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Longitudinal monitoring of the dynamics of infections due to *Bartonella* species in UK woodland rodents

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SUMMARY

Blood samples were repeatedly collected from 12 sympatric woodland rodents over a 12-month period and DNA extracts from each were incorporated into a bartonella-specific PCR targeting a fragment of the 16S/23S rRNA intergenic spacer region (ISR). The composition of each amplicon was analysed using restriction enzyme analysis (REA) and base sequence comparison. Bartonella DNA was detected in 70 of 109 samples. Eleven samples contained DNA derived from more than one strain. Sequence analysis of 62 samples found 12 sequence variants (ISR genotypes) that were provisionally assigned to 5 different species, 2 of which were newly recognized. Up to five different species were detected in each animal. On about two-thirds of occasions, a species detected 1 month was not there the next, but never was a genotype superseded by another of the same species. However, a genotype could be re-encountered months later in the same animal, even if interim samples contained other genotypes. Our results suggest that although most animals are bacteraemic most of the time, specific infections are often superseded and that a complex and dynamic epidemiology of bartonella bacteraemias exists in woodland rodents.

INTRODUCTION

The potential role of parasites and pathogens in the population dynamics of their hosts is now becoming more widely recognized [1]. However, although there is little doubt that most natural populations support endemic microparasite infections that have no obvious or widespread effects on mortality, empirical data to clarify the importance of such infections for the dynamics of their hosts have only recently begun to emerge. *Bartonella* species, which have been demonstrated to parasitize a high proportion of the woodland rodent population in the UK and elsewhere, potentially provide very useful model for addressing this shortfall.

Bartonella species are Gram-negative cocco-bacillary *Proteobacteria* that parasitize a range of mammalian hosts including man. To date, 13 species have been formally proposed and for most a reservoir, in which infection appears not to induce recognizable disease, has been identified [2–5]. Infections due to *Bartonella* species can be transmitted between hosts by ectoparasites, and a range of arthropods has been

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implicated including fleas, lice, sandflies and ticks [2, 6]. Surveys of small woodland mammals in the UK and elsewhere have demonstrated a high prevalence of bartonella within circulating erythrocytes, with, typically, 40–60% of animals yielding isolates on blood culture [7, 8]. In the UK, four *Bartonella* species have so far been identified among isolates recovered from bacteraemic animals; these are not host-specific among sympatric small mammal species [7, 9]. Furthermore, almost all animal populations studied to date concurrently support multiple species of *Bartonella* [7, 8].

Previous surveys to detect and characterize *Bartonella* species in small mammals, however, have been horizontal. The only longitudinal study in UK woodland rodents to date used non-specific staining to identify intra-erythrocytic infections [10] and, while able to demonstrate that infections were encountered throughout the year and that a seasonal variation in prevalence occurred, this study did not attempt to differentiate between the *Bartonella* species observed. Hence, there are many fundamental but unanswered questions. Is the high prevalence of infection an indication that infection is chronic or is there a rapid turnover of acute infections? Do *Bartonella* species commonly coexist within an individual host as well as within host populations? Does infection with one *Bartonella* species, or strain within a species, afford protection against subsequent infection with other species or strains?

Longitudinal sampling of small rodents for bartonella-induced bacteraemia is feasible only if small volumes of blood can be collected without detriment to the animal. The fastidious nature of *Bartonella* species requires that if culture-based surveillance schemes are to be employed, blood samples be aseptically collected, but in practical terms, such a manipulation in the field is difficult. The advent of PCR-based DNA amplification methodologies has circumvented the requirement to isolate an organism as a means for its detection and as a prerequisite to its identification. Several PCR-based approaches to the detection of bartonellae in clinical and veterinary samples have been described [2]. We have recently evaluated the use of one of these methods in conjunction with subsequent analysis of amplification products for the detection and differentiation of strains of bartonellae causing bacteraemia in woodland rodents [11]. In the current study we have applied this methodology to a preliminary longitudinal survey of wild rodents in two British woodland populations. The results suggest a complex dynamic of infection and successive re-infection with a diverse range of *Bartonella* species and strains.

