Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all Brucella species, including the vaccine strains

I. López-Goñi, D. García-Yoldi, C.M. Marin, M.J. de Miguel, P.M. Muñoz, J.M. Blasco, Isabelle Jacques, Maggy Grayon, Axel Cloeckaert, A.C. Ferreira, et al.

Journal of Clinical Microbiology

Evaluation of a Multiplex PCR Assay (Bruce-ladder) for Molecular Typing of All *Brucella* Species, Including the Vaccine Strains

I. López-Goñi, D. García-Yoldi, C. M. Marín, M. J. de Miguel, P. M. Muñoz, J. M. Blasco, I. Jacques, M. Grayon, A. Cloeckaert, A. C. Ferreira, R. Cardoso, M. I. Corrêa de Sá, K. Walravens, D. Albert and B. Garin-Bastuji
J. Clin. Microbiol. **2008, 46(10):3484. DOI: 10.1128/JCM.00837-08.**
Published Ahead of Print 20 August 2008.

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2008, p. 3484–3487 Vol. 46, No. 10
0095-1137/08/$08.00+0 doi:10.1128/JCM.00837-08

Evaluation of a Multiplex PCR Assay (Bruce-ladder) for Molecular Typing of All *Brucella* Species, Including the Vaccine Strains▽†

I. López-Goñi,[1]* D. García-Yoldi,[1] C. M. Marín,[2] M. J. de Miguel,[2] P. M. Muñoz,[2] J. M. Blasco,[2] I. Jacques,[3,4] M. Grayon,[4] A. Cloeckaert,[4] A. C. Ferreira,[5] R. Cardoso,[5] M. I. Corrêa de Sá,[5] K. Walravens,[6] D. Albert,[7] and B. Garin-Bastuji[7]

Departamento de Microbiología y Parasitología, Universidad de Navarra, 31008 Pamplona, Spain[1]; Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), 50080 Zaragoza, Spain[2]; Institut Universitaire de Technologie, Université François Rabelais, 29 rue du Pont-Volant, 37082 Tours Cedex 2, France[3]; INRA, UR1282, Infectiologie Animale et Santé Publique, Centre de Recherche de Tours, Nouzilly 37380, France[4]; Laboratório Nacional de Investigação Veterinária, Estrada de Benfica, 701, 1549-011 Lisbon, Portugal[5]; Department of Bacteriology and Immunology, Veterinary and Agrochemical Research Centre, CODA CERVA, B-1180 Ukkel, Belgium[6]; and European Community Reference Laboratory for Brucellosis, Unité Zoonoses Bactériennes, AFSSA, F-94706 Maisons-Alfort Cedex, France[7]

Received 2 May 2008/Accepted 24 July 2008

An evaluation of a multiplex PCR assay (Bruce-ladder) was performed in seven laboratories using 625 *Brucella* strains from different animal and geographical origins. This robust test can differentiate in a single step all of the classical *Brucella* species, including those found in marine mammals and the S19, RB51, and Rev.1 vaccine strains.

