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New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*

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ABSTRACT

Rapid and specific identification of *Brucella suis* at the biovar level is necessary because some of the biovars that infect animals are pathogenic for humans. None of the molecular typing methods described so far are able to discriminate *B. suis* biovars in a single test and differentiation of *B. suis* from *Brucella canis* by molecular approaches can be difficult. This article describes a new multiplex PCR assay, Suis-ladder, for fast and accurate identification of *B. suis* at the biovar level and the differentiation of *B. suis*, *B. canis* and *Brucella microti*. An advancement of the original Bruce-ladder PCR protocol which allows the correct discrimination of all known *Brucella* species is also described.

1. Introduction

Brucella suis, the causative agent of swine brucellosis, is classified in five biovars that infect different animal hosts: biovars 1, 2 and 3 affect domestic pigs, wild boars and hares; biovar 4 infects reindeer and caribou; and biovar 5 infects only rodents. In contrast to biovar 2, biovars 1 and 3 are pathogenic for humans and require high level of biosafety laboratory precautions (Garin-Bastuji and Hars, 2001). The identification and typing of *B. suis* is currently performed by standard bacteriological and biochemistry methods, but these tests are not straightforward in particular for the identification of biovars 1, 2 and 3 (Alton et al., 1988). With the objective of improving the typing of *B. suis*, different PCR-based assays have been proposed. One of these assays is a multiplex conventional PCR (Bruce-ladder) (García-Yoldi et al., 2006; López-Goñi et al., 2008). In general Bruce-ladder performs excellently and has been recommended by the OIE as a rapid and simple one-step molecular test for identification and typing of *Brucella* species (OIE, 2009). However, its only inconvenience is that some *Brucella canis* strains and *Brucella microti*, isolated from common voles and foxes, can be identified erroneously as *B. suis* (López-Goñi et al., 2008; Scholz et al., 2008). The present report describes a novel multiplex conventional PCR assay (called Suis-ladder) that differentiates between all *B. suis* biovars and *B. canis*, and also propose an advancement of the original Bruce-ladder PCR to distinguish between these two species.

2. Materials and methods

2.1. Strains

To ensure an adequate diversity, a representative collection of *B. suis* and *B. canis* reference strains and field

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Table 1
Oligonucleotides used in the Suis-ladder multiplex PCR assay.

Primer[a]	Sequence (5′–3′)	Amplicon size (bp)	Source of genetic differences	References
BMEI1426	TCG TCG GTG GAC TGG ATG AC	774	Deletion of 351 bp in BMEI1426-BMEI1427 in *B. canis*	Zygmunt et al. (2009)
BMEI1427	ATG GTC CGC AAG GTG CTT TT			
BR1080f	CCC TTG GTT TGT AGC GGT TG	197	Deletion of 162 bp in BR1080 in *B. abortus* and *B. melitensis*	Halling et al. (2005)
BR1080r	TCA TCG TCC TCC GTC ATC CT			
BMEI1688	TCA ACT GCG TGA ACA ATG CT	278	Deletion of 20,883 bp in BMEI1674-BMEI1703 in *B. suis* and *B. canis*	Rajashekara et al. (2004), Halling et al. (2005)
BMEI1687	GCG GGC TCT ATC TCA AGG TC			
BMEI0205f	CGT CAA CTC GCT GGC CAA GAG	299,425	Derived from VNTR Bruce 11	Le Flèche et al. (2006)
BMEI0205r	GCA GGA GAA CCG CAA CCT AA	551,614		

[a] Designation are based on the *B. melitensis* (BME) or *B. suis* (BR) genome sequences. f, forward; r, reverse.

isolates from different geographic origins and different animal species was examined (see supplemental material). When needed, *Brucella* reference strains were used. All *Brucella* isolates were typed according to standard procedures (Alton et al., 1988). Growth and harvesting of *Brucella* cells and bacterial DNA extraction were performed as described elsewhere (García-Yoldi et al., 2006, 2007).

