

identification and biovar typing by real-time PCR

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1	Brucella suis identification and biovar typing by real-time PCR
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1 Abstract

2 Fast and accurate identification of Brucella suis at the biovar level is an important issue for public health laboratories because some of the biovars that infect suidae (boars and pigs) are 3 pathogenic for humans while others are not. Since classical biovar typing methods are often 4 time consuming, hard to standardize and require high-level biosafety containment, 5 methodological improvements are desirable. This article describes new single nucleotide 6 polymorphism (SNP) signatures for the rapid identification and biovar characterization of B. 7 8 suis. These SNPs were included together with previously described ones in real-time PCR 9 assays applicable to low-biosafety conditions. Allelic profiles unique for each B. suis biovar were defined and the most relevant signatures were determined on a collection of 137 field 10 11 strains of worldwide origin characterized previously. Biovars assigned with both present and classical methods were globally consistent except for some biovar 3 field strains which 12 13 matched the allelic profile of biovar 1.

14

15 Keywords

16 Single Nucleotide Polymorphism, SNP typing, biovar typing, Real-Time PCR, Brucella suis

3

1. Introduction

1

2 Bacteria of the species Brucella suis typically cause chronic inflammatory lesions in 3 the reproductive organs of susceptible animals that may extend to joints and other organs. The most prominent clinical sign is abortion at any stage of gestation (Godfroid et al., 2005; 4 Olsen, 2004). The species B. suis is currently divided into five biovars, of which only by 1-3 5 infect suidae. B. suis by. 1 and 3 may cause severe diseases in humans and require high 6 biosafety laboratory precautions. These biovars are spread worldwide. In contrast, biovar 2 7 8 appears restricted to Europe where it is frequently isolated from wild boars with no or mild clinical signs, can infect pigs and hares but seems unable to infect healthy humans (Lagier et 9 10 al., 2005). Biovar 4 infects reindeers and caribous throughout the Arctic region and can be transmitted to cattle, canidae and occasionally to humans (Forbes, 1991). Biovar 5, for which 11 a single representative strain is known, was isolated from a rodent in Eastern Europe and may 12 cause diseases in humans as well (Alton et al., 1988). In recent European outbreaks, wild 13 boars (Sus scrofa) and hares (Lepus europeaeus) have been identified as the source of 14 transmission of B. suis by. 2 to pigs reared outdoors (Cvetnic et al., 2003; Garin-Bastuji et al., 15 2000; Godfroid et al., 1994). Given the zoonotic nature of the disease, surveillance of B. suis 16 17 in wildlife has been advocated in the last years. In addition to serological testing, accurate typing of *B. suis* strains recovered from suspect animals is now frequently performed by 18 19 reference laboratories reporting to veterinary and public health authorities. 20 Reference typing methods involve H₂S production, growth on dyes, sensitivity to 21 bacteriophages and agglutination with mono-specific antisera (Alton et al., 1988). It is 22 generally agreed that most of these techniques are time-consuming, difficult to standardize 23 and require well trained personnel. Moreover, these techniques frequently fail in properly 24 differentiating the different *B. suis* biovars. Various molecular typing methods including PCR techniques differentiating Brucella at the species level and/or at the biovar level have been 25

1	described (Al Dahouk et al., 2005; Bricker and Halling, 1994; Ferrao-Beck et al., 2006;					
2	Garcia-Yoldi et al., 2006; Le Fleche et al., 2006; Whatmore et al., 2005; Whatmore et al.,					
3	2007; Whatmore et al., 2006). These methods are usually easy to perform, require little					
4	expertise and provide valuable alternatives to biochemical typing for most of them. None of					
5	these techniques, however, has been set up with the aim of obtaining clear-cut species and					
6	biovar assignment in a very short time for routine laboratory testing. The present report					
7	describes new genetic markers and their use in real-time assays to discriminate rapidly B. suis					
8	from other Brucella species and to identify the different B. suis biovars.					
9						
10	2. Materials and methods					
11	2.1 <u>Bacterial strains and DNA extraction</u>					
12	B. abortus strain 544 biovar 1 (=ATCC 23448) and B. melitensis strain16M biovar 1 (=ATCC					
13	23456) were used as control strains for assay development. All other bacterial strains used in					
14	this study are listed in Table 1. Unless specified, DNA was extracted with the DNA Easy					
15	mini-column system according to the manufacturer's instructions (Qiagen, Valencia, CA).					
16	2.2 <u>Selection and validation of genetic markers</u>					
17	To select suitable markers for biovar typing, DNA sequence alignments of <i>B. suis</i> bv.1					
18	(Paulsen et al., 2002) and <i>B. melitensis</i> (DelVecchio et al., 2002) total genomes were					
19	computed using a dedicated algorithm (Ma et al., 2002). Potential discriminatory markers					
20	were short-listed based on (i) their location in the coding sequence of genes with house-					
21	keeping function, (ii) the presence of at least 4 single nucleotide polymorphisms (SNPs)					

- within 400-bp sequence stretches and (iii) the presence of a homologous gene in *B. abortus*
- 23 (Halling et al., 2005). Five candidates were kept for further study, PCR-amplified and
- 24 sequenced using primers listed in Table 2. For initial SNP validation, DNA from the reference

