Characterization of Polish feline *B. henselae* isolates by multiple-locus tandem repeat analysis and pulse-field gel electrophoresis

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Abstract

Knowledge about molecular epidemiology of *B. henselae* is important for recognizing the geographical distribution of strains and identification of isolates virulent for humans. Eleven Polish feline *B. henselae* isolates were typed, using 2 different techniques: pulse-field gel electrophoresis (PFGE) and multiple-locus variable-number tandem repeat analysis (MLVA). PFGE analysis distinguished 6 different PFGE types, with subtypes within 3 of them, whereas 10 MLVA types were assigned. Global diversity index (D.I.) for MLVA equaled 0.93. For 7 isolates, the results of MLVA confirmed cluster assignments based on PFGE. Both PFGE and MLVA results were in accordance with epidemiological data. Although PFGE has been previously demonstrated to be a suitable method for the differentiation of *B. henselae* isolates/strains, our results show the superiority of MLVA over PFGE with respect to higher discriminatory power, distinguishing genotypes I and II isolates, easier analysis of results, and possibility to compare the numerical data obtained by different laboratories. With MLVA, 7 new profiles were observed, compared to previous results from around the world; whereas 3 known profiles were previously described mainly in European *B. henselae* isolates. Our results confirm that some VNTR profiles can be used as specific geographical markers.

Key words

*Bartonella henselae*, genotyping, MLVA, PFGE

INTRODUCTION

Among the 27 recognized *Bartonella* species, 13 are pathogenic for humans. Animals are usually the asymptomatic carriers of the bacteria, but some disorders of *Bartonella* etiology have been reported, especially in carnivores [1]. *B. henselae* is a common zoonotic bacterial agent, acquired from accompanying animals, mainly cats, which usually present as asymptomatic bacteremia, and their reservoirs. Depending on the country, the prevalence of *B. henselae* in cats varies from 1-56% [2]. In a previous study carried out in the Mazowieckie Voivodship in northeastern Poland, *B. henselae* was isolated from 10% of the tested cats; whereas in a study performed in western France, *B. henselae* was isolated from 1-5% of the tested cats, whereas specific antibodies were present in 45% of these animals [3]. *B. henselae* is the agent of cat scratch disease (CSD), and has also been implicated in other clinical disorders in humans, such as bacillary angiomatosis and peliosis hepatitis in immunosuppressed patients, endocarditis, osteomyelitis, isolated fever, and some neurological forms, e.g. meningitis and encephalitis [2]. Such a wide spectrum of clinical manifestations in humans, but not commonly in cats, raises the question whether some isolates are more likely than others to infect humans, and to cause a particular type of disease. To date, the factors underlying the differences in the pathogenicity of some isolates/genotypes in humans remain unknown.

Various typing methods have been used to determine the genetic relationships among different *B. henselae* isolates. The first level of differentiation of *B. henselae* isolates/strains was achieved using 16S rRNA genotyping, and allowed the characterization of 2 genotypes: type I – Houston-1, and type II – Marseille [4,5]. These types differ from each other in 3 nucleotides of the 16S rRNA encoding gene. Further studies led to sub-classification of genotype I strains into variants Houston-1 and ZF-1, and of genotype II strains into variants Marseille and CAL-1 [6]. This method did not allow identification to a significant level of polymorphism among *B. henselae* strains/isolates.

Much more genetic heterogeneity among *B. henselae* isolates was demonstrated using subsequently developed typing techniques. The use of pulsed-field gel electrophoresis (PFGE) for the typing of *B. henselae* [7,8], for many years was the most powerful method for intraspecies differentiation. Other typing methods, such as ERIC-PCR (enterobacterial repetitive intergenic consensus) or IRS-PCR (infrequent restriction site), were applied only occasionally for *B. henselae* genotyping, with poor results [9]. Plasmids have not been described for *B. henselae*, which excluded plasmid-associated typing methods [9]. More recently, new techniques based on sequencing of small genomic sequences, either house keeping genes (MLST – multilocus sequence typing) [10] or intergenic sequences (MST – multispacer typing [11], have been developed. The last typing technique, successfully applied to differentiate *B. henselae* isolates/strains, is multiple-locus VNTR analysis (MLVA) [12]. This method is based on the polymorphism of repetitive elements known as variable-number tandem repeats (VNTR).
The aims of this study were to estimate the diversity of Polish feline isolates (intrinsic and with reference to the data available from other countries), and to compare the discriminatory power of MLVA vs PFGE for *B. henselae* typing with the same set of isolates.

