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Leptospirosis in Bovine with Special Reference to Mastitis

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Abstract: Ten leptospiral strains were used for the detection of leptospiral agglutinins in a total of 37 serum samples were collected from cattle suffering from mastitis using MAT. Total results of leptospiral serodiagnosis in cows was found in percentage of 78.38% which showed positivity against *L. interrogans* serovars: Pomona, Canicola, Icterohaemorrhagiae, Grippotyphosa and Wolffi, at rates 51%, 37%, 16.2%, 16.2%, 13.5%, respectively in titer ≥1:200. On the other hand mixed seropositivitis were detected with one or more serovars in the same samples in different percentages.

Key words: Leptospirosis · *Leptospira* · bovine · mastitis · MAT

INTRODUCTION

Leptospirosis is now identified as one of the emerging infectious diseases, caused by infection with Spirochaete of the genus *Leptospira* which consists of 60 or more species classified as serogroups and serotypes, affecting domestic and wild animals worldwide. It has been associated with serious financial losses for dairy farmers due to infertility, abortions, higher culling rates, clinical mastitis and decrease in milk yield [1-3]. The disease in cattle has an incubation period of 4 to 10 days followed by leptospiremia lasting few hours to 7 days.

Human is infected through direct or indirect contact with infected animals. Human to human transmission is extremely rare, but congenital Trans-placental infection can occur. It is hard to control transmission of leptospirosis to human because it is zoonosis-affecting people whose connected with wild or domesticated animals.

The majority of leptospiral infections are observed in tropical and subtropical regions and caused by contact with *Leptospirae* contaminated environments during agricultural or recreational practices or when waste disposal systems are infective [4,5]. Leptospirosis is a potentially serious disease, but treatable, also it is considered as an occupational hazard for agricultural, dairy farmers and veterinarians.

Lactating animals may fail to produce milk, or they could have a leptospiral infection in the mammary gland affecting all four quarters that may result in the formation of yellow, clotted, colostrums like or bloody milk. The udder is flaccid with no signs of inflammatory swelling. This manifestation is due to endotoxins.

The purposes of the study reported here were to examine blood samples collected from cows suffering from mastitis; detect leptospiral agglutinins in the sera of cows under examination and detect end-point titer in positive cases.

MATERIALS AND METHODS

I.Materials

1.1. Organisms:

Leptospiral strains: In the present study, ten leptospiral strains were used for the detection of leptospiral agglutinins in the sera of cattle. These strains are shown in Table 1.

These strains were kindly obtained from the bacterial culture collection of Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza.

1.2. Serum samples: In the present work a total of 37 serum samples were collected from cattle suffering mastitis.

1.3. Culture media used for cultivation and maintenance of leptospiral strains:

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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		*Interrogans*	Grippotyphosa	MoskvaV
4		*Interrogans*	Pomona	Pomona
5		*Interrogans*	Pyrogenes	Salinen
6		*Interrogans*	Ballum	Mus 127
7		*Interrogans*	Bataviae	Vantienen
8		*Interrogans*	Wolffi	3705
9		*Interrogans*	Australis	Ballico
10		*Biflexa*	Biflexa	Patoc-1

1.3.1- Ellinghausen medium modified by Johnson [EMJH], Difco, "Ellinghausen and Mc Cullongh [6] and modified by Johnson and Harris [7].

It is a serum free medium which has been used in the continuous subculture of leptospiral strains both for their maintenance and propagation.

A) Medium base:

- Sodium Phosphate Dibasic 1.000 g
- Potassium Phosphate Monobasic 0.300 g
- Ammonium Chloride 1.000 g
- Thiamine 0.005 g

From this base 2.3 g were dissolved in 900 ml distilled water (addition 0.2% purified agar in case of preparation of EMJH semi-solid medium), sterilized by autoclaving for 20 minutes at 121°C, then allowed to cool at room temperature.

