PREVALENCE OF BOVINE BRUCELLOSIS AMONG MILK SUPPLIERS OF A DAIRY INDUSTRY IN ITIRAPUÃ, SÃO PAULO, BRAZIL

PREVALÊNCIA DE BRUCELOSE BOVINA ENTRE REBANHOS FORNECEDORES DE LEITE DE UM LATICÍNIO EM ITIRAPUÃ, SÃO PAULO

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SUMMARY

The aim of this study was to verify the prevalence of bovine brucellosis among milk suppliers of a dairy industry located in Itirapuã, São Paulo, Brazil, and to analyze the risk factors eventually associated with the occurrence of brucellosis among the herds involved in the study. A voluntary sample of 813 animals from 37 herds whose owners agreed to participate in the study was obtained among a population of 942 adult cattle from 55 herds located in the municipalities of Itirapuã and Patrocínio Paulista. A questionnaire about risk factors that could be associated with the prevalence rates was applied. Serum samples from all 813 animals were collected and tested by the rose Bengal plate test (RBPT); positive samples were then tested by the complement fixation test (CFT). Of the 813 serum samples, 26 (3.2%) tested positive in the serial application of RBPT and CFT, and of the 37 herds, 12 (32.4%) had at least one positive animal. Factors that showed association with the prevalence rate were number of animals in the herd, lack of vaccination with strain B19 and frequent introduction of animals.

KEY-WORDS: Bovine brucellosis, rose Bengal plate test, complement fixation.

RESUMO

O estudo teve por objetivo determinar a prevalência da brucelose bovina entre fornecedores de leite de um laticínio situado no Município de Itirapuã, Estado de São Paulo, e analisar fatores de risco eventualmente associados à ocorrência de brucelose nos rebanhos estudados. De uma população de 942 bovinos adultos distribuídos em 55 rebanhos, situados nos municípios de Itirapuã e de Patrocínio Paulista, foi obtida uma amostra voluntária composta por 813 animais, distribuídos em 37 rebanhos cujos proprietários aceitaram participar do estudo. Foi aplicado um questionário, para a obtenção de informações sobre fatores que pudessem estar associados às taxas de prevalência. Foram colhidas amostras de soro sanguíneo de todos os animais, as quais foram submetidas ao teste do antígeno acidificado tamponado (AAT), e as amostras positivas nesse teste foram submetidas à reação de fixação de complemento (RFC). Das 813 amostras de soro sanguíneo colhidas, 26 (3,2%) resultaram positivas na aplicação em série dos testes de triagem (AAT) e confirmatório (RFC), e das 37 propriedades estudadas, 12 (32,4%) apresentaram pelo menos um animal positivo. Os fatores que se mostraram associados à ocorrência de brucelose foram número de animais no rebanho, não utilização da vacina B19 e compra frequente de animais.

PALAVRAS-CHAVE: Brucelose bovina, antígeno acidificado tamponado, fixação de complemento.

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INTRODUCTION

Brucellosis is a disease of great economic importance due to the damage it can cause to several species, in addition to the fact that its agent is also pathogenic to humans, causing a disease that can be severe and difficult to treat. Although there are resources and technical knowledge to eradicate animal brucellosis as it has been done in many developed countries, this disease is still endemic in most of Brazil (POESTER et al., 2002), causing economic losses and posing a threat to people who are in contact with infected animals and their products. Therefore, the Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento, MAPA) approved in January, 2001, the National Program for Control and Eradication of Brucellosis and Tuberculosis (Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose, PNCEBT), in order to decrease the prevalence of infection in cattle and buffaloes and to increase the supply of products that pose low threat to public health (BRASIL, 2001; 2004). Following the creation of PNCEBT, the legislation that regulates milk production in Brazil established that all dairy cattle producing milk type A and B must be certified brucellosis free (BRASIL, 2002).

Before the PNCEBT was created, a nationwide survey was conducted to determine the prevalence of bovine brucellosis (BRASIL, 1977). Since the program was created, several prevalence studies were performed in different states such as in São Paulo (DIAS et al., 2009) and Minas Gerais (GONÇALVES et al., 2009). Several other studies can be found in the literature, but very localized. In the area where this study was conducted, a survey had been previously conducted by Murakami et al. (2003) in the municipalities of Altinópolis and Santo Antônio da Alegria.

