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Development of a *16S rRNA* PCR-RFLP Assay for Bartonella Identification: Applicability in the Identification of Species Involved in Human Infections

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Abstract We designed a *16S rRNA* gene PCR-RFLP scheme to identify all currently described *Bartonella* spp. The *16S rRNA* genes of all *Bartonella* spp. were *in-silico* analyzed in order to design a RFLP technique able to discriminate among different species. The restriction enzymes selected were *Mae*III, *Mse*I, *Sau96*I, *Bsa*AI, *Drd*I, *Fok*I, *Bss*HII, *Bst*UI, *Alu*I, *Tsp*DTI and *Hph*I which, according to a decision-making tree, facilitated the differentiation of all the currently described species of *Bartonella*.The technique was experimentally tested in different species of *Bartonella*, including human pathogenic *B. bacilliformis* and *B. henselae* with a 100% of concordance with the *in-silico* predicted patterns.This novel RFLP assay could be used to identify both human and non-human pathogenic *Bartonella* in diagnostic, phylogenetic and epidemiologic studies.

Keywords *Bartonella*, PCR-RFLP, *16s rRNA* Gene, Identification

1. Introduction

Bartonella spp. are vector-transmitted Gram-negative bacteria which are members of the α-proteobacteria group phylogenetically close to the Rickettsiie, as well as *Brucella* spp., *Agrobacterium* spp., and *Afipia* spp. [1-3].

The number of species of the *Bartonella* genus described has been raised greatly in the last years. To date more than 29 species belonging to the genera *Bartonella* have been described most of them as a cause of infections in humans and animals [1,4-10]. Thus, *Bartonella bacilliformis* is the causative agent of Carrion's disease, a two-phase human infectious disease (acute or hematic phase, known as Oroya Fever that may be fatal in the absence of treatment, and an eruptive or tissue phase, known as Peruvian Wart) which is endemic in Andean areas of Peru, Ecuador and Colombia [9]. Another relevant illness due to *Bartonella* species are Cat-Scratch disease by *Bartonella henselae* or Trench Fever due to *Bartonella quintana.* Additionally a series of other species such as *Bartonella grahamii*, *Bartonella bovis* or *Bartonella rochalimae* among others have been isolated as a causative of different infections [4, 9, 11, 12].

Currently, standard identification techniques for *Bartonella* spp. include serology methodologies, culture techniques to recover viable organisms or PCR detection of specific sequences from DNA in blood and tissue samples [4]. However, these techniques are cumbersome, or do not provide univocal identification or are only able to detect some specific *Bartonella* spp. among other limitations [4]. Additionally, the members of the *Bartonella* genus have a fairly neutral biochemical profile, except for the production of peptidases, which may vary among species [11]. In absence of molecular studies, this fact may results in incorrect identification problems. The more suitable molecular tools, as DNA sequencing or MALDI-TOFF [13, 14] remains unavailable in a long series of areas from low and middle income countries, especially in those placed far of main cities. In these areas a gap in the knowledge of etiological causes of febrile syndromes is present. This lack of technical resources usually results in clinical symptoms based diagnosis [14], which may drive to misdiagnosis [14]. Thus, the development of techniques which will be able to be

implemented in regional health centers will be a valuable tool in order to determine the relevance of the role of *Bartonella* spp. as a cause of illness in rural low and middle income areas.

Amplification of conserved genes (as *16S rRNA* or *rpoB* genes) followed by PCR-products digestion and the analysis of the resulting Restriction Fragment Length Polymorphisms (RFLP) has been successfully used to identify different bacterial genus at a species level [16-18]. In this line, the construction of a decision-making tree [19] with a series of consecutive restrictions, leading to a specific RFLP pattern in one of the consecutive steps has the advantage of early discrimination of species of special interest.

Although different authors designed PCR-RFLP assays for a rapid identification of different *Bartonella* spp. in the 90's [7, 20], these studies did not evolve in parallel with the description of new *Bartonella* species and are currently unable to discriminate between a series of newly described species. However, the PCR-RFLP is a flexible technique that may be modified incorporating the current and future newly described species, by selecting new restriction enzymes, or by adding a novel species in its own unique branch in one of the described restrictions of the decision-making tree.

In the present work, we designed an alternative *16S rRNA* gene PCR-RFLP scheme to identify 29 currently described *Bartonella* spp. easily applicable to *Bartonella* spp. with clinical interest such as *B. bacilliformis, B. quintana* or *B. henselae.*

2. Materials and Methods

2.1. Bioinformatic Analysis of the Restriction Sites for *16S rRNA* in *Bartonella* spp.

Representative DNA sequences of *Bartonella* spp. *16S rRNA* genes were selected from GenBank (Table 1). Regions of 1452 bp, flanked by the oligonucleotides 8F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1510R (5´-GGTTACCTTGTTACGACTT-3') [18] were aligned and visualized using ClustalW (http://www.clustal.org) and Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) software respectively. In some sequences, the end regions were lack, in these cases these regions were considered as conserved.