B) EMJH enrichment: One hundred ml of albumin fatty acid enrichment (Bacto - *Leptospira* enrichment EMJH) was added aseptically to 900 ml of pre-autoclaved and cooled EMJH base. This was supplied in bottles containing 100ml of enrichment solution, which is composed of:

- Bovine albumin fraction V 10.0 g
- Calcium chloride 2 H_2O aqueous solution 1% 1.0 ml
- Magnesium chloride 6H_2O aqueous solution1% 1.0 ml
- Zinc sulphate 7 H_2O aqueous solution 2% 1.0 ml
- Glycerol aqueous solution 10% 1.0 ml
- Copper sulphate 5 H_2O 0.3% aqueous 1.0 ml
- Ferrous sulphate 7 H_2O 0.5 % aqueous 1.0 ml
- Vitamin B_{12}, 0.02 % aqueous 1.0 ml
- Polysorbate (Tween 80), 10% aqueous 12.5ml
- Distilled water to 100ml

The whole medium was then dispensed into sterilized screw capped tubes each containing 5 ml aseptically.

2. Methods

2.1. Maintenance and subculture of *Leptospira* strains:

2.1.1. In semi-solid medium: The 10 leptospiral strains used in the present work were maintained in EMJH semi-solid medium. Subculture was repeated every three months by taking 1ml of a well grown culture including the disk of concentrated leptospiral growth (Dinger's zone) with a sterilized pipette and inoculation of two new tubes of EMJH medium each with 0.5 ml. The tubes were incubated at 30°C and examined by naked eye for observation of growth of any contaminating bacteria or fungus in the first few days. The growth was then indicated by the formation of the characteristic ring of leptospiral growth after about one week to ten days of incubation. One drop was taken by a sterilized pipette from this ring, placed on a clean slide and examined under the dark field microscope using high power lens and a cover slip. Tubes that showed no ring were also examined microscopically for the presence of *Leptospirae*.

The tubes were discarded after four weeks if no leptospiral growth appeared or when they showed any pellicle or turbidity indicating growth of contaminating bacteria or fungus.

2.1.2. In liquid medium: Subculture was carried out weekly by transfer of 0.5 ml of well grown *Leptospira* culture into two tubes of [EMJH]. Tubes were incubated at 30°C and examined daily by naked eye in the first three days. Presence of heavy turbidity within 24-48 hours from inoculation indicated the presence of contaminating bacteria.

In the third day one drop was taken by a platinum loop wire, placed and slightly spread on a clean slid and examined under the dark field microscope using the low power (magnification 150X). When growth occurred, actively motile *Leptospirae* were seen. If no *Leptospira* could be seen, further incubation was done up to one week. After one week incubation re-examination was done and contaminated tubes were discarded.

Method of serological examination

2.2.1. Collection and preparation of sera: The blood samples were taken from bovine by jugular vein puncture. Five ml of blood without anti-coagulant were collected in clean test tube and transferred to the laboratory where sera were separated by centrifugation at 3000 r.p.m for 10 min. Two ml of each serum sample were taken by a

sterilized Pasteur pipette to a sterilized Eppindorf tube and stored in the deep freezer at – 20°C, until tested.

2.2.2. Serum dilution: 990 µl of saline were dispensed in Eppindorf tubes. To each tube 10 µl of serum sample were added.

2.2.3. Preparation of antigens: Leptospiral antigens used in the (MAT) during the present work, where young (4-6 days old) actively growing cultures propagated in (EMJH) liquid medium. Each of the above ten mentioned strains was subcultured weekly. Selection of a tube that showed good growth and density was done by microscopic examination prior to its use as antigen in (MAT) [8].

2.2.4. Procedure of microscopic agglutination test (MAT): It was carried out in 2 steps as follow according to Ismail *et al.* [9]:

I- Screening test:

- It was performed for screening sera at 1:100 serum (final dilution 1:200 serum antigen mixture) using 96 wells U-shape micro-titer plated by adding 50 µl of each diluted serum sample (1:100) and then loaded by 50 µl of live *Leptospira* cell suspensions from each serovar.
- The serum antigen mixtures were examined using a dark-field microscope at a magnification of 150X after incubation at room temperature (30°C) for 1.5-2 hours.
- Positive reactions were recorded when 50% or more of *Leptospira* cell were agglutinated.

II- Titration of positive samples: Samples that showed positively at 1:200 against one or more leptospiral serovars were titrated as follow:

- Serial 2 fold dilutions were done from each serum sample by adding equal volume of saline and diluted serum sample (1:100) in one well, mixed well and transferred to the next well which contains the same volume of saline and so on.
- Loading the serially diluted serum sample by equal volume of the different serovars which give positive results in screening test, to obtain 2 fold serial dilutions as follow 1:200, 1:400, 1:800, 1:1600 and 1:3200.
- The reported titers were calculated as the reciprocal of highest serum dilutions that agglutinated at least 50% of the cells for each serovar used.

