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Full Length Research Paper

Prevalence of *Leptospira hardjo* antibody in bulk tank milk in some dairy herds in Mashhad suburb

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Bulk tank milk samples were collected from 47 dairy herds in Mashhad suburb. From 19 herds, one sample was collected at each season in 2009 and from the rest of the 28 dairy herds one sample was collected only at autumn, 2009 and samples was tested by ELISA method for detecting antibodies to *Leptospira interrogans* serovar *hardjo.* These herds, which had not been vaccinated against leptospirosis within the previous five years, were categorised according to the herds' regions. Nine herds (19.15%) out of 47 had a positive ELISA result. The antibody level in the herd milk samples were affected by the herd size (*P*<0.05). Region and season had no significant effect on antibody level (*P*>0.05). Larger herds were significantly more likely to have higher mean concentrations of antibody. It was concluded that a number of unvaccinated dairy herds in Mashhad suburb are exposed to infection with *Leptospira hardjo*.

Key words: Cattle, *Leptospira*, antibodies, ELISA, hardjo.

INTRODUCTION

Leptospirosis is a zoonotic disease which affects a wide range of economically important livestock. Cattle are maintenance hosts for leptospires belonging to serovar hardjo, of which there are two species: *Leptospira interrogans* serovar *hardjo* (prajitno) and *Leptospira borgpetersenii* serovar *hardjo* (bovis) (Radostits, 2007). Clinical signs of hardjo infection include abortion and milk drop syndrome. Whilst there are genetic, epidemiological and pathogenic differences between the two species, the two microorganisms are indistinguishable by serological tests (Pritchard, 1999). Positive cases of leptospira infection in livestock have been reported from some area of Iran including: Mashhad, Shiraz, Shahrekord and Ahvaz (Ebrahimi et al., 2004; Firouzi and Vandyousefi, 2001; Garoussi et al., 2003; Hajikolaei et al., 2007). All the surveys have been done in Iran for anti-leptospiral antibodies based on testing of individual blood samples. However, with the advent of an enzyme–linked immunosorbent assay (ELISA), which can be used on milk samples (Pritchard, 1999), herd screening can now be carried out on bulk tank milk samples. Although microscopic agglutination test (MAT) is used widely, this test uses live organisms and thus requires specialized laboratories in which to continuously culture and handle these hazardous agents. Because of the limitations of the MAT, including the low success rate, the labour and time-intensiveness associated with the culture of these organisms, alternative serological methods are being developed for the detection of economically important serovars of Leptospira (Surujballi and Elmgren, 2000). There are reports from a number of countries that hardjo continues to cause substantial reproductive losses in cattle through abortion (Anon, 1999; Haessig and Lubsen, 1998; Langoni et al., 1999) and infertility (Guitian et al., 1999). Thus, it is possible that losses in unvaccinated Mashhad dairy herds may be underestimated. It has been suggested that where vaccination has been discontinued, problems with leptospiral infection recur (Anon, 2001). In addition to possible losses in these herds due to animal disease, transmission of infection to humans could also occur. In a survey that was performed

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Table 1. Herd size and *L. hardjo* percentage positivity (PP) reading in bulk tank milk of 19 dairy herds in four seasons and two regions.

Herd size	Lactating cows	Winter PP	Spring PP	Summer PP	Autumn PP	Mean PP	Region	Result
70	35	23.1	21.8	35.5	15.5	24	E[a]	N[c]
90	35	14.5	23.1	25.2	18.8	20.4	E	N
130	50	97.9	95.4	127.8	13.1	108.5	E	P[d]
140	50	32	37.6	21.4	8.9	25	E	N
165	70	36	27.3	40	6.1	27.3	E	N
170	68	267	304.2	277.5	280	282.2	E	P
190	70	9.2	26	11.1	24.4	17.7	W[b]	N
190	80	13.5	9.2	20.4	13.1	14	E	N
335	126	6.8	8.6	4.7	15.5	8.9	E	N
350	120	91.8	39.1	16.4	16.4	40.9	W	N
420	170	125.5	111.3	142.8	58.2	109.4	W	P
450	170	17.8	9.2	4.3	14.6	11.5	W	N
600	170	8.3	12.9	15.6	20.2	14.2	W	N
700	240	13.6	10.2	13.1	17.8	13.7	W	N
850	350	20.3	16.8	2.6	20.4	15	W	N
950	370	274.8	171.1	220.3	210	219	W	P
1500	500	241.6	247.8	188	200	219.3	E	P
1500	500	285.7	318.1	265.4	290	290	E	P
1700	670	304.5	310	320.7	315	312.5	E	P