**MATERIAL AND METHODS**

Isolates and culture. Eleven feline *B. henselae* isolates, collected in Poland between February 2004 - May 2005, were selected. The cats originated from urban areas of Warsaw and its suburbs. Two isolates were from the same household. The reference strain Houston 1 – ATCC 49882 was included in the study as a positive control for MLVA studies. Before typing by PFGE or MLVA, the isolates/strains were cultivated on chocolate agar plates with sheep blood supplemented with Vitox (Choc V, Oxoid) at 37°C in 5% CO₂ – atmosphere for 9 days.

16S rRNA type-specific PCR. Reaction conditions and primers BH1 and BH2 in conjunction with the broad-host-range primer 16SF were used, according to the method of Bergmans et al. [4].

PFGE. Bacteria were harvested from chocolate agar suspended in SE buffer (75 mM NaCl, 25mM EDTA, pH 8.0) and centrifugated at 8,000 g for 10 min. Agarose blocks were prepared by adding 200 μl of cell suspension in SE to 200 μl of 2% PFGE agarose (Bio-Rad). The solidified blocks were incubated in lysis buffer (50 mM TRIS, 50mM EDTA, 1% Sarcosyl, pH 8.0 and 0.1mg per ml proteinase K) at 54°C for 4 hours. After washing with TE buffer (10 mM TRIS, 1mM EDTA, pH 8.0) blocks were incubated with 60 U *Sma I* at 25°C overnight (MBI Fermentas, Lithuania). PFGE was undertaken with CHEF DR II electrophoresis unit (BioRad, Munich, Germany). Agarose gel (1%) was prepared in 0.5 x TBE running buffer. Electrophoresis was performed for 27 hours at 6 V/cm with pulse times from 3-12 at 11°C. Pulse Marker 50-1,000 kb (Siga-Aldrich, Saint Louis, USA) was used as a molecular weight standard. Agarose gels were stained with ethidium bromide.

Macronrestriction profiles were interpreted, as described by Tenover et al. [13]. Isolates were classified to the same type and considered closely related if there were no differences in more than 1-3 fragments in their PFGE patterns. An isolate was classified as unrelated if there were differences in more than 4 fragments. Such isolates were reported as belonging to separate types.

MLVA. Bacteria were scraped from the agar plates and suspended in 500 μl sterile water. The suspensions were boiled for 10 min and centrifugated at 3,000 g for 15 minutes. Amplifications were conducted for 5 VNTRs, called BHV-A, BHV-B, BHV-C, BHV-D, BHV-E (*B. henselae* VNTR), as previously described by Monteil et al. [12]. Platinum Pfx DNA polymerase (Invitrogen) was applied. PCR was performed under the following conditions: 40 cycles of denaturation for 30s at 94°C, annealing 30s at 50°C for BHV-A, BHV-B, BHV-C, BHV-D, and at 53°C for BHV-E, extension at 72°C for 1 min, and final elongation at 72°C for 7 min. *B. henselae* H1 strain was used as a positive control.

The PCR products were separated on 1-2% agarose gel and visualized by staining with ethidium bromide. For BHV-A and BHV-B, long gels (30 cm) and long migration times (up to 27 hrs) were used. A combination of different molecular markers from 100 bp to 1 kb was applied. The sizes of the amplicon products obtained were translated to repeat copy numbers for each isolate. The determined alleles corresponded to a given number of repeat units for BHV/locus, expressed as the ratio of size of obtained amplicon to the known basic unit length for a particular locus [12]. For incomplete units, the calculated values were rounded up or down to the closest whole number, which means numbers with decimal fraction ≥ 0.5 were regarded as the next integer, e.g. 19.8 was considered to be 20 whereas numbers with decimal fraction ≤ 0.4 were regarded as the closest preceding integer, e.g. 9.3 was regarded as 9.

For the evaluation of discriminatory power of the applied MLVA scheme typing for all Polish isolates, discrimination index (D.I.) was evaluated [14].

Comparison with other *B. henselae* isolates/strains. Comparison of the VNTR profiles of the Polish *B. henselae* isolates was performed against 178 isolates/strains previously tested with the MLVA method [12,15].

**RESULTS**

16S rRNA type-specific PCR. Ten of the 11 isolates were delineated to 16S rRNA type II, and only one isolate belonged to genotype I (Tab. 1).