2.2.5. End point determination: In positive reactions agglutination and clumping of *Leptospirae* appeared in various grades representing by:

++++: Complete agglutination in which more than 75% of cells were agglutinated.
+++: Partial agglutination in which 50–75% of cells were agglutinated.
++: Trace agglutination in which 25–50 % of cells were agglutinated.
+: Presence of occasional small clumps or small stellate aggregations of *Leptospirae.*

Negative: None of the cells were agglutinated i.e. similar to the negative control.

The end point reaction was the highest final dilution of serum in the serum-antigen mixture in which 50% or more of *Leptospirae* were agglutinated. Sera, which had 1:200 and more, were considered as having significant titers.

RESULTS

The increase in incidence is due to mixed infection with more than one serovar in the same sample.

The most predominant seropositivity was detected against *L. int.* Pomona (51.35%) in mastitic cows and the lowest percent was reported against *L. int.* Wolffi (13.5%). Seropositivities against four other leptospiral serovars were also reported namely, *L. int.* Canicola (37%), *L. int.* Icterohaemorrhagiae and *L. int.* Grippotyphosa (16.2%) of each.

Table 2: Percentages of *Leptospira* seropositive cases among mastitic cows

		Positive	
Cows with	Total number	No.	%
Mastitis	37	29	78.38

Table 3: Results of serodiagnosis of leptospirosis in case of mastitis among cows

		Mastitis (n = 37)	
Serial no.	Leptospiral serovars	No.	%
1	*L. int.* Pomona	19	51.35
2	*L. int.* Canicola	7	37
3	*L. int.* Icterohaemorrhagiae	6	16.2
4	*L. int.* Grippotyphosa	6	16.2
5	*L. int.* Wolffi	5	13.5
6	*L. int.* Australis	-	0
7	*L. int.* Ballum	-	0
8	*L. int.* Bataviae	-	0
9	*L. int.* Pyrogenes	-	0
10	*L. biflexa* Patoc 1	-	0
Total		43	116.21

Table 4: Distribution of positive titers against different leptospiral serovars in mastitic cows

Serial no.	Leptospiral serovars	Total positive sera samples	Titers 1:200 No.	%	1:400 No.	%	1:800 No.	%
1	*L. int.* Pomona	19	9	47.36	10	52.63	-	0
2	*L. int.* Canicola	7	2	28.57	4	57.4	1	14.28
3	*L. int.* Grippotyphosa	6	6	100	-	0	-	0
4	*L int.* Icterohaemorrhagiae	6	6	100	-	0	-	0
5	*L. int.* Wolffi	5	5	100	-	0	-	0
6	*L. int.* Australis	-	-	0	-	0	-	0

Table 5: Mixed seropositivity of leptospiral serovars in sera of cows suffering from mastitis (n = 37)

Serial no.	Leptospiral serovars	No.	%	Titers 1:200	1:400
1	*L. int.* Icterohaemorrhagiae &			4	-
	L. int. Pomona	4	10.8	2	2
2	*L. int.* Pomona&			-	3
	L. int. Grippotyphosa	3	8.10	3	-
3	*L. int.* Pomona &			3	-
	L. int. Canicol	3	8.10	2	1
4	*L. int.* Pomona&			2	-
	L. int. Wolffi	2	5.4	2	-
5	*L. int.* Icterohaemorrhagiae &			1	-
	L. int. Canicola	1	2.7	-	1
6	*L. int.* Grippotyphosa&			1	-
	L. int. Icterohaemorrhagiae	1	2.7	1	-
7	*L. int.* Pomona,			1	-
	L. int. Wolffi			1	-
	& *L. int.* Canicola	1	2.7	-	1
8	*L. int.* Icterohaemorrhagiae, *L.*			*1*	-
	int. Grippotyphosa			1	-
	&*L. int.* Pomona	1	2.7	-	-
9	*L. int.* Canicola &			-	1
	L. int. Wolffi	1	2.7	1	-
10	*L. int.* Grippotyphosa, &			-	1
	L. int. Canicola	1	2.7	1	-

Table 4 shows that the highest titer of seropositivity was detected against serovars *L. int.* Grippotyphosa, *L. int.* Icterohaemorrhagiae and *L. int.* Wolffi (1:200), while in case of *L. int.* Pomona was 1:400 and in case of *L. int.* Canicola was 1:800.

