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1 **Genotyping of *Chlamydophila abortus* strains by Multilocus VNTR Analysis**

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22

23 ABSTRACT

24

25 *Chlamydomphila* (*C.*) *abortus* is the causative agent of ovine enzootic abortion with zoonotic potential
26 whose epidemiology has been held back because of the obligate intracellular habitat of the bacterium. In
27 the present study we report on a molecular typing method termed Multiple Loci Variable Number of
28 Tandem Repeats (VNTR) Analysis (MLVA) for exploring the diversity of *C. abortus*. An initial analysis
29 performed with 34 selected genetic loci on 34 ruminant strains including the variant Greek strains LLG
30 and POS resulted in the identification of 5 polymorphic loci, confirming the widely held notion that *C.*
31 *abortus* is a very homogeneous species. Analysis of additional 111 samples with the selected 5 loci
32 resulted in the classification of all strains into 6 genotypes with distinct molecular patterns termed
33 genotypes [1] through [6]. Interestingly, the classification of the isolates in the 6 genotypes was partly
34 related to their geographical origin. Direct examination of clinical samples proved the MLVA to be
35 suitable for direct typing. Analysis of the genomic sequences in 6 *C. abortus* prototypes of amplicons
36 generated with each of the 5 selected VNTR primers revealed that variation between genotypes was
37 caused by the presence or absence of coding tandem repeats in 3 loci. Amplification of *C. psittaci*
38 reference strains with the 5 selected VNTR primers and of the 6 *C. abortus* prototype strains with the 8
39 VNTR primers established for the typing of *C. psittaci* (Laroucau *et al.*, 2008) showed that both MLVA
40 typing systems were species-specific when all respective VNTR primer sets were used.

41 In conclusion, the newly developed MLVA system provides a highly sensitive, high-resolution and easy-
42 to-perform tool for the differentiation of *C. abortus* isolates of different origin, which is suitable for
43 molecular epidemiological studies.

44

45 **KEY WORDS:** *Chlamydomphila abortus*, intra-species differentiation, MLVA typing, molecular
46 epidemiology.

47

48 1. INTRODUCTION

49 The family *Chlamydiaceae* comprises a group of obligate intracellular bacteria that are distributed
50 worldwide and can infect both humans and animals. Two genera have been defined within the family,
51 the genus *Chlamydia* including the species *Chlamydia trachomatis*, *Chlamydia muridarum* and
52 *Chlamydia suis* and the genus *Chlamydophila* (*C.*), consisting of the species *C. psittaci*, *C. abortus*,
53 *C. caviae*, *C. felis*, *C. pecorum* and *C. pneumoniae* (Everett *et al.*, 1999).

54 *C. abortus* is the causative agent of abortion and foetal loss in sheep, goats and cattle in many countries
55 around the world (Aitken and Longbottom, 2007). In addition, the bacterium has been isolated from
56 cases of epididymitis, pneumonia, arthritis, conjunctivitis, and from the faeces of healthy sheep and
57 goats (Denamur *et al.*, 1991; Souriau *et al.*, 1993; Salinas *et al.*, 1995; Salti-Montesanto *et al.*, 1997). *C.*
58 *abortus* has also been associated with cases of abortion in horses, rabbits, guinea pigs and mice
59 (Everett *et al.*, 1999). The bacterium is known to possess zoonotic potential. Pregnant women may
60 suffer severe infections, including spontaneous abortion, following exposure to animals infected with *C.*
61 *abortus* (Buxton, 1986; Pospischil *et al.*, 2002; Walder *et al.*, 2005).

62 Until now, typing methods using monoclonal antibodies, RFLP analysis of the *ompA* gene, ribosomal
63 RNA and *ompA* gene sequencing have consistently failed to reveal any differences between *C. abortus*
64 isolates, whatever the host, the associated disease or the geographical origin (Denamur *et al.*, 1991;
65 Salinas *et al.*, 1995; Everett *et al.*, 1999). Exceptions to this were the Greek strains LLG and POS that
66 were characterized as variants based on inclusion morphology, antigenic and molecular diversity
67 (Vretou *et al.* 1996 and 2001; Siarkou *et al.*, 2002). However, a more sophisticated molecular typing
68 tool, the amplified fragment length polymorphism analysis (AFLP), allowed discerning the French strains
69 from others even when ribosomal RNA gene and *ompA* sequences were highly conserved (Boumedine
70 and Rodolakis, 1998).

71 With the increasing availability of whole bacterial genome sequences, new molecular tools based on
72 genome-wide screening have been developed. The typing method termed Multiple Loci Variable
73 Number of Tandem Repeats (VNTR) Analysis (MLVA) is based on the detection of tandem repeat
74 polymorphisms in the bacterial genome (Vergnaud and Pourcel, 2006). The method has been used
75 successfully for typing many pathogens including *C. psittaci* the causative agent of avian chlamydiosis.
76 This recently developed MLVA system was capable of classifying a panel of 150 *C. psittaci* isolates into
77 20 clusters based on 8 VNTR loci and their respective PCR amplified sizes (Laroucau *et al.*, 2008).

78 The aim of the present study was to explore the potential of MLVA for revealing the molecular diversity
79 of *C. abortus* isolates and to assess its usefulness for future epidemiological investigations. Since *C.*
80 *abortus* and *C. psittaci* are genetically closely related we also aimed to assess the potential of the MLVA
81 systems to discriminate between the 2 species.

82

83 2. MATERIALS AND METHODS

84 **2.1 Bacterial strains and isolates**

85 *C. abortus* strains, isolates and DNA samples were obtained from INRA (Nouzilly, France), the
86 Friedrich-Loeffler-Institut (Jena, Germany), the Hellenic Pasteur Institute (Athens, Greece), the Institute
87 for Hygiene and Infectious Diseases of Animals (Giessen, Germany), the Moredun Research Institute
88 (Edinburgh, Scotland), and the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia
89 Romagna (Pavia, Italy). The characteristics of the strains investigated, including origin and source, are
90 presented in Table 1. Sixty-seven strains originated from Germany and 19, 16, 12 and 10 strains were
91 from France, the UK, Italy and Greece, respectively. Extra-European samples were from USA (3
92 strains), Namibia (4 strains) and Tunisia (4 strains). Finally, 10 strains were of unknown origin. In total,
93 145 samples were investigated. All the strains were isolated from clinically affected animals except
94 Mo907, iC1 and 71 which were faecal isolates from apparently healthy animals.

