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Surveillance of Bovine Leptospirosis: Isolation and Serodiagnosis

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Abstract: The aim of the present study was the isolation and serodiagnosis of leptospiral strains form 625 cows and 200 rats surrounding bovine farms. Twelve leptospiral serovares were used in the Microscopic Agglutination Test (MAT) for the detection of leptospiral agglutinins in the sera of the examined cows and rats. Isolation study identified 7 isolates from cows and 9 isolates from rats and serotyped as *L. interrogans* Icterohaemorrhagiae and *L.interrogans* Pomona. The most predominant serovars were aganist *L. interrogans* Pomona, *L. interrogans* Icterohaemorrhagiae, *L. interrogans* Pyrogenes, *L. interrogans* Hardjo, *L. kirschneri* Grippotyphosa and *L. weilii* Celledoni. This information obtained from such surveillance is helpful to determine the strategy of leptospirosis prevention and control in humans and animals in Egypt.

Key words: Leptospirosis · Bovine · Isolation · Serodiagnosis

INTRODUCTION

Leptospirosis is a zoonotic disease of global importance that is caused by pathogenic spirochete bacteria of the genus *Leptospira*. It is now recognized as an emerging infectious disease [1, 2]. It was estimated that more than 500,000 cases of severe leptospirosis are reported each year, with case fatality rates exceeding 10% [3]. Leptospirosis occurs particularly in tropical and subtropical region, where environmental conditions favor the survival and transmission of leptospires [4]. Beside the economic losses caused by this bacterium to animal production, its zoonotic character makes it an important public health hazard [5, 6].

The economic importance of bovine leptospirosis is the result of both direct and indirect costs including costs of abortion, loss of milk production and related veterinary costs and human infection. Moreover, even in absence of disease, the ability of domestic livestock to act as reservoir for pathogenic leptospira represents a significant health risk to a wide range of workers, veterinarians and slaughterhouse workers [7]. The pathology of the milk drop syndrome in leptospiral infection has not been studied, but appears to be associated with the bacteraemic phase and an increase in leucocytes in milk [8, 9].

Domestic animals in contact with reservoir rodents or the contaminated urine of infected animals may acquire the infection, which may be maintained as an enzootic disease within the herd. The rate of transmission between mammals by indirect contact largely depends upon those environmental conditions that favor the survival of leptospires [10].

The Microscopic Agglutination Test (MAT) is considered as the reference test among the several serological methods for leptospirosis diagnosis [6]. Although the isolation of the pathogen allows definitive diagnosis and provides epidemiological studies of this disease, isolation of leptospira from tissues and fluids of animals is difficult and depends upon the availability of culture media [11-13]. They stated that the main problem in culturing leptospires is contamination with other microorganisms, especially when attempting to culture from non-sterile sources such as urine and fetal tissues. Thiermann [12] concluded that the most important factors for isolation of leptospira are aseptically collected material, quick processing, culture medium suitability and selective antibiotics.

The aim of the current investigation was to do a serosurvey on bovine leptospirosis to stand on the most prevalent serovar (s) affecting bovine species as well as trying to isolate the pathogen from cattle and rodents

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trapped from the region surrounding the investigated cattle farms. This information will guide us to determine the strategy of leptospirosis prevention and control in humans and animals in Egypt.

MATERIALS AND METHODS

***Leptospira* Serovars Used for Detection of Antibodies:** In the present study, twelve leptospiral strains were used in the MAT for the detection of leptospiral agglutinins in the sera of the examined cattle and rats (Table 1). These strains were kindly obtained from the Bacterial Culture Collection of the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Table 1: *Leptospira* serovars used for detection of antibodies by MAT