2.2. Molecular typing

Isolates were subjected to different PCR-based typing techniques: PCR-RFLP for *omp2a*, *omp2b* and *omp31* genes (Cloeckaert et al., 1995; Vizcaíno et al., 1997), multiplex AMOS-*ery*-PCR (Ocampo-Sosa et al., 2005), original multiplex Bruce-ladder (García-Yoldi et al., 2006; López-Goñi et al., 2008), and *Brucella* MLVA (Le Flèche et al., 2006). A new multiplex PCR (Suis-ladder) able to discriminate among all five biovars of *B. suis* and *B. canis* was developed. For this, four pairs of oligonucleotide primers were designed and selected based on biovar-specific genetic differences (Table 1). Amplification of DNA was performed in 25 μl of a reaction mixture containing 1 μl of template DNA, 200 μM of each dNTP (Promega Corp.), 2 mM of $MgCl_2$, 1 U of Immolase DNA polymerase and its amplification buffer (Bioline Ltd.), and 12.5 pmol of each primer. After an initial denaturation at 95 °C for 7 min, the PCR profile was set as follows: 35 s of template denaturation at 95 °C, 45 s of primer annealing at 63 °C, and 60 s of primer extension at 72 °C, for a total of 30 cycles, with a final extension at 72 °C for 6 min. PCR products were analyzed by standard 1.5–2.0% agarose electrophoresis.

3. Results and discussion

A representative example of the multiplex Suis-ladder PCR result is presented in Fig. 1. PCR using *B. suis* DNAs from the five reference biovars gave different band profiles: biovar 1 amplified three fragments of 774, 425 and 197 bp; biovar 2 amplified three fragments of 774, 551 and 278 bp; biovar 3 amplified three fragments of 774, 299 and 197 bp; biovar 4 amplified three fragments of 774, 614 and 197 bp; and biovar 5 amplified four fragments of 774 bp, 614, 278 and 197 bp. Then, the Suis-ladder PCR was applied to a total of 99 *B. suis* field isolates (see supplemental material). All isolates from *B. suis* biovars 1, 2 and 4 displayed the same PCR profiles to the corresponding type strains (Fig. 1), consistent with the biovar assigned by biochemical and molecular characterization. The only exceptions were few *B. suis* field isolates identified by standard microbiological tests as biovar 3 (see supplemental material). Two of these isolates (refs. 03-3081-02 and 01-1825-04) were typed as biovar 3 also by MLVA but were classified as biovar 1 by PCR-RFLP for *omp* genes, AMOS-*ery*-PCR and the new Suis-ladder. On the other hand, three isolates (refs. 04-3025-03, 04-1361-S4, 04-1361-S5) were typed as biovar 3 only by standard microbiological procedures but as biovar 1 by all other molecular techniques. The existence of *B. suis* field strains typed as biovar 3 with the classical biochemical scheme that fit with the genetic profiles of biovar 1 has been described by others (Ferrao-Beck et al., 2006; Le Flèche et al., 2006; Whatmore et al., 2006; Fretin et al., 2008; Huber et al., 2009) and confirms that the taxonomical situation of *B. suis* biovar 3 can be questioned (Fretin et al., 2008).

When the Suis-ladder PCR was applied to the *B. canis* reference strain a specific PCR profile clearly different from *B. suis* was obtained: only two fragments of 614 and 197 bp were amplified (Fig. 1). Interestingly, the 774 bp fragment common to all *B. suis* biovars was not obtained with *B. canis*

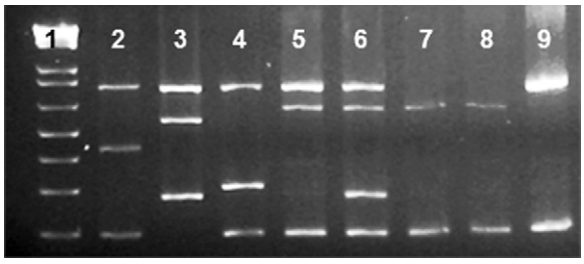

Fig. 1. Suis-ladder multiplex PCR: differentiation of all *B. suis* biovars, *B. canis* and *B. microti*. Lane 1, molecular marker 1 kb plus DNA ladder (Invitrogen Ltd.); lane 2, *B. suis* biovar 1 strain 1330; lane 3, *B. suis* biovar 2 strain Thomsen; lane 4, *B. suis* biovar 3 strain 686; lane 5, *B. suis* biovar 4 strain 40; lane 6, *B. suis* biovar 5 strain ELT80; lane 7, *B. canis*, with a *B. canis* profile in the original Bruce-ladder; lane 8, *B. canis*, with a *B. suis* profile in the original Bruce-ladder; lane 9, *B. microti*.

