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Genotyping of *Chlamydomophila abortus* strains by Multilocus VNTR Analysis

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ABSTRACT

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

KEY WORDS: *Chlamydophila abortus*, intra-species differentiation, MLVA typing, molecular epidemiology.

1. INTRODUCTION

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

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

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

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

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

2. MATERIALS AND METHODS

2.1 Bacterial strains and isolates

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

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

2.2 Clinical samples

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

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

2.3 MLVA genotyping method

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

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

2.4 Data analysis

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

2.5 Sequencing of PCR products

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

3. RESULTS

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

Thirty four tandem repeats with a repeat unit equal to or larger than 6 bp were identified by the program Tandem Repeat Finder (TRF) in the whole genome sequence of *C. abortus* strain S26/3 (Table 3). In order to evaluate the polymorphism within these potential VNTR markers, primers were chosen on both sides of the repeats and PCRs were performed on an initial set of 34 *C. abortus* strains (Table 1). Gel analysis of the size of the amplified fragments showed that only 5 of the tandem repeat loci, namely ChlaAb_457, ChlaAb_581, ChlaAb_620, ChlaAb_914 and ChlaAb_300 were polymorphic for these 34 strains (Figure 1 and data not shown). Furthermore, it was possible to cluster the 34 *C. abortus* strains into 5 distinct genotypes based on the size of the amplified PCR products, highlighting thus the suitability of the selected 5 tandem repeat loci for establishing and developing a genotyping system for *C. abortus* (Figure 1 and Table 1). The repeat units were localized in CAB267, the coding sequence of the polymorphic outer membrane protein pmp5E, in CAB398 coding for a histone-like protein, in CAB502 coding for a putative exported protein with a lysine decarboxylase motif, in CAB541 coding for a conserved membrane protein (pseudogene), and in CAB786 coding for the 30S ribosomal protein S18 for markers ChlaAb_300, ChlaAb_457, ChlaAb_581, ChlaAb_620, and ChlaAb_914, respectively.

3.2 Evaluation of the selected VNTRs for MLVA analysis

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

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

3.3 MLVA genotyping of *C. abortus* isolates

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

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

3.4 Direct typing of clinical samples.

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

3.5 Sequence analysis of PCR products

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

ChlaAb_300, where the polymorphism is due to deletion in the targeted sequence for POS, the repeat unit was localized similarly in all other sequences and its presence or absence in the sequence resulted into the experimental polymorphism (Figure 2). The observed repeat size was a multiple of 3 nucleotides for all VNTRs contributing thus to the coding sequence of the loci. An exception to this was ChlaAb_620 (pseudogene CAB541). Consequently, the polymorphism in this locus was due to a stop in translation in one allele (2 copies of a 11 bp repeat) and to a frameshift in another allele (3 copies), as compared to the allele with a single copy of the repeat. In CAB502 (ChlaAb_581) the 15-mer TAGTTAAACATGGGT coding for peptide LVKHQ, was repeated in strains B577, BAF and S26/3 but not in strains C9/98, AB7 and POS, whereas in CAB786 (ChlaAb_914) the 15-mer GCCTGTTTCATAATAA coding for peptide KPVHN was repeated only in strain S26/3. In the histone-like protein *hctB* (CAB398, ChlaAb_457) the 24-mer peptide AKKTATRKPAVKKAVRKTAACKAT was repeated once in strain POS.

Summarized, the results showed that the MLVA typing method developed on the analysis of 5 VNTR loci was capable of clustering 145 *C. abortus* strains in 6 genotypes and that variation among genotypes was caused in 3 loci by the presence or absence of coding tandem repeats.

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

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

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

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

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

4. DISCUSSION

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

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

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

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

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

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

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

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

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314 CONFLICT OF INTEREST STATEMENT

315 None of the authors has a financial or personal relationship with other people or organisations that could
316 inappropriately influence or bias this paper.

