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The bacteriological prevalence of leptospiral infection in cattle and buffaloes in West Malaysia

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SUMMARY

A cross-sectional bacteriological survey of cattle in West Malaysia revealed 14·4% (32/222) had leptospiral infection. Isolates were obtained from all except one herd with prevalence of infection in herds ranging from 0–44·8%. A small number of buffalo urine samples were examined and all of them were found to be negative. A leptospiral isolate obtained from a bovine kidney proved to be a new serovar of *Leptospira interrogans* and the name *unipertama* was assigned to it. Six other leptospiral serovars were isolated, namely *canicola*, *australis*, *javanica*, *ballum*, *pomona* and *hardjo*. All six serovars were isolated for the first time in cattle in Malaysia.

Cattle in Malaysia appear to be the maintenance host for serovar *hardjo*. The presence of the other serovars in cattle was probably due to contact with the maintenance hosts, pigs for serovar *pomona* and rodents for the other three serovars. It appears that the epidemiology of leptospiral infection in cattle in Malaysia is similar to that reported overseas.

INTRODUCTION

Leptospirosis was first described in Malaysia as early as 1926 by Fletcher (1928) who was responsible for the initiation and establishment of leptospirosis research in this country. In addition to finding leptospires in humans, dogs and rats, Fletcher (1928) was also successful in the isolation of leptospires from water supplies. Since then, 37 pathogenic leptospiral serovars, mainly from rodents and man, have been isolated in Malaysia (Bahaman & Ibrahim, 1987). Up to this present study, none has been isolated from livestock. The importance of leptospiral infection in domestic animals and man in Malaysia is slowly becoming recognized and a serological survey to determine the importance of leptospiral infection in the domestic animals in West Malaysia has been conducted. Cattle and buffaloes were shown to have high serological prevalence of leptospiral infection when compared to the other animal species (Bahaman *et al.* 1987).

Titres detected using the microscopic agglutination test (MAT) are difficult to

interpret. It is not possible to distinguish between past or current infection or, because of cross reaction between serovars, to determine the identity of the infecting strain. This can only be achieved by isolating and identifying the infecting strain. As infected animals are known to excrete leptospires in large number in their urine for long periods of time (Hellstrom, 1978), urine is probably the most appropriate and accessible source of leptospires from which to attempt isolation. The aim of this study was to determine the bacteriological prevalence of leptospiral infection in cattle and buffaloes in Malaysia and to identify the causal serovars.

MATERIALS AND METHODS

Urine samples

Urine samples were collected from cattle on seven farms. The first 30 or more animals that came into the chute on the study farms were chosen for the serological and bacteriological examination. Urine samples were collected from female yearlings by manual stimulation of the perineal region and from male animals while they were restrained in a cattle chute. The 35 urine samples from buffaloes in this study were from established farms where strong restraining chutes are available for easy control of the animals.

Kidney samples

Eleven kidney samples were collected from the local abattoir at Shah Alam. Kidneys obtained were placed in clean individual polythene bags and brought back as quickly as possible to the laboratory to be processed.

Culture procedure

The urine samples were cultured as quickly as possible as any leptospire present in the samples might be inhibited by the urine itself. One drop of the urine samples was inoculated into each of two bottles of semi-solid Johnson and Seiter (JS) medium (Johnson & Seiter, 1977) one with 200 μg 5-fluorouracil (5FU)/ml and the other with 400 μg 5FU/ml using a sterile Pasteur pipette. The urine sample was then diluted tenfold with Stuarts basal medium and a drop each of the diluted urine sample was inoculated into another two bottles of semi-solid JS medium, making four bottles of culture for every urine sample examined.

The surface of each kidney was first disinfected with 70% alcohol and about 10 g of the kidney were then excised and macerated in 40 ml Stuart's basal medium with the aid of a kitchen blender. A drop of the macerated kidney was then inoculated into semi-solid JS medium containing 5FU to control microbial contamination. Following that, the macerated kidney was then diluted tenfold with Stuart's basal medium before further inoculation into another two bottles of culture media.

Initially, cultures were examined weekly for the first 3 weeks and then fortnightly till the 12th week by dark-field microscopy for the presence of leptospires. After it was found that few cultures were visibly positive within the first week of incubation, dark-field examination on the 7th day of incubation was omitted. Aliquots of the culture containing leptospires were subcultured into

liquid JS medium. Positive cultures were consecutively subcultured into liquid JS medium until a pure culture was obtained.