95 *C. psittaci* reference strains Loth, CP3, GR9, NJ1, Cal10 and VS225 for avian serotypes A through F
96 were from AFSSA (Maisons-Alfort, France). DNA was obtained as previously described (Laroucau *et al.*,
97 2008).

98

99 **2.2 Clinical samples**

100 A panel of nine clinical samples collected from French cases of abortion from sheep, cattle and goat
101 were directly subjected to DNA extraction using the QIAamp DNA Mini Kit (Qiagen, Courtaboeuf,
102 France). DNA was eluted with 150 µl of AE buffer and stored at -20°C before analysis (Table 2).
103 Infection by *C. abortus* was detected by a *C. abortus*-specific real-time PCR targeting the *ompA* gene
104 (Pantchev *et al.*, 2008). The protocol included primers Cpa-F (5'- GCAACTGACACTAAGTCGGCTACA
105 -3'), Cpa-R (5'- ACAAGCATGTTCAATCGATAAGAGA -3'), and probe Cpa-p (FAM-5'-

106 TAAATACCACGAATGGCAAGTTGGTTTAGCG-3' -TAMRA). Each reaction mix contained 2 µl of
107 sample DNA template, 10 µl of Universal Mastermix 2x (Applied Biosystems), 0.5 µl of each primer
108 (25 µM), 2 µl of the probe (1 µM), and 5 µl deionized water. The temperature-time profile was 95°C for
109 10 min, followed by 45 cycles at 95°C for 15 s, and at 60°C for 60 s.

110

111 **2.3 MLVA genotyping method**

112 Tandem repeat sequences within the whole genome sequence of the *C. abortus* strain S26/3 (GenBank
113 acc.no. CR848038; Thomson *et al.*, 2005) were determined using the Tandem Repeat Finder (TRF)
114 program (<http://tandem.bu.edu/trf/trf.html>) (Benson, 1999). Thirty-four tandem repeats with a repeat unit
115 equal to or larger than 6 bp were identified and primers for PCR amplification were chosen on both sides
116 of the repeats (Table 3). PCRs were performed on an initial set of 34 *C. abortus* strains underlined in
117 Table 1.

118 For the VNTR amplifications, PCR was performed in a total volume of 15 µl containing 5-10 ng of DNA,
119 1x PCR reaction buffer, 1 U of Hot Start Taq DNA polymerase (Qiagen), 200 µM of each
120 deoxynucleotide, and 0.3 µM of each flanking primer. VNTR sites were selected based on the whole
121 genome sequence of *C. abortus* S26/3 and primers were designed to amplify these sites (Table 3). The
122 initial denaturation step at 95°C for 15 min was followed by 40 cycles consisting of denaturation at 95°C
123 for 30 s, primer annealing at 58°C for 30 s depending on the primers, and elongation at 72°C for 45 s.
124 The final extension step was at 72°C for 10 min. Five microliters of amplification product were loaded
125 onto a 4% Metaphor/standard (50-50) agarose gel. Gels stained with ethidium bromide were visualized
126 under UV light, and photographed. The size marker used was a 100-bp ladder (Mbi, Euromedex,
127 France).

128

129 **2.4 Data analysis**

130 Amplicon size was determined manually using the 100 bp size marker. The number of motifs in each
131 allele was derived from the amplicon size. The resulting data were analyzed with Bionumerics software
132 package version 4.6 (Applied-Maths, Saint-Martens-Latem, Belgium) as a character dataset. Clustering
133 analysis was done using the categorical parameter and the UPGMA coefficient.

134

135 **2.5 Sequencing of PCR products**

136 The 5 targeted genomic regions of *C. abortus* strains C9/98, AB7, B577, BAF, S26/3, and POS were
137 amplified with primers ChlaAb_457, ChlaAb_581, ChlaAb_620, ChlaAb_914 and ChlaAb_300 and the
138 PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). DNA sequencing of these
139 PCR products was done at MWG (Biotech France, Roissy, France). Sequences have been deposited in
140 GenBank database. Accession numbers listed in Figure 2.

141

142 3. RESULTS

143 **3.1 Selection of VNTRs for MLVA analysis from an initial set of 34 *C. abortus* strains**

144 Thirty four tandem repeats with a repeat unit equal to or larger than 6 bp were identified by the program
145 Tandem Repeat Finder (TRF) in the whole genome sequence of *C. abortus* strain S26/3 (Table 3). In
146 order to evaluate the polymorphism within these potential VNTR markers, primers were chosen on both
147 sides of the repeats and PCRs were performed on an initial set of 34 *C. abortus* strains (Table 1).

148 Gel analysis of the size of the amplified fragments showed that only 5 of the tandem repeat loci, namely
149 ChlaAb_457, ChlaAb_581, ChlaAb_620, ChlaAb_914 and ChlaAb_300 were polymorphic for these 34
150 strains (Figure 1 and data not shown). Furthermore, it was possible to cluster the 34 *C. abortus* strains
151 into 5 distinct genotypes based on the size of the amplified PCR products, highlighting thus the
152 suitability of the selected 5 tandem repeat loci for establishing and developing a genotyping system for
153 *C. abortus* (Figure 1 and Table 1). The repeat units were localized in CAB267, the coding sequence of
154 the polymorphic outer membrane protein pmp5E, in CAB398 coding for a histone-like protein, in
155 CAB502 coding for a putative exported protein with a lysine decarboxylase motif, in CAB541 coding for
156 a conserved membrane protein (pseudogene), and in CAB786 coding for the 30S ribosomal protein S18
157 for markers ChlaAb_300, ChlaAb_457, ChlaAb_581, ChlaAb_620, and ChlaAb_914, respectively.