Previous studies have shown that the factors most commonly associated with bovine brucellosis are sex (NICOLETTI, 1980; ACHA & SZYFRES, 2001), herd density (KELLAR et al., 1976; NICOLETTI, 1980; SALMAN & MEYER, 1984; DIAS et al., 2009), lack of vaccination (KELLAR et al., 1976; GONÇALVES et al., 2009) and careless introduction of new animals in the herd (VAN WAVEREN, 1960; NICOLETTI, 1980; KELLAR et al., 1976; DIAS et al., 2009; GONÇALVES et al., 2009).

The concern for animal and public health has motivated careful monitoring of the epidemiological situation of animal brucellosis, especially in dairy cattle, since it can be an important means of transmission to humans. The animals are the carriers of the etiologic agent and therefore, the occurrence of disease in humans depends on the occurrence in animals. Hence, this study was performed to determine the prevalence of brucellosis among milk suppliers of a dairy industry (OU DAIRY PLANT?) located in Itirapuã, a major dairy region in São Paulo state, and to analyze the risk factors eventually associated with the occurrence of brucellosis in the studied herds.

MATERIAL AND METHODS

Herds

This research involved dairy cattle herds that supply milk to a dairy industry located in Itirapuã, São Paulo. This dairy industry received milk from herds located in Itirapuã and Patrocínio Paulista, in the northeast of São Paulo. Itirapuã is located at 20º 38' 27''S and 47º 13' 09'' W, 865 m altitude and occupies an area of 161.9 Km^2. Patrocínio Paulista is located at 20º 38' 22'' S and 47º 16' 54'' W, 743 m altitude and area of 601.7 Km^2 (Figure 1).

Figure 1 – Location of Itirapuã and Patrocínio Paulista, São Paulo, Brazil.

The target population of this study consisted of all animals aged 24 months or older from the herds that supplied milk to this dairy industry, among which a voluntary non-probabilistic sample was sought. The dairy was supplied by 55 herds made up of 942 adult animals. The owners of the 37 herds that accepted to participate in the study had a total of 813 animals, from which 22 farms were in Itirapuã and 15 in Patrocínio Paulista. Blood samples were drawn from animals aged 24 months or older of all the herds that participated in the study, a total of 792 females and 21 males, during the first semester of 2007.

The blood samples were drawn by puncture of the jugular vein into sterile glass flasks, without anticoagulant. These flasks were kept at room temperature during 30 minutes and then cooled. After clot retraction, the material was centrifuged, blood serum was separated, placed in 1.5 mL microtubes and kept at -20°C until used.

The owners were asked to fill out a form in order to verify the correlation between brucellosis and risk factors.

Serological tests

Initially, the blood serum of all sampled animals underwent the rose Bengal plate test (RBPT), performed as described in the technical manual of PNCEBT (BRASIL, 2006). It consisted of briefly mixing in a glass plate, 30 µL of serum with 30 µL of antigen stained with rose Bengal, homogenizing, and after waiting 4 minutes while stirring, check for agglutination. The used antigen was prepared using *Brucella abortus* supplied by the Instituto Biológico in São Paulo. Those with positive screening test (RBPT) underwent complement fixation test (CFT) used as confirmation test, as required by PNCEBT. The CFT was performed by the micro-technique 50% hemolysis, incubated at 37°C in two stages, as described by Alton et al. (1988). The same antigen of the standard agglutination test was used. The antigen dilution was determined by block titration, and the dilution chosen was half of the optimum reactivity dilution. As complement, it was used a mixture of blood serum of several guinea pigs. This complement was titrated as described by Alton et al. (1988), using 20 times the volume employed in the micro-technique, to determine the volume that contained a 50% hemolytic unit. To be used in the test, the complement was diluted to contain five 50% hemolytic units. The hemolytic system consisted of a suspension of sheep red blood cells standardized in a spectrophotometer for the concentration of 0.95 g of hemoglobin per 100 mL, plus an equal volume of hemolysin suspension that consisted of rabbit antibodies against sheep red blood cells (ALTON et al., 1988). The titer was obtained by the reciprocal of the highest serum dilution with at least 25% complement fixation at 1:4 dilution (ALTON et al., 1988). The herd was considered infected if at least one animal was positive to the complement fixation test.

Data analysis

True prevalence was calculated based on the apparent prevalence that was determined by serological diagnosis, using the following formula (DOHOO et al., 2003):

$$TP = \frac{AP - (1 - Sp)}{1 - [(1 - Sp) + (1 - Se)]}$$

where: TP = true prevalence; AP = apparent prevalence; Sp = specificity; and Se = sensitivity.

