Original Article

Seroprevalence and risk factors of *Leptospira* serovar Pomona and *Leptospira* serovar Hardjo infection in dairy cows in Jordan

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Abstract
Introduction: This study was carried out to determine the seroprevalence and risk factors associated with *Leptospira* serovars Pomona and Hardjo infection in dairy cows.
Methodology: Seroprevalence was determined using ELISA using 160 healthy and 80 recently aborted cows. Risk factor assessment was carried out using a pre-validated questionnaire.
Results: The true farm seroprevalence of *Leptospira* serovars Pomona and Hardjo was 92.3% (95% CI: 66%-98%). In healthy cows, the true and apparent cow seroprevalence of *Leptospira* serovars Pomona and Hardjo were 26.9 (95% CI: 20–34%), 26.25% (95% CI: 20–33%) and 28.75% (95% CI: 22–36%) and 27.5% (95% CI: 21–35%), respectively. Semi-intensive management system (OR = 11.43; P < 0.01), surface water as a source of drinking water (OR = 1.21; P < 0.03), lack of special wear for visitors (OR = 1.39; P < 0.05), and previous history of abortion (OR = 1.02; P < 0.05) were associated with high rate of seropositivity against *Leptospira* serovars Pomona and Hardjo. In recently aborted cows, the true and apparent seroprevalence rates of *Leptospira* serovars Pomona and Hardjo were 53.25% (95% CI: 47.5–62%), 53.75% (95% CI: 48.5–63.2%) and 56% (95% CI: 49–61%), 56.25% (95% CI: 49.8–61.2%), respectively.
Conclusions: Leptospirosis is an endemic disease in Jordan and further studies are required to effectively control the disease in dairy cows.

Key words: Leptospirosis; dairy cows; abortion; public health; biosecurity.

Introduction

Leptospirosis affects a wide range of hosts including cattle, sheep, goats and wild animals [1]. The disease has been reported in many parts of the world with variable prevalence rates ranging from 3% to 50% [2,3]. It results in significant negative impact on health and production of livestock due to the high cost of treatment and prevention programs, loss of milk production, abortion, repeated breeding, stillbirth, and retained placenta [4,5]. Clinically, affected animals may be asymptomatic carriers; however, some animals may show fulminant disease characterized by fever, jaundice, pink stained milk, hemoglobinuria, and death [4,5].

The disease is considered an important occupational zoonotic disease [6]. In severe forms, clinical signs are related to multi-organ dysfunction involving the cardiopulmonary, hepatic, and renal systems (Weil syndrome) [7]. The worldwide estimated annual mortality rate due to leptospirosis is around 0.84 deaths per 100,000 people [8].

The most commonly reported risk factors of leptospirosis in dairy herds are age, large herd size, co-grazing with other infected animals, contaminated water sources, use of natural breeding, inadequate husbandry practices, purchase of replacement heifers from infected herds, access of dogs and cats to pastures, contact of rodents with animal feed, presence of calf rearing facility on the farm with close contact with adult cows, and dirty drenching equipment [9-12].

In Jordan, the prevalence and associated risk factors of leptospirosis in apparently healthy cows and in recently aborted cows have not been reported before. Therefore, the objectives of this study were to determine the seroprevalence rate of leptospirosis caused by *Leptospira* serovar Pomona and serovar Hardjo among apparently healthy dairy cows and cows with recent history of abortion in Northern Jordan and

to determine possible risk factors that might be associated with seropositivity against *Leptospira* spp. in dairy cows.

Methodology

Ethical approvals

All procedures performed in this study were approved by the Jordan University of Science and Technology Animal Use and Care Committee (JUST-ACUC approval number 16.3.3.147).

Study area

This study was conducted in dairy herds in Al-Dulail region of Jordan. Al-Dulail region is located in the North East of Jordan. More than 55% of national dairy milk is produced in this area [13]. Dairy farms in this region are managed mainly using the intensive production system, but semi-intensive systems are also practiced. The average annual rainfall in Al-Dulail region is less than 200 mm.