In-silico analysis for the restriction sites in the *16S rRNA* gene was carried out using Restenzym software (http://www.enzim.hu/~tusi/restric/index.html) in order to select the appropriate restriction enzymes. The fragments of the aforementioned *in-silico* RFLP were separated in a virtual electrophoresis using the pDraw32 software (http://www.acaclone.com/). The fragments smaller than 50 bp were not considered in the analysis.

Additionally, closely related microorganisms such as *Brucella melitensis*, *Ricketsia rickectsi*, *Erlichia muris* and *Chlamydia trachomatis* were included in the *in-silico* analysis.

2.2. Bacteria Culture

B. bacilliformis CIP 57.17, *B. henselae* CIP 103737, *B. bovis* CIP 106692, *B. clarridgeiae* CIP104772, acquired from the Collection of the Institute Pasteur (Paris, France) were cultured in Columbia Agar plates adding 10% of sheep blood and incubated at 30 ºC with 5% of CO_2 for 15-45 days. Additionally 7 *Bartonella* spp. clinical isolates, recovered from blood samples of patients with febrile syndrome, were also included in the study.

2.3. Purification of genomic DNA

The cells were collected from the plates and washed with buffer phosphate for three times by centrifugation at 13000g during 2 min. The DNA was obtained with the Wizard® Genomic Purification Kit (Promega, Madison, Wi) following the manufacturer's instructions.

An aliquot of DNA was analyzed by establishing the A260:A280 and A260:A230 ratios and electrophoresis at 1% agarose gel to evidence the quality of purified DNA. The genomic DNA was stored at -20 ºC.

2.4. Amplification of the 16S rRNA and Digestion with Restriction Enzymes

A fragment of 1452 bp of the *16S rRNA* gene, equivalent to that analyzed *in-silico*, was amplified using previously described primers and conditions [18]. The amplified products were digested with the *in-silico* selected restriction enzymes following the manufacturer's instructions and RFLP was visualized by electrophoresis in a 2.5 % agarose gel.

3. Results and Discussion

3.1. Design of *in-silico* decision-making tree

The *16S rRNA* gene possesses a highly conserved DNA sequence and thus has been previously used to identify microorganisms at species level [15, 18, 19]. In the case of *Bartonella* genus an alignment matrix of the sequences showed 97-99 % of homology between species (Table 2). Thus, in this study an easy and rapid technique is proposed to identify *Bartonella* species based on the development of serial restrictions of the *16S rRNA* gene to obtain a RFLP pattern associated with a unique species of the genus. Additionally, this technique has the potential to be used in low or middle resources countries, in which other tecniques may be non-disposable or too expensive to be applied.

The present *in-silico* analyses focused on *Bartonella* spp., result in the selection of *Mae*III, *Mse*I, *Sau*96I, *Bsa*AI, *Drd*I, *Fok*I, *Bss*HII, *Bst*UI, *Alu*I, *Tsp*DTI and *Hph*I restriction enzymes. Using these enzymes in the sequential manner proposed the 29 *Bartonella* species currently present in GenBank may be unambiguously identified (Fig 1-6).

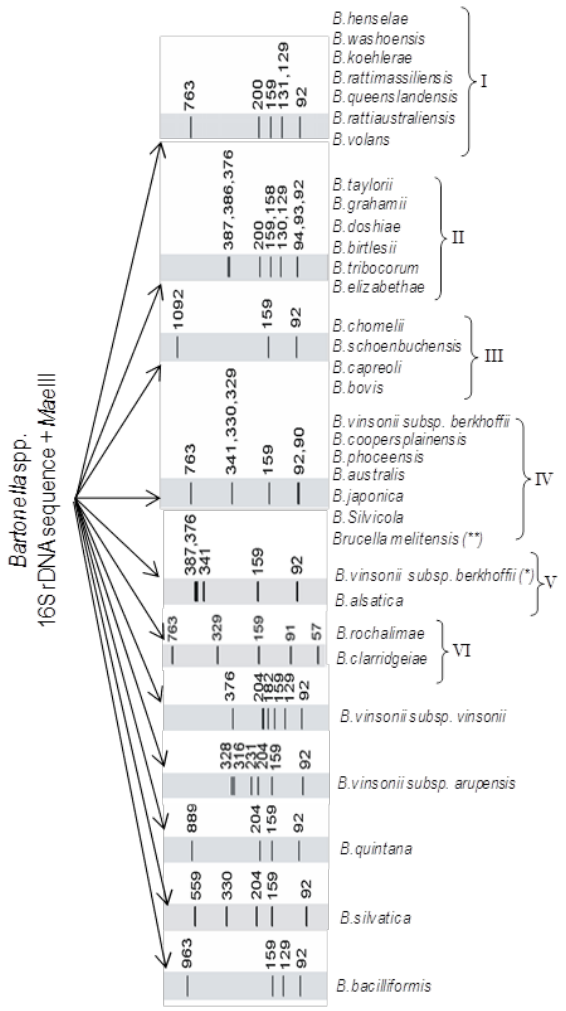

Figure 1. Identification and establishment of clusters of *Bartonella* species. The patterns were obtained using restriction with *Mae*III. *Isolate G7464 (ATCC 35685) of *B.vinsonii* subsp. *berkhoffii*. ** Strain ATCC 23457 (CP001489) of *Brucella melitensis*. In this figure, as in Figures 2 to 7 the proportions have been altered in order to best disposition of the samples sizes.