- 5
- 1 (type) strains of each *B. suis* biovar was used as a template for PCR amplification and DNA
- 2 sequencing.
- 3 **2.3** <u>PCR and DNA sequence analysis of the selected markers</u>

PCRs were performed in 50 µl using 5 ng of template DNA, 0.5 µM PCR primers, 2 4 units of *Taq* DNA polymerase with buffer, MgCl₂ and nucleotides concentrations 5 recommended by the manufacturer (Invitrogen, Carlsbad, CA). Primers are listed in Table 2. 6 Amplification consisted in an initial denaturation step of 5 min at 94°C followed by 30 cycles 7 at 94°C for 45 s, 64.8°C for 30 s and 72°C for 1 min. PCR products were purified on mini-8 columns according to the manufacturer's instructions (Machery-Nagel, Düren, Germany). 9 10 Sequencing reactions were conducted and analyzed on a CEQ8000 automated instrument according to the manufacturer's standard instructions (Beckman Coulter, Fullerton, CA). 11

12 **2.4** *Real-time PCR assays*

Four Real-Time PCR assays were set up that could discriminate 4 critical SNPs 13 (ptsP-1677, pyrH-816-817, rpoB-244 and dnaK-1005). The software Beacon Designer 14 (Premier Biosoft International, Palo Alto, CA) was used to compute primers and TaqMan 15 probes. Four Locked Nucleic Acids (LNA) positions were substituted in each probe to 16 17 optimize their discriminatory potential while reducing their length (see Table 2). Real-time PCR assays were performed with 10 ng of template DNA, 2 PCR primers (0.4 μ M each) and 18 19 1 or 2 probes (0.1 μ M each) matching either *dnaK*, *ptsP*, *pyrH* or *rpoB* in a final volume of 50 µl on an IQ5 instrument using 2-fold concentrated premixed reagents (Biorad laboratories, La 20 Jolla, California). DMSO (10% v/v) was added to improve the probe specificity and Tris (25 21 mM, pH7) to optimize the stability of the Cy-5 label in samples assayed with the ptsP and 22 *rpoB* probes. Thermal cycling conditions for the *ptsP* and *pyrH* assays were as follows: 3 min 23 24 at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 59°C and 45 s at 72°C. For the *dnaK*

1	and <i>rpoB</i> assays, the annealing temperature was adjusted to 66.4°C and 57°C, respectively.
2	Fluorescence was measured during the extension step at 72°C.

- 3
- 3. Results
- 5

4

Polymorphisms were observed in three of the five short-listed markers, namely *ptsP*, 6 *pyrH* and *malG*. By contrast, *cycH* and *mccA* displayed no polymorphism (data not shown). 7 8 As such, the SNP allelic profiles issuing from these three polymorphic markers were not sufficient to assign a unique signature to each of the B suis biovar. However, when combined 9 with SNPs described previously in *rpoB* and *dnaK* (Marianelli et al., 2006; Whatmore et al., 10 2007), such unique signatures could be observed (Table 3). In *dnaK*, a SNP at position 1005 11 allowing the differentiation of B. suis 1, 2, 3 and 4 from other brucellae and identified during 12 the Multiple Locus Sequence Typing (MLST) survey reported by Whatmore et al. was used 13 as a species indicator (Whatmore et al., 2007). A single drawback was noticed for the closely 14 related species *B. canis*, which is undistinguishable from *B. suis* by. 4 in our typing scheme. 15 16 The biovar-specific allelism observed on single type strains was further assessed by 17 DNA sequencing or by real-time PCR applied to a collection of 137 field strains of *B. suis* and *B. canis* with various origin. Each probe proved to hybridize specifically to the target 18 19 SNP alleles, showing little or no cross-reactivity with the non-specific SNP alleles. When 20 plotted against each other, fluorescence data clustered in well-defined zones corresponding to 21 one allele each (Fig. 1). Nearly all tested field strains displayed SNP allele profiles identical 22 to that of the corresponding type strains, consistent with the biovar assigned by biochemical 23 characterization (Table 1). Exceptions are field strains of *B. suis* identified by standard 24 microbiological tests as by. 3, which fitted the SNP allele profile of by. 1, and one strain with unclear biovar which fitted the SNP allele profile of bv. 4 (strain VLA83/3). Alternate 25

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1	methods based on PCR (Bricker and Halling, 1994; Ferrao-Beck et al., 2006; Garcia-Yoldi et
2	al., 2006), variable non-tandem repeats (VNTR) analysis (Le Fleche et al., 2006) or O-
3	polysaccharide antigen typing (Cloeckaert et al., 1998) confirmed these inconsistencies,
4	which are therefore not restricted to the genetic typing scheme assessed here (data not shown).
5	DNA sequence of the genetic markers determined in each B. suis biovar was submitted to
6	GenBank and accession numbers were assigned as listed in Table 3.
7	
8	4. Discussion
9	
10	This work describes single nucleotide polymorphisms suitable for the characterization
11	of Brucella suis at biovar level. Most relevant SNPs were assayed on 137 field strains of
12	diverse origin. The selected genetic markers arose from our biocomputing analyses as well as
13	from previously reported data (Marianelli et al., 2006; Whatmore et al., 2007). These markers
14	were selected within house-keeping genes, known to have better phylogenetic value than
15	repeat-based markers which are by contrast invaluable in epidemiological studies. Four of
16	these markers were included in real-time PCR assays with the aim of (i) ruling out rapidly
17	non-biovar 2 B. suis strains and the subsequent risk for human health, while (ii) providing an
18	easy-to-perform complementary method to classical biovar typing based on genetic markers
19	with sound phylogenetic relevance.
20	The taxonomic position of <i>B. suis</i> within the genus <i>Brucella</i> is the subject of an
21	ongoing debate, complicated by the high level of relatedness displayed by members of the
22	Brucella genus in general. However, a number of genetic observations supported by
23	independent studies have demonstrated that, with the exception of <i>B. suis</i> by. 5, all <i>B. suis</i> and
24	B. canis strains form a consistent group of organisms within the Brucella cluster. Independent
25	studies revealed that B. suis by. 5 is more related to Brucella species infecting marine

