras em qualquer que seja a ilustração, o título deve figurar na parte superior da mesma, seguida de seu número de ordem de ocorrência em algarismo arábico, ponto e o respectivo titulo.

Indicar a fonte consultada abaixo da tabela ou figura (elemento obrigatório). Utilizar fonte menor (Times New Roman 10).

Citar a autoria da fonte somente quando as tabelas ou figuras não forem do autor.

Ex: Fonte: IBGE (2014), ou Source: IBGE (2014).

Preparação dos manuscritos

Artigo científico:

Deve relatar resultados de pesquisa original das áreas afins, com a seguinte organização dos tópicos: Título; Título em inglês; Resumo com Palavras-chave (no máximo seis palavras, em ordem alfabética); Abstract com Key words (no máximo seis palavras, em ordem alfabética); Introdução; Material e Métodos; Resultados e Discussão com as conclusões no final da discussão ou Resultados; Discussão e Conclusões separadamente; Agradecimentos; Fornecedores, quando houver e Referências Bibliográficas. Os tópicos devem ser destacados em negrito, sem numeração, quando houver a necessidade de subitens dentro dos tópicos, os mesmos devem ser destacados em itálico e se houver dentro do subitem mais divisões, essas devem receber números arábicos. (Ex. Material e Métodos... *Áreas de estudo...1. Área rural...2.Área urbana*).

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6. Conclusões: Devem ser claras e de acordo com os objetivos propostos no trabalho.
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a) Os manuscritos devem obedecer aos critérios estabelecidos nos Códigos Internacionais de cada área.
b) Utilizar o Sistema Internacional de Unidades em todo texto.
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d) Utilizar um espaço simples entre as unidades, g L-1, e não g.L-1 ou gL-1.
e) Usar o sistema horário de 24 h, com quatro dígitos para horas e minutos: 09h00, 18h30.
8. Citações dos autores no texto
Deverá seguir o sistema de chamada alfabética seguidas do ano de publicação de acordo com os seguintes exemplos:
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b) De acordo com Santos et al. (1999), o efeito do nitrogênio.....
c) Beloti et al. (1999b) avaliaram a qualidade microbiológica.....
d) [...] e inibir o teste de formação de sincício (BRUCK et al., 1992).
e) [...]comprometendo a qualidade de seus derivados (AFONSO; VIANNI, 1995).
Citações com dois autores
Citações onde são mencionados dois autores, separar por ponto e vírgula quando estiverem citados dentro dos parênteses.
Ex: (PINHEIRO; CAVALCANTI, 2000).
Quando os autores estiverem incluídos na sentença, utilizar o (e)
Ex: Pinheiro e Cavalcanti (2000).
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Indicar o primeiro autor seguido da expressão et al.

Dentro do parêntese, separar por ponto e vírgula quando houver mais de uma referência. Ex: (RUSSO et al., 2000) ou Russo et al. (2000); (RUSSO et al., 2000; FELIX et al., 2008).

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As outras categorias de trabalhos (Comunicação científica, Relato de caso e Revisão) deverão seguir as mesmas normas acima citadas, porém, com as seguintes orientações adicionais para cada caso:

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Uma forma concisa, mas com descrição completa de uma pesquisa pontual ou em andamento (nota prévia), com documentação bibliográfica e metodologias completas, como um artigo científico regular. Deverá conter os seguintes tópicos: Título (português e inglês); Resumo com Palavras-chave; Abstract com Key words; Corpo do trabalho sem divisão de tópicos, porém seguindo a sequência - introdução, metodologia, resultados (podem ser incluídas tabelas e figuras), discussão, conclusão e referências bibliográficas.

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Descrição sucinta de casos clínicos e patológicos, resultados inéditos, descrição de novas espécies e estudos de ocorrência ou incidência de pragas, microrganismos ou parasitas de interesse agronômico, zootécnico ou veterinário. Deverá conter os seguintes tópicos: Título (português e inglês); Resumo com Palavras-chave; Abstract com Key words; Introdução com revisão da literatura; Relato do (s) caso (s), incluindo resultados, discussão e conclusão; Referências Bibliográficas.