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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	C9/98 ^c
							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^b</u> , MB ^b , <u>VPG^b</u>
							Gr	sheep	MA ^b , MD ^b , <u>ME^b</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	Z1 ^b
							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>
							De	sheep	DC4 ^c
							Gr	sheep	<u>FAS^b</u>
							It	goat	4PV ^a
[5]	1	2	3	2	3	18	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 ⁱ , <u>A22^a</u> , A22 ⁱ , S3/3 ⁱ , S95/3 ⁱ , S152/3 ⁱ , S124/3A ⁱ , S82/3 ⁱ , 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	CCATACGCAAAATCCTGATATTACA	CGCCCTGATTTGCCGAT	21	2.3	171
ChlaAb_76	CTCGTCAGGCAGTAGCACAAATT	TCTGTGTGAAGTTCCAAAAGCATC	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	CTTTCTTTATCACACAGTTGTCGCTATAC	CGTGCTTGATTCCTATTAAAAACC	6	4.8	230
ChlaAb_242	TACCATCGGATGCTGTAGGGA	CGATAATAAGCCTGCGACTCG	7	3.3	224
ChlaAb_256	ATATCGCTTGCGGCAAGTTTA	CCTAGGCGGAGACGACTTTG	11	3.2	338
ChlaAb_283	TTTTGCTGCGCTCATAGGC	TGGGAAGGATCTTGCTCTTCA	9	3.3	231
ChlaAb_294	CGTACGTAGCCCCGAAAGAG	GCGATAACGCAACTCCGAA	6	4.0	277
ChlaAb_300	AGACCTAAAGCGCCACCTTCA	ATGCGCCAATCTATACGCTGA	9	3.0	227
ChlaAb_304	GATTTATGGTTTGGGCAGCAA	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	CTATTTTACAAGGCGTTACTTAGCG	AGGTATCTTCAGGGAACATCACG	15	2.0	179
ChlaAb_494	CCTCCCGTGCATCCTAAGC	AACCATTGAGGATTCAGCCG	6	4.2	276
ChlaAb_509	CTTTGACTTGTGGGTAGTGACAT	TTACAACAGCATATCTTCAGTCGCT	18	2.0	152
ChlaAb_533	CCGTCATGCCTAAATTGGAAA	TGCTTGCCTTAGCTTTAAA	6	3.3	275
ChlaAb_546	GGTAGTATTTTCACTACGCTTTCCAG	TTAGAATCAAATCTCAATCTGAAGTGAAT	6	3.5	223
ChlaAb_575	GGAAATACCATGTAGGACGTATACC	CAATCCGACTAATTTGGTCTCAATT	9	3.1	231
ChlaAb_581	ACAGCACCAGCATTAGCCG	TGGATAGTTGTCGCTGGTGG	15	2.1	161
ChlaAb_620	ATGCTATAATTGCTTAGTTTTTTTAACTTG	CACATGCCGCCCTGAAC	11	3.6	163
ChlaAb_656	TAGGACTCATGCAGCCCAATC	TTTTTGGCCTCCACTCAGCT	6	3.5	233
ChlaAb_805	AATGTTTGTGAGCAAGCCC	TCCCCTGCAACCTCATGAAT	7	3.0	272
ChlaAb_839	GATTTGCTCCCCACGTTT	ATTTGCGTTTGGGAGCAGG	7	3.6	227
ChlaAb_873	TCCGGCATGACGCTACAAC	AAGGCACATGATCCCAAAAA	8	3.8	281
ChlaAb_914	TTTAAAGTTTCCGTATCTTTGTAATCGAT	TTTTAGAATTGCGCATATTACCAGAA	15	2.3	174
ChlaAb_924	CCCAGGAGAGAATTGGCAA	GCAATGCCACCCCTTATCAA	6	4.2	177
ChlaAb_940	TGGAGAGCTATGCCAATGCTT	GCGAGCCGTTTTCAATACTG	6	4.2	230
ChlaAb_1077	GTGGTTTCCATTGTTGCGG	GCAGTTTTTATGGCTTGCATCAC	33	1.9	191