8

mammals than to the four other *B. suis* biovars (Marianelli et al., 2006; Whatmore et al., 1 2 2007; Whatmore et al., 2006). Hence, B. suis by. 5, for which very few strain representatives 3 are known, turns out to be most probably misnamed. The designation of a distinct biovar for *B. suis* by, 4 is grounded by the natural host 4 specificity of this biovar (mainly restricted to rangifers). However, there is only few 5 microbiological and genetic evidences to support its distinction from by. 3. Separate genetic 6 clustering of *B. suis* by. 3 and 4 could be illustrated by VNTR (Le Fleche et al., 2006) but not 7 8 by MLST (Whatmore et al., 2007). On the other hand, with the noticeable exception of the B. suis by. 3 reference strain, most – if not all – B. suis field strains typed by. 3 with the classical 9 10 biochemical scheme fit the genetic profiles of by. 1, such as exemplified with Croatian strains included in the present study (Table 1). Similar findings were made by other groups using 11 totally unrelated genetic typing procedures (Cloeckaert et al., 1998; Le Fleche et al., 2006; 12 Whatmore et al., 2006). In fact, we did not succeed in finding a single field strain of B. suis 13 that matched both microbiological and genetic profiles of the by. 3 reference strain nor a 14 single published article stating so. The taxonomical relevance of B. suis biovar 3, or at least 15 the representativity of its reference strain, can thus be questioned. 16

17 The range of specificity covered by the assays described herewith is probably largely sufficient for the identification and typing of *B. suis* strains causing the so-called "swine 18 19 brucellosis" disease, irrespective of the taxonomical debate associated with the B. suis biovars 20 definition. A single drawback persist for the distinction of B. suis by. 4 from B. canis using 21 the present typing scheme, since both of them display the same SNP allele profiles. Apart 22 from the epidemiological context of their respective isolation, which should be quite evident, 23 B. canis can be easily differentiated from B. suis by. 4 due to differences in their respective 24 LPS structure (Alton et al., 1988). Moreover, a SNP reported by Whatmore et al. at position

CR P ٠

1	3715 of <i>omp25</i> and unique to <i>B. canis</i> , could later be included in the typing scheme
2	(Whatmore et al., 2007).
3	Comparative studies are under way in our laboratory to evaluate the applicability of
4	the present assays as direct identification and typing tests on biological samples recovered
5	from animals suspected of <i>B. suis</i> infection (farm pigs, wild boars, hares).
6	
7	5. Acknowledgements
8	
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1 References

2	Al Dahouk, S., Tomaso, H., Prenger-Berninghoff, E., Splettstoesser, W.D., Scholz, H.C.,
3	Neubauer, H., 2005. Identification of Brucella species and biotypes using polymerase
4	chain reaction-restriction fragment length polymorphism (PCR-RFLP). Crit. Rev.
5	Microbiol. 31, 191-196.
6	Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M., 1988, Techniques for the brucellosis
7	laboratory, 1 st Edition. Institut National de la Recherche Agronomique (INRA), Paris.
8	Bricker, B.J., Halling, S.M., 1994. Differentiation of Brucella abortus bv. 1, 2, and 4,
9	Brucella melitensis, Brucella ovis, and Brucella suis bv. 1 by PCR. J. Clin. Microbiol.
10	32, 2660-2666.
11	Cloeckaert, A., Weynants, V., Godfroid, J., Verger, J.M., Grayon, M., Zygmunt, M.S., 1998.
12	O-Polysaccharide epitopic heterogeneity at the surface of Brucella spp. studied by
13	enzyme-linked immunosorbent assay and flow cytometry. Clin. Diagn. Lab. Immunol.
14	5, 862-870.
15	Cvetnic, Z., Mitak, M., Ocepek, M., Lojkic, M., Terzic, S., Jemersic, L., Humski, A., Habrun,
16	B., Sostaric, B., Brstilo, M., Krt, B., Garin-Bastuji, B., 2003. Wild boars (Sus scrofa)
17	as reservoirs of Brucella suis biovar 2 in Croatia. Acta Vet. Hung. 51, 465-473.
18	DelVecchio, V.G., Kapatral, V., Redkar, R.J., Patra, G., Mujer, C., Los, T., Ivanova, N.,
19	Anderson, I., Bhattacharyya, A., Lykidis, A., Reznik, G., Jablonski, L., Larsen, N.,
20	D'Souza, M., Bernal, A., Mazur, M., Goltsman, E., Selkov, E., Elzer, P.H., Hagius, S.,
21	O'Callaghan, D., Letesson, J.J., Haselkorn, R., Kyrpides, N., Overbeek, R., 2002. The
22	genome sequence of the facultative intracellular pathogen Brucella melitensis. Proc.
23	Natl. Acad. Sci. U.S.A 99, 443-448.
24	Ferrao-Beck, L., Cardoso, R., Munoz, P.M., de Miguel, M.J., Albert, D., Ferreira, A.C.,
25	Marin, C.M., Thiebaud, M., Jacques, I., Grayon, M., Zygmunt, M.S., Garin-Bastuji,

