Bartonella henselae exists as a mosaic of different genetic variants in the infected host

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Bartonella henselae is a fastidious bacterium associated with infections in humans and cats. The mechanisms involved in the long-term survival of bartonellae despite vigorous host immune responses are poorly understood. Generation of genetic variants is a possible strategy to circumvent the host specific immune responses. The authors have recently demonstrated the coexistence of different genetic variants within the progeny of three primary *B. henselae* isolates from Berlin by PFGE analysis. Aims of the present study were to determine whether coexistence of different variants is a common feature of *B. henselae* isolates worldwide and whether the genetic variants originally emerged *in vivo*. Thirty-four primary isolates from different geographical regions were analysed by subjecting multiple single-colony-derived cultures to PFGE analysis. Up to three genetic variants were detected within 20 (58.8%) isolates, indicating that most primary isolates display a mosaic-like structure. The close relatedness of the genetic variants within an isolate was confirmed by multi-locus sequence typing. In contrast to the primary isolates, no genetic variants were detected within the progeny of 20 experimental clones generated *in vitro* from 20 primary isolates, suggesting that the variants were not induced *in vitro* during the procedure of PFGE analysis. Hence, the genetic variants within a primary isolate most likely originally emerged *in vivo*. Consideration of the mosaic structure of primary isolates is essential when interpreting typing studies on *B. henselae*.

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INTRODUCTION

Bartonella henselae is a Gram-negative, facultatively intracellular bacterium associated with a broad spectrum of chronic disease manifestations in humans, including cat scratch disease, bacillary angiomatosis, endocarditis and prolonged fever and bacteraemia (Anderson & Neuman, 1997; Jacobs & Schutze, 1998; Raoult *et al.*, 1996). The domestic cat represents the main host and reservoir for *B. henselae*. Infected cats develop a relapsing bacteraemia that may persist for up to 2 years (Breitschwerdt & Kordick, 2000). Previous investigations have shown that *B. henselae* induces specific humoral and cell-mediated immune responses in naturally or experimentally infected hosts, including cats, humans and mice (Abbott *et al.*, 1997; Arvand *et al.*, 1998a, 2001a; Chenoweth *et al.*, 2004; Guptill *et al.*, 1999; Regnath *et al.*, 1998). The mechanisms involved in the long-term survival of the bacteria despite vigorous host immune responses are not entirely understood. It has been postulated that *Bartonella* spp. may persist in an

intracellular niche, from where they are occasionally released to invade host cells (Dehio, 2001; Mandle *et al.*, 2005; Schulein *et al.*, 2001). However, the role of other factors contributing to the persistence of bartonellae in the infected host has not been studied in detail.

Generation of genetic and antigenic variants represents another strategy used by different pathogens such as *Trypanosoma* spp. (Dubois *et al.*, 2005), *Helicobacter pylori* (Kraft *et al.*, 2006) and *Anaplasma marginale* (Brayton *et al.*, 2005) to circumvent the host specific immune responses and maintain a persistent infection. Several studies suggest that *B. henselae* may display a marked genetic and antigenic variability. Kabeya *et al.* (2002) reported on genetic variability among *B. henselae* isolates obtained from recurrent bacteraemic peaks of naturally infected cats. However, due to the study design, they could not rule out the possibility of a reinfection of the cats with a different *B. henselae* strain. Maruyama *et al.* (2004) detected two different genetic variants among *B. henselae* cultures obtained from the lymph node of a patient with cat scratch disease. However, it was postulated that the patient

Abbreviation: MLST, multi-locus sequence typing.

was infected by two different *B. henselae* strains. Kyme *et al.* (2003) demonstrated that *B. henselae* may undergo phase variation *in vitro*, a process that is characterized by the emergence of antigenic variants. More recently, the complete genome of *B. henselae* was found to contain several regions with numerous short repeats in tandem that are prone to rearrangement and may be implicated in phase variation (Alsmark *et al.*, 2004).

We have recently demonstrated the coexistence of different genetic variants within the community of three primary *B. henselae* isolates from Germany by PFGE analysis of multiple single-colony-derived cultures (Arvand *et al.*, 2006). No variants were detectable among the cultures obtained from isolates with a long passage history, suggesting that the inhomogeneous structure was restricted to primary isolates. We hypothesized that many, if not all, primary *B. henselae* isolates may comprise different genetic variants that might emerge *in vivo*.

