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LEPTOSPIROSIS IN BRUSHTAIL POSSUMS: IS *LEPTOSPIRA INTERROGANS* SEROVAR *BALCANICA* ENVIRONMENTALLY TRANSMITTED?

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ABSTRACT: In New Zealand, the biological control of introduced brushtail possums (*Trichosurus vulpecula*) may be the only affordable option for achieving a significant long term reduction in pest numbers on a national scale. *Leptospira interrogans* serovar *balcanica* is among the potential biocontrol agents and vectors currently being investigated for this purpose. As the transmission pathways of *L. interrogans* serovar *balcanica* between possums are poorly understood, the objective of the study was to determine whether infection could result from exposure to contaminated environments. Sixteen individually housed, uninfected possums, in three groups, were regularly exposed over a period of 32 days to contaminated cages or grass enclosures of 16 other experimentally infected possums all shedding leptospires in their urine. None of the 16 challenged possums developed serological evidence of *L. interrogans* serovar *balcanica* infection. These results suggest that this organism is unlikely to be transmitted environmentally, supporting previous circumstantial evidence that social contact may be required for transmission of *L. interrogans* serovar *balcanica* between possums.

Key words: Brushtail possum, *Trichosurus vulpecula*, *Leptospira interrogans* serovar *balcanica*, biological control, environmental transmission.

INTRODUCTION

Brushtail possums, *Trichosurus vulpecula*, are considered New Zealand's most serious vertebrate pest (Parliamentary Commissioner for the Environment, 1994). Initially introduced from Australia in the mid 19th century to establish a fur trade (Pracy, 1974), possums have spread to occupy more than 90% of New Zealand, with the population recently estimated to be between 60 and 70 million (Batchelor and Cowan, 1988). Possums cause severe damage to New Zealand's native forests by selective browsing (Nugent, 1995) and, through competition and predation of young, adversely affect native fauna (Innes, 1995). Possums are also important vectors, maintaining and spreading bovine tuberculosis (Tb) to cattle and deer (Livingstone, 1991). Present methods of controlling possums based on poisons and traps involve ongoing expense and are inadequate for long term national possum control (Atkinson and Wright, 1993). Biological control is considered to be the only affordable option for achieving a long-term major reduction in possum numbers throughout New Zealand (Jolly et al., 1992).

Control might be achieved either through the introduction of a disease not currently present in possum populations in New Zealand or by an infectious agent engineered to control reproduction or impair some other aspect of possum biology. Such an agent should be humane, unable to survive away from possums, infect only possums and have a possum-specific mode of action (Jolly, 1993). One strain of leptospirosis (*Leptospira interrogans* serovar *balcanica*) is among potential organisms presently under investigation.

Leptospires are obligate parasites which require a host species for survival (Hathaway, 1978). Two serovars from the serogroup Hebdomadis are present in New Zealand: *L. interrogans* serovars *hardjo* and *balcanica*. The first isolations of leptospires in New Zealand possums were thought to be isolates of *L. interrogans* serovar *hardjo* (Brockie, 1975; de Lisle et al., 1975) but were later identified as *L. interrogans* serovar *balcanica* (Marshall et al., 1976; Hathaway et al., 1978). *Lepto-*

spira interrogans serovar *balcanica* is thought to have been introduced to New Zealand by infected possums from Victoria and New South Wales, Australia (Presidente, 1984). Restriction enzyme analysis of New Zealand *L. interrogans* serovar *balcanica* has shown its genotype to differ from European *L. interrogans* serovar *balcanica* isolates (Robinson et al., 1982).

The possum is considered to be the maintenance host for *L. interrogans* serovar *balcanica* infection in New Zealand (Hathaway, 1978). A lack of clinical signs (Cowan et al., 1991), the failure to demonstrate biochemical changes characteristic of renal damage and the mild nature of kidney lesions (Hathaway, 1981a) all suggest it has low pathogenicity to possums. A high percentage of many North Island possum populations are seropositive (Horner et al., 1996; Cowan et al., 1991; Pearson and Ashby, 1995) and among captive possums the disease appears highly infectious (Pearson and Ashby, 1995).