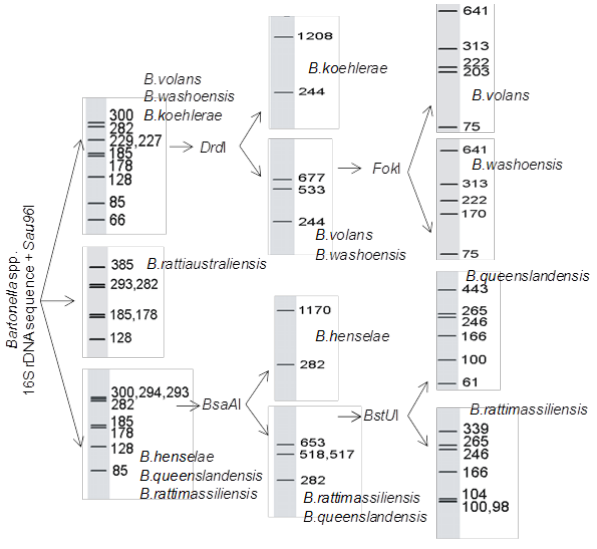

Figure 2. Identification of species included in cluster I. Each enzymatic digestion is always carried out on the *16S rRNA* amplicon.

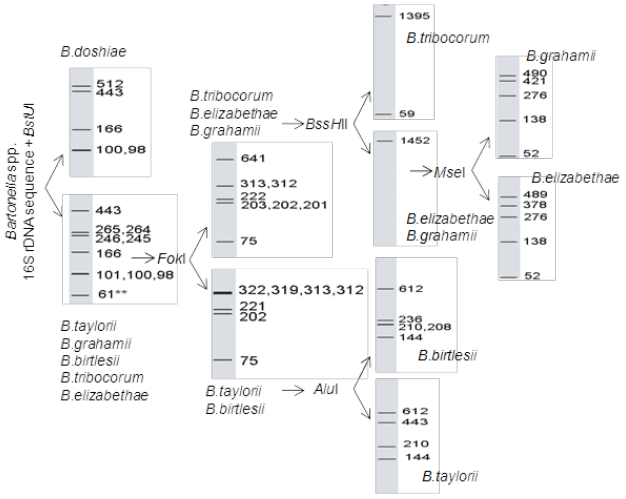

Figure 3. Identification of species included in cluster II. Each enzymatic digestion is always carried out on the *16S rRNA* amplicon.

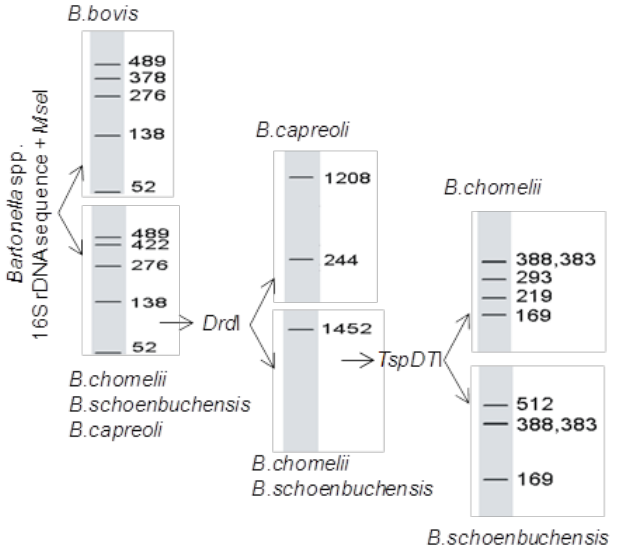

Figure 4. Identification of species included in cluster III. Each enzymatic digestion is always carried out on the *16S rRNA* amplicon.

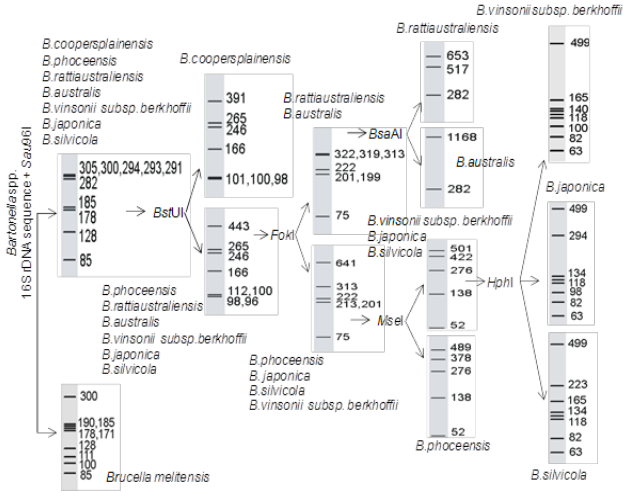

Figure 5. Identification of species included in cluster IV. Each enzymatic digestion is always carried out on the *16S rRNA* amplicon.