The present study was conducted to determine whether (i) all primary *B. henselae* isolates are composed of different genetic variants, and (ii) the genetic variants originally existed *in vivo* and were not induced during the procedure of PFGE analysis. To test these hypotheses, 34 primary *B. henselae* isolates from different geographical regions were evaluated by PFGE analysis of multiple single-colony-derived cultures. The relationship among the genetic variants within a distinct isolate was further evaluated by multi-locus sequence typing (MLST), which has been shown to be an accurate method for the evaluation of genetic relatedness among *B. henselae* isolates (Arvand & Viezens, 2007; Iredell *et al.*, 2003). In addition, 20 *in vitro*-generated clones of *B. henselae* were subjected as single-colony-derived cultures to PFGE analysis in order to determine whether genetic variants might also be detected within the progeny of *in vitro*-generated clones. Our results suggest that genetic variants occur frequently within primary *B. henselae* isolates and most likely emerge *in vivo*.

METHODS

Bacterial isolates

Primary isolates. Thirty-four primary *B. henselae* isolates collected by different investigators in various geographical regions were analysed (Table 1). All primary isolates were originally conserved as a subculture obtained from a sweep of colonies of the primary growth and stored without additional *in vitro* passages at -20°C or -80°C . The primary isolates were grown on Columbia blood agar with 5% sheep blood (CBA, Becton Dickinson) for 7–14 days at 37°C in 5% CO_2 . Four or five morphologically different or randomly selected colonies (colonies A–D or E) were picked from each isolate, inoculated to CBA and incubated for 7–10 days to obtain single-colony-derived cultures.

***In vitro* clones.** Experimental clones of *B. henselae* were generated by picking a single colony (e.g. colony B) of a primary isolate, as described above, and growing it to a pure culture (e.g. clone Gigi-B). The experimental clones were stored without additional *in vitro* passages at -20°C . An aliquot was thawed and grown on CBA for

7–10 days. From these cultures (e.g. Gigi-B), four or five single-colony-derived cultures were obtained (e.g. Gigi-B1 to B5) and subjected separately to PFGE analysis.

PFGE. PFGE was performed as described previously with some modification (Arvand *et al.*, 1998b). Bacteria were harvested from CBA, washed with 0.9% NaCl and embedded in 1% SeaPlaque GTG Agarose (BioWhittaker Molecular Applications). Restriction was performed with *Sma*I (Fermentas) according to the manufacturer's instructions. Electrophoresis was conducted in 1% Seakem GTG Agarose (BioWhittaker) in a CHEF DR III unit (Bio-Rad) at 5.5 V cm^{-1} with 3–14 s ramp times at 14°C for 22 h. Gels were stained with ethidium bromide and photographed with an INTAS Gel Jet imager (Intas Science Imaging Instruments). The lambda ladder PFGE marker (Cambrex Bioscience) was used as molecular mass standard in each gel. Banding patterns were analysed by visual comparison of the restriction patterns. A one-band difference was defined as the presence or absence of a specific band in the PFGE pattern.

MLST. MLST was performed as previously described (Arvand *et al.*, 2006; Iredell *et al.*, 2003). In brief, the partial sequences of eight genetic loci, including 16S rRNA-DNA, *batR*, *ftsZ*, *gltA*, *groEL*, *nlpD*, *ribC* and *rpoB*, were analysed. The PCR products were purified and sequenced on both strands with an ABI 3730 XL apparatus (AGOWA, DNA sequencing service, Berlin, Germany). The DNA sequences were analysed by using the DNASTAR Lasergene. Alleles and sequence types (STs) were assigned in accordance with the published data (Arvand & Viezens, 2007; Iredell *et al.*, 2003).

RESULTS

PFGE analysis reveals a mosaic-like structure in most primary isolates

In order to determine whether genetic variants may exist within all *B. henselae* isolates, 34 primary isolates from different geographical regions were analysed by PFGE. Differences in banding patterns were detected among the single-colony-derived cultures of 20 (58.8%) isolates (Fig. 1). Two distinct variants were detected within the progeny of 18 isolates, whereas three variants were found within the community of two isolates (Table 2). The PFGE patterns of the genetic variants of an isolate displayed one to three band differences. In detail, a one-band difference was observed within the single-colony-derived cultures of 10 isolates, while a two- and three-band difference was detected among the progeny of 9 and 2 isolates, respectively (Table 2). No genetic variants were detectable among the single-colony-derived cultures of 14 (41.2%) isolates.