Leptospira interrogans serovar *balcanica* infection is found predominantly in possums over 18 mo of age and the prevalence of infection does not appear to differ between sexes (Cowan et al., 1991; Pearson and Ashby, 1995). The prevalence varies between habitats in New Zealand (Hathaway, 1978), with a higher prevalence in pastoral habitats than in forests (Cowan et al., 1991). Several aspects of possum behavior in pastoral habitats, such as larger home ranges, nest sharing and the possibility of increased contact with infected material on the ground (see references in Cowan et al., 1991) may account for the increase in prevalence.

In many species (e.g., cattle), contact with infected urine by an uninfected animal is the principal transmission pathway of leptospires; leptospires shed in urine may survive and remain infective in water, pasture and damp soil for up to 74 days under favorable conditions (Faine, 1994). They remain infectious and viable, even after they lose motility (Hathaway, 1981a). However, other species (e.g., hedgehogs) are thought to transmit leptospires through sexual behavior around puberty (Hathaway, 1981c). Hathaway (1978) suggested that environmental transmission of *L. interrogans* serovar *balcanica* between possums was less likely than social transmission, based on the age-specific prevalence of *L. interrogans* serovar *balcanica* in sexually mature possums. Sexually mature captive possums that have been experimentally infected with *L. interrogans* serovar *balcanica* by intraperitoneal inoculation have been shown to infect other possums when in social contact (Day, 1996). However, the possibility of environmental transmission has not yet been systematically investigated. The present study was designed to determine whether *L. interrogans* serovar *balcanica* could be environmentally transmitted between possums, as a first step in assessing its potential as a biocontrol vector.

MATERIALS AND METHODS

Possums used in this study were trapped in October 1994 and January 1995 using box traps from the Huiarau Range in the Urewera National Park (38°37′S, 177°04′E) and Kawau Island (36°26′S, 174°50′E), New Zealand. Possums in both areas are reportedly free of *L. interrogans* serovar *balcanica* infection (Horner et al., 1996; Pearson and Ashby, 1995). Animals were transported in individual sacks to the Animal Behaviour and Welfare Research Centre (AgResearch Ruakura, New Zealand) where they were placed in individual wire cages (550 × 560 × 1050 mm high) suspended from the ceiling, containing a feed tray, nest sack, shelf (300 mm from top of cage) and water nipple. Each cage was separated by an opaque PVC divider, providing a physical barrier between possums. Possums were fed on a daily diet of wet mash (200 g of cereal-based possum pellets (Northern Rolling Mill, Auckland, New Zealand) and water at a 1:1.5 ratio) and a single apple. Animals were kept on a fixed 12:12 hr day/night light regime and the temperature in the room was maintained above 10 C. All possums were aged by tooth wear (Winter, 1980) during routine veterinary checks. Serological testing for evidence of *L. interrogans* serovar *balcanica* infection was examined using a modified version of the Microscopic Agglutination Test (MAT) by the Central Animal Health Laboratory (AgResearch Wallaceville, New Zea-

land). Cross absorption procedures (Faine, 1982) were used to discriminate between *L. interrogans* serovar *balcanica* and other serovars. Positive tests were reported as the greatest serum dilution at which serum showed a reaction. If no reaction was seen in a 1:50 dilution, results were reported as negative. All procedures used in the present study were approved by the AgResearch Ruakura (Hamilton, New Zealand) and University of Waikato (Hamilton, New Zealand) Animal Ethics Committees prior to experimentation.

