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## THE SEROLOGIC AND CULTURAL PREVALENCE OF *Leptospira interrogans* SEROVAR *balcanica* IN POSSUMS (*Trichosurus vulpecula*) IN NEW ZEALAND.

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**Abstract:** In a serologic and cultural survey of 127 brush-tailed possums (*Trichosurus vulpecula*) occupying pasture land in New Zealand, leptospire of the *Hebdomadis* serogroup were obtained from 48 (38%) of the animals sampled. Eight isolates were identified by cross-absorption agglutination studies as being *Leptospira interrogans* serovar *balcanica*. There was a marked age difference in prevalence with 41 positive cultures from 64 mature adults (64%) and no recoveries being made from juveniles. Isolation of leptospire was aided by the use of a new technique involving the homogenation of whole kidneys in gamma sterilized plastic bags in a "Coleworth Stomacher". The use of this apparatus allowed the processing of whole kidneys and the technique was efficient in both the recovery of leptospire and the prevention of contamination.

In view of the fact that serovar *balcanica* has been recorded previously only in East Europe in man, cattle and pigs, the high prevalence of infection in a wild animal population in New Zealand is an interesting development in the world distribution of this serovar.

### INTRODUCTION

The fauna of New Zealand is unique in that the only indigenous mammals are two species of native bats. All other species have been introduced, mostly in the last 100 years, and have occupied vacant ecological niches.<sup>18</sup>

The brush-tailed possum (*Trichosurus vulpecula*), first introduced from Australia in 1937, has been particularly successful at colonization and is now present in high numbers on farmland in many parts of the country, with reported population densities of up to 40 per hectare.<sup>13</sup>

In 1975, a single isolation of *Leptospira interrogans* serovar *balcanica*, a member of the *Hebdomadis* serogroup, was made from a small sample of possums from the Palmerston North region by Marshall *et al.*<sup>12</sup> This serovar previously had been recorded only from Eastern Europe, where it was isolated from a man with

symptoms of leptospirosis in Bulgaria,<sup>1</sup> and from cattle and pig kidneys during a survey of material from abattoirs in Russia.<sup>14</sup>

Serovar *hardjo*, another member of the *Hebdomadis* serogroup, appears to cause widespread infection in New Zealand with isolations having been reported from cattle<sup>9</sup> and humans.<sup>3</sup> Serologic titres against this serovar also have been found in horses (unpubl. data) and sheep.<sup>14</sup> This organism recently was reported from the possum in this country by de Lisle<sup>10</sup> and Brockie.<sup>2</sup>

Serovars *hardjo* and *balcanica* are closely related antigenically, and routine serologic tests cannot differentiate the two organisms. Bearing these facts in mind a survey based on both cultural and serologic techniques was undertaken in the southern half of the North Island of New Zealand to determine the prevalence of the specific serovars in-

volved in *Hebdomadis* serogroup infections in pastoral possum populations.

#### MATERIALS AND METHODS

Field samples were collected during the autumn months of March, April and May from the Palmerston North, Awahanga, Dannevirke and South Wairarapa regions, where possums in pastoral environments had a moderate population density. Wherever possible, possums were head-shot at night using .22 calibre rifles and spotlights, and blood samples were taken immediately. Otherwise, traps baited with aniseed oil and flour were used in which case animals were stunned and bled from the jugular vein.

Following bleeding, carcasses were tagged for identification and sex and age-group recorded. Animals were divided into three age groups: juveniles, transitional stage adults, and mature adults. Differentiation was based on body weight and conformation, and state of sexual development. A scrotal sac length of more than 18 mm in the male and pouch development in the female indicates sexual maturity.<sup>17</sup> Juveniles were sexually immature, while those classified as transitional stage adults were sexually mature but lacked the body weight and conformation of a full grown animal, indicating recent development of sexual maturity.

Serum was stored at -20 C until used in the leptospiral microscopic agglutination test (MAT) according to the method of Galton *et al.*,<sup>5</sup> with the initial serum dilution being 1:24. The six leptospiral serovars so far isolated in New Zealand, *viz.*, *hardjo*, *balcanica*, *pomona*, *ballum*, *tarassovi* and *copenhageni*; were used as live antigens in the MAT and, in addition, 30% of the sera collected were screened against serovars *canicola*,

*bataviae*, *grippotyphosa*, *pyrogenes*, *autumnalis*, *australis* and *biflexa*.