MLST confirms that PFGE variants represent genetic variants within the progeny of a primary isolate

According to the interpretation criteria for the PFGE typing of bacterial pathogens (Tenover *et al.*, 1995), isolates displaying one to three band differences are considered to be closely related and represent a unique strain. Therefore, we hypothesized that the PFGE variants among the single-colony-derived cultures, which displayed one to three band differences and were each isolated from a

Table 1. Clinical source and geographical origin of the primary *B. henselae* isolates studied

Isolate	Clinical source	Geographical origin	Isolated by (Reference)
Berlin-1	Human, BA*	Germany, Berlin	Our group (Arvand <i>et al.</i> , 1998b)
Berlin-2	Cat	Germany, Berlin	Our group (Arvand <i>et al.</i> , 2001b)
K111	Cat	Germany, Berlin	Our group (Arvand <i>et al.</i> , 2001b)
K143	Cat	Germany, Berlin	Our group (Arvand <i>et al.</i> , 2001b)
K161	Cat	Germany, Berlin	Our group (Arvand <i>et al.</i> , 2001b)
FR96/BK74	Cat	Germany, Freiburg	A. Sander (Sander <i>et al.</i> , 1998)
FR96/BK75	Cat	Germany, Freiburg	A. Sander (Sander <i>et al.</i> , 1998)
FR96/BK78	Cat	Germany, Freiburg	A. Sander (Sander <i>et al.</i> , 1998)
FR96/BK79	Cat	Germany, Freiburg	A. Sander (Sander <i>et al.</i> , 1998)
8697/1	Cat	Italy, Pavia	M. Fabbi (Fabbi <i>et al.</i> , 2004)
123930/2	Cat	Italy, Pavia	M. Fabbi (Fabbi <i>et al.</i> , 2004)
118549/4	Cat	Italy, Pavia	M. Fabbi (Fabbi <i>et al.</i> , 2004)
G169	Cat	UK, south-west	R. Birtles (Birtles <i>et al.</i> , 2002)
G338	Cat	UK, south-west	R. Birtles (Birtles <i>et al.</i> , 2002)
G450	Cat	UK, south-west	R. Birtles (Birtles <i>et al.</i> , 2002)
G641	Cat	UK, south-west	R. Birtles (Birtles <i>et al.</i> , 2002)
Budda	Cat	USA, California	L. Guptill (Guptill <i>et al.</i> , 2004)
Levi	Cat	USA, California	L. Guptill (Guptill <i>et al.</i> , 2004)
Norman	Cat	USA, California	L. Guptill (Guptill <i>et al.</i> , 2004)
Saki	Cat	USA, California	L. Guptill (Guptill <i>et al.</i> , 2004)
Sunday	Cat	USA, California	L. Guptill (Guptill <i>et al.</i> , 2004)
Cody	Cat	USA, California	L. Guptill (Guptill <i>et al.</i> , 2004)
Kodie	Cat	USA, Chicago	L. Guptill (Guptill <i>et al.</i> , 2004)
Tascha	Cat	USA, Chicago	L. Guptill (Guptill <i>et al.</i> , 2004)
Aaron	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Gigi	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Rocket	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Zipper	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Buster Brown	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Sam	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Red Dog	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Molly	Cat	USA, Florida	L. Guptill (Guptill <i>et al.</i> , 2004)
Sweetie	Cat	USA, Washington DC	L. Guptill (Guptill <i>et al.</i> , 2004)
Tabatha	Cat	USA, Washington DC	L. Guptill (Guptill <i>et al.</i> , 2004)

*Cutaneous bacillary angiomatosis lesions of an HIV-infected patient.

unique host, most probably represented genetic variants within the community of a distinct strain, rather than a co-infection of the host by two or more different *B. henselae* strains. To test this hypothesis, two different single-colony-derived cultures from 10 primary isolates were subjected to additional typing by MLST, which is a robust, sequence-based typing technique for the analysis of genetic relatedness among *B. henselae* isolates. Both single-colony-derived cultures of each primary isolate studied displayed an identical sequence type, indicating that they belonged to the same strain (Table 3).

Genetic variants do not occur within the progeny of *in vitro*-generated clones of *B. henselae*

We next analysed whether the genetic variants observed within the community of a primary isolate were originally present *in vivo* or had emerged during the procedure of

PFGE analysis *in vitro*. Twenty experimental clones were obtained from 20 primary *B. henselae* isolates as described in Methods and four or five single-colony-derived cultures of each clone were subjected separately to PFGE analysis (Fig. 2). Genetic variants could not be detected within the progeny of any experimental clone (Table 4), indicating that the variants were not induced *in vitro* during the preparation of subcultures for PFGE analysis.