Sample size calculation, inclusion and exclusion criteria

This study targeted small to medium sized dairy herds located in Al-Dulail region in Jordan. Small to medium dairy herds were defined as all dairy herds located in the targeted region with a total of 25 to 500 lactating dairy cows. The total number of farms that fitted this definition in Al-Dulail region was 34 with a total number of lactating animals of 6700 cows [14]. Only farms with no history of vaccination against *Leptospira spp.* within the last 5 years were selected for the study. According to this criteria, the sample size for an infinite population would have been 380 cows assuming a confidence interval of 95%, and confidence level of 5 (https://www.surveysystem.com/sscalc.htm). However, the number of dairy farms that did not use *Leptospira spp.*-containing vaccines were only 13 farms (around 4000 cows). Then the sample size was recalculated to adjust for the actual population of 4000 cows with a confidence interval of 95%, and confidence level of 7.5. Therefore, the actual sample size was 160 cows.

Sample collection

All cows were subjected to a complete physical examination and only apparently healthy cows were enrolled in the study. Approximately, 10 mL of whole blood was collected aseptically via Jugular venipuncture using vacutainer needle and placed in plain blood tubes and allowed to clot in room temperature. Serum was then obtained by centrifugation of clotted samples at 3000g for 10 minutes. Obtained serum samples were stored at -20 ºC until laboratory analysis was performed.

Whole blood samples were also collected from 80 cows that were presented to the Veterinary Health Center at Jordan University of Science and Technology for evaluation of the reproductive system after a recent incident of abortion. Cows belonged to several farms that were located in Northern Jordan. To be included in the study, the cow must have had an incident of abortion within the last 3 to 4 weeks prior to the presentation and must be found healthy otherwise. Therefore, cows were subjected to a thorough physical examination before blood samples were collected.

Questionnaire

A pre-tested and pre-validated questionnaire was administered in the enrolled farms by personal interview at the time of blood sampling. Data that were collected included herd size, management system, feeding management, semen source, history of abortion, repeat breeders, cleaning and disinfection practices, vaccination protocols, deworming protocols, presence or access of rats and other domestic and wild animals to the farm, water source, and availability of veterinary services.

Laboratory analysis

In apparently healthy cows, samples were analyzed to determine serum concentrations of immunoglobulin G (IgG) against *Leptospira* serovar Pomona and serovar Hardjo using commercially available quantitative sandwich IgG-specific ELISA kit according to manufacturer's recommendations (Sincere Biotech, Beijing, China).

For aborted cows, samples were analyzed to determine serum concentrations of immunoglobulin M (IgM) against *Leptospira* serovar Pomona and serovar Hardjo using commercially available quantitative sandwich IgM-specific ELISA kit according to manufacturer's recommendations (Sincere Biotech, Beijing, China).

Positive and negative sera controls were provided with the ELISA kit and 3 replicates of each control were used per plate. The minimum detection limits for IgM and IgG of this ELISA kit were 61.5 pg/mL and 27.5 pg/mL, respectively. The minimum limits were used to classify the results as positive and negative. The ELISA plate was read at 450 nm wave length using a microplate reader (Thomas Scientific, New Jersey, USA).

Table 1. Seroprevalence and serum IgG concentrations against infection caused by *Leptospira* serovar Pomona and serovar Hardjo in apparently healthy dairy cows (N = 160).

Parameters	*L.* Pomona		*L.* Hardjo	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Farm level prevalence	12 (92.3)	1 (7.7)	12 (92.3)	1 (7.7)
Cow level prevalence	43 (26.9)	117 (73.1)	46 (28.75)	114 (71.25)
IgG (pg/mL)	1156 ± 305	Undetectable	1504 ± 306	Undetectable
IgG range (pg/mL)	206-20400	Undetectable	395-20700	Undetectable
Parity	2.7 ± 1.3	2.7 ± 1.3	2.7 ± 1.3	2.7 ± 1.3
Pregnant animals	30 (69.8)	13 (30.2)	30 (65.2)	16 (34.8)
History of abortion	25 (58)	18 (42)	24 (52.2)	22 (47.8)

Table 2. Seroprevalence of infection caused by *Leptospira* serovar Pomona and serovar Hardjo in apparently healthy dairy cows in Jordan.