Brucellosis is caused by a facultative intracellular bacterium of the genus *Brucella*, and it is one of the most frequent bacterial zoonoses in low-income countries, where the control programs have not succeeded in eradicating this neglected zoonosis. The disease is a major cause of direct economic losses and an impediment to trade and exportation. The genus *Brucella* consists of six recognized species, designated on the basis of differences in pathogenicity and host preference: *B. melitensis* (goats and sheep), *B. abortus* (cattle and bison), *B. suis* (infecting primarily swine but also hares, rodents, and reindeer), *B. ovis* (sheep), *B. canis* (dogs), and *B. neotomae* (wood rats) (7). The discovery of *Brucella* in a wide variety of marine mammals has led to the proposal of two new species: *B. ceti* (cetaceans) and *B. pinnipedialis* (pinnipeds) (8). Some of these species include several biovars, which are currently distinguished from each other by an analysis of approximately 25 phenotypic characteristics, including requirement for CO_2, H_2S production, sensitivity to dyes and phages, and other metabolic properties (1). However, all these tests are time-consuming, require skilled technicians, and are not straightforward, and some reagents are not commercially available. In addition, handling of this microorganism represents a high risk for laboratory personnel, since most *Brucella* strains are highly pathogenic for humans. Accurate diagnostic and typing procedures are critical for the success of the eradication and control of the disease, and therefore the identification of the different species is of great epidemiological importance. In order to overcome most of these difficulties, PCR-based assays have been employed for molecular typing of *Brucella* species. However, one of the challenges of using DNA-based techniques for differentiating the various *Brucella* species and strains is their high degree of genetic homology (16). This article describes the evaluation of a new multiplex PCR assay (10), named Bruce-ladder, in seven different European laboratories. The PCR protocol was standardized previously (10), and the same protocol was used in all laboratories (see the supplemental material). The selection of the DNA sequences to design the PCR primers was based on species-specific or strain-specific genetic differences (Table 1). Each laboratory used its own *Brucella* strain collection, typed by standard bacteriological procedures (1). A total of 625 *Brucella* strains were used (see the complete list in the supplemental material). The collection included the reference strains of all biovars of *B. abortus*, *B. melitensis*, *B. suis*, and *B. ovis*, *B. canis*, *B. neotomae*, the *B. abortus* S19, *B. abortus* RB51, and *B. melitensis* Rev.1 vaccine strains, and the recently proposed *B. pinnipedialis* and *B. ceti* (8). To ensure adequate diversity, isolates from different geographic origins and different animal species, including humans, were selected (Table 2). Genomic DNA was extracted from pure cultures by using standard microbial DNA isolation kits or by heat lysis of bacterial cell cultures. Bruce-ladder identification was based on the numbers and sizes of seven products amplified by PCR. A representative example of the multiplex PCR result is presented in Fig. 1. PCR using DNA from *B. abortus* strains amplified five fragments, of 1,682, 794, 587, 450, and 152 bp in size; with *B. melitensis* DNA, an additional 1,071-bp fragment was amplified; and *B. ovis* was distinguished by the absence of the 1,682-bp fragment and *B. suis* by the presence of an additional 272-bp fragment (also present in *B. canis* and *B. neotomae*). PCR with *B. abortus* S19 DNA did not produce the 587-bp fragment common to all *Brucella* strains tested, and *B. abortus* RB51 was readily distinguished by the absence of the 1,682-bp and 1,320-bp fragments and by a specific additional 2,524-bp fragment. The *B. melitensis* Rev.1 vac-

* Corresponding author. Mailing address: Departamento de Microbiología y Parasitología, Universidad de Navarra, c/ Irunlarrea no. 1, 31008 Pamplona, Spain. Phone: (34) 948 425600. Fax: (34) 948 425649. E-mail: ilgoni@unav.es.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 20 August 2008.