158

159 **3.2 Evaluation of the selected VNTRs for MLVA analysis**

160 The stability of the 5 selected tandem repeat markers was assessed by analysis of 3 *C. abortus* strains
161 (A22, S26/3 and B577) stored in different laboratories (INRA, Moredun Research Institute, and/or
162 Hellenic Pasteur Institute) and by analysis of different passages of the AB7 strains (2 and 7 passages).
163 The results indicated that the analysis was independent from the performing laboratory and from the
164 passage history of the strains under examination (data not shown). To assess the reproducibility of the

165 MLVA analysis, three *C. abortus* strains (AB7, S26/3 and POS) were genotyped in parallel in two
166 different laboratories, in AFSSA (Maisons-Alfort) and in INRA (Nouzilly). The results were identical in
167 both places.

168

169 **3.3 MLVA genotyping of *C. abortus* isolates**

170 The set of the selected 5 markers was used to examine a panel of 111 additional *C. abortus* isolates
171 from different ruminant hosts and from different geographical origins (Table 1). Among this larger group
172 of samples, strain C9/98 was identified as a new genotype.

173 Overall, clustering of all examined strains by similarity of the sizes of the PCR products resulted into 6
174 genotypes termed [1] through [6] (Table 1). The distinction between genotypes [1] through [5] was
175 based on differences within one marker. Among these 6 genotypes, genotypes [2], [3] and [5] appear to
176 be predominant including 115, 8 and 18 isolate samples, respectively. The other 3 genotypes comprised
177 either a single strain as genotypes [1] and [4] or two strains as genotype [6]. The 2 Greek strains
178 (genotype [6]) were the most distinct ones, reconfirming previous results obtained with various typing
179 tools (Vretou *et al.*, 1996 and 2001; Siarkou *et al.*, 2002). Interestingly, none of the UK strains was
180 represented among genotypes [2] and [3], and no French strain was represented among genotypes [3]
181 and [5]. No correlation could be found with the animal species involved or with the infection status of the
182 animals.

183

184 **3.4 Direct typing of clinical samples.**

185 A panel of nine French clinical samples that were tested positive using the *C. abortus*-specific real-time
186 PCR were genotyped using the 5 selected markers ChlaAb_457, ChlaAb_581, ChlaAb_620,
187 ChlaAb_914 and ChlaAb_300. All samples amplified very efficiently and were found to belong to the
188 genotype [2], except sample 07-525/J, which belonged to genotype [1] (Table 2). This result highlighted
189 the suitability of the novel MLVA for direct typing of clinical samples without previous propagation of the
190 bacteria.

191

192 **3.5 Sequence analysis of PCR products**

193 Genomic sequences were generated for each PCR product amplified with the 5 selected markers for the
194 6 strains C9/98, AB7, B577, BAF, S26/3 and POS, representing the 6 genotypes. With the exception of

195 ChlaAb_300, where the polymorphism is due to deletion in the targeted sequence for POS, the repeat
196 unit was localized similarly in all other sequences and its presence or absence in the sequence resulted
197 into the experimental polymorphism (Figure 2). The observed repeat size was a multiple of 3 nucleotides
198 for all VNTRs contributing thus to the coding sequence of the loci. An exception to this was ChlaAb_620
199 (pseudogene CAB541). Consequently, the polymorphism in this locus was due to a stop in translation in
200 one allele (2 copies of a 11 bp repeat) and to a frameshift in another allele (3 copies), as compared to
201 the allele with a single copy of the repeat. In CAB502 (ChlaAb_581) the 15-mer TAGTTAAACATGGGT
202 coding for peptide LVKHQ, was repeated in strains B577, BAF and S26/3 but not in strains C9/98, AB7
203 and POS, whereas in CAB786 (ChlaAb_914) the 15-mer GCCTGTTTCATAATAA coding for peptide
204 KPVHN was repeated only in strain S26/3. In the histone-like protein *hctB* (CAB398, ChlaAb_457) the
205 24-mer peptide AKKTATRKPAVKKAVRKTAAKKAT was repeated once in strain POS.
206 Summarized, the results showed that the MLVA typing method developed on the analysis of 5 VNTR
207 loci was capable of clustering 145 *C. abortus* strains in 6 genotypes and that variation among genotypes
208 was caused in 3 loci by the presence or absence of coding tandem repeats.

209

210 **3.6 Evaluation of MLVA typing for discerning between *C. psittaci* and *C. abortus***

211 Since *C. psittaci* and *C. abortus* are genetically closely related species it was important to assess the
212 capability of the developed MLVA to distinguish between the 2 species. To this end 6 *C. psittaci*
213 reference strains corresponding to the 6 avian serotypes, namely strains Loth, CP3, GR9, NJ1, Cal10
214 and VS225 were amplified with the 5 *C. abortus* VNTR primer sets ChlaAb_300, ChlaAb_457,
215 ChlaAb_581, ChlaAb_620, and ChlaAb_914 (Figure 3). No amplification product was observed with
216 primers ChlaAb_581, ChlaAb_620, and ChlaAb_914 for any avian strain, while the size of the amplified
217 fragments with primer ChlaAb_457 (histone-like protein CAB 398) was distinct for the avian strains. The
218 amplification product with primer ChlaAb_300 (CAB267, pmp5E) was similar to the PCR product of the
219 variant *C. abortus* strain POS.

220 Of equal importance was to evaluate the performance of a recently developed MLVA system for
221 genotyping *C. psittaci* strains that was based on 8 selected VNTR loci identified in the genomic
222 sequence of strain 6BC (Laroucau *et al.*, 2008). To this end the 6 *C. abortus* prototypes C9/98, AB7,
223 B577, BAF, S26/3 and POS were amplified using the 8 *C. psittaci* primer sets ChlaPsi_280,
224 ChlaPsi_480, ChlaPsi_605, ChlaPsi_810, ChlaPsi_222, ChlaPsi_281, ChlaPsi_929 and ChlaPsi_1778,

225 Amplification patterns for all 6 *C. abortus* prototypes were obtained with only 3 of the 8 avian primer
226 sets, ChlaPsi_480, ChlaPsi_281 and ChlaPsi_1778, while strains C9/98, S26/3 and POS were also
227 amplified with ChlaPsi_280. Amplifications with the other 4 primers ChlaPsi_605, ChlaPsi_222,
228 ChlaPsi_810 and ChlaPsi_929 were of low efficiency and were not consistently observed (Figure 4).
229 The PCR products with primers ChlaPsi_480 and ChlaPsi_281 corresponded to 1 and 3 repeat units,
230 respectively. The amplified products of strains POS and LLG with marker ChlaPsi_1778 corresponded
231 to 4 repeat units whereas the size of the PCR products in all other *C. abortus* strains was unique, never
232 encountered before in *C. psittaci* (Figure 4).