1	B., Blasco, J.M., Sa, M.I., 2006. Development of a multiplex PCR assay for
2	polymorphism analysis of Brucella suis biovars causing brucellosis in swine. Vet.
3	Microbiol. 115, 269-277.
4	Forbes, L.B., 1991. Isolates of Brucella suis biovar 4 from animals and humans in Canada,
5	1982-1990. Can. Vet. J. 32, 686-688.
6	Garcia-Yoldi, D., Marin, C.M., de Miguel, M.J., Munoz, P.M., Vizmanos, J.L., Lopez-Goni,
7	I., 2006. Multiplex PCR assay for the identification and differentiation of all Brucella
8	species and the vaccine strains Brucella abortus S19 and RB51 and Brucella
9	melitensis Rev1. Clin. Chem. 52, 779-781.
10	Garin-Bastuji, B., Hars, J., Calvez, D., Thiébaud, M., Artois, M., 2000. Brucellose du porc
11	domestique et du sanglier sauvage due à Brucella suis biovar 2 en France. Epidémiol.
12	Santé Anim. 38, 1-5.
13	Godfroid, J., Cloeckaert, A., Liautard, J.P., Kohler, S., Fretin, D., Walravens, K., Garin-
14	Bastuji, B., Letesson, J.J., 2005. From the discovery of the Malta fever's agent to the
15	discovery of a marine mammal reservoir, brucellosis has continuously been a re-
16	emerging zoonosis. Vet. Res. 36, 313-326.
17	Godfroid, J., Michel, P., Uytterhaegen, L., De Smedt, C., Rasseneur, F., Boelaert, F.,
18	Saegerman, C., Patigny, X., 1994. Enzootic brucellosis due to Brucella suis biotype 2
19	in wild boars (Sus scrofa) in Belgium. Ann. Med. Vet. 138, 263-268.
20	Halling, S.M., Peterson-Burch, B.D., Bricker, B.J., Zuerner, R.L., Qing, Z., Li, L.L., Kapur,
21	V., Alt, D.P., Olsen, S.C., 2005. Completion of the genome sequence of Brucella
22	abortus and comparison to the highly similar genomes of Brucella melitensis and
23	Brucella suis. J. Bacteriol. 187, 2715-2726.

1	Lagier, A., Brown, S., Soualah, A., Julier, I., Tourrand, B., Albert, D., Reynes, J., Garin-
2	Bastuji, B., 2005. Brucellose aiguë à <i>B. suis</i> biovar 2 chez un chasseur de sanglier.
3	Méd. Mal. Infect. 35, 185.
4	Le Fleche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nockler, K.,
5	Neubauer, H., Guilloteau, L.A., Vergnaud, G., 2006. Evaluation and selection of
6	tandem repeat loci for a Brucella MLVA typing assay. BMC Microbiol. 6, 9.
7	Ma, B., Tromp, J., Li, M., 2002. PatternHunter: faster and more sensitive homology search.
8	Bioinformatics 18, 440-445.
9	Marianelli, C., Ciuchini, F., Tarantino, M., Pasquali, P., Adone, R., 2006. Molecular
10	characterization of the rpoB gene in Brucella species: new potential molecular
11	markers for genotyping. Microbes Infect. 8, 860-865.
12	Olsen, S., 2004, Porcine Brucellosis, In: Manual of Diagnostic Tests and Vaccines for
13	Terrestrial Animals. OIE, Paris, France.
14	Paulsen, I.T., Seshadri, R., Nelson, K.E., Eisen, J.A., Heidelberg, J.F., Read, T.D., Dodson,
15	R.J., Umayam, L., Brinkac, L.M., Beanan, M.J., Daugherty, S.C., Deboy, R.T.,
16	Durkin, A.S., Kolonay, J.F., Madupu, R., Nelson, W.C., Ayodeji, B., Kraul, M.,
17	Shetty, J., Malek, J., Van Aken, S.E., Riedmuller, S., Tettelin, H., Gill, S.R., White,
18	O., Salzberg, S.L., Hoover, D.L., Lindler, L.E., Halling, S.M., Boyle, S.M., Fraser,
19	C.M., 2002. The Brucella suis genome reveals fundamental similarities between
20	animal and plant pathogens and symbionts. Proc. Natl. Acad. Sci. U S A. 99, 13148-
21	13153.
22	Whatmore, A.M., Murphy, T.J., Shankster, S., Young, E., Cutler, S.J., Macmillan, A.P., 2005.
23	Use of amplified fragment length polymorphism to identify and type Brucella isolates
24	of medical and veterinary interest. J. Clin. Microbiol. 43, 761-769.