Experimentally infected possums consisting of 16 animals (mean ± SD age = 3.8 ± 1.6 yr; 7 females, 9 males), in three groups, were anaesthetised and inoculated intraperitoneally with 0.5 ml *L. interrogans* serovar *balcanica* inoculum (about 1×10^8 organisms). The inoculum was cultured from an infected New Zealand possum at the Department of Veterinary Pathology and Public Health at Massey University (New Zealand) and confirmed as New Zealand possum *L. interrogans* serovar *balcanica* isolate, using restriction endonuclease analysis and DNA verification. The first two groups (group 1 and 2), each of four possums, were confirmed to be shedding leptospires 28 days post inoculation (pi), by dark field (DF) microscopy (400×) of mid-stream urine samples. The third group of eight possums (group 3) were shown to be infected with *L. interrogans* serovar *balcanica* using MAT serology at 24 days pi. MAT serology was found to be more reliable for detecting establishment of infection than DF microscopy, hence it was used in group 3.

The infected possums were used as a source of environmental *L. interrogans* serovar *balcanica* contamination in cages and in grass enclosures, to which uninfected possums were exposed. The three groups of infected and three groups of uninfected possums used in this study were balanced for sex ratio, origin and mean age.

Four recently infected possums (group 1) (3.8 ± 2.2 yr; 2 females, 2 males) were used to contaminate cages with *L. interrogans* serovar *balcanica*. For this experiment, sacks were laid on the cage floor, allowing urine, faeces, food scraps and shed leptospires to accumulate within the cages. Nest and floor sacks were not changed for the duration of the experiment. Fluid trapped by the sacks was examined under DF microscopy (400×) for leptospires 14 days after swapping began. Four uninfected possums (4.3 ± 1.9 yr; 2 females, 2 males) were regularly exposed to the cages of the infected possums. Over 32 days, each uninfected animal was interchanged with each infected possum's cage eight times (each of 24 hr duration). The cage swapping was designed to correspond with the maximum period of leptospire shedding after infection (27 to 60 days pi; Hathaway 1981a). Swapping began on day 29 pi and ended on day 62 pi. The entire procedure was then replicated with another group (group 2) of four infected (4.8 ± 1.5 yr; 2 females, 2 males) and four uninfected (3.9 ± 1.8 yr; 2 females, 2 males) possums in another set of cages.

Urine samples were collected from uninfected possums throughout the cage swapping period and were examined by DF microscopy (400×) for leptospires. A urine sample was taken 18 days after swapping began and subsequently at four day intervals until exposure ended. Blood samples were taken from uninfected possums between 14 and 28 days after the end of the experiment and tested by MAT serology for *L. interrogans* serovar *balcanica* infection.

Urine from the four infected possums in group 1 was also sampled on day 62 pi to ascertain if leptospiruria was still present. Urine from the four infected possums in group 2 was examined by DF microscopy every 7 days throughout the swapping period. Blood for serology was collected from the six surviving infected possums at 152 and 186 days pi.

Eight individually fenced, outdoor grass enclosures (ranging from 51 m^2 to 130 m^2), containing a wooden nest box and one or two climbing logs, were used in this experiment. Also used, were eight indoor concrete pens (3 to 5 m^2), again with a wooden nest box in each pen. Eight recently infected possums (group 3) (3.4 ± 1.3 yr; 3 females, 5 males) were transferred (on day 22 pi) from individual cages in the quarantine facility to individual enclosures, seven days prior to the beginning of the experiment. Each enclosure was subject to natural weather conditions and the grass (approximately 300 mm long) was not mown during the experimental period. Possums were able to contact each other through the wires of neighbouring grass enclosures, but not between indoor pens or between indoor pens and enclosures. Each infected possum alternated daily between the grass enclosure and the indoor pen. In a similar procedure to that used with cages, each of eight uninfected possums (3.9 ± 2.0 yr; 4 females, 4 males) was alternated with eight infected possums, between the indoor pens and outside enclosures daily. All eight uninfected possums were exposed in sequence to both of the environments of all eight infected possums over an 18 day period. The experiment was designed so that all infected animals were in the outside enclosures on one day and all uninfected possums were outside