TABLE 1. Oligonucleotides used in Bruce-ladder multiplex PCR assay

Primer[a]	Sequence (5′–3′)	Amplicon size (bp)	DNA target	Source of genetic differences	Reference(s)
BMEI0998f	ATC CTA TTG CCC CGA TAA GG	1,682	Glycosyltransferase, gene *wboA*	IS*711* insertion in BMEI0998 in *B. abortus* RB51 and deletion of 15,079 bp in BMEI0993–BMEI1012 in *B. ovis*	9, 15
BMEI0997r	GCT TCG CAT TTT CAC TGT AGC				
BMEI0535f	GCG CAT TCT TCG GTT ATG AA	450 (1,320)[b]	Immunodominant antigen, gene *bp26*	IS*711* insertion in BMEI0535–BMEI0536 in *Brucella* strains isolated from marine mammals	5
BMEI0536r	CGC AGG CGA AAA CAG CTA TAA				
BMEII0843f	TTT ACA CAG GCA ATC CAG CA	1,071	Outer membrane protein, gene *omp31*	Deletion of 25,061 bp in BMEII826–BMEII0850 in *B. abortus*	17
BMEII0844r	GCG TCC AGT TGT TGT TGA TG				
BMEI1436f	ACG CAG ACG ACC TTC GGT AT	794	Polysaccharide deacetylase	Deletion of 976 bp in BMEI1435 in *B. canis*	13
BMEI1435r	TTT ATC CAT CGC CCT GTC AC				
BMEII0428f	GCC GCT ATT ATG TGG ACT GG	587	Erythritol catabolism, gene *eryC* (D-erythrulose-1-phosphate dehydrogenase)	Deletion of 702 bp in BMEII0427–BMEII0428 in *B. abortus* S19	14
BMEII0428r	AAT GAC TTC ACG GTC GTT CG				
BR0953f	GGA ACA CTA CGC CAC CTT GT	272	ABC transporter binding protein	Deletion of 2,653 bp in BR0951–BR0955 in *B. melitensis* and *B. abortus*	11, 12
BR0953r	GAT GGA GCA AAC GCT GAA G				
BMEI0752f	CAG GCA AAC CCT CAG AAG C	218	Ribosomal protein S12, gene *rpsL*	Point mutation in BMEI0752 in *B. melitensis* Rev.1	6
BMEI0752r	GAT GTG GTA ACG CAC ACC AA				
BMEII0987f	CGC AGA CAG TGA CCA TCA AA	152	Transcriptional regulator, CRP family	Deletion of 2,203 bp in BMEII0986–BMEII0988 in *B. neotomae*	13
BMEII0987r	GTA TTC AGC CCC CGT TAC CT				

[a] Designation are based on the *B. melitensis* (BME) or *B. suis* (BR) genome sequences. f, forward; r, reverse.
[b] Due to a DNA insertion in the *bp26* gene, the amplicon size in *Brucella* strains isolated from marine mammals is 1,320 bp.

cine strain was readily distinguished from other *B. melitensis* strains by a specific additional 218-bp fragment. *B. canis* was distinguished by the absence of the 794-bp fragment and *B. neotomae* by the absence of the 152-bp fragment. Finally, when DNA from *Brucella* strains isolated from marine mammals (*B. pinnipedialis* and *B. ceti*) was used, a specific additional 1,320-bp fragment was amplified whereas the 450-bp fragment was absent. The same results presented in Fig. 1 were obtained with all *Brucella* strains assayed, with the only exception being some *B. canis* strains. Nine out of 21 *B. canis* strains showed the same PCR profile as *B. suis*. Typing of these nine strains was confirmed by classical typing and multiple-locus variable-number tandem-repeat analysis (data not shown). Interestingly, these *B. canis* strains with a *B. suis*-like profile were resistant to basic fuchsin and safranin (a variant of the classical *B. canis* phenotypic pattern). However, these findings do not detract from the utility of the Bruce-ladder PCR, since it is very simple, using sensitivity to dyes and phage susceptibility to differentiate *B. canis* (a rough species) from *B. suis* (a smooth species). In addition, *B. canis* is always isolated from dogs, and *B. suis* has never been found in this host.

Although this PCR assay cannot differentiate among biovars from the same species, Bruce-ladder was species specific and all the strains and biovars from the same *Brucella* species gave the same profile. The practical interest of Bruce-ladder for typing purposes is evident since some of the cumbersome and long-lasting microbiological procedures currently used could be avoided. The specificity of the Bruce-ladder PCR has been tested previously (10), using as targets DNA from 30 strains phylogenetically or serologically related to *Brucella*. The Bruce-ladder PCR worked equally well irrespective of the cultural conditions, DNA extraction methods, or thermocyclers used. The same results were obtained in seven different laboratories with brucellae obtained from the five continents and

TABLE 2. Hosts and geographic origins of the 625 *Brucella* strains tested by Bruce-ladder PCR