Table 1. Species of *Bartonella* used in the restriction bioinformatic analysis.

Species	Strain	*16S rRNA* gene	
		Collection No.	GenBank No.
B. alsatica	IBS382^T	CIP 1054773	AJ002139
B. australis	AUST/NH1^T	CIP 108978^T	DQ538394
B. bacilliformis	KC583^T	ATCC 35685	NC_008783
B. birtlesii	IBS 325^T	CIP 106294^T	AF204274
B. bovis	91-4T	CIP 106692^T	AF293391
B. capreoli	IBS 193^T	CIP 106691^T	AF293389
B. chomelii	A828^T	CIP 107869^T	AY254309
B. clarridgeiae	94-F40		U64691
B. coopersplainensis	AUST/NH20	CIP 109064	EU111759
B. doshiae	R18^T	NCTC 12862	Z31351
B. elizabethae	F9251^T	ATCC 49927	L01260
B. grahamii	V2	NCTC 12860	Z31349
B. henselae	Houston-1^T	ATCC 49882	NC_005956
B. japonica	Fuji 18-1^T	JCM 15567^T=CIP 109861^T	AB440632
B. koehlerae	C-29^T	ATCC 700693	AF076237
B. phoceensis	16120	CIP 107707^T	AY515119
B. queenslandensis	AUST/NH12	CIP 109057	EU111754
B. quintana	Toulouse		NC_005955
B. rattiaustraliensis	AUST/NH4	CIP 109051	EU111749
B. rattimassiliensis	15908	CIP 107705^T	AY515120
B. rochalimae		ATCC BAA-1498	Contig12 (12399-13873)
B. schoenbuchensis	R1^T	NCTC 13165^T	AJ278187
B. silvatica	Fuji 23-1^T	JCM 15566^T=CIP 109862^T	AB440636
B. silvicola	Cul-9		EF616480
B. taylorii	M6^T	NCTC 12861	Z31350
B. tribocorum	IBS 506^T	CIP 104576	AJ003070
B. vinsonii subsp. arupensis	OK 94-513^T	ATCC 700727	AF214558
B. vinsonii subsp. berkhoffii	93 CO1^T	ATCC 51672	L35052
B. vinsonii subsp. berkhoffii (*)	strain G7464	ATCC 35685	U26258
B. vinsonii subsp. vinsonii	Baker^T	ATCC VR-152	Z31352
B. volans	FSq-1	ATCC BAA-1451	EU294521
B. washoensis	NVH1		AF070463

A total of 29 species of *Bartonella* were analyzed in this study, for the case of *B.vinsonii* were analyzed three subspecies. Negative controls were used in the *in-silico* analysis of RFLP patterns: *Chlamydia trachomatis* strain HAR-13 (D89067), *Brucella melitensis* ATCC 23457 (CP001489), *Ehrlichia muris* AS145 (U15527), *Rickettsii rickectsi* strain R (L36217). (*) Isolate G7464 of *B.vinsonii* subsp. *berkhoffii* used in other publications (11) close related to type strain 93 CO1T of *B.vinsonii* subsp. *berkhoffii.*

Table 2. *16S rRNA* similarity values obtained from a multiple–sequence alignment.