PFGE. Digestion of DNA from *B. henselae* isolates/strains with *Sma I* led to 14 - 22 chromosomal fragments for each isolate. The molecular sizes of the majority of the *Sma I* fragments ranged between 20 - 200 kb. Among the 11 examined isolates, 9 different restriction patterns were observed (Fig. 1). According to Tenover’s criteria, they

![Figure 1. Sma I pulsed-field gel electrophoresis of 11 Polish *B. henselae* isolates.](image-url)
Table 1. Typing results of feline Polish B. henselae isolates with MLVA and PFGE

<table>
<thead>
<tr>
<th>Cat/Strain ID #</th>
<th>Date of blood collection</th>
<th>Age</th>
<th>Location</th>
<th>16S rRNA Genotype</th>
<th>BHV A</th>
<th>BHV B</th>
<th>BHVC</th>
<th>BHV D</th>
<th>BHV E</th>
<th>MLVA group</th>
<th>Profile*</th>
<th>PFGE type (subtype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>27.08.03</td>
<td>5 years</td>
<td>Warsaw</td>
<td>II</td>
<td>9</td>
<td>14</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>A</td>
<td>New</td>
<td>Type A (A1)</td>
</tr>
<tr>
<td>130</td>
<td>15.01.05</td>
<td>8 years</td>
<td>Józefów</td>
<td>II</td>
<td>9</td>
<td>15</td>
<td>18</td>
<td>1</td>
<td>1</td>
<td>A</td>
<td>New</td>
<td>Type A (A1)</td>
</tr>
<tr>
<td>129</td>
<td>15.01.05</td>
<td>8 years</td>
<td>Józefów</td>
<td>II</td>
<td>9</td>
<td>15</td>
<td>18</td>
<td>1</td>
<td>1</td>
<td>A</td>
<td>Europe</td>
<td>Type A (A1)</td>
</tr>
<tr>
<td>159</td>
<td>22.03.05</td>
<td>1 year</td>
<td>Warsaw</td>
<td>II</td>
<td>9</td>
<td>15</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>A</td>
<td>Europe</td>
<td>Type A (A2)</td>
</tr>
<tr>
<td>154</td>
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<td>1 year</td>
<td>Otwock</td>
<td>II</td>
<td>12</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>B</td>
<td>New</td>
<td>Type B</td>
</tr>
<tr>
<td>28</td>
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<td>1 year</td>
<td>Warsaw</td>
<td>II</td>
<td>10</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>A</td>
<td>Europe</td>
<td>Type C (C1)</td>
</tr>
<tr>
<td>150</td>
<td>unknown</td>
<td>6 years</td>
<td>Warsaw</td>
<td>II</td>
<td>9</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>A</td>
<td>Europe + USA</td>
<td>Type C (C2)</td>
</tr>
<tr>
<td>12</td>
<td>20.01.04</td>
<td>7 months</td>
<td>Warsaw</td>
<td>II</td>
<td>11</td>
<td>20</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>B</td>
<td>New</td>
<td>Type D</td>
</tr>
<tr>
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<td>05.04.05</td>
<td>8 years</td>
<td>Józefów</td>
<td>II</td>
<td>14</td>
<td>24</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>B</td>
<td>New</td>
<td>Type E</td>
</tr>
<tr>
<td>13</td>
<td>27.01.04</td>
<td>1 year</td>
<td>Warsaw</td>
<td>I</td>
<td>12</td>
<td>24</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>B</td>
<td>New</td>
<td>Type F (F1)</td>
</tr>
<tr>
<td>163</td>
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<td>6 months</td>
<td>Warsaw</td>
<td>II</td>
<td>12</td>
<td>23</td>
<td>18</td>
<td>5</td>
<td>3</td>
<td>B</td>
<td>New</td>
<td>Type F (F2)</td>
</tr>
</tbody>
</table>

Remarks:
- Cats 3 and 150 lived in the same shelter.
- Cats 129 and 130 belonged to the same owner and lived in the same household.
- * Profile: New - never described before; Europe - already found in Europe; Europe + USA - already found in Europe + USA [4].

DISCUSSION

MLVA revealed a high diversity among the feline B. henselae isolates from Poland. Ten MLVA profiles of the 11 tested B. henselae isolates were assigned, and the diversity index was very high (0.93) at the scale of only 11 isolates. Macrorestriction endonuclease analysis of genomic DNA by Sma I PFGE revealed the presence of 6 types among the same isolates.