on the following day. This ensured that no social contact could be made between infected and uninfected possums. Swapping continued for 32 days (day 29 pi to day 60 pi) so that each uninfected possum was exposed to each infected environment two to three times. Possums were fed daily with mash and an apple in their indoor pen or outside enclosure. Fluid samples (urine and water) from the concrete floor of indoor pens and the base of climbing logs in the grass enclosures (collected by pipette into sterile jars) were examined under DF microscopy for leptospires once between 14 and 21 days after exposure began. Urine samples were taken from the eight infected group 3 possums at days 28, 43 and 71 pi.

Two urine samples were taken from each of the eight uninfected possums during the swapping period (days 52 pi and 62 pi) and examined under DF microscopy. Blood samples were taken from the uninfected possums 38 days after swapping ended to determine if titres to *L. interrogans* serovar *balcanica* infection were present in any possums.

Temperature and rainfall data were collected at the Ruakura Meteorological Station (Hamilton, New Zealand) located approximately 1 km from the enclosure complex. Dewfall and frosts on the grass of the enclosures were recorded daily.

RESULTS

Using DF microscopy, fluid samples taken from sacks on the bottom of cages (14 days post exposure) indicated all eight cages contained non-motile leptospires. Similarly, fluid sampled from all eight indoor pens and eight outdoor enclosures between 14 and 21 days after exposure began contained non-motile leptospires.

During the exposure period rain fell on 20 of the 32 experimental days. The maximum period without rainfall was 2 days. Temperatures ranged from −2 C to 17 C in the outdoor enclosures with frosts over 3 nights. In the cage environment the temperature ranged between 10 and 19 C.

All captured possums were confirmed to be free of *L. interrogans* serovar *balcanica* infection before the experiments began. None of the 16 uninfected possums that were exposed to cages or enclosure environments containing leptospires showed positive titres to *L. interrogans* serovar *balcanica* in MAT serology tests. No leptospires were seen under DF microscopy in any urine samples from uninfected possums during or after exposure to the cages or enclosures.

TABLE 1. Serological Microscopic Agglutination Test titres and leptospiruria pattern of experimentally infected possums.

Possum	MAT titre (day 24 pi)	DF urine analysis (day pi)		
		28	43	71
1	1:800	M[a]	N/M[b]	X[c]
2	1:1600	M	N/M	N/M
3	1:1600	M	N/M	N/M
4	1:1600	M	N/M	N/M
5	1:3200	M	N/M	N/M
6	1:1600	M	X	N/M
7	1:1600	M	N/M	N/M
8	1:800	M	X	N/M

[a] Motile leptospires detected.
[b] Non-motile leptospires detected.
[c] No leptospires detected.

In group 1, all four infected possums were shedding motile leptospires in the urine at day 28 pi. Three of the four infected possums showed leptospiruria (non-motile leptospires) at the next sampling on day 62 pi. In group 2, weekly urine samples from day 28 pi showed that leptospiruria continued intermittently throughout the swapping period. Shedding was seen in all group 2 possums at day 62 pi, although none of the possums showed leptospiruria in every weekly urine sample. Leptospires detected in the urine from group 2 possums were motile at day 28 pi; non-motile leptospires were seen in all further weekly urine samples. MAT serology for the six group 1 and 2 possums tested at 152 and 186 days pi showed positive titrations to *L. interrogans* serovar *balcanica* ranging from 1:200 to 1:400.

Group 3 infected possums, used in enclosure swaps, showed positive serological *L. interrogans* serovar *balcanica* titres at day 24 pi (Table 1). Motile leptospires were detected in urine samples from all eight individuals at day 28 pi. Only non-motile leptospires were detected after day 28 pi, although not in all samples (Table 1).

