

A SURVEY OF MICROBIAL PARASITES IN LUNDY BROWN RATS (*RATTUS NORVEGICUS*)

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ABSTRACT

The object of this study was to determine the parasite profile of Lundy brown rats. A combination of methods comprising culture, polymerase chain reaction (PCR) and immunofluorescence assay serology (IFA) were used to analyse past and present infection by seven micro-parasites. The results of this study show that the sampled rats were infected with two of the seven taxa of organisms tested for: *Bartonella* spp. and *Trypanosoma* spp. The observed prevalence and possible reasons for presence and absence of the different organisms tested for are discussed, together with the possible implications of these infections at the individual and population level.

Keywords: *Lundy, brown rat, microbial parasites.*

INTRODUCTION

Rodents act as reservoirs for a wide range of parasitic organisms some of which can be defined as zoonotic, meaning that they are capable of infecting humans. Rats of the genus *Rattus* have been well characterised as reservoirs for zoonoses such as *Yersinia pestis*, the causative agent of bubonic plague, and *Leptospira interrogans*, the agent of Weil's disease (Gerke & Rump, 2003). Whilst *Yersinia pestis* is no longer endemic in the UK, several other zoonotic diseases, such as cowpox and rodent-specific parasites, can be identified in the rat population.

Parasites that can infect rodents may also infect other organisms, as part of their natural cycle, with rodents representing a transient host for a single stage of the life cycle. Parasites with such complex life cycles require the hosts for all stages of the cycle to be present and easily accessible. Often parasites are transmitted between hosts by arthropod vectors, such as fleas and ticks. Not all parasitic species are

transmitted to new hosts by this route. Some are transmitted by direct contact with the reservoir, through the respiratory tract, or by contact with an infected host's urine or faecal material. Ectoparasites can have a significant impact on the life cycle through their specificity for either the parasite or the reservoir host(s).

Lundy is interesting to look at in terms of microbial parasites, as small islands often have considerably fewer vertebrate species than similar habitats on the mainland, therefore, fewer potential hosts for parasites and ultimately a less diverse range of parasites (MacArthur & Wilson, 1967; Milazzo *et al.*, 2003). Lundy differs from many other islands as all except two mammals, the grey seal and pygmy shrew, are non-native to the island and in the case of the Sika deer, are non-native to the UK. This would almost certainly have an effect on the range of parasites capable of living in this environment. Microbial parasite load may have interesting implications on the health of the host population, particularly fecundity. A great deal of research exists examining the effect of epidemic infections but little has been published on the effects of endemic infections. Feore *et al.* (2004) examined the effect of cowpox infections on the reproductive success of bank voles and wood mice and concluded that fecundity was significantly reduced in infected animals. Work is ongoing in Feore's research group to examine the effects of endemic infections, which do not have clinical symptoms or generally affect survival, and will include some of the bacterial species included in this report.

Microbial parasites that are either known, or suspected, to infect species of the genus *Rattus* were selected for prevalence testing, these comprised viruses cowpox, MHV-68 and Lymphocytic choriomeningitis virus (LCMV), bacterial species of *Bartonella* and *Anaplasma* and the Protozoa *Babesia* and *Trypanosoma*.

All three viruses naturally infect rodents, although only Cowpox has been proven to naturally exist in rat populations. MHV-68 is a rodent-specific virus (Blasdell *et al.*, 2003), whilst cowpox and LCMV are known to be zoonotic (Armstrong & Sweet, 1939; Baxby *et al.*, 1994), with Cowpox capable of infecting a wide range of mammalian hosts (Baxby *et al.*, 1979). However, little work has been done on the natural host reservoirs of these viruses, particularly in regards to LCMV and MHV-68. The work reported here is part of a larger study investigating the range of natural reservoir hosts for these virus species.

Both *Bartonella* and *Anaplasma* have representatives that exist naturally in wild rodent populations. There are at least five species of *Bartonella* that have rodents as their reservoir host (Birtles *et al.*, 1994), including *B. elizabethae*, which is also known to be zoonotic. Of the species of *Anaplasma* found in the UK, *A. phagocytophila* is capable of infecting humans and species of *Anaplasma* have been isolated from rodent species (Bown *et al.*, 2003). Like the bacteria, both protozoan groups also contain species that reside in rodent populations. At least two species of *Babesia* naturally infect rodents in the UK, including *B. divergens*, a zoonotic pathogen (Zintl *et al.*, 2003), which, along with *B. microti*, is known to infect rats, (Akinboade *et al.*, 1981).