233 Taken together, the results showed that, though few VNTR primers produced amplification products with
234 the heterologous species, amplification with the total number of VNTR primer sets was restricted to
235 members of the homologous species, indicating that the developed MLVA typing systems were species-
236 specific.

237

238 4. DISCUSSION

239 Epidemiological studies of *C. abortus* isolates have been hampered by the requirement of culturing this
240 fastidious obligate intracellular bacterium. Here, we present a new MLVA-based molecular typing
241 system for the discrimination of *C. abortus* isolates that does not require culture and can therefore be
242 performed directly on DNA extracted from field/clinical specimens. MLVA typing is a reproducible
243 method which can be standardized and performed at low cost, thus facilitating large-scale molecular
244 epidemiological investigations. Out of 34 tandem repeat loci identified in the *C. abortus* S26/3 genome
245 by the program Tandem Repeat Finder, 5 were polymorphic in a preliminary PCR screen, thus
246 confirming the high genetic homogeneity of this chlamydial species. By comparison, 8 VNTRs were
247 found in *C. psittaci* using just 20 primer pairs (Laroucau *et al.*, 2008). The final analysis of about 145 *C.*
248 *abortus* strains and field isolates resulted in a total of 6 distinct patterns designated genotype [1] through
249 [6]. Strains belonging to genotype [1] through [5] were more closely related to each other than to the two
250 Greek variant strains LLG and POS, constituting genotype [6]. These findings were in accordance with
251 previous studies demonstrating significant differences of the two strains (Vretou *et al.*, 1996).
252 Interestingly, the commercial vaccine strain 1B was classified as genotype [2]. In this sense MLVA could

253 provide the means to differentiate between vaccinated and naturally infected animals if local strains
254 were of a different genotype.

255 Three of the VNTR markers identified in the present study were located within genes or ORFs encoding
256 predicted membrane or exported proteins (markers ChlaAb_300, ChlaAb_581 and ChlaAb_620
257 (pseudogene)). A further marker was located in *hctB*, a histone-like protein gene involved in establishing
258 the nucleoid structure of chlamydial elementary bodies (CAB398, marker ChlaAb_457). In *C.*
259 *trachomatis*, Hc1 is conserved among serovars while Hc2 (encoded by *hctB*) varies in size between
260 strains due to internal deletions encoding lysine-and alanine-rich pentameric repeats (Hackstadt *et al.*,
261 1993). Indeed, different numbers of repetitive elements and point mutations were recently detected in
262 *hctB* in genital clinical samples, leading to high-resolution intra-serotype variation in *C. trachomatis* (Klint
263 *et al.*, 2008). Furthermore, polymorphic sites including *hctB* were responsible for the pathogenic
264 diversity of *C. trachomatis* trachoma strains (Kari *et al.*, 2008). In the present study the *hctB* differed in
265 the Greek strains LLG and POS bearing an 72 nucleotide repeat encoding a 24mer peptide. Previous
266 studies had shown that the polypeptide profiles of strains LLG and POS were characterized by the
267 presence of a band at 26.5 kDa that was absent from the other *C. abortus* strains, and by the absence
268 of a polypeptide at 22-23 kDa present in the other strains. It had been suggested by the same authors
269 that these peptides could represent histone-like proteins that could eventually prove useful
270 epidemiological tools for the identification of LLG-like strains (Vretou *et al.*, 1996).

271 A series of previous studies based either on the reactivity with monoclonal antibodies (Salinas *et al.*,
272 1995) or on ribosomal and *ompA* sequences have highlighted the remarkable homogeneity within the
273 species *C. abortus* (Denamur *et al.*, 1991; Everett *et al.*, 1999). However, when AFLP, a technique
274 based on whole-genome analysis not requiring knowledge of the genomic sequence, was applied to *C.*
275 *abortus* strains, it was possible to accomplish differentiation within this species and identify a cluster of
276 strains comprising French chlamydial isolates (Boumedine and Rodolakis, 1998). All strains studied by
277 AFLP (n=12) were included in the present study, except strain PS22. Clustering by MLVA was similar to
278 that by AFLP (Boumedine and Rodolakis, 1998). However, the French strains, AC1 and AV1 could be
279 distinguished by AFLP, but not by MLVA. MLVA is based on the detection of polymorphic repeat units,
280 whereas AFLP identifies point mutations dispersed throughout the genome. Both techniques seem to be
281 complementary as already observed for the genotyping of *C. psittaci* (Laroucau *et al.*, 2008).

282 Interestingly, MLVA classified UK strains only in genotypes [4] and [5] and French strains in genotype
283 [2]. Only one French clinical sample was classified in genotype [1].

284 Apart from genotype [6], the number of tandem repeats was higher in genotype [5] than in all other
285 genotypes. Genotype [5] includes the sequenced S26/3 strain (Thomson *et al.*, 2005) from which the
286 present MLVA marker panel has been deduced. Availability of genome sequences from other MLVA
287 groups, e.g. AB7 or C9/98, will most probably give access to an additional number of tandem repeats
288 and thus will increase the discriminatory power of MLVA. Variability observed in short repeated motifs,
289 i.e., up to 9 bp, is thought to be caused by slipped-strand mispairing, which may occur in combination
290 with inadequate mismatch repair pathways (Bichara *et al.* 2006). However, there are many indications of
291 the way in which larger sequences are duplicated.