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Deve envolver temas relevantes dentro do escopo da revista. O número de artigos de revisão por fascículo é limitado e os autores somente poderão apresentar artigos de interesse da revista mediante convite de membro(s) do comitê editorial da Revista. No caso de envio espontâneo do autor (es), é necessária a inclusão de resultados relevantes próprios ou do grupo envolvido no artigo, com referências bibliográficas, demonstrando experiência e conhecimento sobre o tema.

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Appendix

Authors: These minimum items of information are needed by our referees and Editors to evaluate your manuscript. Additional information may be appropriate, depending on your study design and objectives.

Excellent guidelines for standardizing and strengthening the reporting of biomedical research are available from the CONSORT, MOOSE, PRISMA, REFLECT, STARD,

and STROBE statements. We strongly urge you to consult these guidelines before submitting papers to Preventive Veterinary Medicine. The guidelines are freely available (with considerable elaborations and explanations) at the following websites:
http://www.consort-statement.org (for clinical trials; there are elaborations for abstracts, cluster designs, reporting of harms, herbal interventions, non-inferiority and equivalence studies, trials of non-pharmacologic interventions, and pragmatic trials)
http://jama.ama-assn.org/cgi/reprint/283/15/2008 (for MOOSE: Meta-analysis of Observational Studies in Epidemiology: A Proposal for Reporting, Donna F. Stroup et al.; published in J Am Med Assoc 2000; 283:2008-2012)
http://prisma-statement.org (for meta-analyses and systematic reviews)
http://reflect-statement.org (for clinical trials in livestock)
http://www.stard-statement.org (for evaluations of diagnostic tests)
http://www.strobe-statement.org (for observational studies; there is an elaboration for studies of genetic associations) 1. For ALL descriptive and comparative studies:
a. Source of subjects
b. Eligibility criteria
c. Sample-size justification appropriate for the study design and primary hypothesis. This should include details of adjustment for clustering (including the levels of clustering, the assumed cluster size, and either the design effect or the intracluster correlation) if clustering was present.
d. Methods by which the data were acquired
e. Diagnostic sensitivity and specificity of any tests used. (Analytic sensitivity and reproducibility
might be appropriate alternatives for some studies.) Correction to the true prevalence is expected
for e.g., seroprevalence studies.
f. Descriptions of the observed data (including measures of subject-level variation), stratified onthe outcome implied by the primary hypothesis. These descriptions should include time, place, "demographics," and relevant management and health information.
g. Declaration of the experimental unit
h. Descriptions of the formal random mechanism (e.g., lottery or table of random numbers)and the list frame (enumerating every eligible subject and/or cluster) used at any step claimed to be "random"
i. Descriptions of the pilot, repeatability, and validation testing of any questionnaire used to acquire data for the study. Also needed are: the language of the survey instrument, thetime it took to complete, how it was administered, the types of questions (e.g., closed, semi-closed, open), and the training of any persons administering the survey. Making a copy available to the review process is desirable (in English as well as the language of administration).
2. For comparative studies (including both observational and intervention studies):
a. Numerical descriptions of all tested risk factors or pre-intervention characteristics of the subjects, stratified on the primary hypothesis/outcome of the study
b. Descriptions of how blindness was accomplished for all subjective evaluations
3. For randomized controlled trials and other intervention studies:
a. Approval by your institution's animal-welfare committee and description of measures taken for rescue analgesia or rescue euthanasia. b. Methods by which the owners of the animals gave informed consent for their animals to bein the trial
c. Methods used for allocation concealment after the animals were determined to be eligiblefor random assignment to the various experimental or control groups