Kidneys to be used for attempted isolation of *Leptospira* were aseptically removed from possums within three hours of death. Each kidney was placed in a gamma-sterilized plastic bag containing 50 ml of Stuart's transport media and homogenized in a Coleworth Stomacher 400<sup>□</sup> for 30 seconds. Three serial ten-fold dilutions of homogenate supernatant were made in Stuart's media, and from these dilution tubes 0.3 ml was inoculated into EMJH<sup>□</sup> semisolid media. An additional series of EMJH semisolid media containing 200 ug/ml of 5-Fluorouracil as a selective agent<sup>6</sup> also was inoculated. The homogenation and inoculation procedures were carried out as rapidly as possible.

Culture tubes were held at 30 C for 10 weeks and examined at two-weekly intervals by dark field microscopy. Positive cultures were subcultured in liquid EMJH medium until there was sufficient growth for typing using standard antisera for serogroup identification.

Antisera against eight isolates were prepared in rabbits using the method recommended by Kmety.<sup>8</sup> For serovar identification, paired antisera and isolates were sent to the WHO/FAO Leptospirosis Reference Centre, Centre for Disease Control, Atlanta, Georgia, USA, for cross-agglutination-absorbition studies.

#### RESULTS

Serologic results revealed a high prevalence of *Hebdomadis* serogroup reactions in possums sampled from pastoral localities, with 60 of 127 sera (47%) positive against serovar *hardjo* antigen and 54 of 127 sera (43%) positive

<sup>□</sup> Coleworth Stomacher, A.J. Seward & Co. Ltd. 6 Stamford St., London, SE 19 U6, England.

<sup>□</sup> Difco Laboratories, Detroit, Michigan, USA.

against serovar *balcanica* antigen (Table 1).

A marked age difference in prevalence of serologic reactions was seen, the mature adults having the highest prevalence of positive titres, the transitional-stage adults having a lower prevalence, and the juveniles showing only one positive reaction (1:24 against *hardjo*).

Mature male and female and transitional-stage male subgroups had geometric mean titres (GMT) of the same order for serologically positive sera. In comparison, the transitional-stage female subgroup had a GMT considerably higher than that of the other adult subgroups, indicating recent infection.

Leptospiral isolations were obtained from 48 of 127 animals collected (38%). All were typed against standard antisera as belonging to the *Hebdomadis* serogroup. Eight isolates sent to the WHO/FAO Leptospirosis Reference Centre in Atlanta, USA, were all identified as *Leptospira interrogans* serovar *balcanica*.

The results of the culture examinations revealed a similar age related prevalence to those obtained in the serologic studies. Isolates were obtained from 41 of 64 mature adult possums (64%) and from 7 of 33 transitional-stage adult animals (21%). No recoveries were made from juveniles (Table 1). In the mature adult group, seven isolations were made from serologically negative animals.

There was no statistically significant increase ( $P.05$ ) in the number of isolates obtained from media containing 5-Fluorouracil compared with media without this selective agent. Culture contamination occurred in only 2.5% of tubes inoculated.

Serovar *hardjo* antigen gave a consistently higher titre compared with *balcanica* antigen in all sera tested. There was a strong positive correlation ( $r = 0.80$ ) between titres against the two antigens. The reason for this paradoxical

TABLE 1. Serologic and cultural prevalence by age and sex of serogroup *Hebdomadis* leptospiral infections in possums in the North Island of New Zealand.

Subgroup	No. collected	No. seropositive		GMT of positive sera		No. culture positive	No. sero. and/or culture positive
		<i>hardjo</i>	<i>balcanica</i>	<i>hardjo</i>	<i>balcanica</i>		
Mature male	32	25 (78)*	23 (72)	603	182	23 (72)	28 (86)
Mature female	32	22 (69)	19 (60)	563	115	18 (56)	26 (81)
Transitional-stage male	15	7 (47)	7 (47)	631	156	4 (27)	7 (47)
Transitional-stage female	18	5 (28)	5 (28)	1351	288	3 (17)	5 (28)
Juvenile male	15	0	0	0	0	0	0
Juvenile female	15	1 (7)	0	12	0	0	1 (7)
TOTAL	127	60 (47)	54 (43)	603	154	48 (38)	67 (53)

\*percentage in parentheses.

reaction is unexplained and is being investigated further. Serologic reactions against other serogroup antigens occurred in less than 2% of sera.