292 The present study complements and expands the recent report on the development and application of
293 MLVA for typing strains of the species *C. psittaci*. (Laroucau *et al.*, 2008). Both MLVA studies do not
294 only provide means to type strains or field isolates within the species, but also provide markers that
295 discern between the 2 genetically related species, as presented in Figures 3 and 4. This is of particular
296 importance since *C. abortus* has also been detected in bird infections (see Pantchev *et al.*, 2008).
297 However it is highly recommended to determine the chlamydial species before performing MLVA
298 genotyping as suggested by the same authors.

299 In summary, our findings show that MLVA is a new typing method providing enhanced discrimination of
300 *C. abortus* isolates from various hosts and geographical origins. The biological significance of the coding
301 tandem repeats identified in the putative lysine decarboxylase (CAB502), in the 30S ribosomal protein
302 S18 (CAB786), in *pmp5E* (CAB267) and in the *hctB* gene (CAB398) is unknown. Coding tandem
303 repeats are believed to enhance specific properties of bacterial virulence factors. It is anticipated that
304 application of MLVA to a large number of pathological isolates will not only prove useful to future
305 veterinary surveys but will also might help establishing a possible association of these markers of
306 diversity with pathogenic traits.

307

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313

314 CONFLICT OF INTEREST STATEMENT

315 None of the authors has a financial or personal relationship with other people or organisations that could
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FIGURES

Figure 1. VNTR amplicon sizes. PCR amplification of *C. abortus* strains C9/98 (A), AB7 (B), B577 (C), BAF (D), S26/3 (E) and POS (F) with ChlaAb_457, ChlaAb_581, ChlaAb_620, ChlaAb_914 and ChlaAb_300 (see Table 1).

A 100-bp ladder (100 to 1,000 bp) is run on both sides of each group of samples. The number of repeat units within each allele is indicated.

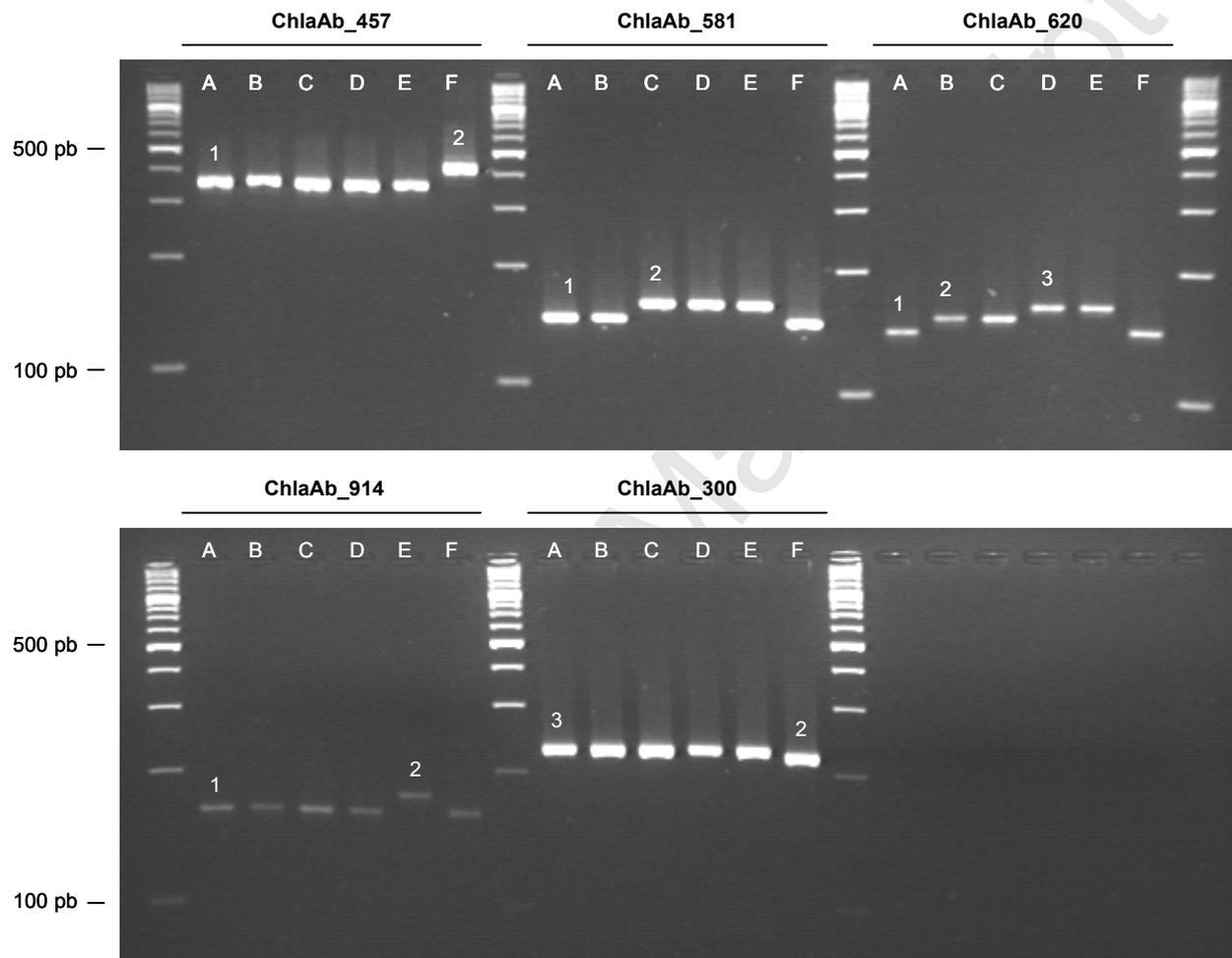


Figure 3. *C. abortus* MLVA scheme on *C. psittaci* DNA. PCR amplification of the 5 VNTRs ChlaAb_457, ChlaAb_581, ChlaAb_620, ChlaAb_914 and ChlaAb_300 using DNA from 3 *C. abortus* strains POS (A), AB7 (B) and S26/3 (C) and from the 6 *C. psittaci* reference strains Loth (D), CP3 (E), GR9 (F), NJ1 (G), Cal10 (H) and VS225 (I). Negative control is in well J. A 100-bp ladder (100 to 1,000 bp) is run on both sides of each group of samples. The number of repeat units within each allele is indicated.