Organism	*B.bacilliformis*	*B.schoenbuchensis*	*B.henselae*	*B.vinsonii subsp. vinsonii*	*B.vinsonii subsp. arupensis*	*B.vinsonii subsp. berkhoffii*	*B.clarridgeiae*	*B.tribocorum*	*B.quintana*	*B.alsatica*	*B.rattimassiliensis*	*B.phoceensis*	*B.birtlesii*	*B.capreoli*	*B.bovis*	*B.elizabethae*	*B.washoensis*	*B.koehlerae*	*B.australis*	*B.rattiaustraliensis*	*B.queenslandensis*	*B.coopersplainensis*	*B.grahamii*	*B.chomeli*	*B.taylorii*	*B.doshiae*	*B.japonica*	*B.silvatica*	*B.rochalimae*	*B.silvicola*	*B.volans*	*B.vinsonii subsp. berkhoffii (*)*
B.bacilliformis	ID																															
B.schoenbuchensis	98.	ID																														
B.henselae	98.	98.	ID																													
B.vinsonii subsp. vinsonii	98	98.	98.	ID																												
B.vinsonii subsp. arupensis	97.	98	98.	98.	ID																											
B.vinsonii subsp. berkhoffii	97.	97.	98.	98.	98	ID																										
B.clarridgeiae	97.	98	98	98	97.	97.	ID																									
B.tribocorum	97.	98	98.	99	98.	98.	97.	ID																								
B.quintana	98	98.	98.	98.	98.	97.	97.	98.	ID																							
B.alsatica	97.	98.	98.	99.	98.	98.	97.	98.	98.	ID																						
B.rattimassiliensis	98.	98.	99	98.	98.	98.	98	99	98.	98.	ID																					
B.phoceensis	97.	98.	98.	98.	98.	97.	97.	98.	98.	98.	99	ID																				
B.birtlesii	98.	98.	98.	99.	98.	98.	98	99.	98.	99.	98.	98.	ID																			
B.capreoli	98.	99.	99.	99.	98.	98	98.	98.	98.	98.	98.	98.	98.	ID																		
B.bovis	98.	99.	98.	98.	97.	97.	97.	97.	98.	98.	98.	98.	98.	99.	ID																	
B.elizabethae	97.	97.	98.	98.	98.	98	97.	99.	98	98.	98.	98.	98.	98	97.	ID																
B.washoensis	98.	98.	99.	98.	98.	98.	97.	98.	99	98.	98.	98.	98.	98.	98.	98.	ID															
B.koehlerae	98.	98.	99.	99.	98.	98.	98.	98.	98.	98.	99	98.	99	99.	98.	98.	99.	ID														
B.australis	98.	97.	98	97.	97.	97.	98.	97.	97.	97.	98.	98	98.	98	97.	97.	97.	98	ID													
B.rattiaustraliensis	98.	98.	99.	99	98.	98.	98.	99	98.	98.	99.	98.	99.	98.	98.	98.	98.	99.	98.	ID												
B.queenslandensis	98.	98.	99.	99.	98.	98.	98.	99.	98.	99	99.	98.	99.	99.	98.	98.	98.	99.	98.	99.	ID											
B.coopersplainensis	97.	97.	98.	98.	97.	97.	97.	98.	98	98	98.	98.	98.	98.	97.	97.	98	98.	97.	98.	98.	ID										
B.grahamii	98.	98.	99	99.	98.	98.	98	99.	98.	99	99.	98.	99.	98.	98.	98.	98.	99	98	99.	99.	98.	ID									
B.chomeli	98.	99.	98.	98.	97.	97.	97.	97.	97.	97.	97.	97.	97.	99	99.	97.	97.	98.	97.	98.	98.	97.	97.	ID								
B.taylorii	98.	98.	98.	99.	98.	98	97.	99	98.	98.	98.	98.	99.	98.	98	98.	98.	98.	98	98.	99	98	99.	97.	ID							
B.doshiae	98	98.	98.	99.	98.	98.	98	99	98.	98.	98.	98.	99.	99	98.	98.	98.	99.	97.	99	99.	98.	99.	98	99.	ID						
B.japonica	97.	98	98.	98.	98.	98.	98.	98.	98.	98.	99.	98.	98.	98.	97.	98.	98.	98.	98.	98	99	99.	98.	97.	98.	98.	ID					
B.silvatica	97.	98.	98.	98.	98.	97.	98	98.	98.	98.	99.	99.	98.	98.	98.	98.	98.	98.	98	98.	98.	98.	98.	97.	98.	98.	99	ID				
B.rochalimae	97.	97.	97.	97.	97.	96.	99.	97.	97.	97.	97.	97.	97.	97.	97.	97.	97.	97.	98.	98	98	97.	97.	97.	97.	97.	98	97.	ID			
B.silvicola	98	98.	98.	99	98.	98.	98.	99	98.	98.	99	98.	99	98.	98.	98.	98.	98.	98	99	99.	98.	99.	97.	98.	98.	98.	98.	98	ID		
B.volans	98	98.	98.	98.	98.	98	97.	98.	98.	98.	98.	98.	98.	98.	98	98.	99.	99.	97.	98.	98.	97.	98.	97.	98.	98.	98.	98.	97.	98.	ID	
B.vinsonii subsp. berkhoffii	97.	97.	98.	98.	98.	99.	97.	98.	98	98.	98.	97.	98.	98.	97.	98.	98.	98.	97.	98.	98.	97.	98.	97.	98.	98.	98.	97.	97	98.	98	ID

* Isolate G7464 (ATCC 35685) of *B.vinsonii* subsp. *berkhoffii*

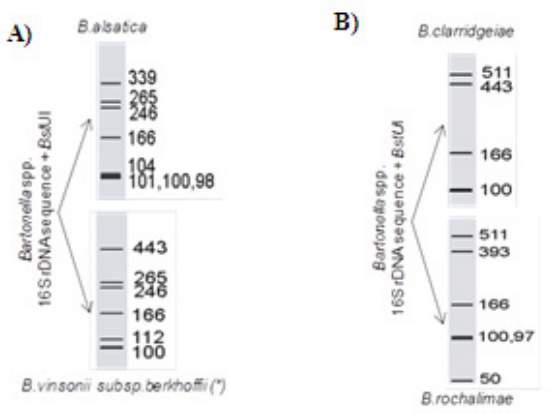

Figure 6. A) Identification of species included in cluster V. (*) Isolate G7464 (ATCC 35685) of *B.vinsonii* subsp. *berkhoffii*. B) Identification of species included in cluster VI. Each enzymatic digestion is always carried out on the *16S rRNA* amplicon.

