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Original article

Emergence of distinct genetic variants in the population of primary *Bartonella henselae* isolates

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Abstract

Bartonella henselae isolates from different hosts display a marked genetic heterogeneity, as determined by pulsed-field gel electrophoresis (PFGE). The aim of the present study was to determine whether different genetic variants may coexist within the population of distinct *B. henselae* isolates and could be detected by PFGE. Three primary *B. henselae* isolates and the *B. henselae* reference strains ATCC 49793 and 49882 were subjected as single colony derived cultures in quadruplicate to PFGE analysis upon restriction with *Sma*I or *Not*I. Up to 4 fragment differences were found among the cultures obtained from each primary isolate, indicating the coexistence of genetic variants in the population of primary *B. henselae* isolates. The clonal relatedness of the genetic variants was confirmed by arbitrarily primed PCR and multi-locus sequence typing. In contrast to the primary isolates, no variants were detected among the single colony derived cultures of the high-passage ATCC strains. We hypothesized that the coexistence of different genetic variants may represent a feature that is restricted to primary or low-passage *B. henselae* isolates. The primary isolates were serially passed in vitro and then subjected as single colony derived cultures to PFGE analysis, which now revealed identical patterns among the quadruplicate cultures of each high-passage isolate. These results suggest that the population of a primary *B. henselae* isolate is composed of distinct genetic variants, which may disappear upon repeated passages on artificial culture media. Generation of genetic variants by *B. henselae* may represent an escape mechanism to circumvent the host specific immune responses.

Keywords: *Bartonella henselae*; Primary isolate; Genetic variants; Molecular typing; Pulsed-field gel electrophoresis; Arbitrarily primed PCR; Multi-locus sequence typing

1. Introduction

The spectrum of human diseases caused by *Bartonella henselae* is continuously expanding and includes cat scratch disease (CSD), bacillary angiomatosis and peliosis hepatis, endocarditis, and prolonged bacteremia and fever in children [1–6]. Domestic cats represent the natural reservoir for the bacterium [7,8]. Infected cats develop relapsing bacteremia, which may persist for up to two years [9,10]. *B. henselae* induces specific humoral and cell-mediated immune responses in naturally or experimentally infected cats, human patients, and experimentally infected mice [4,11–17]. Thus, the question arises as to how Bartonellae can evade the specific immune responses of the infected host to maintain a chronic infection. It has been proposed that Bartonellae may persist in an as yet unidentified intracellular niche, from where they are occasionally released to invade host erythrocytes [18,19]. Recently, human hematopoietic progenitor cells were proposed to represent such an intracellular niche for *B. henselae* [20]. Generation of genetic and antigenic variants represents another common strategy to circumvent the host specific immune responses. Recent reports revealed some variation within individual *B. henselae* or *Bartonella quintana* isolates that may contribute to the chronic or relapsing course of the infection by these pathogens [21,22].

A marked genetic diversity has been found among different *B. henselae* isolates by using several typing techniques

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[23,24]. PFGE is referred to as gold standard among the band-based typing methods and has frequently been used for epidemiological studies [24–27]. Interestingly, some previous observations based on PFGE analysis point toward the existence of genetic variants within the population of a distinct *B. henselae* isolate. For example, Sander et al. [24] found minute differences between the *Sma*I restriction fragments obtained from the isolates FR96/BK36 and FR96/36II grown from the blood of an infected cat in Germany. The question whether the cat was infected with one or two different strains was not addressed in this study. Kabeya et al. [10] demonstrated some variations among the *Not*I restriction patterns of *B. henselae* isolates derived from recurrent bacteremic peaks of naturally infected cats. However, interpretation of those variations was hampered by the limited number of restriction fragments generated by *Not*I (five fragments) and the fact that no additional restriction enzyme was used. Furthermore, because of the study design, the possibility of a co-infection or re-infection of cats with an additional *B. henselae* isolate could not be ruled out. Thus, the question remained unanswered whether the observed differences in PFGE patterns represented different *B. henselae* strains or different genetic variants, which belonged to the same clonal lineage. More recently, Maruyama et al. [28] found two different *B. henselae* variants among different single colony derived cultures obtained from the lymph node of a patient with CSD. Again, *Not*I was used as a single restriction enzyme, and it was postulated that the patient had been co-infected or was re-infected with two different *B. henselae* strains [28].