Species	Biovar	Host(s)	Origin	No. of strains tested
B. abortus	1	Cattle, buffalo, humans	Spain, Portugal, France, Belgium, Brazil, USA,[a] Algeria	39
	2	Cattle	Costa Rica. Greece	3
	3	Cattle, humans, sheep	Spain, Portugal, France, Belgium, Greece	40
	4	Cattle	France, Italy, Equator	14
	5			1
	6	Cattle	France	3
	7	Cattle	Mongolia, Turkey	2
	9	Cattle	Belgium, France	7
B. melitensis	1	Sheep, goats, cattle, humans	Spain, Portugal, Belgium, USA, Mexico	45
	2	Humans, goat, cattle	Spain, Turkey, Lebanon	8
	3	Sheep, goats, cattle, humans, swine, chamois	Spain, Portugal, France, Belgium, Morocco, Croatia	163
B. suis	1	Wild boars, swine, hares, humans	Croatia, China, United Kingdom, Portugal, France, French Polynesia, Wallis and Futuna islands	25
	2	Wild boars, swine, hares, humans, cattle	Spain, Croatia, Portugal, Belgium, France, Germany	83
	3	Wild boars, swine, horses, humans	Croatia, Wallis and Futuna islands	10
	4	Humans, caribou, musk oxen	Canada	4
	5	Human	USA	1
B. ovis		Sheep	Spain, Croatia, France, Argentine, USA	25
B. canis		Dogs	Romania, Canada, Costa Rica, Madagascar, France, Spain, Brazil, Serbia	21
B. neotomae				1
B. ceti		Dolphins, whales, porpoises	Scotland, Norway, Costa Rica, France	38
B. pinnipedialis		Seals	Scotland, Greeland Sea	27
B. abortus	RB51	Cattle	Spain, Portugal, USA	27
B. abortus	S19	Cattle	Spain, France, USA	3
B. melitensis	Rev.1	Sheep, goats, cattle, humans	Spain, France, South Africa, Portugal, Belgium, Israel, USA	35

[a] USA, United States.

from humans and both domestic and wild animals (Table 2), demonstrating without a doubt the reproducibility and robustness of the PCR assay proposed.

One of the most popular PCR assays for the differentiation of *Brucella* species, designated AMOS PCR (3), was based on the polymorphism arising from species-specific localization of the insertion sequence IS*711* in the *Brucella* chromosome and can differentiate *B. abortus* (biovars 1, 2, and 4), *B. melitensis*