MATERIALS AND METHODS

The samples used in this study were collected from sympatric *Clethrionomys glareolus* (bank vole) and *Apodemus sylvaticus* (wood mouse) populations inhabiting two mixed woodland sites on the Wirral in North West England: Manor Wood and Rake Hey. Sampling of these communities began in March 1995, using methods described in detail elsewhere [12]. Briefly, animals were caught in traps placed at permanent trapping stations 10 metres apart arrayed as a 100 m² square grid. Each month, two traps were set at each trapping station over three consecutive nights. Traps were checked each morning during the monthly trapping period and all captured animals were scanned for the presence of an electronic transponder (Avid). Newly encountered animals were identified to species, weighed, sexed and tagged. The tail-tip of each animal was then clipped and a few drops of blood were collected into a sterile Eppendorf tube prior to the animal's release. Tagged animals that had not previously been encountered during the monthly trapping period were also weighed and sexed, and a blood sample collected. Finally, tagged animals, which had already been encountered during the ongoing trapping period were immediately released.

All blood samples were centrifuged and serum was removed. The remaining cell pellets were stored at −20 °C. Testing of all blood pellets collected in August and September 1996 for the presence of bartonella DNA allowed infected animals in those samples to be identified, and 12 of these were selected on the basis of the best availability of samples from the subsequent 12 months. In total, 109 blood samples were tested from these animals, with between 7 and 12 samples being available for each for the period between August 1996 and July 1997.

DNA extracts were prepared from the blood pellets [13] and subjected to a bartonella genus-specific PCR, based on the oligonucleotide primer pair QHVE1 and QHVE3 [14]. The success of each reaction was determined by examination of UV-irradiated 1% agarose gels containing ethidium bromide on which amplification products had been electrophoretically resolved. Products were then assessed by restriction endonuclease analysis (REA); 25 µl of product was mixed with 4 µl of 10 × reaction

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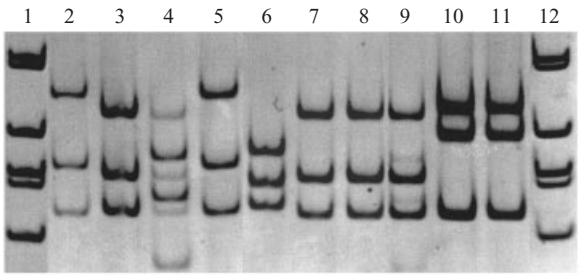

Fig. 1. Restriction profiles obtained following resolution of *Hae*III digests of QHVE1/3 amplification products obtained from animal 12. The profiles obtained are as follows: lane 2, N40 (genotype A6); lane 3, *B. taylorii* (A1 or A5); lane 4, *B. taylorii* (A1 or A5) and *Bartonella* sp. (B); lane 5, N40 (genotype A6); lane 6, *B. doshiae* (C2); lane 7, *B. taylorii* (A1 or A5); lane 8, *B. taylorii* (A1 or A5); lane 9, *B. taylorii* (A1 or A5) and (faintly) *Bartonella* sp. (B); lane 10, *B. grahamii* (B); lane 11, *B. grahamii* (B). Lanes 1 and 12 contain molecular weight markers, with bands at 154 base pairs (bp), 220 bp, 234 bp, 298 bp, 394 bp and 453 bp (molecular weight marker VI, Boehringer Mannheim).

buffer, 1 µl (10 U) of *Hae*III and 10 µl of distilled water then incubated at 37 °C for 3 h. Digestion products were electrophoretically resolved on an 8% polyacylamide gel (mini Protan II, BioRad) at 100 V for 1 h then visualized by UV illumination following ethidium bromide staining.

The remainder of each amplification product was purified (QIAquick kit, Qiagen) and its base sequence was determined using cycle sequencing reactions containing either QHVE1 or QHVE3 as previously described [11]. Alignment of the partial ISR sequences obtained above with one another and with ISR sequences of the characterized *Bartonella* species was attempted using version V of the CLUSTAL multi-sequence alignment program [15]. The hypervariablity of ISR sequences belonging to different *Bartonella* species prevented their accurate alignment and thus quantitative comparison of these data could not be made. However, if a new ISR sequence was encountered, an attempt to infer the phylogenetic position of the bartonella strain from which it was derived was made by citrate synthase gene (*gltA*) analysis. Briefly, an approximately 700 base pair 3′ fragment of the gene was amplified using PCRs incorporating the previously described primers 440f/1137r [9]. The base sequences of the products obtained were determined then compared (% similarities calculated) with partial *gltA* fragments available for previously characterized *Bartonella* species and strains.