DISCUSSION

The data presented here indicate that primary *B. henselae* isolates worldwide are commonly composed of several distinct genetic variants. We believe that the variants, which were isolated from a unique host, do not represent different strains, because they display only one to three band differences in PFGE patterns, which strongly suggests

Fig. 1. Representative gel showing macrorestriction patterns of single-colony-derived cultures of five primary *B. henselae* isolates upon PFGE analysis. The isolate designations are shown above the brackets and the letters A–E refer to the single-colony-derived cultures of each isolate. λ , Lambda ladder PFGE marker.

that they are closely related to each other and represent one strain. In contrast, ‘unrelated’ strains usually display seven or more band differences (Tenover *et al.*, 1995). The close relatedness of the variants was confirmed by a second typing method, MLST, which is discriminatory for *B. henselae* to the strain level. The frequency of genetic variants within the community of a unique isolate appears to be high, since two or three different variants were detectable among the progeny of approximately 59 % of the isolates by screening four or five single-colony-derived cultures of each isolate. Although genetic variants were not found among the single-colony-derived cultures of 41 % of the isolates studied, we can not exclude that they might have existed within those isolates but remained undetected because of the limited sensitivity of our screening method.

Most primary isolates included in this study were of feline origin. Only one human isolate was available as a primary isolate and evaluated. Different genetic variants were observed within most feline isolates and the only human-derived isolate analysed, suggesting that a mosaic-like genetic structure is a feature shared by feline and human *B. henselae* isolates. It is noteworthy that all primary isolates investigated here were originally conserved as a subculture from a sweep of colonies of the primary growth, which seems to be crucial for the conservation of the mosaic-like structure of *B. henselae* isolates *in vivo*.

We further analysed the prevalence of genetic variants within the progeny of *in vitro*-generated clones of *B. henselae* in order to rule out that the genetic variants observed above were induced by one or two *in vitro* passages or a freeze-and-thaw cycle, which are prerequisites for PFGE analysis. We could not detect any variants among the progeny of the *in vitro* clones, indicating that the genetic variants within primary isolates must have

originally emerged in the mammalian host and were not artificially induced *in vitro*.

Generation of genetic and antigenic variants may contribute to the persistence of a pathogen in the infected host or in the community in different ways. Emergence of genetic variants may lead to a more effective adaptation to an individual host and specific niches within a host, as shown for *Helicobacter* and *Bacteroides* (Kraft *et al.*, 2006; Cerdeno-Tarraga *et al.*, 2005). Antigenic variation may alter surface-coat proteins and contribute to the evasion of specific immune responses such as B cell recognition (Dubois *et al.*, 2005), which can result in prolonged bacteraemia and a more effective transmission to other hosts. Our results suggest that *B. henselae* undergoes genetic and therefore may undergo antigenic variation in the mammalian host. They are in accordance with recent data of Lindroos *et al.* (2006) that revealed evidence for extensive rearrangements within the *B. henselae* genome. It is likely that the variations observed in our study are caused by genetic rearrangement. Further investigation is required to identify the genetic loci and antigenic structures that are predominantly affected by these events.

The fact that primary *B. henselae* isolates are composed of different genetic variants might be of particular importance for the interpretation of future typing studies on *B. henselae*. We found up to three different genetic variants within a unique isolate. A careful interpretation of the PFGE data by using the guidelines for interpretation of PFGE typing results was necessary to obtain a reliable classification of different PFGE patterns to the variant, isolate or strain level. Different genetic variants within the community of *B. henselae* isolates might also be detectable by other typing techniques, especially those based on analysis of repetitive elements or hypervariable genomic regions such as multi-spacer typing or variable number of tandem repeats typing. Therefore, a careful interpretation of the latter typing results and comparison with standard typing methods such as PFGE or MLST is strongly recommended to ensure a correct classification of different patterns to the variant, isolate or strain level.

Consideration of the mosaic-like structure of primary isolates might also be important for studies on pathogenicity of *B. henselae*, since different variants could vary in production of virulence factors. Furthermore, it is possible that a distinct variant may eventually overgrow the other variants within the community of an isolate under certain environmental conditions, giving rise to a high-passage isolate with different genetic or antigenic properties than the original one. This could explain the observation of several investigators who found that the copy of the Houston-1 isolate used in their laboratory was different from the previously described Houston-1 isolate (Alsmark *et al.*, 2004; Kyme *et al.*, 2003; Riess *et al.*, 2007). Further studies are required to test this hypothesis, e.g. by monitoring the genetic alterations within the progeny of a primary *B. henselae* isolate upon serial *in vitro* passages.