NUSCRIPT ACCEP A

1	Whatmore, A.M., Perrett, L.L., MacMillan, A.P., 2007. Characterisation of the genetic
2	diversity of Brucella by multilocus sequencing. BMC Microbiol. 7:34.
3	Whatmore, A.M., Shankster, S.J., Perrett, L.L., Murphy, T.J., Brew, S.D., Thirlwall, R.E.,
4	Cutler, S.J., MacMillan, A.P., 2006. Identification and characterization of variable-
5	number tandem-repeat markers for typing of Brucella spp. J. Clin. Microbiol. 44,
6	1982-1993.
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1 Figure captions

2

Figure 1: Typical allelic discrimination plots. Fluorescence values were collected from the 3 30th real-time PCR cycle and plotted against each other while assaying the reference strains 4 of B. suis by. 1 (O), by. 2 (\Box), by. 3 (X), by. 4 (Δ), by. 5 (+) and no-template controls (\Diamond). 5 6 Dual-probe SNP typing of *ptsP*1677 is shown in A, of *pyrH*816-817 in B and of *rpoB*244 in 7 C. Single-probe SNP typing of *dnaK*1005 was assayed on the above *B. suis* strains as well as on the type strains of B. canis (‡), B. abortus by. 1-9 (.) and B. melitensis 1-3 (.) as shown in 8 D. Each point falls within one out of two delineated clusters corresponding to one SNP allele 9 10 each.

Table

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Table 1 : Origin and SNP typing summary of the *Brucella* strains used in this study

					Single Nucleotide polymorphisms			18	
Strain ID	Biovar ^a	Animal	Origin	Year	<i>ptsP</i> -1677	<i>pyrH</i> - 816/817	rpoB-244	<i>malG-</i> 963/984 ^b	<i>dnaK</i> -1005 ^c
1330 (Ref)	1	swine	USA	NK	А	GT	G^{d}	СА	Т
AFSSA-01-5744	1	swine	Polynesia	2001	А	GT	G	ND	T ^e
AFSSA-04-2987	1	human	Polynesia	2004	А	GT	G	ND	ND
AFSSA-F3/03	1	human	Polynesia	2003	А	GT	G	ND	ND
AFSSA-F6/04 79/0349	1	human	Polynesia	2004	А	GT	G	ND	ND
AFSSA-F6/04 79/1616	1	human	Polynesia	2004	А	GT	G	ND	ND
VLA-298	1	NK	USA	NK	А	GT	G	ND	ND
VLA-63/166	1	swine	Israel	1963	А	GT	G	ND	ND
VLA-63/169	1	NK	Germany	1963	А	GT	G	ND	Т
VLA-63/170	1	swine	Argentina	1963	А	GT	G	ND	Т
VLA-63/171	1	swine	Former USSR	1963	А	GT	G	ND	ND
VLA-63/172	1	swine	Bulgaria	1963	Α	GT	G	ND	ND
VLA-63/176	1	human	Tunisia	1963	А	GT	G	ND	T ^e
VLA-63/177	1	human	USA	1963	A	GT	G	ND	Т
VLA-63/178	1	NK	Switzerland	1963	Α	GT	G	ND	Т
VLA-63/180	1	swine	Poland	1963	A	GT	G	ND	ND
VLA-63/233	1	NK	Bulgaria	1963	Α	GT	G	ND	Т
VLA-63/24	1	NK	South	1963	A	GT	G	ND	ND
VLA-63/32	1	NK	South Africa	1963	Α	GT	G	ND	Т
VLA-63/49	1	NK	Poland	1963	Α	GT	G	ND	Т
VLA-64/24	1	swine	USA	1964	Α	GT	G	ND	T ^e
VLA-74/10	1	cow	Columbia	1974	А	GT	G	ND	Т
VLA-74/8	1	swine	Columbia	1974	А	GT	G	ND	Т
VLA-74/9	1	cow	Columbia	1974	А	GT	G	ND	ND
VLA-76/4	1	human	Australia	1976	А	GT	G	ND	ND
VLA-79/224	1	human	New Zealand	1979	А	GT	G	ND	Т
VLA-80/147	1	NK	Argentina	1980	А	GT	G	ND	ND
VLA-81/27	1	NK	Brazil	1981	А	GT	G	ND	ND
VLA-86/27	1	human	Mexico	1986	А	GT	G	ND	ND
VLA-88/114	1	human	China	1988	А	GT	G	ND	ND
VLA-88/45	1	swine	China	1988	А	GT	G	ND	Т
VLA-92/29	1	human	Mexico	1992	А	GT	G	ND	T ^e
VLA-A403 SA	1	NK	Portugal	NK	А	GT	G	ND	Т
VLA-F7/03S1	1	wild boar	Croatia	NK	А	GT	G	ND	T ^e
VLA-F7/03S2	1	wild boar	Croatia	NK	А	GT	G	ND	Т
VLA-M17SA	1	NK	Portugal	NK	А	GT	G	ND	Т
Thomsen (Ref)	2	swine	Denmark	NK	G	AG	G^{d}	ND	Т
AFSSA-00-4898	2	bovine	France	2000	G	AG	G	CG	ND
AFSSA-00-5952-1	2	swine	Portugal	2000	G	AG	G	ND	ND
AFSSA-00-9182	2	hare	France	2000	G	AG	G	ND	ND
AFSSA-01-1107	2	wild boar	Germany	2001	G	AG	G	ND	ND
AFSSA-01-3017-5	2	swine	Portugal	2001	G	AG	G	ND	ND
AFSSA-03-1483-8	2	wild boar	France	2003	G	AG	G	ND	ND
AFSSA-03-2067-203	2	swine	France	2003	G	AG	G	ND	ND
AFSSA-04-115	2	hare	France	2004	G	AG	G	ND	ND
AFSSA-04-1918-01	2	wild boar	Switzerland	2004	G	AG	G	ND	ND
AFSSA-04-3025-1	2	swine	Croatia	2004	G	AG	G	ND	ND
AFSSA-04-770	2	wild boar	Italy	2004	G	AG	G	ND	ND
AFSSA-92-11580-4528	2	hare	France	1992	G	AG	G	ND	ND
AFSSA-92-13000	2	hare	France	1992	G	AG	G	ND	ND
AFSSA-96-9635	2	swine	France	1996	G	AG	G	ND	ND