Risk factors	N	*L.* Pomona		*L.* Hardjo		*L.* Pomona & *L.* Hardjo	
		Positive	%	Positive	%	Positive	%
Management system							
Intensive	85	13	15	19	22	7	9
Semi-intensive	75	30	40*	27	36*	19	25*
Water source							
Underground wells	133	7	5	1	0.7	1	0.7
Municipality water	15	1	7	3	20	2	13
Rivers or streams as water source	12	2	17*	4	33*	2	17*
Farm-owned dogs							
Yes	107	29	27	28	26	18	17
No	53	14	26	18	34	8	15
Dog vaccination							
Yes	52	19	37	16	31	11	21
No	108	24	22	30	28	15	14
Presence of ticks							
Yes	22	4	18	5	23	3	14
No	138	39	28	41	30	23	17
Special wear for visitors							
Yes	36	10	27	8	22	6	17
No	124	57	46*	38	39*	20	16
Pregnancy							
Yes	104	30	29	30	29	18	17
No	56	13	23	16	29	8	14
Semen source							
Public veterinary services	36	13	36	16	44	6	17
Private sources	124	30	24	38	31	20	16
History of abortion on the farm							
Yes	49	25	32*	24	30	15	19
No	40	18	22	22	27	11	14
Parity							
1	16	8	24	8	24	6	18
2	33	16	33	17	35	10	20
3	12	5	15	7	21	3	9
4	15	7	35	8	40	4	20
5	13	7	29	6	25	3	13

* indicate significant difference.

According to the manufacturer, there are no significant cross-reactivity or interference between the bovine *Leptospira* serovar Pomona and serovar Hardjo IgM and IgG-specific ELISA and any soluble receptors, binding proteins, or any other cytokines that may present in biological samples including other *Leptospira* spp. Based on personal communications with the ELISA kit manufacturer, they indicated that the sensitivity and specificity of both kits were 100% and 96%, respectively. Intra-assay and inter-assay CVs were less than 10 and 15, respectively. Correlation coefficient (R) of linear regression of the tested samples was more than 0.92.

Statistical analysis

The data is presented as means ±SD. Observed and true prevalence data after adjusting for ELISA kits sensitivity and specificity were reported. Initially, data were analyzed using univariate analysis (Chi-Square). Significant associations between risk factors and leptospirosis status were then determined using multivariable logistic regression analysis. P value < 0.05 was considered statistically significant. Analysis was performed using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, USA).

Results

Apparently healthy dairy cows

Table 1 shows the seroprevalence and serum IgG concentrations against infection caused by *Leptospira* serovar Pomona and serovar Hardjo in apparently healthy dairy cows. A total number of 160 cows were tested. The average parity of cows involved in the study was 2.7 ± 1.3. The number and percentages of pregnant animals that were positive for *Leptospira* serovar Pomona and serovar Hardjo were 30 (69.8%) and 30 (65.2%), respectively. The number and percentages of cows with previous history of abortion that were tested positive for *Leptospira* serovar Pomona and serovar Hardjo were 25 (58%) and 24 (52.2%), respectively.

The true farm prevalence rate of infection caused by *Leptospira* serovar Pomona and serovar Hardjo was 92.3% (95% CI: 66%-98%). The true and apparent individual cow prevalence rates of infection caused by *Leptospira* serovar Pomona and serovar Hardjo were 26.9 (95% CI: 20–34%) and 26.25% (95% CI: 20–33%) and 28.75% (95% CI: 22–36%) and 27.5% (95% CI: 21–35%), respectively. The true and apparent individual cow prevalence rate of combined infection with *Leptospira* serovar Pomona and serovar Hardjo were 26.25% (95% CI: 18.3–31.0%) and 25% (95% CI: 21–36%) (data not shown in table). The mean ± SD and ranges of IgG serum concentrations against infection caused by *Leptospira* serovar Pomona and serovar Hardjo were 1156 ± 305 pg/mL (206-20400 pg/mL) and 1504 ± 306 pg/mL (395-20700 pg/mL), respectively.

Univariate analysis identified farm semi-intensive management system, water source, availability of special wear for visitors, and history of abortion as risk factors associated with *Leptospira* serovar Pomona and serovar Hardjo infections (Table 2). Table 3 shows the multivariate analysis of risk factors associated with *Leptospira* serovar Pomona and serovar Hardjo in apparently healthy dairy cows. The analysis revealed that cows raised in semi-intensive management system were significantly at higher risk of becoming seropositive to the infection (OR = 11.43; P < 0.01). Farms that used rivers or stream water as a source of drinking water were at significantly higher risk of becoming seropositive to the infection (OR = 1.21; P <

Table 3. Multivariate analysis of risk factors associated with infection caused by *Leptospira* serovar Pomona and serovar Hardjo in apparently healthy dairy cows in Jordan.