For the series testing protocol adopted, we considered 95% sensitivity and 95% specificity according to Dias et al. (2009). The correlation between the analyzed factors and infection prevalence was verified by univariate analysis. To this end, it was calculated the prevalence ratio (PR) and confidence interval (CI) with 95% probability (SCHWABE et al., 1977). Also, statistical significance was determined based on Fisher's exact test, whose calculations were performed using spreadsheet available at www.physics.csbsju.edu/stats/fisher.form.html. A significant probability equal or higher than 95% was adopted to determine if the association observed was not by chance.

RESULTS

Of the 55 milk suppliers of the dairy, 37 (67.3%) agreed to participate and thus, 813 (86.3%) of 942 adult animals of the target population were examined (Table 1).

Brucellosis tests showed that of the 813 animals, 26 were positive to the AAT and CFT, an apparent prevalence rate of 3.2% (Table 1). Taking into account the sensitivity and specificity of serological diagnosis, the true prevalence was estimated at 2.9% of infected animals. Among the 37 studied herds, 12 had at least one animal with a positive result in serological diagnosis, thus 32.4% of the herds had an infection source (Table 1).

Comparing the prevalence rates of animals with positive serological diagnosis observed in both municipalities, it can be seen that in Patrocínio Paulista 4.5% of the animals were reactive, and in Itirapuã, 2.5% (Table 2). The prevalence ratio was 1.79, with confidence interval between 0.84 and 3.81. The probability of this difference to be by chance was 5.1% (Table 11).

The rate of females positive to brucellosis was 3.3%, while among the 21 bulls tested there was no positive animal (Table 3). With this result, it was not possible to calculate the prevalence ratio, but the Fisher exact test indicated that the observed difference is not statistically significant, since the probability of this difference being by chance was 50.1% (Table 11).

Table 1 – Prevalence of brucellosis in dairy herds in Itirapuã, São Paulo, 2007.

	Total	Examined	Positives		Negatives	
			Number	%	Number	%
Animals	942	813	26	3.2	787	96.8
Herds	55	37	12	32.4	25	67.6

Table 2 - Prevalence of brucellosis according to the municipality where the herds were located .

Municipality	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
Patrocínio Paulista	13	4.5	278	95.5	291 (100%)
Itirapuã	13	2.5	509	97.5	522 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 3 – Prevalence of brucellosis according to sex .

Sex of animal	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
Female	26	3.3	766	96.7	792 (100%)
Male	0	0	21	100	21 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 4 – Prevalence of brucellosis according to the fact whether the animal was lactating or not .

Lactation	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
No	13	4.3	285	95.7	298 (100%)
Yes	13	2.6	481	97.4	494 (100%)
Total	26	3.3	766	96.7	792 (100%)

Table 5 – Prevalence of brucellosis according to herd density.

Number of animals per herd	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
1 to 30	20	4.6	415	95.4	435 (100%)
≥ 31	6	1.6	372	98.4	378 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 6 – Prevalence of brucellosis according to use or not of vaccine B19 in the herd

Use of vaccine strain B19	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
No	25	3.8	638	96.2	663 (100%)
Yes	1	0.7	149	99.3	150 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 7 – Prevalence of brucellosis according to the requirement of brucellosis testing before introducing a new animal in the herd.

Brucellosis testing requirement	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
No	25	3.7	643	96.3	668 (100%)
Yes	1	0.7	144	99.3	145 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 8 – Prevalence of brucellosis according to the requirement of brucellosis diagnosis testing

Brucellosis diagnosis testing	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
Yes	16	4.2	365	95.8	381 (100%)
No	10	2.3	422	97.7	432 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 9 – Prevalence of brucellosis according to how often new animals were introduced in the herd.

Introduced new animals often in the herd	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
Yes	21	4.4	463	95.6	484 (100%)
No	5	1.5	324	98.5	329 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 10 – Prevalence of brucellosis in farms where other species were raised

Presence of other species in the farm	Brucellosis				Total
	Yes		No		
	Nº	%	Nº	%	
Yes	26	3.5	727	96.5	753 (100%)
No	0	0	60	100	60 (100%)
Total	26	3.2	787	96.8	813 (100%)

Table 11 – Prevalence ratio (PR), confidence interval (CI – 95%) of the ratio and the probability that this association may be by chance (P), calculated by Fisher exact test for each factor analyzed .