Table 1. *Identify and distribution of* Bartonella *species and ISR genogroups encountered in the study*

Site*	Host†	Month 8/96	9/96	10/96	11/96	12/96	01/97	02/97	03/97	04/97	05/97	06/97	07/97
MW	*A.s*	—	*Bg* (B1)‡	*Bg* (B1)	*Bg*+N40	N40 (A2)	*Bt* (A1)	0	0	—	N40 (A2)	0	0
MW	*A.s*	—	—	—	—	—	0	*Bd* (C2)	0	D3	0	0	0
MW	*A.s*	0	N40	0	0	0	0	*Bd* (C2)	0	*Bt* (A3)+*Bd* (C2)	*Bt* (A3)+*Bd* (C2)	0	0
MW	*A.s*	*Bd* (C2)	*Bd* (C2)+*Bt* (A1)	*Bd* (C2)	*Bt* (A3)	N40 (A2)	0	*Bd* (C2)	0	0	*Bd* (C2)	*Bg* (B1)	*Bg* (B1)
MW	*A.s*	—	D2	0	*Bg* (B1)	*Bg* (B1)	0	*Bd* (C2)	0	—	*Bd* (C2)	—	—
MW	*A.s*	*Bt*+N40	*Bt*+N40	*Bd* (C2)	—	*Bd* (C2)+D	N40 (A4)	*Bd* (C2)	0	0	D2	0	—
MW	*C.g*	—	*Bg*+N40	0	0	0	0	—	—	0	0	—	—
MW	*C.g*	Bt (A3)	—	*Bg* (B1)	*Bg*(B1)+N40 (A2)	N40 (A2)	*Bd* (C2)	—	—	*Bd* (C2)	*Bt* (A3)	—	—
RH	*A.s*	—	*Bg* (B1)	*Bg* (B1)	—	0	N40 (A6)	—	*Bt* (A1)	*Bt* (A1)	N40 (A6)	N40 (A6)	—
RH	*A.s*	—	*Bt* (A5)	*Bt* (A5)	—	D2	0	0	0	E	0	*Bd* (C2)	—
RH	*A.s*	—	*Bt* (A5)	*Bt* (A5)	—	—	0	—	N40 (A6)	D1	*Bd* (C2)	*Bd* (C2)	*Bd* (C2)
RH	*C.g*	—	N40 (A6)	*Bt* (A5)	*Bt*+D	N40 (A6)	*Bd* (C2)	*Bt* (A5)	*Bt* (A5)	*Bt* (A5)+D	*Bg* (B1)	*Bg* (B1)	—

* MW, Manor Wood; RH, Rake Hey. † *A.s*, *Apodemus sylvaticus*; *C.g*, *Clethrionomys glareolus*. ‡ *Bg*, *Bartonella grahamii*; *Bt*, *B. taylorii*; *Bd*, *B. doshiae*. Key to different ISR genotypes: Initial letter (A–E) indicates the similarity cluster to which the genotype belongs. The following number (1–6) indicates the specific genotype within the similarity cluster. If no ISR genotype is given, the PCR product was analysed by REA only and therefore a sequence-based genotype could not be designated. 0, no bartonella DNA detected. —, no blood sample collected.

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RESULTS

Preliminary work demonstrated that *Hae*III-based REA of ISR amplification products was clearly able to differentiate between the type strains of the four species of *Bartonella* currently associated with UK woodland rodents and thus that this methodology provided a useful means for assessing the presence of mixed species infections in individual blood samples.

A total of 70 of 109 blood pellets yielded amplification products. REA demonstrated 59 to comprise a single profile and 11 to comprise two superimposed profiles. Most of these 11 mixed profiles consisted of strong bands derived from one profile and weaker bands from a second (Fig. 1). Three previously unrecognized profiles were observed. Sequence analysis was attempted on all amplification products. Nonsensical data were obtained from 8 samples, all of which had yielded mixed profiles on REA but data for the remaining 62 samples were unambiguous (Table 1).

A total of 12 different sequences were obtained. Five of these had been encountered previously among sequences derived from isolates of *Bartonella taylorii* (A-1 and A-3), *B. grahamii* (B-1) and N40 (A-2 and A-4) [11]. Two of the new ISR genotypes (A-5 and A-6) could be aligned and were found to be very similar to those obtained for *B. taylorii* and N40. As these two species possess very similar ISR genotypes, we were unable to specifically assign either of these new genotypes to one or other of these species. However, *gltA* analysis was able to assign to species the strains from which these two ISR genotypes were derived. A partial *gltA* sequence that was identical to that of the *B. taylorii* type strain was obtained from three blood samples that had yielded ISR genotype A-5, and a partial *gltA* sequence identical to that of the N40 representative strain was obtained from three blood samples that had yielded ISR genotype A-6. One of the remaining ISR genotypes (C-2) could be aligned with that of the *B. doshiae* type strain and a partial (654 base pair) *gltA* sequence comparison revealed 96% similarity. This value is markedly higher than the similarities of 93% or lower that are observed between the *gltA* sequences of different *Bartonella* species [9].