Both MLVA and PFGE showed high discriminatory power. However, each of these typing methods resulted in different clustering of the isolates, with the exception of 2 isolates originating from the 2 cats living in the same household.
that were typed to the same cluster by both techniques. The congruence between epidemiological data and the MLVA profiles of the tested isolates proves also the high reliability of the technique. Our results are in accordance with other studies in which the same clusters were defined when PFGE and MLVA were applied on groups of isolates from owners and their cats. This demonstrates the reliability of MLVA for molecular epidemiological studies [16]. Furthermore, MLVA enables a clear separation between 16S rRNA genotypes I and II, since no MLVA profile was shared by 16S rRNA genotype I and genotype II isolates [15].

Although PFGE has been previously shown to be a suitable method for differentiation of *B. henselae* isolates/strains [17], the results of our study confirm the superiority of the MLVA technique over PFGE, as already underlined by Bouchouicha et al. [15], in such points as higher discriminatory power, distinguishing between 16S rRNA genotypes I and II isolates, and ease of interpretation of the results. MLVA provides simple numerical data that enable comparison of BHV profiles of isolates/strains obtained by different laboratories. Conversely, one major problem of PFGE is lack of a standardized method that would determine bacterial concentration, selection of the appropriate digestion enzyme, and optimization of the electroforetic parameters. In addition, interpretation and exchange of PFGE-typing data is complicated because it depends on banding patterns and subjective decision regarding the true existence of discrete bands. Furthermore, some genes of *B. henselae* can mutate frequently, and this phenomenon influences the PFGE results by changing the restriction place for the enzyme restriction pattern obtained in PFGE for *Bartonella*, as shown by Xu et al. [18]. This has also been illustrated by Chang et al. [18], who compared the PFGE profiles of isolates obtained simultaneously from one human patient and his 7 cats. The profiles were close, but differences could be observed. However, when tested by MLVA, identical profiles were observed [15,16].

The present results are of interest when compared to data obtained from other parts of the world. The feline isolates from Poland appear very diverse, as many as 10 different MLVA profiles were obtained from 11 isolates. Indeed, these isolates are limited in number and are far from being representative of Polish *B. henselae* isolates. Nevertheless, 3 of the profiles correspond to profiles already observed in Europe, of which one appears predominant in Europe (17.5% of the European feline profiles) and has never been described outside Europe [15]. As these 3 profiles are close to each other, and as the structure of *B. henselae* populations is still considered to be clonal, even though horizontal transfer can occur [7], this suggests that a European diffusion of some related clones or local emergences of derived subpopulations from a parental clone, originally present somewhere in Europe, have taken place in the past and could be used as specific geographic markers. Such data will need to be confirmed through analysis of a larger and more representative sample of isolates.

Our results also tend to confirm the geographical predominance of the 16SrRNA genotype II and group A in European cats, as the majority of the Polish isolates were delineated to this type and to this group. 16S rRNA genotype I isolates may be more virulent for humans than genotype II variants, as 16S genotype I was more frequently detected than genotype II *B. henselae* in human samples, especially in lymph nodes from CSD patients, even in countries where genotype II is more frequent in cats than genotype I [9,20], and in severe forms of bacillary angiomatosis [19]. However, this relationship was not systematically confirmed [6]. In Western Europe, Australia, and the Western United States, feline isolates mostly belong to the 16SrRNA genotype type II, whereas in Asia (Japan, Philippines), they mainly belong to 16S rRNA genotype I [1]. These 2 genogroups can be delineated by MLVA, and isolates belonging to group A may not be zoonotic [15], as the zoonotic potential of *B. henselae* isolates could vary according to their genogroup [7,10,19]. In view of previous results, it would be interesting to investigate whether: a) human *B. henselae* isolates in Poland also belong to group B; b) human cases are associated to restricted genetic diversity; c) the most severe clinical cases are associated to genotype I.

At present, MLST or MLVA seem to be the most appropriate tools for genetic differentiation of *B. henselae* isolates present in human samples. MLST data are unambiguous and can be easily transferred electronically between laboratories. Both MLST and MLVA can potentially be applied directly to clinical specimens and are not strictly dependent on culture. The problems with MLST are related to the lack of *Bartonella* MLST web site (in preparation) and the high cost of sequencing [7]. MLVA overcomes these limitations. In addition, MLVA demonstrates a higher discriminatory power as it was able to distinguish isolates within those found to be identical by MLST [16].

**CONCLUSIONS**

Our results confirm that some VNTR profiles can be used as a very efficient molecular tool for differentiation *B. henselae* strains. Unfortunately, the use of MLVA for *B. henselae* typing has not yet been adopted by laboratories that have access to human samples. Collaboration between physicians and veterinarians is therefore needed in order to answer the numerous questions about *B. henselae* determinants for zoonotic potential and virulence, both in humans and animals.

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**REFERENCES**