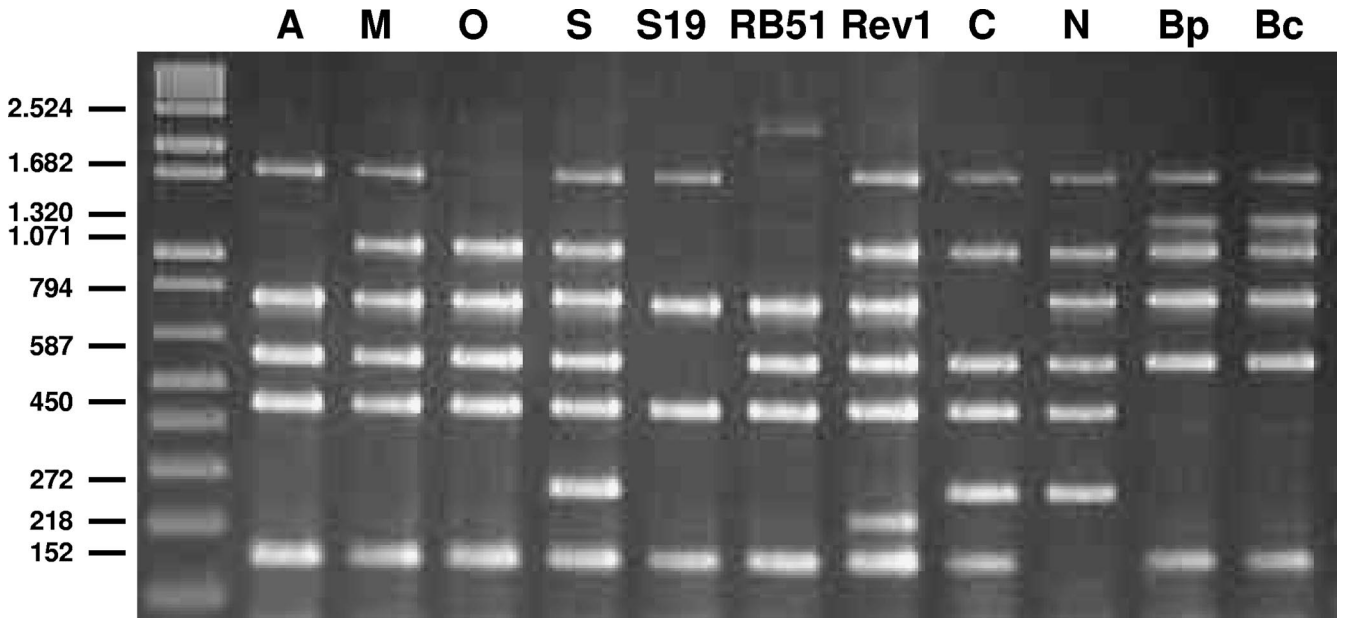

FIG. 1. Differentiation of all *Brucella* species and S19, RB51, and Rev.1 vaccine strains by Bruce-ladder multiplex PCR. Lane 1 (A), *B. abortus*; lane 2 (M), *B melitensis*; lane 3 (O), *B. ovis*; lane 4 (S), *B. suis*; lane 5 (S19), *B. abortus* S19 vaccine strain; lane 6 (RB51), *B. abortus* RB51 vaccine strain; lane 7 (Rev.1), *B. melitensis* Rev.1 vaccine strain; lane 8 (C), *B. canis*; lane 9 (N), *B. neotomae*; lane 10 (Bp), *B. pinnipedialis*; lane 11 (Bc), *B. ceti*.

(biovars 1, 2, and 3), and *B. ovis* and *B. suis* (biovar 1). Modifications of this assay have been introduced over the years to improve performance, and additional strain-specific primers were incorporated for identification of the *B. abortus* S19 and RB51 vaccine strains (2, 4). However, other *Brucella* species (such as *B. canis*, *B. neotomae*, *B. pinnipedialis*, and *B. ceti*) and some particular biovars (*B. abortus* biovars 3, 5, 6, 7, and 9 and *B. suis* biovars 2, 3, 4, and 5) cannot be detected by AMOS PCR. A major advantage of the Bruce-ladder PCR assay over previously described multiplex PCR tests is that it can identify and differentiate for the first time all of the *Brucella* species and the vaccine strains in the same test. In contrast to AMOS PCR, Bruce-ladder PCR is also able to detect DNA from *B. canis*, *B. neotomae*, *Brucella* isolates from marine mammals, *B. abortus* biovars 3, 5, 6, 7, and 9, and *B. suis* biovars 2, 3, 4, and 5. Other advantages are speed (the PCR can be performed in less than 24 h), minimal sample preparation (it works with whole-cell lysates), and reduced risks (PCR can be carried out with *Brucella* colonies, limiting the manipulation of live *Brucella*). In conclusion, Bruce-ladder PCR can be a useful tool for the rapid identification of *Brucella* strains of animal or human origin, not only in reference centers but also in any basic microbiology laboratory worldwide.

We express our gratitude to Sylvie Malbrecq, Damien Desqueper, Patrick Michel, Martine Thiébaud, and Martine Lapalus for their technical skill and to Geoff Foster, Morten Tryland, and Zeljko Cvetnic for providing some of the *Brucella* isolates.