A previous study found ISRs that could not be meaningfully aligned with one another were derived from different *Bartonella* species [11]. Thus, on this basis, we assigned the remaining four previously unencountered ISR genotypes to two new putative species. *GltA* analysis supported this assignment. All three variants of ISR genotype D possessed an identical *gltA* sequence that shared less than 92% similarity with those available for other *Bartonella* species. However, our efforts to obtain a *gltA* sequence from the blood sample that yielded ISR genotype E failed and exhaustion of the DNA extract prevented a repeat attempt. In summary, the 12 ISR genotypes were assigned to 5 putatively distinct *Bartonella* species, as follows: *B. taylorii* (ISR genotypes A-1, A-3, A-5), *B. grahamii* (B-1), *Bartonella* N40 (A-2, A-4, A-6), *B. doshiae* (C-2), *Bartonella* species D (D-1, D-2, D-3) and *Bartonella* species E (E).

The distribution of these 12 genotypes among the 70 amplification products is presented in Table 1. It should be borne in mind when examining the table that the genotypes were characterized in a random order and without their provenance being known. Thus, the clustering of a single species or strain within a chronological sequence of samples from the same host, for example, was not the result of these isolates themselves being characterized sequentially. The 12 genotypes were not encountered with the same frequency. Species E, for instance, was detected only once, whereas *B. doshiae* (ISR genotype C-2) was amplified from 21 samples and *B. grahamii* from 20. Bearing this in mind, there is no evidence of any *Bartonella* species being specific either to one species of host or to one of the two sites. Nor was there any suggestion of any *Bartonella* species being disproportionately found in either host or at either site. Furthermore, even over the course of the sampling period, more than 1 species of *Bartonella* was detected from all host individuals, 4 species were detected in the cases of 5 hosts, and from 1 host, 5 were detected. Conversely, with the caveat that the sample size is small, there is a suggestion that at least some strains may be site-specific: *B. taylorii* ISR genotype A-3 and N40 ISR genotype A-6, for instance, were both isolated on several occasions and from both host species at Rake Hey, but never at Manor Wood.

Chronic infection, over a period of months, was relatively infrequently encountered. In 22 cases, the same species was detected in an animal over 2 or even 3 consecutive months, but more often (42 cases), a species detected during 1 month was no longer there during the next. A species' presence was followed by, in roughly equal proportions, either an infection due to a different species (22 occasions) or by no detectable infection (20 occasions). On no occasion, however

(with the caveat that mixed infections were occasionally not characterized to strain) was a strain within a species immediately superseded by a different strain of the same species. In 7 of the 12 animals, however, a species detected during the early part of the study but then replaced either by a different species or by no apparent infection, was re-encountered in later samples. The delays between initial encounter and re-encounter varied but were as short as 2 months.

Mixed infections were apparently uncommon. Among the four *Bartonella* species that were common enough for meaningful comparisons to be made (*B. taylorii*, *B. grahamii*, N40 and *B. doshiae*), out of 6 possible comparisons between pairs of species, 5 showed fewer than expected mixed infections, of which 3 came very close to statistical significance (N40 and *B. doshiae*, χ^2_1 (with the Haber correction for continuity) = 3·51, $P \leqslant 0{\cdot}065$; *B. grahamii* and *B. taylorii*, $\chi^2_1 = 3{\cdot}38$, $P \leqslant 0{\cdot}07$; *B. grahamii* and *B. doshiae*, $\chi^2_1 = 3{\cdot}26$, $P \leqslant 0{\cdot}075$). In line with the patterns of successive infections (above), none of the mixed infections comprised two strains of the same species. In eight instances, a blood sample from an individual was tested in the month preceding a mixed infection. In seven of these, at least one of the species in the mixed infection was also present in the preceding sample. In all 11 mixed infections, a blood sample was tested in the immediately following month. In only five of these were at least one of the species in the mixed infection also present in the subsequent sample. On four occasions both were replaced by a single, different species, whereas on two occasions no subsequent infection could be detected.

The novel partial *gltA* sequences obtained in this study have been submitted to the GenBank database under the following accession numbers: uncultured *B. doshiae* strain (ISR genotype C-2) (wbs013), AF207827, and uncultured *Bartonella* spp. D (ISR genotypes D-1, D-2 and D-3) (wbs089), AF207828.