Analyzed Factor	Table	PR	C I(95%)	P
Municipality	2	1.79	0.84 – 3.81	0.051
Female	3	------	---------------	0.501
Non lactating	4	1.66	0.78 – 3.53	0.067
Up to 30 animals in the herd	5	2.9	1.78 – 4.72	0.011
Non- vaccinated	6	5.66	0.77 – 41.44	0.032
Brucellosis testing not required	7	5.4	0.74 – 39.53	0.038
Brucellosis diagnosis testing	8	1.81	0.83 – 3.94	0.093
Animals were bought often	9	2.85	1.09 – 7.48	0.018
Other species were raised in farm	10	------	----------------	0.132

Of the 298 females that were not lactating, 13 (4.3%) were positive, and among lactating females the apparent prevalence observed was 2.6%, according to Table 4. The ratio between these two prevalence rates was 1.66, confidence interval (95%) ranging from 0.78 to 3.53 and no significant difference (P = 0.067) by Fisher's exact test (Table 11).

The analysis of the prevalence of brucellosis in relation with herd size was statistically significant (P = 0.011). In the herds that had up to 30 animals, 4.6% were positive, while the herds that had more than 31 animals, this rate was 1.6% (Table 5). The prevalence ratio was 2.9, confidence interval between 1.78 and 4.72 (Table 11).

The frequency of reactive animals in the herds where the owners did not vaccinate the animals with strain B19 was 3.8%, while among those who reported vaccinating the animals with B19, the frequency of positives was 0.7% (Table 6). The prevalence ratio was 5.66 (interval 95% from 0.77 to 41.44) and were significantly different by Fisher exact test (P = 0.032), according to Table 11.

In herds whose owners said they did not require brucellosis testing before introducing new animals, the frequency of positives was 3.7% and among herds whose owners required the test, the rate was 0.7% (Table 7). Prevalence ratio was 5.4 and the confidence interval was 0.74 to 39.53 (Table 11), which was statistically significant (P = 0.038).

There was a prevalence rate of 4.2% of reactive animals among those whose owners declared to perform the brucellosis test to control the disease. Among animals whose owners declared not to perform the test, the prevalence rate was 2.3% (Table 8). The ratio between these two rates was 1.81 (083 – 3.94), and the difference between the two was not significant, since P = 0.093 (Table 11).

In herds where the owners reported buying animals more often, the observed prevalence rate was 4.4% and herds where animals were not bought so often the rate was 1.5% (Table 9). The prevalence ratio was 2.85, confidence interval 1.09 to 7.48 (Table 11) and statistically significant (P = 0.018).

We did not observe any reactive animals among those from farms where only cattle are raised, while in other farms where other species were raised the prevalence rate was 3.5% (Table 10). Prevalence ratio was not calculated, but the difference between these two situations was not significant and could be attributed to chance with 13.2% probability (Table 11).

DISCUSSION

A survey conducted in the 70's (BRASIL, 1977) showed a prevalence rate of 7.5% of brucellosis in the southeast region of Brazil. In São Paulo, it was reported that 22.7% of the farms had infected animals and that 7.4% of the females were reactive. In Minas Gerais, the same study reported 17.7% of the herds had positive animals and 6.3% of the females were reactive.

After such a long time and the differences in the methodology used now make difficult a comparison between that study and this investigation, which showed an apparent prevalence of 3.2% and true prevalence estimated at 2.9%. The prevalence of infected herds, that is, herds with at least one reactive animal, was 32.4%.

In another serological survey conducted in the same region, in the municipalities of Altinópolis and Santo Antônio da Alegria, using methodology similar to this study, it was found that 3.15% of the animals were reactive and 16.6% of the herds had at least one reactive animal (MURAKAMI et al., 2003). The comparison between the results shows similar rates of reactive animals, but different with relation to infected herds, since in the present study it was observed almost two times more the presence of infected herds compared to the study in the neighboring municipalities.

The results reported here can also be compared with those of the survey conducted for the PNCEBT. In São Paulo, the prevalence of positive animals was estimated at 3.8%, while the proportion of infected herds was 9.7%. More specifically, in the region where the herds of the present study are found (Producer circuit 4) the reported prevalence rate was 5.52%, higher than what is reported here, and a prevalence of infected herds of 11.11%; the prevalence of infected dairy herds was 17.4% (DIAS et al., 2009). The prevalence rates of infected herds were lower than what was observed in the present study. By comparing the data it is noted that although the prevalence of reactive animals was lower, brucellosis was more widespread, since practically one third of the herds had at least one positive diagnosis.