Serial	Genus	Species	Serovars	Strains
1	*Leptospira*	*interrogans*	Canicola	Ruebush
2		*interrogans*	Icterohaemorrhagiae	RGA
3		*kirschneri*	Grippotyphosa	Moskva V
4		*interrogans*	Pomona	Pomona
5		*interrogans*	Pyrogenes	Salinen
6		*borgpetersenii*	Ballum	Mus 127
7		*interrogans*	Bataviae	Van Tienen
8		*interrogans*	Wolffi	3705
9		*interrogans*	Australis	Ballico
10		*interrogans*	Hardjo	Hardjo prajitno
11		*weilii*	Celledoni	
12		*biflexa*	Patoc 1	

Table 2: No. of different sources and sites from which specimens have been collected

Source of samples	Number	Body sites
Rats	200	Blood, Kidneys, Urine
Cows	625	Blood, Urine, Milk, Uterine discharges
Total	825	

Animals: A total of 200 rats were captured from inside and surrounding the dairy farms using traps. They were used to isolate and identify leptospiral serovars. Meanwhile, a total of 625 cows from different dairy farms and from individual animals in scattered areas were also used to isolate and identify leptospiral serovars (Table 2). A total 509 cows were mastitic and the remainders 116 suffered abortion.

Isolation of Leptospiral Strains: One drop from each blood, milk and/or urine samples was inoculated into 2-3 tubes containing EMJH broth medium containing 5-fluorouracil (5-FU 200 µg/ml) as selective agent to minimize contamination. This agent can inhibit contaminating bacteria, while leptospires can withstand the used concentration. Collected organs (rat kidneys) were macerated by passage through a 3ml syringe without a needle and inoculated in the same media. All cultures were incubated at 30°C and examined weekly for up to 6 weeks using dark field microscope. All leptospiral strains were purified and maintained on Fletcher's medium [14], while continuous sub culturing and isolation of leptospiral strains was done using serum free EMJH semi-solid medium (BD, USA), according to Ellinghausen and McCullough [15] and modified by Johnson and Harris [16]. All media were sterilized by autoclaving for 20 minutes at 121°C and allowed to cool at room temperature.

Microscopic Agglutination Test (MAT): MAT was applied on the collected sera from cattle and rats to detect serologically reactive cases. On the other hand, MAT was used for serotyping of positive cultures. MAT is based on the old agglutination-lysis test which is modified by Cole *et al*. [17].

Antigen Preparation: The antigens for the MAT were live cell suspensions prepared from 4 to 7 days old broth cultures of the previously mentioned strains. The turbidity was adjusted to match 0.5 McFarland equivalents. Any leptospiral strains showing microscopic auto-agglutinating clumps in the absence of serum were excluded.

Serum Preparation: Serum specimens were thawed in a 37°C water bath; then vortexed gently and left to cool at room temperature (25°C ± 2°C) before use. For screening, Phosphate buffered saline (PBS) was used to dilute serum samples starting with 1:100 and then incubated for complement inactivation at 56°C water bath for 30 min. Then two folds serial dilutions were applied for antibody titration.

Screening Assay: Live leptospira cell cultures representing the tewelve serovars were added to the diluted serum specimens (1:100) in 96-well flat-bottomed microtiter plates. Samples were examined for agglutination by dark-field microscope at a total magnification of 100 X. The test was considered negative when free motile leptospires with no clumps were observed, while positive result was recorded if at least 50% of the screened leptospires were agglutinated.

Titration Assay: Positive sera in the screening assay were serially diluted and titrated (two fold serial dilutions). A reactive MAT was determined by titers = 1:200 for diagnosis) [18], while reported end point titers were calculated as the reciprocal of highest serum dilutions that agglutinated at least 50% of the cells for each serovar used.