Risk factors	Standard error	Odds ratio	P value	95% confidence interval	
				Lower	Upper
Semi-intensive management system	0.63	11.43	0.01	2.44	28.58
Rivers or streams as water source	1.19	1.21	0.03	0.22	11.75
Farm-owned dog	0.66	0.75	0.37	0.15	2.06
Dog vaccination	0.12	0.25	0.61	0.20	5.69
Presence of ticks	0.95	1.24	0.26	0.45	18.63
No special wear for visitors	0.90	1.39	0.05	0.45	19.56
Pregnancy	0.41	0.02	0.88	0.47	2.40
Semen source	0.73	0.57	0.33	2.02	35.12
History of abortion	0.40	1.02	0.05	0.43	12.04
Parity 1	0.70	0.001	0.97	0.25	3.79
Parity 2	0.71	0.04	0.84	0.29	4.57
Parity 3	0.62	0.361	0.43	0.14	1.64
Parity 4	0.75	0.92	0.33	0.11	2.11

0.03). Farms that did not provide special wear for visitors were at significantly increased risk of becoming seropositive to the infection (OR = 1.39; P < 0.05). Farms with previous history of abortion were at significantly higher risk of becoming seropositive to the infection (OR = 1.02; P < 0.05).

Cows with history of abortion

Seroprevalence and serum IgM concentrations against infection caused by *Leptospira* serovar Pomona and serovar Hardjo in recently aborted dairy cows are shown in Table 4. A total number of 80 recently aborted dairy cows were tested. All cows appeared healthy on presentation. The average days since abortion incident was 22 (range 13 to 35 days). The average parity of cows involved in the study was 2.3 ± 1.2. The average DIM of cows that were tested positive for *Leptospira* serovar Pomona and serovar Hardjo infection were 220 ± 60 and 223 ± 56, respectively. The average body condition score of cows that were tested positive for *Leptospira* serovar Pomona and serovar Hardjo infection were 2.9 ± 0.30.

The true and apparent seroprevalence rates of *Leptospira* serovar Pomona and serovar Hardjo infection in recently aborted cows were 53.25% (95% CI: 47.5–62%), 53.75% (95% CI: 48.5–63.2%) and 56% (95% CI: 49–61%), 56.25% (95% CI: 49.8–61.2%), respectively. The true and apparent seroprevalence rate of combined *Leptospira* serovar Pomona and serovar Hardjo infection in recently aborted dairy cows was 15.75% (95% CI: 8.5-22%) and 16% (95% CI: 9-21%), respectively (data not shown in table). The mean $\pm$ SD and range of serum IgM concentrations in cows that were tested positive for *Leptospira* serovar Pomona and serovar Hardjo infection were 289 ± 125 pg/mL (138-630 pg/mL) and 737 ± 490 pg/mL (340-2300 pg/mL), respectively.

Univariate analysis identified days in milk (DIM) 151-200 and 251-300 as associated with a significantly (P < 0.05) higher risk of abortion associated with seropositivity to *Leptospira* serovar Pomona infection.

Discussion

ELISA test is commonly used laboratory method for initial screening of leptospirosis [15]. It is characterized by high sensitivity and specificity compared to the microscopic agglutination test (MAT), the gold standard technique [15]. Unlike MAT, ELISA can differentiate between individual immunoglobulin class and therefore can be used to detect infections in early stages as well as older infections. It has been reported that IgM can be detected as early as 4 to 5 days and persists up to 6 months after the onset of *Leptospira* infection in cattle, long before immunoglobulin G and agglutinating antibodies can be detected [15]. Therefore, in this study, IgM- and IgG-specific ELISA kits were used to determine the seroprevalence of antibodies against *Leptospira* serovar Pomona and serovar Hardjo infection in recently aborted cows (acute infection) and in apparently healthy cows (convalescent), respectively.

This is the first study to report the seroprevalence and risk factors of *Leptospira* serovar Pomona and serovar Hardjo infection in dairy cows in Jordan. *Leptospira* serovar Pomona and serovar Hardjo are considered among the most prevalent *Leptospira spp*. in cattle populations in the world and therefore, these 2 species were targeted in this study [16]. The farm prevalence rate of *Leptospira* serovar Pomona and serovar Hardjo infection was 92.3% and the individual-cow prevalence rates were 26.9% and 28.75%, respectively. Furthermore, the prevalence of combined infection with *Leptospira* serovar Pomona and serovar Hardjo infection in apparently healthy cows in this study was 26.25%. These results are in congruence with previous observations that indicate that the majority of cows become infected with one serovar, however infection with multiple serovars is also possible [17]. Since, vaccination against *Leptospira spp*. in the sampled herds involved in this study has never been practiced, therefore, the high infection prevalence rates reported here indicate most likely natural infection. Although the manufacturer of the ELISA kits used here claim there are no cross reactivity between different

Table 4. Seroprevalence and serum IgM concentrations against infection caused by *Leptospira* serovar Pomona and serovar Hardjo in recently aborted dairy cows (N = 80).