					Single Nucleotide polymorphisms				IS
Strain ID	Biovar ^a	Animal	Origin	Year	<i>ptsP</i> -1677	<i>pyrH</i> - 816/817	rpoB-244	<i>malG-</i> 963/984 ^b	<i>dnaK</i> -1005 ^{<i>c</i>}
AFSSA-97-9757	2	swine	France 19		G	AG	G	ND	ND
AFSSA-98-6335	2	swine	France	1998	G	AG	G	ND	ND
AFSSA-98-7296-4204	2	hare	France	1998	G	AG	G	ND	ND
AFSSA-96/5693	2	wild boar	France	1996	G	AG	G	ND	ND
AFSSA-96/6594	2	wild boar	France	1996	G	AG	G	ND	ND
AFSSA-97/4088	2	swine	France	1997	G	AG	G	ND	ND
AFSSA-97/4924	2	swine	France	1997	G	AG	G	ND	ND
AFSSA-97/7247	2	swine	France	1997	G	AG	G	ND	ND
AFSSA-97/8800	2	swine	France	1997	G	AG	G	ND	ND
AFSSA-97/9266	2	swine	France	1997	G	AG	G	ND	ND
AFSSA-97/9757	2	swine	France	1997	G	AG	G	ND	ND
AFSSA-98/12418	2	swine	France	1998	G	AG	G	ND	ND
AFSSA-98/4537	2	wild boar	France	1998	G	AG	G	ND	ND
AFSSA-98/6335	2	hare	France	1998	G	AG	G	ND	ND
AFSSA-98/7926	2	swine	France	1998	G	AG	G	ND	ND
BW-140	2	wild boar	Germany	2000	G	AG	G	CG	Т
BW-141	2	swine	Germany	2000	G	AG	G	CG	Т
BW-142	2	hare	Switzerland	1998	G	AG	G	CG	Т
BW-143	2	wild boar	Germany	2000	G	AG	G	CG	Т
BW-144	2	wild boar	Germany	2001	G	AG	G	CG	Т
BW-145	2	wild boar	Germany	2001	G	AG	G	CG	Т
BW-146	2	wild boar	Germany	2001	G	AG	G	CG	Т
BW-147	2	swine	Germany	2000	G	AG	G	CG	Т
BW-148	2	hare	Switzerland	NK	G	AG	G	CG	Т
BW-149	2	swine	Germany	1998	G	AG	G	CG	Т
BW-150	2	swine	Germany	1998	G	AG	G	CG	Т
BW-151	2	wild boar	Germany	2001	G	AG	G	CG	Т
BW-152	2	wild boar	Germany	2001	G	AG	G	CG	Т
BW-153	2	wild boar	Germany	2002	G	AG	G	CG	Т
BW-154	2	swine	Germany	2002	G	AG	G	CG	Т
BW-155	2	wild boar	Germany	2002	G	AG	G	CG	Т
BW-156	2	wild boar	Germany	2002	G	AG	G	CG	Т
BW-157	2	wild boar	Switzerland	2001	G	AG	G	CG	Т
BW-158	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-159	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-160	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-161	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-162	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-163	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-164	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-165	2	wild boar	Germany	1999	G	AG	G	CG	ND
BW-166	2	wild boar	Germany	1999	G	AG	G	CG	ND
BW-167	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-168	2	wild boar	Germany	2003	G	AG	G	CG	ND
BW-169	2	wild boar	Germany 20		G	AG	G	CG	ND
BW-170	2	wild boar	Germany	2004	G	AG	G	CG	ND
BW-1/1	2	wild boar	Germany	2004	G	AG	G	CG	ND
VAR- \$5-11	2	wild boar	France	1997	G	AG	G	ND	ND
VAK-0111602/4+9	2	wild boar	Belgium	2002	G	AG	G	ND	ND
VAK-CIUB/	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-CI1B4	2	wild boar	Belgium	2003	G	AG	G	ND	ND

Table 1 : Origin and SNP typing summary of the *Brucella* strains used in this study