Those reports and our own observations of (i) morphological differences among different colonies of a primary *B. henselae* isolate, and (ii) sporadic inter-assay variability among the PFGE fingerprints obtained from a distinct *B. henselae* isolate raised the question as to whether genetic variants may coexist within the natural population of a *B. henselae* isolate and serve as possible explanation for those observations. In order to address this question, three primary *B. henselae* isolates were subjected as single colony derived cultures to PFGE analysis. Two reference *B. henselae* strains from ATCC were studied in parallel. The clonal relatedness of the PFGE variants among the progeny of the primary isolates was evaluated by using two additional, sequence-based typing techniques, i.e. arbitrarily primed (AP)-PCR and multi-locus sequence typing (MLST). This work represents the first systematic analysis of genetic variations within the population of individual *B. henselae* isolates.

2. Materials and methods

2.1. Bacterial strains

B. henselae isolates Berlin-1, Berlin-2, and K111 were isolated in Berlin, Germany. The Berlin-1 isolate was originally grown from the skin biopsy specimen of a patient with bacillary angiomatosis [29]. The Berlin-2 isolate was isolated from the blood of a cat owned by a patient with CSD [27,29]. The K111 isolate was grown from the blood of a naturally infected cat in Berlin [27]. All the isolates from Berlin were conserved without any additional in vitro passages after isolation (primary isolates) as follows. A sweep of colonies from the first growth on Columbia blood agar (CBA) was inoculated into *Brucella* broth supplemented with hemin and Fildes [30] and stored at −70 °C until use. The *B. henselae* strains ATCC 49793 and 49882 (Houston-1) were originally isolated from the blood of two human patients [2,31] and obtained from ATCC.

2.2. Preparation of single colony derived cultures

The primary isolates and the ATCC strains were grown from frozen stocks on CBA at 37 °C in 5% CO_2 in ambient air. Four distinct colonies with different morphological characteristics, or selected on random, of each isolate were picked, inoculated separately to CBA plates and grown to lawns prior to the isolation of bacterial DNA.

For one set of experiments, *B. henselae* isolates from Berlin were serially passed on CBA as follows. Bartonellae were grown on CBA and a sweep of each isolate containing at least 10 different colonies was inoculated to the next CBA plate and grown to a lawn. After 19 passages, four different colonies were selected on random, grown to separate cultures, and then subjected as subculture 20 isolates to PFGE analysis.

2.3. PFGE analysis

Macrorestriction fragments of genomic DNA were separated by PFGE as described previously [29]. Briefly, bacterial DNA was embedded in 1% Seaplaque GTG Agarose (BioWhittaker Molecular Applications, Walkersville, USA) and restriction was performed with *Sma*I or *Not*I (Fermentas, St. Leon Roth, Germany) according to the manufacturer's instructions. Electrophoresis was conducted in a CHEF II or CHEF III chamber (BioRad, Munich, Germany) under the following conditions: 5.5 V, 3–12 s, 22 h, 16 °C for *Sma*I, and 6 V, 5–30 s, 33 h, 14 °C for *Not*I. The PFGE patterns were analysed by visual comparison of banding patterns.

2.4. Arbitrarily primed PCR

AP-PCR using the core sequence of the phage M13 as a single primer was performed as described elsewhere [24]. PCR products were visualized after electrophoresis in a 1.5% agarose gel by staining with ethidium bromide. The banding patterns were evaluated by visual analysis.

2.5. Multi-locus sequence typing

MLST was performed by partial sequencing (300–500 bp) on both strands of the genes 16S rRNA–DNA, *bat*R, *glt*A, *fts*Z, *gro*EL, and *rib*C as described previously [32]. The amplification products were purified and sequenced directly by using an ABI 3730 xl or ABI 3700 sequencer (AGOWA, DNA sequencing service, Berlin, Germany). The sequence data were analysed by ClustalW multiple sequence alignment