The RFLP decision-making tree is started using *Mae*III. This should allow among *B.bacilliformis*, *B.quintana*, *B.silvatica*, *B.vinsonii* subsp. *vinsonii*, and *B.vinsonii* subsp. *arupensis* to be unambiguously and accurately differentiated from the remaining *Bartonella* (Fig. 1) in a single step.

The interest of this point is specially related to the clinical interest of *B. bacilliformis* and *B. quintana* as human pathogens. Thus, *B. bacilliformis* and *B. quintana* are the etiologic agents for Carrion´s disease (acute Oroya fever and angiomatosis, endocarditis, chronic bacteremia, pericarditis), respectively [4]. Referring to non-*Bartonella* included in the analysis, only *Brucella melitensis* results in a common pattern with some *Bartonella* species (cluster IV) in the *Mae*III restriction, but was quickly separates in the cluster analysis (Fig 5).

3.2. Applicability of the PCR-RFLP identification

This rapid technique may be of special interest to describe more in depth the real role of different *Bartonella* spp. in human infections being of specially interest in limted-resources countries. Until early 1990, only one *Bartonella* species (*B. bacilliformis*) was implicated in human pathology, but currently around ten species have been related to different human pathologies [4]. Moreover, identifications based on clinical findings may not be correct as in a suspicious case of Oroya's Fever, pathology related to *B. bacilliformis*, which was described as produced by *B. rochalimae* [12].

The use of molecular tools may overcome these erroneous bacterial identifications obtained on the basis of clinical presentations or classical techniques [12, 18, 21] This problem is especially relevant in fastidious microorganisms such as the members of the *Bartonella* genus.

Despite it has been described that some members of the *Bartonella* genus are able to grown from blood stored at 4ºC, even being maintained for more than 2 years [22], the growth and isolation of *Bartonella* species on blood agar plates generally requires a prolonged incubation period (an average of 21 days) and is rarely successful. This fact may be considered as a pitfall, because the need of dispose of cultured bacteria. However, the RFLP method provides a broad application, even in culture-negative infected material [23]. Moreover, the amplification of the *16S rRNA* gene has been successfully used as rapid *Bartonella* spp. diagnosis tool [14]. Thus, RFLP analysis of the *16S rRNA* gene in *Bartonella* can be a useful molecular tool for the identification of *Bartonella* species.

An interesting possibility may be the presence of two different *16S rRNA* sequences in the same microorganism. This fact has been showed in the case of *B. henselae*, in which 2 different *16S rRNA* sequences have been found, and isolates presenting both together have been described [24, 25]. This *16S rRNA* gene variants has been namely as type 1, with has *B. henselae* Houston-1 as representative strain, and type 2, with has *B. henselae* Urlly8 as a representative strain (GenBank access: AF214556).

The small differences among these two variants (2 bases plus 1 gap) do not affect the proposed decision-making tree. Moreover, despite the strongly identity between these two variants, the use of the restriction enzymes *Dde*I or *Bsu36*I may allow arriving to determine the exact variant(s) presents in the sample, because the presence of a specific digestion point in the *16S rRNA* gene belonging to the type 1 which is absent in those belonging to type 2.

3.3. Experimental application of the PCR-RFLP to identify several *Bartonella* spp

Experimentally, we have run the RFLP analysis of the *16S rRNA* gene in *B.bacilliformis*, *B.henselae*, *B.bovis* and *B.clarridgeiae* (Fig. 7). In all cases, the electrophoresis pattern of the fragments obtained in the digestion with *Mae*III was different in each species and occurred in accordance to the predicted pattern obtained using bioinformatics tools.

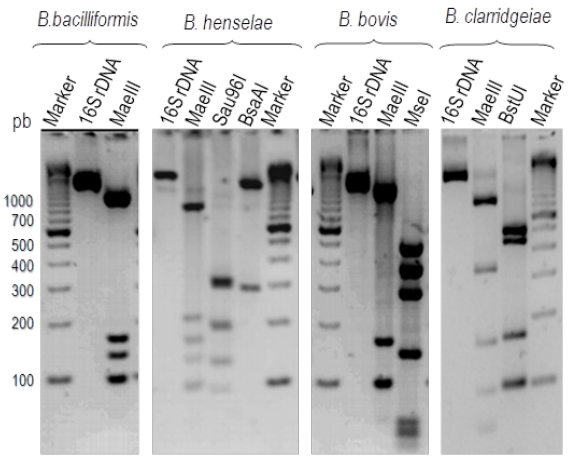

Figure 7. Restriction profiles obtained after endonuclease digestion (*Mae*III, *Mse*I, *Sau*96I, *Bsa*AI, or *Bst*UI) of the amplified *16S rRNA* gene with the universal primer set 8F and 1510R. Products were electrophoresed in 2.5% (w/v) agarose and visualized with Sybr Safe (Invitrogen, Eugene, OR). Molecular size marker was a 100bp (Invitrogen).