## DISCUSSION

This investigation demonstrates that leptospiral infection is very common in possums in New Zealand. Based on combined serologic and cultural results, there was a prevalence of over 80% in mature adults.

The recovery and identification of *L. interrogans* serovar *balcanica* from possums in New Zealand establishes this animal as a primary host-reservoir. This is an interesting development in the world distribution of this serovar, in that *balcanica* has only been reported previously in Eastern Europe. Other wildlife species, including rodents, Mustelidae, hedgehogs (*Erinaceus europaeus*) and waterfowl, have been investigated by the authors for infection with *Hebdomadis* organisms in New Zealand (unpubl. data), but no isolations of leptospire in this serogroup have been made.

It is significant that recoveries of leptospire were made from 48 of 127 mixed-age possums (38%) using the homogenation technique described. This is a much higher prevalence than that recorded in an earlier field report by Brockie<sup>2</sup> in which a kidney plug technique was used and tissue macerated in a Griffith tube. In that case, isolates were obtained from only 2 of 146 possums cultured (1.4%).

It is believed that these results indicate the greater efficiency of the whole kidney homogenation technique used in the present survey. With the use of sterile disposable plastic bags in the Coleworth Stomacher, whole kidneys can be homogenised rapidly, efficiently, and with minimum contamination. The apparatus was especially suited for the immediate processing of fresh samples in the field. Although the stomacher was designed for the bacteriologic examina-

tion of meat samples, these results illustrate another important use of this apparatus.

Isolations were made in all cases from sexually mature adults, and positive serologic reactions were also almost entirely restricted to this group. This pattern has been observed in several wildlife leptospirosis surveys from other countries<sup>4,16,19</sup> and may be a feature of endemic host-adapted leptospiral infection in wild animal reservoir populations. It also demonstrates the importance of age-differentiation when surveying wildlife populations for the prevalence of leptospirosis.

The female possum in New Zealand has a relatively well-defined mating season. The main reproductive activity occurs during March and April,<sup>11,17</sup> although small numbers of females may come into oestrus throughout the year. Consequently, the transitional-stage female group was for the first time engaging in mating and the associated changes in social behaviour at the time of sampling.

In contrast, the adult male population has the potential for sexual activity throughout the year.<sup>7</sup> Due to the extremely variable age at which juveniles mature, the transitional-stage males as a group may exercise their mating potential several months earlier than their female counterparts. Consequently, as a group, transitional-stage males at the time of sampling were not mating for the first time.

The high GMT for serologically positive animals seen in the transitional-stage female subgroup, suggestive of recent infection, indicates that it is during first mating that infection becomes established. This hypothesis is supported by the absence of infection in sexually-immature possums although these animals are fully susceptible to experimental infection (unpubl. data). The GMT's for other adult subgroups are much lower, suggesting later-stage infec-

tion, and are within a similar range to each other.

Previous reports of *Hebdomadis* isolations from possums in New Zealand have provisionally identified the serovar involved as *L. hardjo*.<sup>2,10</sup> This serovar is endemic in bovines and possibly other domestic stock in this country and the epidemiological implications of a possum wildlife reservoir of *hardjo* infection were discussed by these workers.

As *hardjo* is indistinguishable from *balcanica*, without the employment of

cross-absorption tests, it would be more likely in light of the present survey that the organisms isolated by Brockie<sup>2</sup> and de Lisle<sup>10</sup> were in fact *balcanica*.

Although *balcanica* infections have been recorded from man and cattle in East Europe, similar infections have not been recorded in New Zealand as previously all *Hebdomadis* reactions in humans and bovines were assumed to be due to *hardjo*. It appears that *hardjo* is the main leptospiral infection of cattle, but it is possible that some *Hebdomadis* reactions of man and cattle could be due to *balcanica*.

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