There are three species of trypanosome that infect rodents in the UK, of which *T. lewisi* is thought to be rat-specific and non-zoonotic (Hoare, 1972). Although both species of rats native to the UK (*Rattus norvegicus*, brown rat; *R. rattus*, black rat) have been found on Lundy, this survey is restricted to the Lundy brown rat due to sample availability.

METHODS

Sample preparation

42 brown rats (*Rattus norvegicus*) stored at -20°C, were obtained from Lundy Island courtesy of the 'Seabird Recovery Project'. These were dissected and a sample of blood (obtained by cardiac puncture) and the spleen were taken from each animal. The spleens were finely chopped and placed in enough Eagle's Minimum Essential Medium (EMEM), containing 1% foetal calf serum (FCS), to cover the sample. Both blood and spleen were kept at -80°C until required.

DNA extraction

50µl of blood and 50ml of EMEM containing spleen from each animal were used for DNA extraction. Samples were diluted in 500ml 1.25% Ammonia solution and heated at 100°C for 20 minutes. Samples were vortexed and returned to the heating block for a further 25 minutes, this time with the lid of each tube open to allow evaporation and hence concentration of the DNA extract, (Bown *et al.*, 2003). All samples were diluted 1:10 in double-distilled H₂O. Stock DNA extracts were placed at -80°C for storage, whilst diluted samples were placed at -20°C until required.

Bacterial culture for *Bartonella*

A 1µl loopful of rat blood was streaked onto a Columbia agar plate containing 10% defibrinated horse blood. The plates were then incubated at 37°C with 5% CO₂ saturation, in a moist environment, for between 7 and 14 days until colonies were visible. Samples were prepared ready for PCR by resuspending bacteria in water. Samples for which plates remained clear for more than 10 days were considered as being sterile.

Polymerase chain reaction

Whole-cell preparations or a small quantity of DNA extracted from blood or spleen were added to a commercially prepared PCR enzyme mix (Abgene) and primers, short nucleotide sequences complementary to known DNA sequences in the target

parasite, in 0.2ml thin walled tubes (Axygen). The samples were then processed through a series of heating cycles, using a thermocycler (Thermohybrid). PCR reactions were subjected to gel electrophoresis using a 1% agarose gel, containing 5µl of ethidium bromide at a concentration of 10mg/ml and visualised using UV light. Details of the primers used are illustrated in Table 1.

Table 1. Details of the primers used to test for the presence of the different bacterial and protozoan taxa.

TARGETED SPECIES	PRIMER NAME	TARGET REGION	STAGE	DIRECTION	SEQUENCE
<i>Bartonella</i> (Birtles & Raoult, 1996; Norman et al., 1995)	CS440F	gltA gene	1 st stage	Forward	GCTATGTCTGC ATTCTATCA
	CS1137R			Reverse	AATGCAAAAAG AACAGTAAACA ¹
<i>Anaplasma</i> (Massung et al., 1996)	GE 3a	16srRNA region	1 st stage	Forward	CACATGCAAGTCG AACGGATTATTC
	GE 10R			Reverse	TTCCGTTAAGAA GGATCTAATCTCC
	GE 9F		Nested stage	Forward	AACGGATTATTC TTTATAGCTTGCT
	GE 2			Reverse	GGCAGTATTA AGCAGCTCCAGG
<i>Babesia</i> (K. Bown, pers. comm.)	Bm F1	18S region	1 st stage	Forward	GCGATGTATCA TTCAAGTTTCTG
	Bm R1			Reverse	TGTTATTGCCT TACTTCCTTGC
	Bm F2		Nested stage	Forward	ACGGCTACCAC ATCTAAGGAAGGC
	Bm R2			Reverse	TCTCTCAAGG TGCTGAAGGA
<i>Trypanosoma</i> (Noyes et al., 1999)	TR F	18SRNA region	1 st stage	Forward	GAAACAAGAA ACACGGGAG
	TR R			Reverse	CTACTGGGC AGCTTGA
	SS F		Nested stage	Forward	TGGGATAACA AAGGAGCA
	SS R			Reverse	CTGAGACTGTA ACCTCAAAGC