Additionally, the RFLP-technique was also tested in a series of *Bartonella* spp. clinical isolates proceeding from blood cultures. In all cases the PCR-RFLP results in an unambiguous identification. Thus, 6 cases were identified as *B. bacilliformis*, while the remaining as *B. elizabethae* (data not show). In the seven cases the sequencing of the *16S rRNA* gene confirms this identification.

The RFLP technique has previously been used for rapid identification of *B. henselae* in the Cat-Scratch disease [7]. Endonuclease digestion of the *16S rRNA* gene with *Dde*I resulted in fragments of approximately 410 and 210 bp for *B.henselae*, *B.quintana*, *B.elizabethae*, *B.vinsonii*, and *B.clarridgeiae*. A third fragment of approximately 380 bp was observed in all these strains except *B.clarridgeiae*. Although other species-specific products ranged from 100 to 280 bp, *B.elizabethae* and *B.vinsonii berkhoffii* were barely identical [7], besides further studies showed a similar pattern in *B. bacilliformis* [9]. Additionally, in last years a high number of *Bartonella* species has been described, and, to our knowledge, no study to adapt this proposal has been made.

4. Conclusion

The description of new human pathogenic species of the *Bartonella* genus makes improvement of the molecular techniques in the detection and identification of these species necessary. The *16S rRNA* gene is used to establish the phylogenetic taxonomy of the *Bartonella* genus due to the high interspecies similarity. This same approach has been used to develop a molecular assay by RFLP analysis able to both differentiate among all the currently described *Bartonella* species and to be modified according to future knowledge. This simple, rapid method provides a broad application and can be expected to significant increase our understanding of the epidemiology of this group of important human pathogens.

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REFERENCES

[1] R.J.Birtles, T.G.Harrison, N.A.Saunders, D.H.Molyneux, Proposals to unify the genera Grahamella and Bartonella, with descriptions of Bartonella talpae comb. nov., Bartonella peromysci comb. nov., and three new species, Bartonella grahamii sp. nov., Bartonella taylorii sp. nov., and Bartonella doshiae sp. nov. International Journal of Systematic Bacteriology. Vol: 45, No: 1, 1-8. 1995.

[2] D.J.Brenner, S.P.O'Connor, H.H.Winkler, A.G.Steigerwalt. Proposals to unify the genera Bartonella and Rochalimaea, with descriptions of Bartonella quintana comb. nov., Bartonella vinsonii comb. nov., Bartonella henselae comb. nov., and Bartonella elizabethae comb. nov., and to remove the family Bartonellaceae from the order Rickettsiiles. International Journal of Systematic Bacteriology. Vol: 43, No:4, 777-786, 1993.

[3] W.G.Weisburg, C.R.Woese, M.E.Dobson, E.Weiss, A common origin of Rickettsiie and certain plant pathogens. Science. Vol: 230 No:4725. 556-558. 1985.

[4] J.R.Blanco, I.Jado, M.Marín, I.Sanfeliu, A.Portillo, P.Anda, I.Pons, J.A.Oteo. Diagnóstico microbiológico de las infecciones por patógenos bacterianos emergentes: Anaplasma, Bartonella, Rickettsii, Tropheryma whipplei. Enfermedades. Infecciosas y Microbiologia Clinica. Vol:26, No: 9, 573-580. 2008.

[5] L. Guptill. Bartonellosis. Veterinary Microbiology. Vol: 140, No: 3-4, 347-359. 2010.

[6] E.Huarcaya, C.Maguiña, R.Torres, J.Rupay, L.Fuentes. Bartonelosis (Carrion's disease) in the pediatric population of Peru: an overview and update. Brazilian Journal of Infectious Diseases Vol:8, No:5, 331-339. 2004.

[7] D.L.Kordick,, E.JHilyard, T.L.Hadfield, K.H.Wilson, A.G.Steigerwalt, D.J.Brenner, E.B.Breitschwerdt. Bartonella clarridgeiae, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). Journal of Clinical Microbiology. Vol:35, No:7, 1813-1818. 1997.

[8] D.L.Kordick, B.Swaminathan, C.E.Greene, K.H.Wilson, A.M.Whitney, S.O'Connor, D.G.Hollis, G.M.Matar, A.G,Steigerwalt, G.B.Malcolm., P.S.Hayes, T.L.Hadfield, E.B.Breitschwerdt, D.J.Brenner. Bartonella vinsonii subsp. berkhoffii subsp. nov., isolated from dogs; Bartonella vinsonii subsp. vinsonii; and emended description of Bartonella vinsonii. International Journal of Systematic Bacteriology. Vol:46, No:3, 704-709. 1996.