[a] E = East, [b] W = West , [c] N = Negative, [d] P = Positive.

on 162 workers serum samples from 18 dairy herds at suburb of Mashhad by using MAT, twenty three (14.19%) sera showed positive serological reaction against leptospiral antigens (Garoussi et al., 2003). The prevalence of infection in Mashhad dairy herds may have significant implications for both animals and humans. The objective of this study was to establish the prevalence of anti-leptospiral antibodies in bulk tank milk samples from unvaccinated herds in Mashhad suburb.

MATERIALS AND METHODS

The bulk tank samples were collected from 47 dairy herds that had not been vaccinated against leptospirosis during the previous five years at two regions of Mashhad suburb. The herds were selected by stratified cluster sampling. From 19 herds one sample was collected at each season in 2009 and from the rest of the 28 dairy herds one sample was collected only at autumn, 2009. Tank milk samples were collected in 25 ml screw-top plastic tubes after complete mixing in bulk tank. Samples were combined in approximate proportions from different tanks of one herd. The presence of fat may affect the optical density (OD) measurement used with ELISA, so milk samples were first centrifuged at 2000 g for 10 min at 4°C in the laboratory and then skimmed milk stored at -20°C before analysis by the PrioCHECK ELISA (Prionic, Switzerland). The PrioCHECK *Leptospira hardjo* antibody is an indirect ELISA and detects antibodies against *L. interrogans* serovar *hardjo*. The test has a relative sensitivity and specificity of approximately 98.5%. At first, test plate was pretreated by dispensing 100 µl ELISA buffer to all wells and incubated for 60 min at 37°C and washed 6 times after incubation. After pretreatment, 100 µl of ELISA buffer was dispensed to wells of A1 and B1 of test plate as blanks and 90 µl of ELISA buffer were dispensed to wells C1 to H1. 10 µl of 1:20 diluted reference serum 1 was dispensed to wells C1 and D1 as positive control. 10 µl of 1:20 diluted reference serum 2 was dispensed to wells E1 and F1 as negative control. 10 µl of 1:20 diluted reference serum 3 was dispensed to wells G1 and H1 as weak positive control serum. One hundred µl of bulk milk samples from each herd was dispensed in two wells so the average of optical density for each herd was measured. After dispensing milk samples, test plate was incubated for 60 min at 37°C. After incubation, test plate was washed 6 times and 100 µl of diluted conjugate was dispensed to all wells and incubated and washed as before. One hundred µl of chromogen substrate was dispensed to all wells and test plate was incubated for 15 min at 22°C. One hundred µl of stop solution was added 15 min after the first well was filled with chromogen substrate. Optical density of the wells was measured at 450 nm within 15 min of stopping color development. Optical density readings equivalent to 60% or greater of a positive standard (OD450 of standard 1.00) were considered positive. Results were expressed as percentage positivity of the standard (PP). Statistical analyses were performed using the SPSS package ver. 12 (Chicago, Il, USA). Herd size and mean PP relationship was detected by Pearson correlation test. One way Anova was used to observe the difference of PP among seasons and the difference of PP between regions was detected by T-student test. A P value <0.05 was considered statistically significant for these analyses.

RESULTS

The mean number of lactating cows per herd was 103 with a range of 5 to 670 cows. A positive ELISA antibody result was detected in 9 herds out of 47 (19.15%). Detailed results on prevalence of *L. hardjo*-positive herds according to herd size and region are presented in Tables 1, 2 and 3.