Table 3: Isolated and identified *Leptospira* serotypes

			Serovars (s)	
Animal species	Number	No. of positives (%)	L. Icterohaemorrhagiae	*L.* Pomona
Rats	200	9(4.5)	5	4
Cows	625	7(1.1)	2	5
total	825	16(1.9%)		

Table 4: Percentage of seropositive samples against each serovar in cows and rats

Leptospiral serovars	Cows No. (%)	Leptospiral serovars	Rats No. (%)
Pomona	120(51.1)	Icterohaemorrhagiae	96(56.5)
Icterohaemorrhagiae	110(46.8)	Pomona	70(41.2)
Pyrogenes	3(1.2)	Celledoni	3(1.8)
Hardjo	2(0.9)	Grippotyphosa	1(0.5)
Total	235(100)	Total	170(100)

Table 5: Titration against predominant serovars in cows and rats

	Cows				Rats			
Titers	Icterohaemorrhagiae	Pomona	Hardjo	Pyrogenes	Icterohaemorrhagiae	Pomona	Grippotyphosa	Celledoni
1:400				3		2		
1:800			2		4		6	1
1:1600	10				13			
1:3200	23	10			20	10		
1:6400	77	85			15	42		3
1:12800		25			44	10		
Total	110	120	2	3	96	62	1	3

RESULTS

A total of 9 isolates were obtained from rat samples and serotyped using MAT. Five isolates were belonged to *L. interrogans* Icterohaemorrhagiae and 4 were belonged to *L. interrogans* Pomona. Meanwhile, a total of 7 isolates were obtained from cow samples. Two isolates were belonged to *L. interrogans* Icterohaemorrhagiae and 5 belonged to *L. interrogans* Pomona (Table 3).

Table (4) showed the highest percentage of seropositive samples against *L. interrogans* Pomona followed by *L. interrogans* Icterohaemorrhagiae then *L. interrogans* Pyrogenes and the lowest one was against *L. interrogans* Hardjo in case of cow samples. While in rat samples the highest percentage of seropositive samples was against *L. interrogans* Icterohaemorrhagiae followed by *L. interrogans* Pomona then *L. weilii* Celledoni and the lowest one was against *L. kirschneri* Grippotyphosa. The results of titeration against predominant serovars in cows and rats were presented (Table 5). The results showed that the highest titer of seropositive detected against serovar *L. interrogans* Icterohaemorrhagiae was 1:6400 while in case of *L. interrogans* Pomona was 1:12800, *L. interrogans* Hardjo was 1:800 and *L. interrogans* Pyrogenes was 1:400 in case of cow samples while in case of rat samples. The highest titer of seropositive detected against serovar *L. interrogans* Icterohaemorrhagiae and *L. interrogans* Pomona was 1:12800, while in case of *L. kirschneri* Grippotyphosa was 1:800 and *L. weilii* Celledoni was 1:6400.

DISCUSSION

Pathogenic leptospires are responsible for a world wide zoonosis, leptospirosis, in which humans are occasional hosts in a cycle involving wild and domestic animals. The animal reserve includes mostly rodents; they excrete leptospires in their urine and thus contaminate hydric environment and transmit the disease to other animals or to humans [19, 20].

In the present study the results of isolation (Table 3) from rats captured from inside or surrounding dairy farms showed nine positive isolates from which 5 were *L. interrogans* Icterohaemorrhagiae and 4 were *L. interrogans* Pomona when serotyped by MAT using standard antisera, while in case of cows 7 isolates have been recovered, 2 of them were *L. interrogans* Icterohaemorrhagiae and 5 were *L. interrogans* Pomona. The obtained culture results in case of rats were similar to that obtained in the Azorean islands by Colleras-Pereira *et al*., [21] who isolated *L. interrogans* Icterohaemorrhagiae from house mouse and black rat in rates of 88% and 33%, respectively. Also in Egypt, Samir [22] isolated *L. interrogans* Icterohaemorrhagiae from black rats. Moreover, Stritof Majetic *et al.* [23] in Eastern Croatia obtained 20 isolates from which 10 (50%) belonged to serogroup Pomona.

In case of culture results from cows, the obtained results agreed with that recorded in Brazil by Miraglia *et al.* [24] who characterized the isolates originated from bovine by MAT with polyclonal and monoclonal antibodies and were identified as *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni, also similar to that obtained in Argentina by Draghi *et al.* [25] who isolated a strain of *L. interrogans* Pomona from samples collected from dead calves.