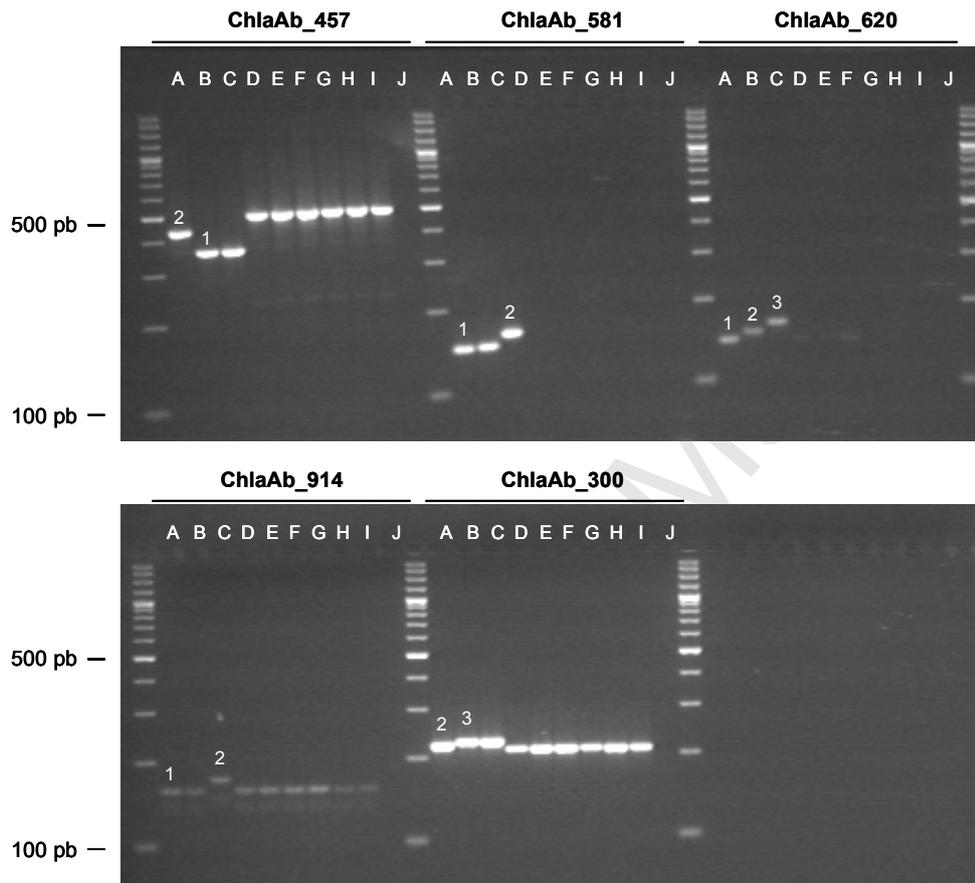
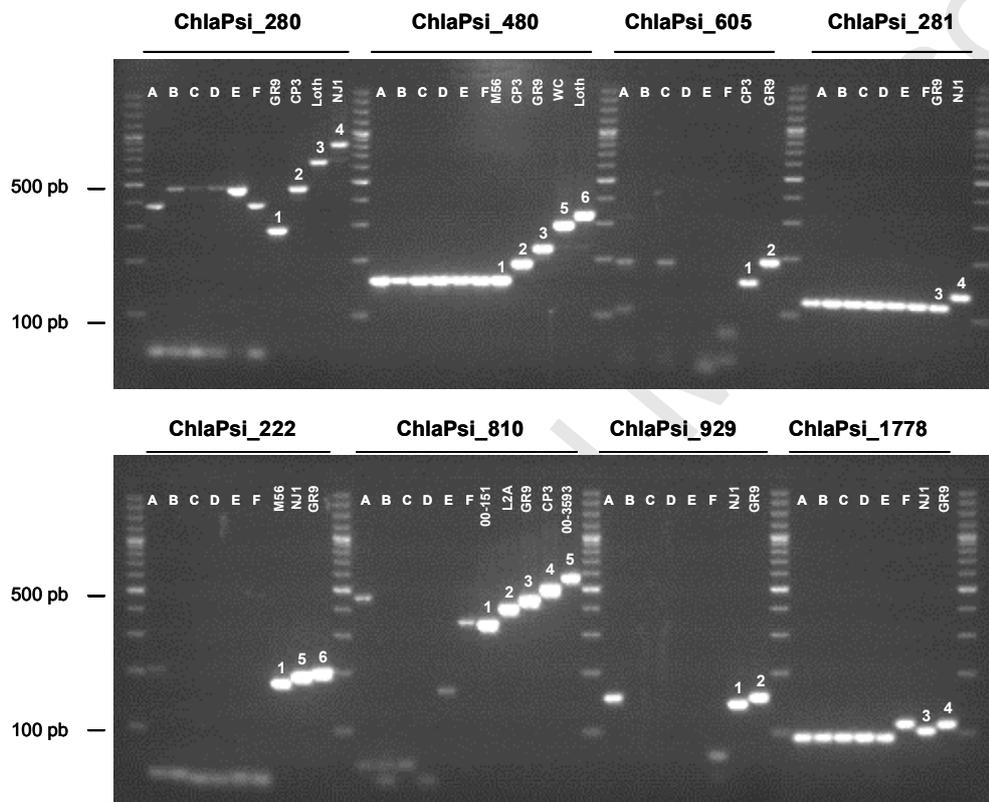


Figure 4. *C. psittaci* MLVA scheme on *C. abortus*. DNA. PCR amplification of the 8 VNTRs ChlaPsi_280, ChlaPsi_480, ChlaPsi_605, ChlaPsi_810, ChlaPsi_222, ChlaPsi_281, ChlaPsi_929 and ChlaPsi_1778 using DNA from 6 *C. abortus* prototypes C9/98 (A), AB7 (B), B577 (C), BAF (D), S26/3 (E), POS (F) and from selected *C. psittaci* strains for comparison. The strains selected for each primer was according to previous patterns and their names are inserted in the figure (Laroucau *et al.*, 2008). The number of repeat units within each allele is indicated.