The antibody level of the bulk tank milk sample were affected by the herd size (P<0.05). There was a positive

Table 2. Herd size and *L. hardjo* percentage positivity (PP) in bulk tank milk of 28 dairy herds in one season and two regions.

Herd size	Lactating cows	Autumn PP	Region	Result
11	6	37.5	W	N
12	5	15.5	W	N
12	5	17.4	W	N
13	5	10.3	W	N
14	5	6.1	W	N
15	6	6.6	W	N
17	8	20.2	W	N
18	8	16.5	W	N
20	9	20.7	W	N
20	10	45.5	W	N
25	12	28	W	N
30	15	39.4	W	N
32	15	27.7	W	N
42	20	19.7	W	N
55	25	56.8	W	N
65	30	33.3	W	N
70	34	95.8	W	P
80	40	17.5	E	N
85	40	13.6	E	N
85	44	16	W	N
90	40	10.3	W	N
105	50	16.9	W	N
110	50	96.7	W	P
110	55	24.4	W	N
130	60	13.6	W	N
160	80	140	W	P
170	90	12.7	E	N
250	120	19.2	E	N

Table 3. *Leptospirosis hardjo* prevalence of 47 dairy herds (autumn samples) in two regions.

	Herd		Total
	West	East	
Positive (%)	5 (13.5)	5 (33.3)	10
Negative (%)	32 (86.5)	10 (66.7)	42
Total	37	15	

correlation between herd size and antibody levels (r = 0.708, $P<0.05$) (Figure 1). There was no significant difference in mean PP among herds in the west and east region ($P>0.05$). There was also no significant difference in PP in each herd at four seasons ($P>0.05$).

DISCUSSION

Mashhad is a mountainous city and is one of major livestock husbandry centers in Iran. Previous studies in Iran have shown the prevalence of different leptospiral serotypes. MAT was the only test that had been used for serological survey of leptospiral infection in Iranian dairy herds and in these studies the lowest prevalence was for hardjo serovar.

In a study that was performed on 400 bovine serum samples from dairy farms of Shahrekord district, central Iran by using MAT, the lowest prevalence was for hardjo serovar (17%) (Ebrahimi et al., 2004). In another study that was performed in Mashhad area on the 389 cow sera from 18 dairy herds by using MAT, ninety-three cow

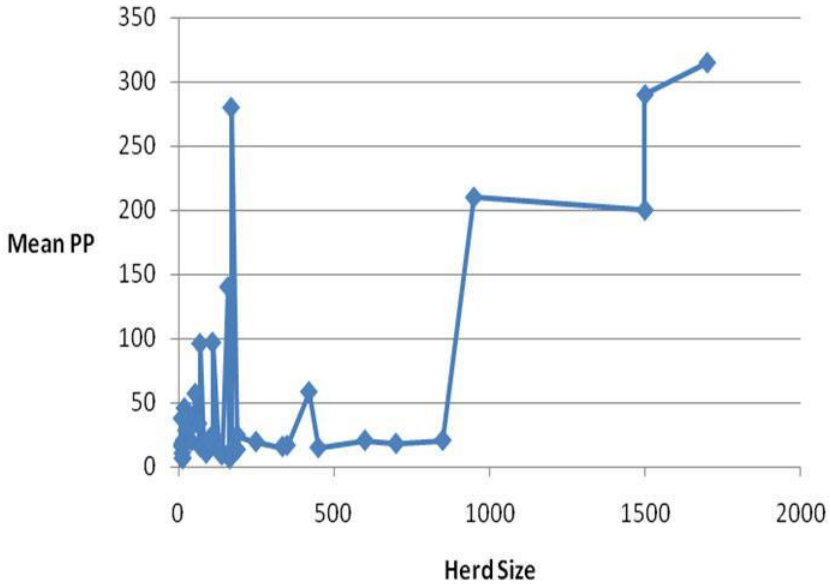

Figure 1. Correlation between mean percentage positivity (PP) and herd size.