DISCUSSION

This study showed that *L. interrogans* serovar *balcanica* was not transmitted to possums by contact with environments containing leptospires. This supports previous predictions that other routes such as social contact, are required for *L. interrogans* serovar *balcanica* transmission (Hathaway, 1978; Durfee and Presidente, 1979).

The experimentally infected possums in this study showed infection characteristics very similar to those described previously by Hathaway (1981a), who also infected possums by intraperitoneal inoculation with similar quantities of inoculum. In both studies, the detection of leptospires in urine was not consistent for all individuals over time. Hathaway (1981b) suggests that the inconsistency may be due to technical difficulties in detecting leptospires in possum urine by DF microscopy.

In a related study, Day (1996) found that when pairs of possums (one possum infected with *L. interrogans* serovar *balcanica* in each pair) were housed together (12 to 69 days pi) in the same grass enclosures that were used in this study, transmission occurred in 50% of male/female pairs. This showed that an infection following intraperitoneal inoculation from the same *L. interrogans* serovar *balcanica* culture was infectious to other possums when in social contact.

The two environments used in this experiment, cages and enclosures, were both shown to contain non-motile leptospires. The loss of motility of leptospires does not affect their infectiousness or viability (Hathaway, 1981a). The survival of leptospires following excretion is influenced by several environmental factors. Leptospires survive best in slightly alkaline conditions with adequate moisture, moderate temperatures and a lack of direct sunlight or high salt concentrations (see references in Hathaway, 1978). When environmental factors are suitable some leptospire serovars may survive and remain infective in water, pasture and damp soil for up to 74 days (Faine, 1994).

In the cages, sufficient moisture, moderate temperatures (10 to 19 C) and continual re-exposure of each environment to infected possums ensured some leptospires were present in each cage throughout the trial period. Based on weather data, each enclosure should also have contained sufficient moisture in the grass, due to the dew fall and rainfall, to ensure leptospire survival. Frosts on three of the 32 days may have reduced the survival of leptospires. Neither environmental salt concentration or pH were recorded but neither would have been likely to have detrimentally affected leptospiral survival.

Several workers have suggested that favorable environmental conditions are important for the maintenance of endemic leptospiral infection in host species (see references in Hathaway, 1978). Other researchers describe population factors of the maintenance host species (e.g., host density, contact rate, social structure) as more important in maintaining infection (see references in Hathaway, 1978). Hathaway (1978) attempted to assess which factors, environmental conditions or possum population characteristics, were more important in maintaining *L. interrogans* serovar *balcanica* in possums. He concluded that the prevalence of this infection in three different populations was not associated with environmental variables in the different ecosystems, and that possum population factors (such as possum contact rate) were important in the maintenance of *L. interrogans* serovar *balcanica*. Hathaway (1978) did not, however, show that this organism could not be spread by contact with contaminated environments.

The data obtained in this study have shown that environmental contamination alone is not sufficient to cause infection in possums. Social contact between possums would therefore appear to be required for *L. interrogans* serovar *balcanica* transmission. However, in the wild, other factors not present in this study (foot pad cuts,

etc.), may enhance the probability of environmental transmission occurring.

Age-specific differences in the seroprevalence of *L. interrogans* serovar *balcanica* (Hathaway, 1978; Pearson and Ashby, 1995) in possums suggest that behaviors associated with sexual maturity may be important in the acquisition of infection. Social transmission of this leptospire between possums would be desirable from the biological control perspective. Social transmission is more likely to be species-specific than environmental transmission. Although environmental transmission would increase the rate at which a biocontrol agent could spread between possums, this would also enhance the possibility of inter-species transmission.

The effects of social variables on *L. interrogans* serovar *balcanica* transmission between possums are currently being investigated. However, further comprehensive research will be required to determine both the specificity of this serovar to possums and whether the specific subtype of *L. interrogans* serovar *balcanica* endemic to New Zealand possums can be transmitted environmentally to other species.

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