Since the studied region borders Minas Gerais, it is worth to compare with the data available for that state. Gonçalves et al. (2009) reported for Minas Gerais 1.1% prevalence of infected animals and 6% prevalence of infected herds. In the region of Minas that borders the area where the studied herds are located (South and Southwest), the prevalence of animals was 0.4%, the lowest of the state, prevalence of infected herds 3.8%, also the lowest in the state, and the prevalence of infected dairy herds was 3.59%. By comparison, it is noted that the prevalence rate in Minas Gerais is lower than in the studied herd and in São Paulo state, in general. This situation is certainly related to the fact that Minas Gerais has started a mandatory vaccination program for calves with *Brucella abortus* B19 back in the mid-1990s, several years before the national program, confirming the importance of vaccination to reduce the prevalence rate of bovine brucellosis.

The comparison between the percentage of reactive animals in both locations that had herds that were part of the target population were not significantly different, although it was close (P = 0.051).

Although it was observed that 3.3% of the females examined had antibodies against *Brucella* and no reactive male was found, the comparison between sexes was not significantly different. Perhaps, this lack of significance may be related to the low number of bulls examined, since the cows, especially those pregnant, are the most susceptible to brucellosis (NICOLETTI, 1980; ACHA & SZYFRES, 2001), which leads to expectation of greater prevalence among females.

The comparison between the percentages of lactating reactive cows and dry cows was also not significantly different, in spite of the slightly higher rate in dry cows, which could be explained by the fact that brucellosis is a disease of reproductive nature and is reflected in the fact whether the animal is lactating or not.

The frequency of reactive animals in farms that require brucellosis test before introducing new animals in the herd, as an unquestionably useful tool to prevent the infection of being introduced in the herd, was 0.7% and lower than that of farms that do not require the test 3.7%; however, the statistical analysis did not show significant difference, probably due to the small number of farmers that make this demand.

The testing for the diagnosis of brucellosis also did not influence significantly the percentage of reactive animals.

There was a lower percentage of reactive animals in herds that had more than 31 animals (1.6%) compared to the herds that had up to 30 animals (4.6%). These data contradict information found in the literature, which shows that in larger herds there is greater likelihood of infection, higher prevalence rates and greater difficulty to eliminate the infection (KELLAR et al., 1976; NICOLETTI, 1980; SALMAN & MEYER, 1984). According to NICOLETTI (1980), as the herd size increases, density also increases, and this increases the risk of contact between susceptible animals and the infection source. The association between prevalence of infected animals and larger herds was also observed in São Paulo. Dias et al. (2009) reported that herd size equal or higher than 87 animals proved to be a factor associated with prevalence rate. However, the results of this study did not confirm the observations of these authors, perhaps because the population studied consisted mostly of small herds, and the number of animals adopted as a reference number to separate the two groups was also small (31) when compared to the 87 adopted by Dias et al. (2009).

Vaccination of calves as established by the PNCEBT regulations is a factor that contributes to decrease the prevalence rates of brucellosis, and the data reported here also point in that direction. In herds that did not adopt vaccination with B19, the percentage of reactive animals was 3.8%, significantly higher than the 0.7% observed for herds that were vaccinated. These observations were also made by Kellar et al. (1976), who in a case-control study (the present study is cross-sectional type, called prevalence study) conducted in Canada found a lower frequency of vaccination in infected herds. Also in Brazil, in Minas Gerais, it was observed the lowest prevalence among herds that were vaccinated with B19 (GONÇALVES et al., 2009). This confirms the importance of vaccination and that this sanitary measure should be emphasized, in order to significantly lower the prevalence of bovine brucellosis in the country.

The most common way of entry of *Brucella abortus* in the herd is by introducing infected animals. The introduction of new animals in the herd without the proper sanitary control has been shown to be a relevant factor by several authors (VAN WAVEREN, 1960; NICOLETTI, 1980). Kellar et al. (1976), who also observed higher risk of brucellosis occurrence in herds whose owners buy animals frequently. In Brazil, this has also been reported by Dias et al. (2009), in São Paulo, and by Gonçalves et al. (2009), in Minas Gerais. These authors emphasized that the risk of introducing the infection is not when purchasing the animals, common practice on farms, but rather the acquisition of animals without the proper sanitary measures, mainly the testing to check if the animal is infected and to know the health condition of the herd of origin. In agreement with all these authors, in the present study, there was a greater percentage of reactive animals in herds whose owners often bought cattle (4.4%) compared to herds whose owners did not purchase animals often (1.5%).

Univariate analysis performed in this study indicated a significant association between the frequency of reactive animals and the following factors: the number of animals in the herd, lack of vaccination with strain B19 and frequent purchase of animals.

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