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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 acompaning 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 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.

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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 (intrinsically 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% CO_2 – 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 (Sigma-Aldrich, Saint Louis, USA) was used as a molecular weight standard. Agarose gels were stained with ethidium bromide.

Macrorestriction 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 its restriction pattern differed from other isolates in 4 or more 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 centrifuged 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 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 consider 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 *SmaI* 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

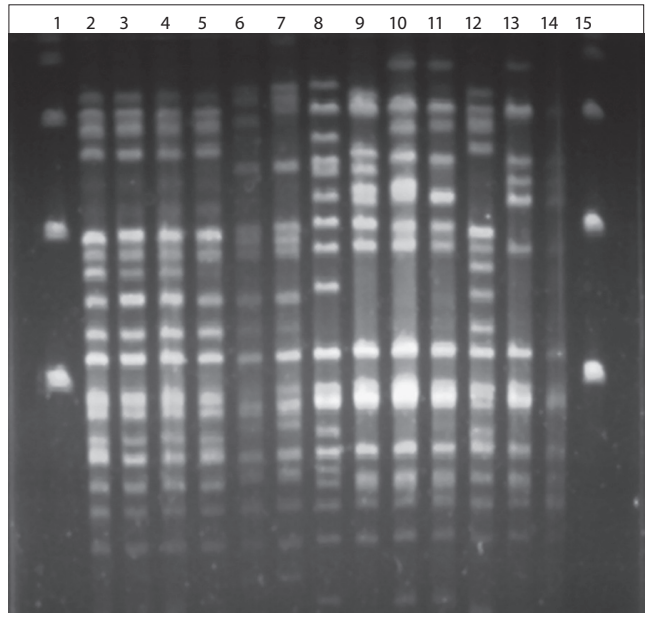

Figure 1. *Sma I* pulsed-field gel electrophoresis of 11 Polish *B. henselae* isolates.

Line 1 Pulse Marker 50-1,000 kb, Line 2 isolate 3 (type A subtype A1), Line 3 isolate 130 (type A subtype A1), Line 4 isolate 129 (type A subtype A1).
Line 5 isolate 159 (type A subtype A2), Line 6 isolate 28 (type C subtype C1), Line 7 isolate 150 (type C subtype C2), Line 8 isolate 12 (type D).
Line 9 isolate 30 (type E), Line 10 isolate 13 (type F subtype F1), Line 11 isolate 163 (type F subtype F2), Line 12 isolate 3 (type A subtype A1).
Line 13 isolate 154 (type B), Line 14 - , Line 15 Pulse Marker 50-1,000 kb.

Table 1. Typing results of feline Polish *B. henselae* isolates with MLVA and PFGE

Cat/Strain ID #	Date of blood collection	MLVA										PFGE type (subtype)
		Age	Location	16S rRNA Genotype	BHV A	BHV B	BHVC	BHV D	BHV E	MLVA group	Profile*	
3	27.08.03	5 years	Warsaw	II	9	14	5	1	2	A	New	Type A (A1)
130	15.01.05	8 years	Józefów	II	9	15	18	1	1	A	New	Type A (A1)
129	15.01.05	8 years	Józefów	II	9	15	18	1	1			Type A (A1)
159	22.03.05	1 year	Warsaw	II	9	15	2	1	1	A	Europe	Type A (A2)
154	unknown	1 year	Otwock	II	12	15	2	2	4	B	New	Type B
28	21.04.04	1 year	Warsaw	II	10	14	2	2	1	A	Europe	Type C (C1)
150	unknown	6 years	Warsaw	II	9	15	2	2	1	A	Europe + USA	Type C (C2)
12	20.01.04	7 months	Warsaw	II	11	20	10	1	2	B	New	Type D
30	05.05.04	8 years	Józefów	II	14	24	10	7	3	B	New	Type E
13	27.01.04	1 year	Warsaw	I	12	24	2	5	3	B	New	Type F (F1)
163	30.03.05	6 months	Warsaw	II	12	23	18	5	3	B	New	Type F (F2)

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].