Immunofluorescence assay serology

A ninety-six well plate (Costar) was prepared so that each well contained a confluent layer of Vero (green monkey kidney) cells. These cells were infected with the relevant virus, either cowpox or MHV-68, and left to grow for three or five days respectively. Blood from each animal was placed onto two wells each at dilutions of 1:40 and 1:20 (total volume 30µl). Antibodies in the blood, resulting from prior infection, bind to virus-infected cells in the wells. The plates were washed three times with Phosphate buffered saline (PBS) and a fluorescently labelled anti-rat antibody (Sigma) diluted 1:20 (total volume 30µl) was added. This meant that only rats that have been infected with a virus previously and therefore had produced antibodies against it would show positive results. Fluorescent-labelled positive samples were detected using an UV microscope (Crouch *et al.*, 1995).

Immunofluorescence assay serology for LCMV Commercially produced slides (Charles River Laboratories) with eighteen wells were used and the blood was tested at a single dilution of 1:2 (total volume 10µl). The wells of these slides contained a confluent layer of cells, both infected and uninfected (mouse cell-line), which had been fixed with acetone prior to purchase.

RESULTS

Bacterial culture

Six of the 40 blood samples yielded *Bartonella*-like colonies (off-white, small, smooth). These bacteria were then subjected to PCR to confirm their identity.

Polymerase chain reaction

The six culture positive samples were subjected to PCR, five of the samples were confirmed to be *Bartonella* each yielding a PCR product of the correct size (740bp) (see Figure 1). Insufficient bacteria were available for testing the sixth sample.

DNA extracted from the blood and spleen from each rat was tested using PCR for *Anaplasma*, *Babesia* and *Trypanosoma*. PCR for *Anaplasma* and *Babesia* were negative. The blood and spleen from three rats and the blood from one further rat were positive for *Trypanosoma* (see Figure 2). The PCR products from the samples found positive for trypanosomes were PCR purified using a DNA purification kit (Qiagen).

Sequencing The five *Bartonella* PCR positive samples and the seven *Trypanosome* PCR positive samples were sequenced commercially (MWG). Forward and reverse sequences were obtained for each sample to produce a double-stranded coverage. Sequence data obtained were then compared to sequences available online (BLAST search). All *gltA* sequences from the Lundy *Bartonella* isolates were identical to one-another and showed 100% similarity with that of *Bartonella tribocorum*. (see Figure 3). However it should be noted that the available sequence for the *B. tribocorum gltA* is somewhat shorter (c330bp) than those obtained by this study (698bp). The Lundy *Bartonella gltA* sequence was also 100% identical over 698bp to sequences obtained previously from isolates obtained from *Rattus norvegicus* in Peru, (R. Birtles, Per. Comm.) and the USA (Ellis *et al.*, 1999, 220-224). The three Trypanosome sequences obtained were also indistinguishable from each other and shared 99-100% identity to that of *Trypanosoma lewisi*.

Immunofluorescence assay serology

All samples for Cowpox virus, MHV-68 and LCMV were negative.