Identification procedures

1. *Determination of species*

The first step after obtaining the leptospiral isolates is to determine whether the isolates were pathogenic or saprophytic. There are several methods available:

(I) Egg yolk test – Fuzi & Csoka, 1961*a*.
(II) Oxidase test – Fuzi & Csoka, 1961*b*.
(III) Inhibition of growth by 8-azaguanine–Johnson & Rogers, 1964.
(IV) Growth at low temperature (13 °C) – Johnson & Harris, 1967.

2. *Initial serogrouping of the isolates*

After obtaining the leptospiral isolates in pure culture, they were then subjected to the microscopic agglutination test (MAT) against 16 rabbit hyperimmune sera (Difco, Michigan) which represent 15 important leptospiral serogroups. The hyperimmune sera were: *australis* (Australis), *autumnalis* (Autumnalis), *ballum* (Ballum), *bataviae* (Bataviae), *canicola* (Canicola), *celledoni* (Celledoni), *cynopteri* (Cynopteri), *grippotyphosa* (Grippotyphosa), *mini* (Hebdomadis), *icterohemorrhagiae* (Icterohemorrhagiae), *javanica* (Javanica), *pomona* (Pomona), *pyrogenes* (Pyrogenes), *sejroe*, *hardjo*, (Sejroe), *tarassovi* (Tarassovi) (names in parentheses represent serogroups).

The hyperimmune sera were firstly diluted twofold in a microtitre plate to give a series of diluted antisera from 1/40 to 1/5120 and to these, an equal volume of a 5- to 7-day culture of the isolate to be serogrouped was added. The plate was incubated for 1·5 h before being examined for evidence of agglutination. The hyperimmune serum which gave the highest titre is considered as the serogroup of the isolate under examination.

3. *Preparation of antisera against isolates*

Apparently healthy rabbits weighing approximately 2·5 kg were inoculated with 5- to 7-day old culture of the leptospiral isolates. The immunization schedule is a modification of the method by Tan (1970*a*). Three successive doses of 4 ml of killed cultures of the leptospiral isolates were inoculated into the rabbit through the marginal ear vein at weekly intervals. The fourth inocula was of live culture given as in the previous three inoculations. The animal was bled 1 week after the last inoculation and the serum tested by MAT for the strength (titres) of agglutinating antibodies.

4. *Serovar identification using the cross-agglutination absorption test*

It is not possible to identify the isolates up to the serovar level by using the MAT. Isolates are usually identified to the serovar level by employing the agglutinin absorption test (Dikken, 1986). This is a tedious procedure which is best left to established reference laboratories. The isolates and their respective rabbit anti-isolate hyperimmune sera from this study were submitted to the Leptospirosis Reference Laboratory, Laboratory of Microbiology and Pathology, Department

Table 1. *Bacteriological prevalence of leptospiral infection in cattle and buffaloes in West Malaysia*

Farms	No. samples examined	Bacterio-logical prevalence (%)	Serovars identified	Serological prevalence (%)
		Bovine urine samples		
1. P. T. Haiwan, Pantai	31	0	—	47·3
2. UPM, Serdang	31	0	(3 *biflexa*)	37·4
3. P. T. Haiwan, Batu Arang	29	44·8	10 *canicola* 2 *australis* 1 *javanica* (1 lost)	40·9
4. Institut Haiwan, Kluang	29	10·3	1 *ballum* 2 *javanica* (2 *biflexa*)	32·5
5. P. T. Haiwan, Air Hitam	30	10·0	3 *hardjo* (1 *biflexa*) (1 unidentified)	65·0
6. P. T. Haiwan, Sg. Siput	35	25·7	5 *pomona* 3 *hardjo* (2 *biflexa*) 1 *australis*	56·4
7. P. T. Haiwan, U. Behrang	38	10·5	4 *hardjo*	57·6
Total	222	14·4	42 isolates	45·5
Bovine kidney samples				
1. Abattoir, Shah Alam	11	9·1	1 *unipertama*	—
Buffalo urine samples				
1. UPM, Serdang	25	0	—	40·2
2. MARDI, Bk. Ridan	10	0	—	25·5

of Health, Brisbane, Australia for definitive identification. First, the Reference Laboratory has to establish the species of the isolates, that is, whether the isolates belong to the *interrogans* or the *biflexa* group and finally, the isolates were subjected to the agglutination–absorption test for definitive identification of their serovars.