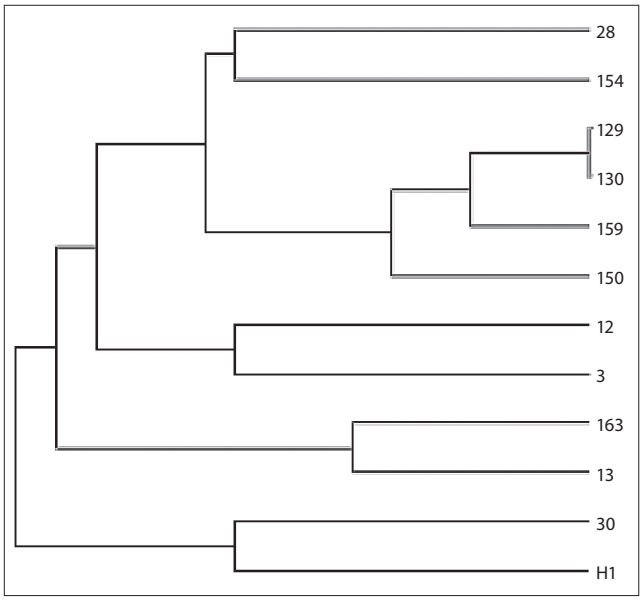

Figure 2. Clustering analysis of 11 *B. henselae* isolates by MLVA using five mark

were considered as 6 types and reported as A, B, C, D, E, F. Among types A, C, F, different subtypes were identified and designated subtypes A1, A2 and C1, C2 and F1, F2 (Tab. 1).

MLVA. VNTR analysis of the 11 Polish *B. henselae* isolates led to the identification of 10 MLVA types (Tab. 1, Fig. 2). The technique revealed a high level of polymorphism between isolates as 4 - 5 alleles per locus were observed with the 5 tested BHVs. The Global Diversity Index D.I equaled 0.93. For 7 of the 11 isolates, the results of MLVA confirmed the cluster assignments based on the PFGE technique, especially for PFGE types B, D, E, which were represented by single isolates. However, some isolates classified by PFGE as subtypes were not related according to their MLVA profile; for example, this was the case for strains 28 and 150, classified as PFGE subtypes C1 and C2, and for strain 3, which belonged to the same PFGE subtype (A1) as strains 129 and 130 (Tab. 1). Only subtypes F1 and F2 were recognized as being closely related by both techniques. Interestingly, one feline isolate displayed a profile very closely related to the one of *B. henselae* H1 strain, which is pathogenic for humans. Comparison of VNTR profiles of the Polish *B. henselae* isolates with 178 isolates/strains previously tested with the MLVA method, revealed for 2 VNTR the presence of alleles that had never been previously described: allele 18 in BHV C and alleles 23 and 24 in BHV B. The alleles' combination for the 5 VNTR generated 7 new profiles for the 11 isolates. Three already described profiles for BHV A-B-C-D-E (9-15-2-1-1, 10-14-2-2-1 and 9-15-2-2-1) corresponded to 3 common European VNTR profile for feline isolates. Interestingly, the 10-14-2-2-1 profile is the one most frequently detected in cats from European countries, mainly Denmark, France and Germany (17.5% of the profiles). This profile and profile 9-15-2-2-1 have been exclusively detected to date in European *B. henselae* isolates, whereas some isolates with the 9-15-2-1-1 profile have been detected in the USA.

Comparison of MLVA *vs* PFGE. Isolate #13, which was the only one with genotype I, was indistinguishable by *Sma I* pulsed-field electrophoresis from others isolates, but it had a unique VNTR profile.

Both results of PFGE and MLVA were in accordance with epidemiological data: 2 isolates (129 and 130) originated from 2 cats living in the same household; the animals had blood collected on the same day. These 2 isolates had the same VNTR profile and belonged to PFGE subtype A1.

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 electrophoretic 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 places 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 sub-populations 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 16 S 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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