Parameters	*L.* Pomona		*L.* Hardjo	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Prevalence	43 (53.75)	37 (46.25)	45 (56.25)	35 (43.75)
IgG (pg/mL)	289 ± 125	Undetectable	737 ± 490	Undetectable
IgG range (pg/mL)	138-630	Undetectable	340-2300	Undetectable
Parity	2.3 ± 1.2	2.4 ± 1.2	2.3 ± 1.2	2.3 ± 1.2
Days in milk (DIM)	220 ± 60	210 ± 50	223 ± 55	200 ± 56
Body condition score (BCS)	2.9 ± 0.30	2.8 ± 0.3	2.9 ± 0.30	2.7 ± 0.30

Leptospira spp., the high percentage of combined infection also could indicate that a high proportion of detected antibodies reacted with both Pomona and Hardjo antigens in ELISA kits.

The disease has been reported in many parts of the world with variable prevalence rates. For example, in a large survey of slaughtered cattle conducted in the Unites States, the prevalence rates were 22% and 15% for *Leptospira* serovar Pomona and serovar Hardjo infection, respectively [18]. In Brazil, the herd-level and cow-level prevalence rates were 100% and 42.86%, respectively [19]. This great variation in the prevalence rates of leptospirosis over the world is most likely due to variation in geographical location, management systems, husbandry practices, different breeds of animals, levels of natural immunity and disease resistance among studied populations [20].

In this study, univariate analysis indicated that cows raised in semi-intensive systems were almost 11 times more likely to become seropositive to the disease than intensively managed cows. This could be attributed to poor husbandry practices and to the fact that infected animals in overcrowded and confined conditions increase the risk of contaminating the environment [21].

In this study, cows raised in farms that used surface water for drinking such as rivers or streams were also at higher risk to become seropositive to the disease than cows that received water from wells or tap water. These results are in complete agreement with previously reported data that implicate communal water sources that are shared between different animal species as an important source of infection [22]. Furthermore, *Leptospira* spp. has been reported to survive in in the soil and bedding in high moisture surfaces for up to 180 days [23].

In this study, the lack of special wear for visitors was associated with higher prevalence of leptospirosis in dairy farms. This goes along with poor hygiene and sanitation practices in some dairy farms with overcrowded populations [20].

Previous history of abortion on the farm was also associated with high risk of seropositivity against leptospirosis. This can be viewed as an evidence of the widespread of *Leptospira* spp. as a cause of bovine abortion in the studied population which was further substantiated by finding high prevalence of IgM seropositivity in recently aborted cows.

This is the first study to report the seroprevalence and risk factors of *Leptospira* serovar Pomona and serovar Hardjo infection in recently aborted dairy cows in Jordan. The prevalence rates of *Leptospira* serovar Pomona and serovar Hardjo infection in recently aborted cows were 53.75% and 56.25%, respectively. These results are similar to previous reports that indicated that 57.7% of cows with history of abortion and reproductive disorders were found seropositive against *Leptospira* spp. [24]. In other parts of the world, approximately 10% of bovine abortions were due to one or more *Leptospira* serovar infection in the United states, 6% in Canada, 50% in Northern Ireland and 63.33% in Pakistan [25,26].

Previous work has identified dogs as a major risk factor for epizootic *Leptospira* abortions in dairy farms [27]. It has been reported that cattle exposed to dogs and other wild animals which act as reservoir for *Leptospira* spp. and warm, moist environment have an increased incidence of *Leptospira* infection [26,27]. In this study, univariate analysis identified days in milk (DIM) as a potential risk factor for abortion caused by *Leptospira* serovar Pomona and serovar Hardjo infection.

Conclusions

Results of this study showed that leptospirosis is widespread in Jordan in apparently healthy and in recently aborted dairy cows. Indeed, these results indicate the dire need to take this disease in consideration in the national animal disease control programs that limit stray dogs and wild carnivores contact with cows and their feed and water sources, and limit exposure of susceptible cows to other reservoirs and contaminated environments. Vaccination using multivalent vaccines is indicated here because little or no cross-protection between *Leptospira* serovars has been reported.

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Conflict of interests: No conflict of interests is declared.