Results of the present serological study on cow sera in tables (4 and 5) showed seropositivity against *L. interrogans* Pomona at a percentage of 51.1%. This serovar is a well recognized cause of abortion in cattle. Studies have already highlighted its role in pregnancy loss [26]. The titers ranged from 1:3,200 to 1:12,800. The obtained results were higher than that reported by Van De Weyer *et al.* [27] who detected antibodies against *L. interrogans* Pomona in percentages of 9.6% and 5.5% in spring and fall season, respectively.

Also the results in tables (4 and 5) showed that seropositivity against *L. interrogans* Icterohaemorrhagiae was 46.8% from total positive reactors at titers ranged between 1:1,600, 1:6,400 which are higher than that obtained by Suepaul *et al.* [28] who determined it in 9.3% of the examined samples also results revealed that 1.2% of positive reactors was against *L. interrogans* Pyrogenes and 0.9% against *L. interrogans* Hardjo at titers of 1:400 and 1:800, respectively which are lower than that detected by Martins *et al.* [29] and Otaka *et al.* [30] who recorded 74.6% and 13.8% of seropositive cattle from the latter respectively and lower than that obtained in Nigeria by Ezeh *et al.* [31] who detected it in a percentage of 11.7% from the former.

In Egypt, Felt *et al.* [32] conducted a survey on 179 animals including rats in Mahalla city (Lower Egypt) where 7% were positive by culture for *L. interrogans* Grippotyphosa, Pyrogenes and Icterohaemorrhagiae. *Leptospira borgptresenii* serovar polonica was isolated for the first time in Egypt from 3 rats, also MAT titers ≥ 1:800 were observed in 11% of other rats.

The results of serological study on the sera of rat trapped from inside or surrounding dairy farms (Tables 4 and 5), showed seropositvity in percentage of 56.5% against *L. interrogans* Icterohaemorrhagiae titer ranged from 1: 800 to 1: 12800 which is higher than that recorded in Kandy, Srilanka [33] and in Kelantan, Malaysia [34] which were 30.8% and 12.3%, respectively.

Results shown in Tables (4 and 5) revealed that *L. interrogans* Pomona was found in 41.2% of total seropositive samples in rat in titers of 1:400, 1:800, 1:3,200, 1:6,400 and 1:12,800 which are higher than that detected in Thailand by Kositanont *et al.* [35] who detected antibody titers against *L. interrogans* Pomona in only 6.4% of the examined samples. The results also showed that there were 1.8% of the reactive sera against *L. interrogans* Celledoni in titer of 1:6400. On the other hand, titers of 1:800 aganist *L. interrogans* Grippotyphosa were detected in 0.5% of samples which was lower than that detected in Egypt by Samir [22] who detected it in 7 rats out of 15.

In the Middle East some countries around Egypt have reported endemic leptospirosis. In Israel, Weil disease, caused by serogroup Icterohemorrhagiae has been recognized and considered endemic. Leptospirosis has been recorded to cause severe illness among children in Eastern Turkey [36] emphasizing the importance of leptospirosis in rural areas where farming is the major source of income. In Jordan, studies have shown that 49.7% of the cattle were seropositive for several serogroups [37]. In Sudan, cattle was found to be positive at titers > 1:400 in 63.5% of the examined samples, namely 50.6% for Tarassovi, 17.1% for Sejroe-Hebdomadis, 9.4% for Bataviae, 1.2-3.5% for Icterohaemorrhagiae, Cynopteri, Autumnalis, Australis, Pomona and Grippotyphosa [38].

The current investigation gives an idea about the existing situation of the epidemiology of such zoonosis in Egypt to prevent the public health hazard. As it was proven in recent studies in Egypt that human can become infected through contact with companion animals [39, 40]. Our results were helpful to determine which isolates could be found in animals in the country. This information is helpful to determine the strategy of bovine leptospirosis prevention and control by discarding the disease in animals (source of infection) when we use the correct vaccination programs.

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