Table 2. Summary of PFGE results of single-colony-derived cultures obtained from primary *B. henselae* isolates

Isolate	No. of cultures analysed	Detection of genetic variants	No. of genetic variants	No. of band differences*
Berlin-1	4	yes	2	1
Berlin-2	4	yes	2	1
K111	4	yes	2	1
K143	5	no	–	–
K161	5	yes	2	3
FR96/BK74	5	yes	2	1
FR96/BK75	5	yes	2	2
FR96/BK79	5	no	–	–
FR96/BK79	5	yes	2	1
8697/1	5	yes	2	1
123930/2	4	yes	2	1
118549/4	4	yes	3	2, 3
G169	4	yes	2	2
G338	5	no	–	–
G450	5	yes	2	1
G641	5	no	–	–
Budda	5	no	–	–
Levi	5	no	–	–
Norman	4	no	–	–
Saki	4	yes	2	1
Sunday	5	yes	2	2
Cody	5	no	–	–
Kodie	4	yes	2	2
Tascha	4	no	–	–
Aaron	5	no	–	–
Gigi	5	yes	2	2
Rocket	5	yes	2	2
Zipper	4	yes	2	2
Buster Brown	5	no	–	–
Sam	5	yes	2	1
Red Dog	5	yes	3	2, 2
Molly	5	no	–	–
Sweetie	5	no	–	–
Tabatha	5	no	–	–

*Difference between the PFGE pattern of a variant compared to the colony A-derived culture.

Table 3. Summary of MLST data of duplicate single-colony-derived cultures obtained from 10 primary *B. henselae* isolates

Isolate	Cultures analysed	16S RNA	<i>batR</i>	<i>gltA</i>	<i>ftsZ</i>	<i>groEL</i>	<i>nlpD</i>	<i>ribC</i>	<i>rpoB</i>	ST*
Berlin-1	A, C	1	1	1	1	1	1	1	1	1
Berlin-2	A, C	2	2	3	2	1	2	4	1	7
8697/1	A, B	1	1	1	1	1	1	1	1	1
123930/2	A, B	1	1	1	1	1	1	1	1	1
Sunday	A, B	1	1	1	1	1	1	1	1	1
Cody	A, C	1	1	1	1	1	1	1	1	1
Buster Brown	A, B	2	1	1	1	2	1	1	1	5
Sam	A, B	1	1	1	1	1	1	1	1	1
Red Dog	A, D	1	1	1	1	1	1	1	1	1
Sweetie	A, B	2	1	1	1	2	1	1	1	5

*ST, sequence type.

Fig. 2. Representative gel showing PFGE patterns of single-colony-derived cultures of five experimentally generated *B. henselae* clones. The designation of the *in vitro* clones is shown above the brackets and the numbers 1–5 refer to the single-colony-derived cultures of each clone. λ , Lambda ladder PFGE marker.

In summary, our results indicate that different genetic variants may coexist within the community of most primary *B. henselae* isolates. They probably emerge *in vivo* and could serve as an escape mechanism to circumvent the host specific immune responses and maintain a chronic infection. Consideration of the coexistence of genetic

Table 4. Summary of PFGE results of the single-colony-derived cultures obtained from *in vitro* clones of *B. henselae*

No genetic variants were detected within the progeny of any of these clones.

<i>In vitro</i> clone	Cultures analysed
Berlin-1-A	A1, A2, A3, A4
Berlin-2-C	C1, C2, C3, C4
K111-D	D1, D2, D3, D4
K161-A	A1, A2, A3, A4, A5
FR96/BK74-C	C1, C2, C3, C4, C5
FR96/BK75-D	D1, D2, D3, D4, D5
FR96/BK79-E	E1, E2, E3, E4, E5
8697/1-A	A1, A2, A3, A4, A5
123930/2-B	B1, B2, B3, B4, B5
118549/4-C	C1, C2, C3, C4, C5
G169-A	A1, A2, A3, A4, A5
G338-D	D1, D2, D3, D4, D5
G450-B	B1, B2, B3, B4, B5
G641-F	F1, F2, F3, F4, F5
Saki-E	E1, E2, E3, E4, E5
Kodie-B	B1, B2, B3, B4, B5
Zipper-F	F1, F2, F3, F4, F5
Gigi-B	B1, B2, B3, B4, B5
Rocket-A	A1, A2, A3, A4, A5
Sam-B	B1, B2, B3, B4, B5

variants within primary isolates is essential when interpreting typing studies on *B. henselae*.

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