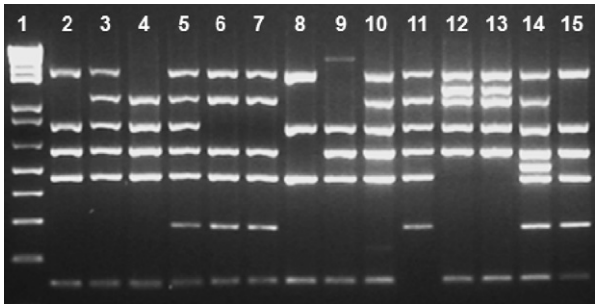

Fig. 2. Bruce-ladder v2.0 multiplex PCR: differentiation of all known *Brucella* species and vaccine strains. Lane 1, molecular marker molecular marker 1 kb plus DNA ladder (Invitrogen Ltd.); lane 2, *B. abortus*; lane 3, *B. melitensis*; lane 4, *B. ovis;* lane 5, *B. suis;* lane 6, *B. canis* isolate with a *B. canis* profile in the original Bruce-ladder; lane 7, *B. canis* isolates with a *B. suis* profile in the original Bruce-ladder; lane 8, *B. abortus* S19; lane 9, *B. abortus* RB51; lane 10, *B. melitensis* Rev.1; lane 11, *B. neotomae*; lane 12, *B. pinipedialis*; lane 13, *B. ceti*; lane 14, *B. microti*; lane 15, *B. inopinata*.

DNA. The Suis-ladder PCR was then applied to a total of 17 *B. canis* field isolates (see supplemental material), included 6 *B. canis* strains that previously were misidentified as *B. suis* by the original Bruce-ladder PCR. All *B. canis* field isolates gave the same PCR profile to the corresponding *B. canis* type strain, confirming the identity of all isolates as *B. canis*. The Suis-ladder PCR was also able to differentiate *B. microti* strain by the presence of two bands of 774 and 197 bp (Fig. 1).

In the original Bruce-ladder protocol PCR seven amplicons (1682, 1071, 794, 587, 450, 272 and 152 bp) were expected from *B. suis* DNA, and *B. canis* was distinguished by the absence of the 794 bp fragment (García-Yoldi et al., 2006). However, as mentioned before, some *B. canis* strains were identified erroneously as *B. suis*. To overcome this problem, here we also propose the advancement of this PCR protocol by substituting the primers BMEI1436f/BMEI1435r in the original primer mix by the new primers BMEI1426/1427 (Table 1), which amplified a 774 bp fragment from the *wbkF-wbkD* region (Zygmunt et al., 2009). In addition, the primer pair identifying *B. microti* described before was also included (Mayer-Scholl et al., 2010). An example of the modified Bruce-ladder PCR (called Bruce-ladder v2.0) is presented in Fig. 2. The new primer pair did not interfere with the other primers present in the cocktail mix. All *B. canis* field strains tested were distinguished to *B. suis* by the absence of the 774 bp fragment, confirming that the deletion of this genetic region is specific for *B. canis* and can be used in the Bruce-ladder v2.0 to discriminate between *B. suis* and *B. canis*.

In conclusion, for the differentiation of all *B. suis* biovars, *B. canis* and *B. microti* we propose a two steps PCR assays. First, by the advancement Bruce-ladder v2.0 PCR it is possible to identify correctly all known *Brucella* species and vaccine strains. Second, by the new Suis-ladder PCR it is possible differentiate the *B. suis* biovars. These rapid and robust multiplex PCR systems are important tools for routine laboratories for effective diagnosis of animal brucellosis in domestic and wild animals, and can contribute to the control and eradication of the disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2011.06.035.

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