A 100-bp ladder (100 to 1,000 bp) is run on both sides of each group of samples.



TABLES

Table 1. *C. abortus* isolates organised by MLVA genotype. Genotypes were based on similarity clustering of PCR product sizes obtained with the 5 selected primer sets; genotypes are characterised by numbers 1 through 6, shown in square brackets. Underlined strains were used for the complete MLVA based on 34 sets of primers.

MLVA genotype	ChlaAb_457	ChlaAb_581	ChlaAb_620	ChlaAb_914	ChlaAb_300	Nb of strains	Country	Species	Strains
[1]	1	1	1	1	3	1	De	sheep	<u>C9/98^c</u>
							De	cattle	03DC34 ^c , 03DC37 ^c , 1274/80 ^d , Tiho 22 ^d
							De	goat	Kra ^b
							De	sheep	C1/98 ^c , C10/98 ^c , C17/98 ^c , C7/98 ^c , DC1 ^c , DC7 ^c , Z 339/84 ^d , Z 375/99 ^d , Z 346/99 ^d , Z 295/99 ^d , Z 241-IV/99 ^d , Z 241-III/99 ^d , Z 236-I/99 ^d , Z 227/99 ^d , Z 203/99 ^d , Z 184/99 ^d , Z 183/99 ^d , Z 174/99 ^d , Z 153/99 ^d , Z 154/99 ^d , Z 81/99 ^d , Z 106/97 ^d , Z 43-I/97 ^d , Z 136/96 ^d , Z 3479/92 ^d , Z 2512/89 ^d , Z 2488/89 ^d , Z 2181/89 ^d , Z 2007/88 ^d , Z 1905/88 ^d , Z 1808/87 ^d , Z 1509/87 ^d , Z 1493/87 ^d , Z 326/84 ^d , Z 319/84 ^d , Z 75/83 ^d , Z 27/83 ^d , 623/83 ^d , 96 ^d , 53 ^d , Vi 1902 ^d , Vi 760 ^d , Vi 1921 ^d , Vi 1825 ^d , Vi 233 ^d , Vi 202 ^d , Vi 155 ^d , Vi 63 ^d , Vi 19 ^d , Vi 3 ^d , Z 201/01 ^d , Z 52/02 ^d , Z 78/02 ^d , Z 178/02 ^d , Z 213/02 ^d , Z 207-2/03 ^d , Z 164/04 ^d , Z 157/04 ^d
[2]	1	1	2	1	3	115	Fr	vaccine	<u>1B^a</u>
							Fr	mutant	<u>1H^a</u>
							Fr	cattle	<u>AV1^a</u>
							Fr	goat	<u>AC1^a</u> , <u>iC1^a</u>
							Fr	sheep	<u>AB1^a</u> , <u>AB2^a</u> , <u>AB7^a</u> , <u>AB8^a</u> , <u>AB9^a</u> , <u>AB11^a</u> , <u>AB13^a</u> , <u>AB15^a</u> , <u>AB16^a</u> , <u>AB22^a</u> , <u>QC1^a</u> , <u>Q18^a</u> , <u>VB1^a</u>
							Fr	sprinkbok	<u>SB1^a</u>
							Gr	goat	<u>FAG^d</u> , MB ^d , <u>VPG^d</u>
							Gr	sheep	MA ^d , MD ^d , <u>ME^d</u>
							It	goat	1107/1 ^e , 1107/2 ^e , 9/2 ^e , 27684 ^e , 36550 ^e , 60172 ^e
							It	sheep	221176 ^e , 3053/1 ^e , 201/2 ^e , 39678 ^e , 38552 ^e
							Namibia	goat	C21/98 ^c , A-57 ^d , A-55 ^d
							Tunisia	goat	532 ^d , 363 ^d , 469 ^d , 15 ^d
							unknown	unknown	Z 947/85 ^d , Z 739/99 ^d , Z 631/99 ^d , Z 74/05 ^d , Z 81/05 ^d , Z 17/06 ^d , Z 22/06 ^d , Z 16/06 ^d
							unknown	sheep	C16/98 ^c
[3]	1	2	2	1	3	8	De	cattle	03DC36 ^c , Z 1215/86 ^d
							Gr	sheep	<u>Z1^b</u>
							Namibia	goat	A-58 ^d
							USA	sheep	<u>B577^a</u> , <u>Mo907^a</u> , B577 ^d
							unknown	cattle	C13/98 ^c
[4]	1	2	3	1	3	1	UK	cattle	<u>BAF^a</u>
[5]	1	2	3	2	3	18	De	sheep	DC4 ^c
							Gr	sheep	<u>FAS^b</u>
							It	goat	4PV ^a
							UK	cattle	<u>BA/M^a</u> , <u>BA1^a</u>
							UK	sheep	<u>H574^a</u> , <u>1H77^a</u> , <u>S26/3^a</u> , S26/3 ¹ , <u>A22^a</u> , A22 ¹ , S3/3 ¹ , S95/3 ¹ , S152/3 ¹ , S124/3A ¹ , S82/3 ¹ , SP9001 ^d , C20/98 ^c
[6]	2	1	1	1	2	2	Gr	sheep	<u>POS^b</u>
							Gr	goat	<u>LLG^c</u>

Laboratories providing *C. abortus* strains: ^aINRA, ^bHellenic Pasteur Institute, ^cFriedrich Loeffler Institute, ^dInstitute for Hygiene and Infectious Diseases of Animals, ^eIstituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", and ^fMoredun Research Institute.

Country abbreviations: France (Fr), Germany (De), Greece (Gr), Italy (It), United Kingdom (UK), United States of America (USA).

Table 2. Direct MLVA typing on French clinical samples. Genotypes were based on similarity clustering of PCR product sizes; they are characterised by numbers 1 through 6, shown in square brackets.