DISCUSSION

An understanding of the epidemiology of *Bartonella* species in their natural hosts is important from several perspectives. In terms of public health, the spectrum of bartonella-associated infections continues to expand together with the number of species implicated as pathogens [2]. Very recent work has linked one of the species that exploit UK woodland rodents to human disease [16]. This finding adds weight to the proposition that all *Bartonella* species are potentially pathogenic to man but that this potential is tempered if contact between man and the species' natural host is seldom made. From an ecological standpoint, the study of bartonella infections in woodland rodents can potentially provide much-needed data to test theoretical models of infectious disease dynamics within naturally susceptible populations and the role of such infections in the regulation of host abundance [1].

More comprehensive follow-up studies will require the testing of large numbers of animals and, due to their simplicity and speed, the methods we have used [11] lend themselves to such work. Nonetheless, even in this preliminary study we have been able to demonstrate, for the first time, a complex *in vivo* epidemiology for *Bartonella* species in their natural hosts. Although our findings require confirmation using culture-based methods, they provide clear evidence in support of a very dynamic microbial ecology within the blood of bank voles and wood mice.

The high prevalence of infection identified in horizontal surveys of natural populations of woodland mammals [7, 8] combined with studies of experimental infections in rodents has led to the notion that individual animals are susceptible to persistent, long-term bacteraemia rather than high incident rates of infection [8, 17]. Our findings, however, suggest that this is not typically the case in natural infections. Furthermore, on this basis, the applicability to field epidemiology of data drawn from laboratory-based studies of bartonella infections in other host species may also be limited. Our samples were collected at monthly intervals. Thus, a detected species may have been present in the animal's blood for several weeks. In some animals we observed the same genotype in consecutive samples, which may be interpreted as an extremely chronic infection. However, as the sensitivity of our differentiation method remains unknown, this same genotype may in fact represent different infecting strains. Furthermore, as a positive PCR result is not an indicator of viability, our assay may be prone to overestimation of bacteraemia duration through the detection of remnant DNA from dead or destroyed bartonellae. These uncertainties notwithstanding, however, our results demonstrate that a continuous turnover of specific bacteraemias occurs in most animals, with both chronic and mixed infection being uncommon.

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Our results also indicated that super-infection could only be achieved by a genotype belonging to a different *Bartonella* species from that of the current infection. This observation is not unexpected if different *Bartonella* species possess distinct immunogenic antigens during natural infections as they do when grown on axenic media [18]. Thus, immunity to one strain should provide cross-protection against other strains of the same species, but not against strains of other species. However, Kosoy and colleagues [8] reported that naturally infected American rodents developed no detectable antibodies, a finding they subsequently suggested may be due to immunotolerence as a consequence of transplacental infection [19]. It is not known if transplacental transmission occurs in our populations, but the dynamic epidemiology of infections reported here makes it unlikely to be the most common route of infection. It may, in any case, be wrong to accept that axenic immunogen expression by bartonellae accurately reflects that occurring *in vivo*. In humans, antibodies raised against one infecting species generally cross-react significantly with antigens derived from other (albeit axenically-grown) species [20]. Furthermore, thermal adaptation of bartonellae, as needs to occur *in vivo* during their transmission from ectoparasite to mammalian host, involves the upregulation of a number of proteins, some of which were identified as being recognized heat-shock proteins with immunogenic properties [21]. Similarly, the ability of bartonellae to adapt to oxidant stress has been demonstrated [22], and this response may also lead to expression of specifically induced immunogens. Despite these concerns, very recent data derived from work on laboratory specific pathogen-free cats has found a lack of heterologous protection between *B. henselae* and *B. clarridgeiae*, and even between different *B. henselae* serogroups/subtypes [23].

Although this study demonstrates there to be a very dynamic epidemiology of *Bartonella* species in their natural hosts, the effect of these repeated infections on either an individual host or the naturally infected population as a whole remain as yet unknown. Indeed, the lack of humoral response [8] may suggest there to be little or no cost to an individual. Furthermore, despite observing that trypanosome and/or bartonella infections in bank voles were associated with lowered haemocrit levels and enlarged spleens, Wiger [24] concluded that such parasites were of little importance as mortality factors in the population ecology of the rodent. However, recent work on *B. henselae* in cats has suggested that although no immediate and overt manifestations result from infection, the ability of infected cats to become or sustain pregnancy may be compromised [25]. Feore and colleagues [26] have recently shown that cowpox infection in bank voles can also induce pregnancy delay/failure and point out that although having no immediate effect on the individual host, such manifestations have a cost to the natural population as a whole through the expense of lost litters. Drawing from the findings of these two studies, it would be pertinent to determine whether rodent bartonellae have a similar effect on their natural hosts.

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