sera showed positive serological reaction against leptospiral antigens. Twenty four samples (18.9%) showed positive reaction to hardjo antigens which it was at the third place after grippotyphosa (25.98%) and icterohaemorrhagie (25.19%) (Garoussi et al., 2003). Microscopic agglutination test is the most widely used test (on serum) for *L. hardjo* and is adopted for international trade purposes. With hardjo serovar, a significant percentage of cattle that are actively infected and shedding leptospires have anti-hardjo antibody titers

100 and they consider as negative to hardjo infection (Ellis, 1986). Therefore, a low antibody titer detected by MAT does not necessarily rule out a diagnosis of leptospirosis. The ELISA measures IgG which does not appear until three or four weeks after infection and persists for about two years. In the other hand, MAT measures mainly IgM which titres peak after 10 to 20 days but decline within 6 to 12 months so MAT just demonstrates recent infection (Pritchard, 2001). ELISA is therefore a better guide to longer term status than MAT.

Since MAT cannot efficiently diagnose infection with hardjo serovar and all the previous studies in Iran was done by using MAT so, it might be that the infection by hardjo serovar in dairy herds of Iran is underestimated.

There have been surveys in larger scale in comparison to this study in Ireland, France and Germany by using ELISA on bulk milk samples that showed existence of infection in these countries (Leonard et al., 2004; Wiseman et al., 2002) but shortage of these studies is that the interpretation is done on a single bulk milk result.

A single bulk milk result can only provide an approximate guide. For monitoring purposes before making major decisions, bulk milk should be retested at least every three months.

In our study, nine herds (19.15%) had PP>60 and considered as positive to *L. hardjo* infection.

There was a positive correlation between herd size and mean PP. The fact that fewer small herds were positive for anti-Leptospira antibody in milk is not surprising. Exposure to infection is more likely to occur in animals in large herds as infection can be transmitted more easily and persist for longer periods in larger intensive herds (Agaev, 1992; Thevenon et al., 1990). Positive correlation between herd size and presence of positive been reported previously for hardjo infection in cattle and for Bratislava infection in sow herds (Ellis, 1994; Lilenbaum and Santos, 1996; Mousing et al., 1995). In contrast, a significant association between herd size and hardjo infection was not detected in a survey of dairy and beef herds in Spain (Alonso-Andicoberry et al., 2001). A positive result indicates exposure of animals in the herd to infection, but there are no published data available at present, which allow correlation of antibody level with the probability of active versus chronic infection in the herd.

Statistical analysis did not show a significant difference in mean PP for each herd among four seasons. Because cattle are a maintenance host for hardjo serovar and ELISA test that had been used in this study, measures IgG which persists for about two years after infection so we expected to see persistent IgG in infected herds among four seasons. We suppose that there is a chronic infection but existence of active infection cannot be rejected because there could be a sporadic occurrence of disease that did not change overall quantity of IgG in bulk tank milk. Cattle are a maintenance host for hardjo serovar so it is expected an endemic transmission and a tendency to cause chronic rather than acute disease and also a low antibody response to infection (Radostits,

2007). The low antibody response to infection may be the reason that no significant difference in mean PP among seasons was observed. There was also no significant difference in PP between east and west of Mashhad. It was expected that there would not be significant impact of environmental risk factors between these regions. We consider that herd management is the most important factor that causes different levels of antibody among herds. It was observed that herds with better management practices like good biosecurity had lower levels of antibody but a detailed epidemiological study would be required to determine the factors affecting herd prevalence in Mashhad. This is the first study that was done in Iran for monitoring the prevalence of hardjo serovar in dairy herds by using ELISA.

In conclusion, the findings of the present study showed that the prevalence of *L. hardjo* infection in cattle herds in Mashhad area with a significant positive correlation between herd size and mean PP. These results emphasize on the importance of prevention and challenge against *L. hardjo* infection in larger dairy herds.

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