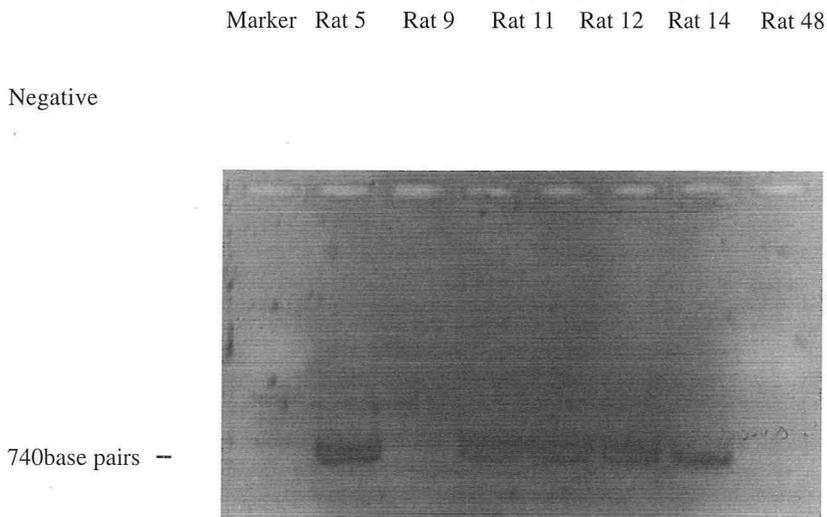


Figure 1. Electrophoresis gel of *Bartonella* samples found positive by culture, indicated by visible 740 base pair bands.

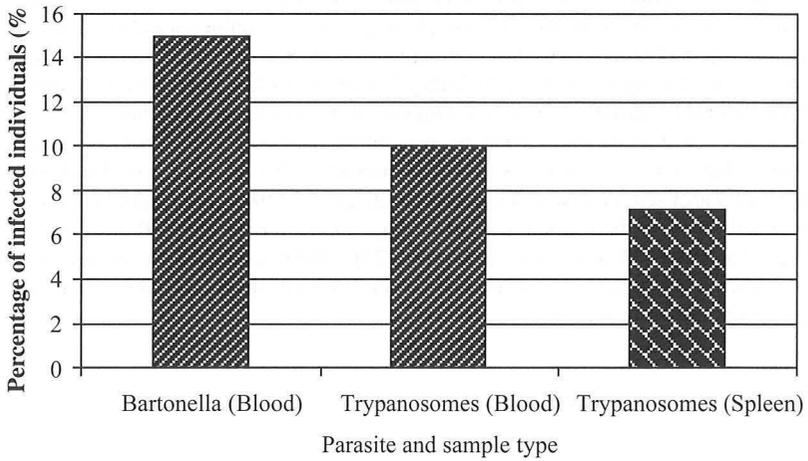


Figure 2. Percentage prevalence of *Bartonella* and *Trypanosoma* in the blood of Lundy rats and also in the spleen for *Trypanosoma*.

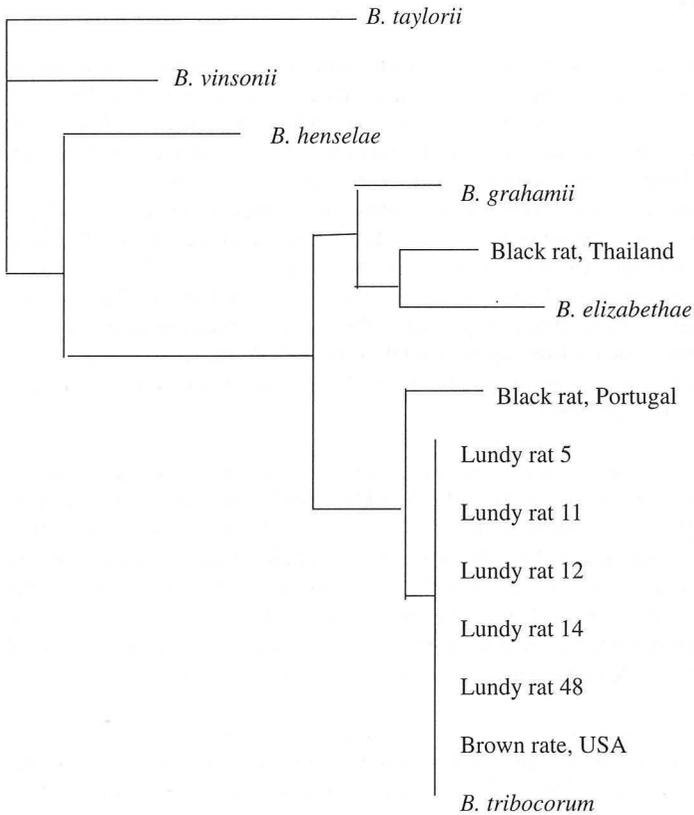


Figure 3. A phylogenetic tree inferred under the assumptions of Juke's Cantor evolutionary relationship from a 285 base pair *gltA* alignment, illustrating the evolutionary relationship between the Lundy rat bartonellae, several other rat *Bartonella* samples and *B. tribocorum*, (sequences downloaded from Genbank. Accession numbers: *B. taylorii*, Z70013; *B. vinsonii*, Z70015; *B. henselae*, L38987; *B. grahamii*, Z70016; Black rat, Thailand, AY277892; *B. elizabethae*, Z70009; Black rat, Portugal, AF086636; Brown rat, USA; AF075164; *B. tribocorum*, AJ005494).