d. Description and justification of the "control" group's "treatment" (e.g., standard therapy, placebo to mimic the delivery system in the absence of a standard therapy, or "do nothing" to mimic both the treatment and its delivery)
e. Methods used for active monitoring for adverse effects ("harms")
4. For simulation studies and risk assessments:
a. Distinction between deterministic and stochastic processes
b. Descriptions of (and justifications for) all choices of distributions and their parameter Values
c. Description of numbers, training, experience, and representativeness of any "experts" used to provide opinions
d. Declaration of the stakeholders for any risk assessment
e. Distinction between assumptions, input data, calculations from intermediate steps in the modeling process, and model predictions
f. Descriptions of the assumed chance variation and assumed knowledge uncertainty in the inputs, and methods used to deal with those sources of total uncertainty
g. Sensitivity analyses of key assumptions and of the input variables that had the greatest uncertainty
h. Descriptions of the variability in the "outputs" from stochastic models
5. For statistical-hypothesis tests:
a. Declarations of the unit of statistical analysis and of the dependent ("outcome") variable
b. Alpha and tails, and any methods used to adjust for multiple comparisons (to protect experimentwise alpha from the problem of multiplicity)
c. Methods used to adjust for clustering within the data
d. Methods used to determine that the statistical assumptions were met (e.g., that the data were Gaussian or that the odds ratio or hazards ratio was constant across the observed range of the risk factor)
e. Methods used to look for collinearities or other interrelationships among the risk factors being tested
f. Methods used to select or to retain risk factors within multivariable models (including the test criterion)
g. Clear declaration of any variables "forced into" the model (not allowed to drop out; this implies a need to account for that factor) or offered to the model on a priori grounds despite any screening results (this implies that the factor was part of a major hypothesis)
h. Description of the goodness-of-fit of any models
i. How missing data were handled
AFTER ACCEPTANCE
Online proof correction
Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors. If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF. We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is

important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

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ATTACHMENT III

Normas do periódico *PESQUISA VETERINÁRIA BRASILEIRA*

Os trabalhos para submissão devem ser enviados por via eletrônica, através do e-mail <jurgen.dobereiner@pvb.com.br>, com os arquivos de texto na versão mais recente do Word e formatados de acordo com o modelo de apresentação disponível no site da revista (www.pvb.com.br. Devem constituir-se de resultados de pesquisa ainda não publicados e não considerados para publicação em outra revista.

Para abreviar sua tramitação e aceitação, os trabalhos sempre devem ser submetidos conforme as normas de apresentação da revista (www.pvb.com.br) e o modelo em Word (PDF no site). Os originais submetidos fora das normas de apresentação, serão devolvidos aos autores para a devida adequação.

Apesar de não serem aceitas comunicações (*Short communications*) sob forma de "Notas Científicas", não há limite mínimo do número de páginas do trabalho enviado, que deve, porém, conter pormenores suficientes sobre os experimentos ou a metodologia empregada no estudo. Trabalhos sobre Anestesiologia e Cirurgia serão recebidos para submissão somente os da área de Animais Selvagens.

Embora sejam de responsabilidade dos autores as opiniões e conceitos emitidos nos trabalhos, o Conselho Editorial, com a assistência da Assessoria Científica, reserva-se o direito de sugerir ou solicitar modificações aconselháveis ou necessárias. Os trabalhos submetidos são aceitos através da aprovação pelos pares (*peer review*).

NOTE: Em complementação aos recursos para edição da revista (impressa e online) e distribuição via correio é cobrada taxa de publicação (*page charge*) no valor de R$ 250,00 por página editorada e impressa, na ocasião do envio da prova final, ao autor para correspondência.

1. Os trabalhos devem ser organizados, sempre que possível, em Título, ABSTRACT, RESUMO, INTRODUÇÃO, MATERIAL E MÉTODOS, RESULTADOS, DISCUSSÃO, CONCLUSÕES (ou combinação destes dois últimos), Agradecimentos e REFERÊNCIAS:

a) o Título do artigo deve ser conciso e indicar o conteúdo do trabalho; pormenores de identificação científica devem ser colocados em MATERIAL E MÉTODOS.

b) O(s) Autor(es) deve(m) sistematicamente encurtar os nomes, tanto para facilitar sua identificação científica, como para as citações bibliográficas. Em muitos casos isto significa manter o primeiro nome e o último sobrenome e abreviar os demais sobrenomes:

Paulo Fernando de Vargas Peixoto escreve Paulo V. Peixoto ou Peixoto P.V.; Franklin Riet-Correa Amaral escreve Franklin Riet-Correa ou Riet-Correa F.; Silvana Maria Medeiros de Sousa Silva poderia usar Silvana M.M.S. Silva, inverso Silva S.M.M.S., ou Silvana M.M. Sousa-Silva, inverso, Sousa-Silva S.M.M., ou mais curto, Silvana M. Medeiros-Silva, e inverso, Medeiros-Silva S.M.; para facilitar, inclusive, a moderna indexação, recomenda-se que os trabalhos tenham o máximo de 8 autores;