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					Single Nucleotide polymorphisms				15
Strain ID	Biovar ^a	Animal	Origin	Year	<i>ptsP</i> -1677	<i>pyrH</i> - 816/817	<i>rpoB-</i> 244	<i>malG-</i> 963/984 ^b	<i>dnaK</i> -1005 ^c
VAR-C13B1	2	wild boar	Relgium	2003	G	AG	G	ND	ND
VAR-C13B3	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C13B4	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C13B5	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C13B6	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C2B11	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C3B3	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-CJB3	$\frac{2}{2}$	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C4DJ VAR-C5B5	$\frac{2}{2}$	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-CSDS	2	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-CODI VAD CODI	$\frac{2}{2}$	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C8B3	$\frac{2}{2}$	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-COB3	$\frac{2}{2}$	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C9DJ VAR-C9B4	$\frac{2}{2}$	wild boar	Belgium	2003	G	AG	G	ND	ND
VAR-C9D4	$\frac{2}{2}$	wild boar	Belgium	1006	G	AG	G	ND	ND
VAR-COSa 15	2	wild boar	Delgium	1990	G	AC	G	ND	ND
VAN-IVIASA U/	2	wild boar	Delgium	1990	G	AG	G	ND	ND
VAR-PY 09 VAR S275	2	wild boar	Belgium	2004	G	AG	G	ND	ND ND
VAR-5275	2	WIIU UUAI	Dengiuili	2004	C	AC	U C	ND	
V LA - 03/201	2	INK	Deninark	1905 NIV	G A	AG		ND CC	
000 (Kel)	3	swille	USA	2002	A	AU CT	A		
AFSSA-03-3081-2	3 2	noise	Croatia	2003	A	CT CT	G	CA	
AFSSA-04-3023-3	3 of	swine	Croatia	2004	A		G	CA	1 T ^e
VLA-83/3	?~ 	swine	Australia	1985 NIZ	A	AG	G		1 T ^e
40 (Ref)	4	reindeer	Former USSR	NK 10(2	A	AG	G		I e
VLA-63/198	4	reindeer	Former USSR	1963	A	AG	G	ND	I T ^e
VLA-63/202	4	reindeer	Former USSR	1963	A	AG	G	ND	I T ^e
VLA-63/219	4	reindeer	Former USSR	1963	A	AG	G	ND	I T ^e
VLA-03/232	4	caribou	USA (Alaska)	1903	A	AG	G		1 T ^e
VLA-79/30	4	numan	Finland	1979 NIZ	A	AG	G	ND TC	Γ^{e}
513 (KeI)	р	mouse	Kussia		G	AG	G	IG	C T
KIMI6/66 (KeI)	B. canis	dog	USA	NK 1007	A	AG	G		
VAR-8/-62	B. canis	dog	Canada	1987	A	AG	G	ND	I T
VAR-8/-65	B. canis	dog	Canada	1987	A	AG	G	ND	I T
VAR-8/-66	B. canis	dog	Canada	1987	A	AG	G	ND	I T
VAR-D519	B. canis	dog	Madagascar	NK 100(A	AG	G	ND	I T
VAR-9	B. canis	dog	Romania	1996	A	AG	G	ND	I
VAR-96-104	B. canis	dog	Romania	1996	A	AG	G	ND	T
VAR-96-105	B. canis	dog	Romania	1996	A	AG	G	ND	T
VAR-96-106	B. canis	dog	Romania	1996	A	AG	G	ND	T
VAR-96-107	B. canis	dog	Romania	1996	A	AG	G	ND	T
VAR-96-121	B. canis	dog	Komania	1996	А	AG	G	ND	Т

Table 1 : Origin and SNP typing summary of the Brucella strains used in this study

^{*a*} *B. suis* biovar as determined with reference (microbiological) tests ^{*b*} SNP profile not determined systematically here (marker described and validated by Whatmore et al., 2007) ^{*c*} SNP profile not required for biovar assignment; used as confirmation marker (bv. 3/5/atypical)

^d Data retrieved from Marianelli et al., 2006

^e Data retrieved from Whatmore et al., 2007

^f Atypical strain displaying microbiological characters of *B. abortus* bv.1 but clustering with *B. suis* bv.3/4 when assayed by any genetic typing method

BW, German Army; VAR, Veterinary and Agrochemical Research Centre; VLA, Veterinary Laboratories Agency; AFSSA, French Food Safety Agency. NK, Not Known; ND, Not Determined; Ref, reference (type) strain