REFERENCES

1. **Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger.** 1988. Techniques for the brucellosis laboratory. INRA, Paris, France.
2. **Bricker, B. J., D. R. Ewalt, S. C. Olsen, and A. E. Jensen.** 2003. Evaluation of the *Brucella abortus* species-specific polymerase chain reaction assay, an improved version of the *Brucella* AMOS polymerase chain reaction assay for cattle. J. Vet. Diagn. Investig. **15:**374–378.
3. **Bricker, B. J., and S. M. Halling.** 1994. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. J. Clin. Microbiol. **32:**2660–2666.
4. **Bricker, B. J., and S. M. Halling.** 1995. Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. J. Clin. Microbiol. **33:**1640–1642.
5. **Cloeckaert, A., M. Grayon, and O. Grepinet.** 2000. An IS*711* element downstream of the *bp26* gene is a specific marker of *Brucella* spp. isolated from marine mammals. Clin. Diagn. Lab. Immunol. **7:**835–839.
6. **Cloeckaert, A., M. Grayon, and O. Grepinet.** 2002. Identification of *Brucella melitensis* vaccine strain Rev.1 by PCR-RFLP based on a mutation in the *rpsL* gene. Vaccine **20:**2546–2550.
7. **Corbel, M. J.** 1988. International committee on the systematic bacteriology-subcommittee on the taxonomy of *Brucella*. Int. J. Syst. Bacteriol. **38:**450–452.
8. **Foster, G., B. S. Osterman, J. Godfroid, I. Jacques, and A. Cloeckaert.** 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. Int. J. Syst. Evol. Microbiol. **57:**2688–2693.
9. **García-Yoldi, D., C. M. Marín, and I. López-Goñi.** 2005. Restriction site polymorphisms in the genes encoding new members of group 3 outer membrane protein family of *Brucella* spp. FEMS Microbiol. Lett. **245:**79–84.
10. **García-Yoldi, D., C. M. Marín, M. J. De Miguel, P. M. Muñoz, J. L. Vizmanos, and I. López-Goñi.** 2006. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. Clin. Chem. **52:**779–781.
11. **Halling, S. M., B. D. Peterson-Burch, B. J. Bricker, R. L. Zuerner, Z. Qing, L. L. Li, V. Kapur, D. P. Alt, and S. S. Olsen.** 2005. Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. J. Bacteriol. **187:**2715–2726.
12. **Paulsen, I. T., R. Seshadri, K. E. Nelson, J. A. Eisen, J. F. Heidelberg, T. D. Read, R. J. Dodson, L. Umayam, L. M. Brinkac, M. J. Beanan, S. C. Daugherty, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, W. C. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S. E. Van Aken, S. Riedmuller, H. Tettelin, S. R. Gill, O. White, S. L. Salzberg, D. L. Hoover, L. E. Lindler, S. M. Halling, S. M. Boyle, and C. M. Fraser.** 2002. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc. Natl. Acad. Sci. USA **99:**13148–13153.
13. **Rajashekara, G., J. D. Glasner, D. A. Glover, and G. A. Splitter.** 2004. Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. J. Bacteriol. **186:**5040–5051.
14. **Sangari, F. J., J. M. García-Lobo, and J. Agüero.** 1994. The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes. FEMS Microbiol. Lett. **121:**337–342.
15. **Vemulapalli, R., J. R. McQuiston, G. G. Schurig, N. Sriranganathan, S. M. Halling, and S. M. Boyle.** 1999. Identification of an IS*711* element interrupting the *wboA* gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin. Diagn. Lab. Immunol. **6:**760–764.
16. **Verger, J. M., F. Grimont, P. A. D. Grimont, and M. Grayon.** 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. **35:**292–295.
17. **Vizcaíno, N., J. M. Verger, M. Grayon, M. S. Zygmunt, and A. Cloeckaert.** 1997. DNA polymorphism at the *omp-31* locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology **143:**2913–2921.