c) o ABSTRACT deverá ser apresentado com os elementos constituintes do RESUMO em português, podendo ser mais explicativos para estrangeiros. Ambos devem ser seguidos de "INDEX TERMS" ou "TERMOS DE INDEXAÇÃO", respectivamente;

d) o RESUMO deve apresentar, de forma direta e no passado, o que foi feito e estudado, indicando a metodologia e dando os mais importantes resultados e conclusões. Nos trabalhos em inglês, o título em português deve constar em negrito e entre colchetes, logo após a palavra RESUMO;

e) a INTRODUÇÃO deve ser breve, com citação bibliográfica específica sem que a mesma assuma importância principal, e finalizar com a indicação do objetivo do trabalho;
f) em MATERIAL E MÉTODOS devem ser reunidos os dados que permitam a repetição do trabalho por outros pesquisadores. Na experimentação com animais, deve constar a aprovação do projeto pela Comissão de Ética local;
g) em RESULTADOS deve ser feita a apresentação concisa dos dados obtidos. Quadros devem ser preparados sem dados supérfluos, apresentando, sempre que indicado, médias de várias repetições. É conveniente, às vezes, expressar dados complexos por gráficos (Figuras), ao invés de apresentá-los em Quadros extensos;
h) na DISCUSSÃO devem ser discutidos os resultados diante da literatura. Não convém mencionar trabalhos em desenvolvimento ou planos futuros, de modo a evitar uma obrigação do autor e da revista de publicá-los;
i) as CONCLUSÕES devem basear-se somente nos resultados apresentados no trabalho;
j) Agradecimentos devem ser sucintos e não devem aparecer no texto ou em notas de rodapé;
k) a Lista de REFERÊNCIAS*,* que só incluirá a bibliografia citada no trabalho e a que tenha servido como fonte para consulta indireta, deverá ser ordenada alfabeticamente pelo sobrenome do primeiro autor, registrando-se os nomes de todos os autores, em caixa alta e baixa (colocando as referências em ordem cronológica quando houver mais de dois autores), o título de cada publicação e, abreviado ou por extenso (se tiver dúvida), o nome da revista ou obra, usando as instruções do "Style Manual for Biological Journals" (American Institute for Biological Sciences), o "Bibliographic Guide for Editors and Authors" (American Chemical Society, Washington, DC) e exemplos de fascículos já publicados (www. pvb.com.br).
2. Na elaboração do texto deverão ser atendidas as seguintes normas:
a) os trabalhos devem ser submetidos seguindo o exemplo de apresentação de fascículos recentes da revista e do modelo constante do site sob "Instruções aos Autores" (www.pvb.com.br). A digitalização deve ser na fonte Cambria, corpo 10, entrelinha simples; a página deve ser no formato A4, com 2cm de margens (superior, inferior, esquerda e direita), o texto deve ser corrido e não deve ser formatado em duas colunas, com as legendas das figuras e os Quadros no final (logo após as REFERÊNCIAS). As Figuras (inclusive gráficos) devem ter seus arquivos fornecidos separados do texto. Quando incluídos no texto do trabalho, devem ser introduzidos através da ferramenta "Inserir" do Word; pois imagens copiadas e coladas perdem as informações do programa onde foram geradas, resultando, sempre, em má qualidade;
b) a redação dos trabalhos deve ser concisa, com a linguagem, tanto quanto possível, no passado e impessoal; no texto, os sinais de chamada para notas de rodapé serão números arábicos colocados em sobrescrito após a palavra ou frase que motivou a nota. Essa numeração será contínua por todo o trabalho; as notas serão lançadas ao pé da página em que estiver o respectivo sinal de chamada. Todos os Quadros e todas as Figuras serão mencionados no texto. Estas remissões serão feitas pelos respectivos números e, sempre que possível, na ordem crescente destes. ABSTRACT e RESUMO serão escritos corridamente em um só parágrafo e não deverão conter citações bibliográficas.
c) no rodapé da primeira página deverá constar endereço profissional completo de todos os autores e o e-mail do autor para correspondência, bem como e-mails dos demais autores (para eventualidades e confirmação de endereço para envio do fascículo impresso);
d) siglas e abreviações dos nomes de instituições, ao aparecerem pela primeira vez no trabalho, serão colocadas entre parênteses e precedidas do nome por extenso;

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