Gene	Primer/Probe	Coordinates ^a	Nucleotide Sequence
	PTSP-F	1359-1378	CGGCTTGCTTGTCGATCTG
ptsP	PTSP-R	1746-1765	CTCGCGTTCGATGATCTCG
	$PTSP-RT-F^{b}$	1545-1562	TGGCGACAAGGTTCTCC
	PTSP-RT-R ^b	1688-1706	GCATGAGCTTCAGTTCACG
	$PTSP-1^{b}$	1671-1685	Cy5-CAC <u>CCGCT</u> G <u>C</u> CTTC- BHQ3
	$PTSP-2^{b}$	1671-1685	TxRd-CAC <u>C</u> C <u>C</u> C <u>C</u> CTTC- BHQ3
	PYRH-F	540-557	GCGTTGATTCCGTGGTGC
	PYRH -R	921-940	GCCTTGCAGAATATCGGCC
	PYRH -RT- F^b	787-802	ACCCGCTTCGACCAG
pyrH	PYRH -RT-R ^b	884-909	TCATGGATGGAATAGACTATTATCG
	PYRH -1^b	807-827	FAM-CCACAAG <u>G</u> AG <u>T</u> TT <u>C</u> TC <u>G</u> ATC-BHQ1
	PYRH -2^b	807-827	HEX-CCACAAG <u>G</u> AA <u>G</u> TT <u>C</u> TC <u>G</u> ATC-BHQ1
malG	MALG-F	827-849	AGGATTGTGAGGACTCCATTGC
	MALG-R	1210-1228	CACTTCTTCCGGCCCATG
avaU	CYCH-F	289-308	TTTGTTGCAGTTCTCGCCG
сусн	CYCH-R	681-701	GCGCAAGATAATATTGCGGAC
mccA	MCCA-F	68-83	TGGCGGCAATCGACG
	MCCA-R	450-471	AAAACCTCATCCTGATTGGGC
	DNAK-RT-F ^b	972-992	CAAGGCTGGCGAAATTGACG
dnaK	DNAK-RT-R ^b	1080-1100	CCACGACTTCATCCGGGTTC
rpoB	RPOB-RT-F ^b	171-191	GGTTTTCAAGTCTGTTTTCC
	RPOB-RT-R ^b	284-301	CGCCGAATAAGTCAGATC
	RPOB-1.2.4 ^b	259-276	FAM-CGGC <u>A</u> TT <u>C</u> ATCAA <u>C</u> ATC-BHQ1
	RPOB-3 ^b	259-276	HEX-CGGC <u>A</u> TT <u>C</u> AT <u>T</u> AA <u>C</u> ATC-BHQ1

Table 2 : Sequence of primers and probes

^{*a*} Coordinates are given according to the numbering of GenBank ORF BR1870 (*ptsP*), BR1160 (*pyrH*), BR1648 (*malG*), BR0060 (*cycH*), BR0019 (*mccA*) and BR1243 (*rpoB*) in *B. suis* strain 1330.

strain 1330. ^b Primers and probes used in real-time PCR assays. Underlined positions identify locked nucleic acids (LNA), bold characters identify polymorphic nucleotides.

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Gene	Species	SNP position ^a				ORF or Accession n ^r	Ref
ptsP		1512 1573 1677 ^b					
	<i>B. suis</i> bv. 1	С	G	А		BR1870	(19)
	<i>B. suis</i> bv. 2	С	G	G		DQ865114	This work
	<i>B. suis</i> bv. 3	С	G	А		DQ865115	This work
	<i>B. suis</i> bv. 4	С	G	А		DQ865116	This work
	<i>B. suis</i> bv. 5	С	G	G		DQ993291	This work
	B. canis	С	G	А		DQ865117	This work
pyrH		798	811	816 ^b	817 ^b		$\langle \rangle$
	<i>B. suis</i> bv. 1	С	А	G	Т	BR1160	(19)
	<i>B. suis</i> bv. 2	С	Α	А	G	DQ865121	This work
	<i>B. suis</i> bv. 3	С	Α	А	G	DQ865122	This work
	<i>B. suis</i> bv. 4	С	А	А	G	DQ865123	This work
	<i>B. suis</i> bv. 5	С	А	А	G	DQ993293	This work
	B. canis	С	А	А	G	DQ865124	This work
malG		954	963	984	1016		
	<i>B. suis</i> bv. 1	Т	С	А	A	BR1648	(19)
	<i>B. suis</i> bv. 2	Т	С	G	G	DQ865118	This work
	<i>B. suis</i> bv. 3	Т	С	G	G	DQ865119	This work
	<i>B. suis</i> bv. 4	Т	С	G	G	DQ865120	This work
	<i>B. suis</i> bv. 5	С	Т	G	G	DQ993292	This work
	B. canis	Т	С	G	G	-	
rpoB		244^{b}					
	<i>B. suis</i> bv. 1	G				BR1243	(19)
	<i>B. suis</i> bv. 2	G				DQ086123	(17)
	<i>B. suis</i> bv. 3	A				DQ086124	(17)
	<i>B. suis</i> bv. 4	G				DQ086125	(17)
	<i>B. suis</i> bv. 5	G				DQ086126	(17)
	B. canis	G				DQ086128	(17)
dnaK		1005 ^b					
	<i>B. suis</i> bv. 1	Т				AM694671	(21)
	<i>B. suis</i> bv. 2	Т				AM694770	(21)
	B. suis bv. 3	Т				AM695040	(21)
	<i>B. suis</i> bv. 4	Т				AM695058	(21)
	<i>B. suis</i> bv. 5	С				AM695112	(21)
	B. canis	Т				AM695121	(21)
	B. $spp.^{c}$	С				-	(21)

Table 3. Summary of SNP allele distribution within the species Brucella suis

^a SNP positions are given according to open reading frame numbering
 ^b SNP used in Real-Time PCR assays
 ^c The same SNP allele is observed in all *Brucella* species but *B. suis* bv. 1-4 and *B. canis* Markers analyzed in this study and exhibiting no polymorphism between the different *B. suis* biovars are not listed (GenBank Accessions DQ865125 through DQ865130).