[9] C.Maguiña, H.Guerra, P.Ventosilla, Bartonellosis. Clinical Dermatology. Vol:27, No:3, 271-280. 2009.

[10] W.Saisongkorh, J.M.Rolain, Y.Suputtamongkol, D.Raoult. Emerging Bartonella in Humans and Animals in Asia and Australia. Journal of the Medical Association of Thailand. Vol:92, No:5, 707-731. 2009.

[11] E.B.Breitschwerdt, D.L.Kordick, Bartonella infection in animals: carriership, reservoir potential, pathogenicity, and zoonotic potential for human infection. Clinical Microbiology Reviews. Vol:13 No:3 428-438. 2000.

[12] M.E.Eremeeva, H.L.Gerns, S.L.Lydy, J.S.Goo, E.T.Ryan, S.SMathew, M.J.Ferraro, J.M.Holden, W.L.Nicholson, G.A.Dasch, J.E.Koehler. Bacteremia, fever, and

splenomegaly caused by a newly recognized bartonella species. New England Journal of Medicine. Vol:356, No:23 2381-2387. 2007

[13] P.E.Fournier, C.Couderc, S.Buffet, C.Flaudrops, D.Raoult. Rapid and cost-effective identification of Bartonella species using mass spectrometry. Journal of Medical Microbiology, Vol:58, No:9, 1154-1159. 2009.

[14] J.Ruiz, W.Silva, C.Tinco, M.J.Pons, L.del Valle, C.Gomez, J.Bazan, M.Vargas, D.Champin, J.del Valle Mendoza. Etiological and molecular diagnostic of Carrion's disease in patients from Cajamarca - Perú. International Journal of Infectious Diseases. Vol:16 No: Suppl1, e252-e254. 2012.

[15] N.Borrell, S.G..Acinas, M.J.Figueras, A.J.Martínez-Murcia. Identification of Aeromonas clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. Journal of Clinical Microbiology Vol:35, No:7 1671-1674. 1997.

[16] M.Ferreira-Tonin, J.Rodrigues-Neto, R.Harakava, S.A.Destéfano. Phylogenetic analysis of Xanthomonas based on partial rpoB gene sequences and species differentiation by PCR-RFLP. International Journal of Systematic and Evolutionary Microbiology. Vol:62, No:Pt 6, 419-424. 2012.

[17] J.B.Messick, L.M.Berent, S.K.Cooper. Development and evaluation of a PCR-based assay for detection of Haemobartonella felis in cats and differentiation of H. felis from related bacteria by restriction fragment length polymorphism analysis. Journal of Clinical Microbiology Vol:36, No2, 462-466. 1998.

[18] E.Z.Salazar de Vegas, B.Nieves, M.Araque, E.Velasco, J.Ruiz, J.Vila. Outbreak of infection with Acinetobacter strain RUH 1139 in an intensive care unit. Infection Control and Hospital Epidemiology. Vol:27. No:4 397-403. 2006.

[19] M.J.Figueras, L.Soler, M.R.Chacón, J.Guarro, A.JMartínez-Murcia. Extended method for discrimination of Aeromonas spp. by 16S rDNA RFLP analysis. International Journal of Systematic and Evolutionary Microbiology. Vol:50 No:Pt 6, 2069-2073. 2000.

[20] R.J. Birtles. Differentiation of Bartonella species using restriction endonuclease analysis of PCR-amplified 16S rRNA genes. FEMS Microbiology Letters. Vol: 129, No: 2-3, 261-266. 1995.

[21] O.Ormen, P.E.Granun, J.Lassen, M.J.Figueras. Lack of agreement between biochemical and genetic identification of Aeromonas spp. APMIS. Vol:113, No:3 203-207. 2005.

[22] J.Ruiz, W.Silva, M.J.Pons, L.J.del Valle, C.R.Tinco, V.D.Casabona, C.Gomes, J.Bazan, V.Zavaleta, H.Cornejo, D.Champin, J.del Valle. Long time survival of Bartonella bacilliformis in blood stored at 4 °C. A risk for blood transfusions. Blood Transfusion. Vol:10, No:4, 563-564. 2012.

[23] D.Rodrick, B.Dillon, M.Dexter, I.Nicholson, S.Marcel, D.Dickeson, J.Iredell. Culture-Negative Endocarditis Due to Houston Complex Bartonella henselae Acquired in Noumea, New Caledonia. Journal of Clinical Microbiology. Vol: 42, No:4, 1846-1848. 2004.

[24] J Viezens J, M.Arvand. Simultaneous presence of two different copies of the 16S rRNA gene in Bartonella henselae. Microbiology. Vol 154, No:9, 2881-2886. 2008

[25] V.V.Ebani, F.Bertelloni, F.Fratini. Occurrence of Bartonella henselae types I and II in Central Italian domestic cats. Research in Veterinary Science. Vol:93 No:4, 63–66. 2012