DISCUSSION

Bacteria of the genus *Bartonella* are known to infect a wide variety of mammalian hosts and several species are known to have rats as a natural reservoir host. *B. tribocorum*, *B. elizabethae* and *B. grahamii* have all been previously isolated from rats (Ellis *et al.*, 1999). Lundy rats were found to be infected with *Bartonella*, which based on available sequence data is likely to be *B. tribocorum*. *B. tribocorum* is a rat-specific *Bartonella* species (Heller *et al.*, 1998). Incomplete sequence is available for the *B. tribocorum gltA* gene, the target of these PCR reactions, but a 100% match was obtained with the available sequence. *B. tribocorum* is genetically related to *B. elizabethae*, based on analysis of several genes, which has been implicated in zoonotic infections (Heller *et al.*, 1998; Daly *et al.*, 1993). Prevalences found in other rat populations have ranged from approximately 0-60% in *Rattus norvegicus*, so the finding of a prevalence of approximately 15% for Lundy rats fits with this data (Birtles *et al.*, 1994; Ellis *et al.*, 1999).

The protozoan species *Trypanosoma lewisi* was identified in the Lundy samples. This parasite species has been identified in both black and brown rats, with prevalences of 11% and 22% respectively (Laakkonen *et al.*, 2003; Linardi & Botelho, 2002). The observed prevalence of 10% (blood) in this population may be slightly low, but may simply be an effect of small sample size, or possibly the poor quality of the samples, due to the samples being taken from animals that had been deceased and stored for an unknown length of time. Both *T. lewisi* and *B. tribocorum* are transmitted by the flea, clearly demonstrating the survival of this vector on Lundy.

The three viruses, cowpox virus, LCMV and MHV-68 tested for were absent. While evidence exists for cowpox and LCMV naturally infecting rats, these are not the primary reservoir hosts. Due to the lack of other rodent species on Lundy, there is less possibility of cross-infection from natural reservoir hosts, voles and mice (Marennikova *et al.*, 1977; M. Bennett, Pers. Comm.). The absence of *Anaplasma* and *Babesia* can more readily be explained by considering seasonal variations in the prevalence of the arthropod vector, *Ixodes ricinus* (Bown *et al.*, 2003; Hartelt *et al.*, 2004). We were unable to confirm the presence or absence of ticks on Lundy, although due to the presence of sheep and deer it seems likely that ticks would be present. Alternatively, any of these parasites may have been absent from the founding population of Lundy rats, although the importance of the founding population would depend on whether rats from the mainland regularly infiltrated the island population.

It is interesting to note that the two micro-parasite species present are both transmitted by fleas, whilst the two non-virus species that are absent are both transmitted by the same tick species, *Ixodes ricinus*. These data confirm that fleas are present in the Lundy animal population and are likely to be acting as vectors for infectious disease. It is unlikely that *Ixodes ricinus* is absent from the island, this tick species is commonly found in woodland habitats, but has been shown to be capable of existing in open

sheep pasture (Walker *et al.*, 2001). Due to the number of different potential host species that are present on the island, it is likely that the ticks would survive on Lundy. Tick populations fluctuate with the seasons, if the rat population was sampled in a seasonal trough period it may affect the disease prevalence. Combined with the poor quality of the original samples and the small sample size this may explain the low numbers of parasites isolated.

It would be interesting to carry out an ectoparasite survey lasting a year on Lundy, combined with a survey of the infectious agents present. Surveying the ectoparasites and micro-parasites of other mammalian species on Lundy would give a complete picture of the microbial profile of mammals on the island. Combining these studies with work determining the effects of endemic infections on the fecundity and survival of mammalian hosts, would provide interesting parallels between Lundy mammals, with an apparently limited parasite burden, and mainland mammals.

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