RESULTS

Altogether 32 leptospiral isolates were obtained from 222 urine samples from cattle on various farms in West Malaysia, giving an overall bacteriological prevalence of 14·4% (Table 1). In comparison, the serological survey on the same animals gave an overall prevalence of 45·5%. Leptospires were seen in the majority of the positive cultures by the third week of incubation. The bacteriological technique used is seen as a sensitive method and was able to isolate leptospires from every study farm except two.

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The bacteriological prevalence of leptospiral infection in individual farms varied from 0 to 44·8% with an average of 14%. The highest prevalence (44·8%) of leptospiral infection is seen in a herd of Jersey and Jersey crosses in a farm close to Kuala Lumpur. Three leptospiral serovars from this farm have been identified and confirmed by the Leptospirosis Reference Laboratory, Brisbane when representative isolates were submitted. Ten of the 14 isolates obtained from this farm were identified as serovar *canicola*, two isolates as serovar *australis* and one as serovar *javanica*. The predominant serovar affecting this herd was shown to be serovar *canicola*. The second highest bacteriological prevalence amongst the herds was 25·7%. This was again an infection of more than one leptospiral serovar in a herd. Five of the isolates were found to be serovar *pomona*, three were serovar *hardjo* and one was serovar *australis*. Another two of the isolates were identified as saprophytic leptospires. Serovar *pomona* is seen as the predominant serovar affecting this second herd.

Altogether six leptospiral serovars were successfully isolated from cattle in this study. These were identified as *canicola*, *australis*, *javanica*, *ballum*, *pomona* and *hardjo*. Serological examination of cattle for leptospiral infection revealed that a majority of the animals had titres to serovar *hardjo* (Bahaman *et al.* 1987). Collating the overall serological prevalence of each serovar obtained, it is seen that serovar *hardjo* is the most common serovar affecting cattle in the study farms and probably true for other farms not covered by this survey. All urine samples from buffaloes were culturally negative.

One of the 11 kidneys from cattle that were killed at an abattoir was positive for leptospires. This is the only leptospiral isolate obtained from bovine kidneys. The Leptospirosis Reference Laboratory, CDC, Atlanta has identified this strain as belonging to the *interrogans* group. However, it could not be matched with any of the established serovars and was considered as a new serovar. The Leptospirosis Reference Laboratory, Brisbane confirmed this isolate as a new strain belonging to the Sejroe serogroup. The name *Leptospira interrogans* serovar *unipertama* was assigned to this new serovar.

DISCUSSION

Studies on a wide range of animal species, particularly rodents, have been done to determine the animal reservoirs of leptospires in Malaysia. Gordon-Smith *et al.* (1961*a*, *b*) found that the principal maintenance hosts of leptospiral infection in Malaysia are the various ground dwelling rats. It is believed that leptospiral infections in domestic animals in Malaysia are frequently the results of rodent contact. However, leptospirosis as a zoonotic problem does not appear to be as serious as it does in many parts of the world (Ungku Omar, 1967).

This study records the isolation of serovars *canicola*, *australis*, *javanica*, *ballum*, *pomona*, and *hardjo* for the first time in livestock in Malaysia. Serovar *pomona* has been isolated from a cat (Gordon-Smith *et al.* 1961*a*) whilst serovar *hebdomadis* has been reported in a dog (Fletcher, 1928). These are the only two reports of culturally proven leptospiral infection in domestic animals in Malaysia. However, both have not been reported in livestock in Malaysia. All six serovars mentioned above except serovar *hardjo* have been frequently isolated from