Table 5 shows that the mixed titers, in mastitic cows, against more than one leptospiral serovar were encountered in animals sera reacting positively against *L. int.* Icterohaemorrhagiae with *L. int.* Pomona and *L. int.* Pomona with *L. int.* Grippotyphosa.

DISCUSSION

Bovine leptospirosis is associated with economic losses in cattle such as abortion [10], lowered fertility [2] and decreased milk production [11].

Limited studies on bovine leptospirosis were carried out in Egypt. Most of the previous studies on this disease reported seropositivities with very little isolation of Leptospires [12].

Serological tests for leptospirosis are used not only for diagnosis of infection in humans, but even more frequently used for serodiagnosis in domestic animals. Tests are also applied for epidemiological studies, which frequently entail retrospective determination of antibodies which may have been provoked many months previously.

In the present study, titers of 1:200 or greater were recorded as positive. According to the report of WHO [13] titers of 1:800 or greater were considered as indicative for an active leptospiral infection even if a single serum titration was conducted

The results in Table 3 showed results of leptospiral serodiagnosis in cows suffering from mastitis against *L. int.* Pomona, *L. int.* Canicola, *L. int.* Icterohaemorrhagiae, *L. int.* Grippotyphosa and *L. int.* Wolffi were 51.35%, 37%, 16.2%, 16.2% and 13.5%, respectively.

Leptospiral infections in cattle have been classified into two major groups, one consisting of serovars adapted and carried by cattle, such as *L.* Hardjo, which is independent of lesions and the second group consists of accidental infection caused by serovars carried by other domestic and free-living animals such as rats and dogs which are dependent on the hygiene status of the farm (water and food pollution as well as the efficacy of disinfection procedures used in the farm.

The results in Table 4 of serological study on cow sera suffering from mastitis revealed that agglutinins against *L.* Canicola were predominant at titers ranged between 1:200, 1:400 and 1:800 in the following percentages were 28.57%, 57.14% and 14.28%, respectively, agglutinins against *L.* Grippotyphosa, *L.* Icterohaemorrhagiae and *L.* Wolffi were predominant at titers 1:200 percentage was 100% of each and agglutinins against *L.* Pomona were predominant at titers ranged between 1:200 – 1:400 in percentages of 47.36% and 52.63%, respectively.

In Brazil, Oliveira *et al.* [14] recorded agglutinins against *L.* Canicola, *L.* Grippotyphosa, *L.* Icterohaemorrhagiae and *L.* Pomona in 0.21%, 0.86%,

1.08% and 0.80%, respectively in dairy cattle suffering from mastitis.

The magnitude and duration of the immune response induced by leptospiral infections is known to be very variable [15], since the seroprevalence is influenced by both the frequency of new infections and the duration. A longer duration of the immune response induced by serovars Icterohaemorrhagiae and Pomona could detected in the present study may explain the relatively high seroprevalences found against these two serovars. Moreover, the renal carrier ship nature of leptospires which tend to nest in the kidneys of infected animal result in continuous stimulation of the immune system which usually result in high titers existing for years.

Mixed seropositivity were detected with one or more serovars in the same sample in different percentages as shown in Table 5. This may be due to either mixed infection of animal with more than leptospiral serovars from more than one source (reservoir animals, i.e. dog, wild rodent, contaminated water, or imported cattle carriers from different countries), in the same time cross-antigenisity between leptospiral serovars which may lead to mixed seropositivity is known in leptospirosis research.

CONCLUSION

Serological examination of cattle sera generally indicates a possible active leptospiral infection particularly with serovars Canicola, Pomona and Icterohaemorrhagiae, such infection may be accidental and caused by serovars carried by domestic and free living animals which are acting as chronic carriers or reservoirs of different Leptospires. Pomona was the predominant serovar. This may be attributed to the wide spreads of wild rats in the area from which cows were under-examination.

The presence of high titers against these two serovars in sera of cattle indicate a significant public health hazard for man and a possible cause of great economic losses in animals. Titers of 1:200 against *L.* Icterohaemorrhagiae, *L.* Grippotyphosa and *L.* Wolffi, 1:400 against *L.* Pomona and to 1:800 against *L.* Canicola in case of mastitis confirm the presence of infection with serotypes which may be located in the kidney of animals for period extended to years and excreted in urine of these animals in an intermitted nature which act as a source of infection to human or farm animals including cattle, buffalo, sheep, etc.

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