Name	Species	Sample	MLVA genotype
08-1301/2	sheep	vaginal swab	[2]
08-1301/9	cattle	placenta	[2]
08-1301/11	cattle	placenta	[2]
08-1301/15	cattle	mucus	[2]
08-1301/18	cattle	placenta	[2]
07-1463/40752	sheep	placenta	[2]
07-1463/40753	sheep	placenta	[2]
07-525/J	sheep	vaginal swab	[1]
08-1250	goat	placenta	[2]

Table 3. Primers for PCR amplification and MLVA. These primers were used to characterize tandem repeats in 34 genetic loci. Primers ChlaAb_457, ChlaAb_581, ChlaAb_620, ChlaAb_914 and ChlaAb_300 were selected for the MLVA. Theoretical repetition numbers and repeat units were detected by the program Tandem Repeat Finder after analysis of the *C. abortus* S26/3 genome.

Name	Forward primer (5'–3')	Reverse primer (5'–3')	Repeat unit (bp)	<i>C. abortus</i> S26/3 theoretical repetition number	whole amplified <i>C. abortus</i> S26/3 fragment size
ChlaAb_22	CCATACGCAAATCCTGATATTACA	CGCCCTGATTTGCCGAT	21	2.3	171
ChlaAb_76	CTCGTCAGGCAGTAGCACAAATT	TCTGTGTGAAGTCCAAAAGCATC	12	2.1	164
ChlaAb_105	CAGGAATCATCGAAGGCTTCA	AGAAGCACACAACGCAAATCC	6	4.3	230
ChlaAb_109	TGTAAGCATCCAAAGTATACATTTATCGT	CCCGATAGAATGGCCTTCATAG	8	3.1	226
ChlaAb_112	GGAGCCTCATGGAAACGTGA	GTAGCCATGATCTGCCCTTCA	7	3.4	276
ChlaAb_165	CGATTCTCCTCGAGCTTTTCA	TTAGTGCTATCAACCGGACG	7	3.1	226
ChlaAb_174	CGGAGACTACGCTTACGGCT	ACCGCAGAATCCGTAACGTT	7	3.4	226
ChlaAb_198	CTTTCTTTATCACACAGTTGTGCGTATAC	CGTGCTTGATTCCTTATTAATAAACCC	6	4.8	230
ChlaAb_242	TACCATCGGATGCTGTAGGGA	CGATAATAAGCCTGCGACTCG	7	3.3	224
ChlaAb_256	ATATCGCTTGGCGCAAGTTTA	CCTAGGCGGAGACGACTTTG	11	3.2	338
ChlaAb_283	TTTTGCTGGCTCATAGGC	TGGGAAGGATCTTGCTCTTCA	9	3.3	231
ChlaAb_294	CGTACGTAGCCCCGAAAGAG	GCGATAACGCAACTCCGAA	6	4.0	277
ChlaAb_300	AGACCTAAAGCGCCACCTTCA	ATGCGCCAATCTATACGCTGA	9	3.0	227
ChlaAb_304	GATTTATGGTTTTGGGCAGCAA	GGGTCTTCTCACACAGCCGTA	7	3.4	227
ChlaAb_312	GGATTCAACGCTTGAGTGCA	TGCCAAGGCTCCTCACTACAA	6	3.5	225
ChlaAb_318	CTAGTGGAGCCGTTGAATCCA	TCACCTGCAAGACACGCAAG	8	3.0	325
ChlaAb_404	GTTTTGTCAATTGTTGGCCTGG	AAAGCGCTCTGTTAATAAACCTTCAT	6	4.2	230
ChlaAb_457	GTACAAAAAAACGTAGCAGCAAGAA	CACGTTGGCAAGAAGCTGTGT	28	2.8	358
ChlaAb_488	CTATTTACAAGGCGTTACTTAGCG	AGGTATCTTCAGGGAACATCACG	15	2.0	179
ChlaAb_494	CCTCCCGTGCATCCTAAGC	AACCATTGAGGATTCAGCCG	6	4.2	276
ChlaAb_509	CTTTGACTTGTGGGTAGTGACAT	TTACAACAGCATATCTTCAGTCGCT	18	2.0	152
ChlaAb_533	CCGTCATGCCTAAATTGGAAA	TGCTTGGCCTTAGCTTTAAA	6	3.3	275
ChlaAb_546	GGTAGTATTTTTCACTACGCTTTCCAG	TTAGAATCAAATCAATCTGAAGTGAAC	6	3.5	223
ChlaAb_575	GGAAATACCCATGTAGGACGTATACC	CAATCCGACTAATTTGGTCTCAATT	9	3.1	231
ChlaAb_581	ACAGCACCAGCATTAGCCG	TGGATAGTTGTCGCTGGTGG	15	2.1	161
ChlaAb_620	ATGCTATAATTGCTTAGTTTTTTTAAACATTG	CACATGCCGCCCTGAAC	11	3.6	163
ChlaAb_656	TAGGACTCATGCAGCCCAATC	TTTTTGGCCTCCACTCAGCT	6	3.5	233
ChlaAb_805	AATGTTTGCTGAGCAAGCCC	TCCCCTGCAACCTCATGAAT	7	3.0	272
ChlaAb_839	GATTTGCTCCCCACGTTT	ATTTGCGTTTGGGAGCAGG	7	3.6	227
ChlaAb_873	TCCGGCATGACGCTACAAC	AAGGCACATGATCCCACAAAA	8	3.8	281
ChlaAb_914	TTTAAAGTTTCCGTATCTTTGTAATCGAT	TTTTAGAATTCGCATCATTACCAGAA	15	2.3	174
ChlaAb_924	CCCAGGAGAGAATTGGCAA	GCAATGCCACCCCTTATCAA	6	4.2	177
ChlaAb_940	TGGAGAGCTATGCCAATGCTT	GCGAGCCGTTTTCAATACTG	6	4.2	230
ChlaAb_1077	GTGGTTCCATTGTTGCGG	GCAGTTTTATGGCTTGCATCAC	33	1.9	191