rodents (Gordon-Smith *et al.* 1961*a*). This is apparently the first isolation of serovar *hardjo* in Malaysia. Serovars *sejroe* and *hebdomadis* have been incriminated for the Hebdomadis serogroup titres seen in domestic animals particularly cattle. It is possible that the Hebdomadis serogroup titres mentioned by previous workers (Arunasalam, 1975; Leong & Maamor, 1975; Joseph, 1979) could possibly be due to serovar *hardjo*. A serological survey of leptospiral infection recently done in cattle and buffaloes indicates that titres to serogroup Sejroe were the predominant ones in these two animal species (Bahaman *et al.* 1987). With the isolation of serovar *hardjo* from cattle on a number of farms in this study, it indicates that serovar *hardjo* is probably the principal serovar responsible for the titres to Sejroe serogroup. A large number of the leptospiral serovars seen in Malaysia have rodents as their maintenance host but this is apparently not true with serovar *hardjo*. It has been mentioned by Ellis *et al.* (1981) that serovar *hardjo* has never been reported in rodents. It appears that, as in other countries, serovar *hardjo* is also being maintained by cattle in Malaysia. Other animal species and human's therefore, would tend to get accidental infection when they come in contact with infected cattle or the environment that has been contaminated with bovine urine.

The primary leptospiral serovars affecting domestic animals in Malaysia as seen in this study are serovars *hardjo* and *pomona*. Although 37 leptospiral serovars have been isolated and identified in the country, it was shown indirectly from this study that these infections were mainly in wildlife, as only sporadic infections were seen in domestic animals. Therefore, infection in domestic animals by a majority of these serovars occur when domestic animals come in contact either directly or indirectly with rodents.

Information on serovar *unipertama* is still lacking. Although it has been isolated from a bovine kidney, it is not necessarily that cattle are the maintenance host of *unipertama* infection. If cattle do maintain serovar *unipertama*, then the epidemiology and subsequent control of leptospirosis in Malaysia would have to be reconsidered. Investigations to determine the prevalence and significance of this new finding are in progress.

Fourteen leptospiral isolates were obtained from a herd of Jersey and Jersey-cross yearlings (8 months to 2 years of age) on an established cattle farm in 1983. The bacteriological prevalence of leptospiral infection of 44·8% seen in this study was the highest recorded. A majority of the animals on this farm were shown to be infected with serovar *canicola* and it appears that the animals on this farm are maintaining the infection. Roth *et al.* (1963) have defined a maintenance population as one where prevalence of infection was approximately unity or below. The present finding of 0·91 (41%/44·0%) fits that definition. Cattle have been established as the maintenance host for serovar *hardjo* (Ellis *et al.* 1981) and if it can be confirmed that the cattle in this study farm were maintaining *canicola* infection, then it would appear that serovar *canicola* is starting to adapt to a new host. So far, there has been no report of cattle maintaining leptospiral serovars other than serovar *hardjo*. On the other hand, this case could possibly be an isolated instance of high prevalence of *canicola* infection in cattle. Experimental infections are therefore required to determine the other features of a maintenance host, such as the length of leptospiruria and the pathogenicity of the serovar

involved, before cattle can be regarded as maintenance host. Serovar *canicola* is normally maintained by dogs but apparently no dogs were seen on this farm. A survey of leptospiral infection in dogs has yet to be done. In the UK, Ellis *et al.* (1982) have isolated a strain belonging to the Canicola serogroup from an aborted bovine foetus and recently, strains belonging to the Canicola serogroup were obtained from calves. This shows that *canicola* infection might be more prevalent than commonly realised. It is proposed that a detailed study should be done on the leptospiral infection in this farm with the objective of determining whether the animals are maintaining the *canicola* infection or not.

All the animals in the study herd appeared healthy and there was no indication of any clinical sign suggestive of leptospirosis. This is also true for the other herds in the other farms where leptospires have been isolated. It shows that leptospiral infection in cattle in Malaysia is inapparent in nature. This lack of clinical signs indicates that the infection is endemic in the herd. Animals in endemic areas are constantly exposed to the infection and this results in some degree of immunity, initially from passive immunity through colostrum and later through natural infection. It is interesting to note that multiple serovar infection is quite common in the herds examined. The difficulties in making a definite leptospirosis diagnosis in man and animals has long been realized (Tan, 1970*b*) and one of the reasons is probably this multiple serovar infection which is further confounded with the inapparent nature of the infection and low serological titres. This study shows that multiple serovar infection in cattle in Malaysia is quite common and recommends that the causal leptospiral serovar be isolated and identified for definitive diagnosis in the event of an outbreak of leptospirosis.

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