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Identification of *Salmonella abortusovis* by PCR Amplification of a Serovar-Specific IS*200* Element

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Field and collection isolates of *Salmonella abortusovis* carry one IS*200* element in a distinct chromosome location. IS*200* is not found in the corresponding region of the chromosome of other *Salmonella* serovars. Sequencing of the boundaries of the *S. abortusovis*-specific IS*200* insertion permitted the design of primers for the amplification of this IS*200* element by PCR. Isolates of *S. abortusovis* are identified by the amplification of a DNA fragment of about 900 bp or larger. PCR amplification of DNA from salmonellae other than *S. abortusovis* yields either a fragment of about 200 bp or no product. The high specificity of the assay is confirmed by the absence of cross-reactivity with the following templates: (i) sheep DNA, (ii) DNAs from abortion-causing agents other than *S. abortusovis*, and (iii) DNAs from microorganisms that do not cause abortion but are common in flocks.

Salmonella abortusovis is a *Salmonella* serovar host-adapted to sheep which causes infectious disease, with abortion as the main symptom (reviewed in references 13, 18, and 21). The disease occurs in the last weeks of pregnancy, and the pathogenic mechanisms involved are not yet understood (21). In areas in which the microorganism is endemic, abortion may occur in up to 50% of the ewes in a flock, usually during the first pregnancy (13). This high incidence of salmonellosis represents a major threat to flocks and may result in important economic losses in regions that depend on sheepherding (18, 27). Although *S. abortusovis* is the serotype most frequently associated with ovine salmonellosis, especially in Europe and the Middle East (13), other *Salmonella* serovars, including *Salmonella typhimurium* (15, 24), *Salmonella dublin* (10), and *Salmonella choleraesuis* subsp. *arizonae* (3, 26), have also been described as agents of abortion. Furthermore, other microbial pathogens, such as *Brucella*, *Campylobacter*, *Chlamydia*, *Coxiella*, *Listeria*, *Toxoplasma*, and *Yersinia*, have been reported to cause ovine abortion (6–8, 14, 16, 17, 20). This variety of agents makes diagnostic tests highly desirable.

Traditionally, the identification of *S. abortusovis* has relied on the use of antisera against O and H antigens (4, 30). However, other *Salmonella* serotypes can have such antigens (1). On the other hand, serological identification of *Salmonella* serovars sometimes faces the problem that recombination of phase 1 flagellin genes can generate new *Salmonella* serovars (26, 32). The use of a biochemical test such as the API 20E system is not an advisable alternative, because this procedure does not distinguish between *S. abortusovis* and *Salmonella typhi* (5, 19). Thus, a molecular identification assay, such as the PCR procedure described below, could be a breakthrough in the field.

Our PCR assay is based on the finding that genomic DNAs from *S. abortusovis* contain a serovar-specific IS*200* copy on the chromosome (25). Because of the existence of this IS*200* copy, PCR amplification of *S. abortusovis* DNA templates can be expected to yield a serovar-specific band.

The serovar-specific IS*200* element was cloned from *S. abortusovis* SS 44. Genomic DNA was isolated according to the method of Ausubel et al. (2), digested with *Pst*I, and size fractionated by agarose electrophoresis. DNA fragments of ~9 kb were recovered, purified with Gene Clean, and cloned into pBluescript I KS(+) (Stratagene, La Jolla, Calif.). The ligation mixture was used to transform *Escherichia coli* DH5α (31) by following the procedure of Inoue et al. (12). The presence of IS*200* among Lac^- Ap^r transformants of *E. coli* DH5α was screened by Southern hybridization with the 0.6-kb *Eco*RI fragment of plasmid pIZ46 as a probe (9, 25, 28). One positive isolate was arbitrarily chosen as the source of plasmid pIZ72. Restriction analysis proved that pIZ72 carried an insert of the expected size (around 9.5 kb).

Digestion of pIZ72 with restriction enzymes that do not cut within pBluescript I KS(+) indicated that *Stu*I digestion was able to split the 9.5-kb insert into two fragments of 6.5 and 3 kb. The 3-kb fragment (but not the 6.5-kb fragment) hybridized against the *Eco*RI-*Hin*dIII and *Taq*I IS*200* probes, suggesting that the IS*200* element was located in the small *Stu*I fragment. The latter was cloned into pBluescript I KS(+) to generate plasmid pIZ73. To reduce the size of the 3-kb *Stu*I fragment of pIZ73, nested deletions were obtained. pIZ73 DNA was isolated by the "boiling" method (11) and purified in Sephadex G-50 columns (22). Two enzymes that do not cut within the insert, *Cla*I and *Apa*I, were used to produce 5′ and 3′ overhanging ends, respectively. Aliquots were treated with exonuclease III, and the reactions were allowed to proceed for various amounts of time. Deleted plasmids were treated with nuclease S1 (to generate blunt ends) and with T4 ligase (to religate deleted plasmids). Transformants of *E. coli* DH5α were then obtained. Plasmid separation by gel electrophoresis indicated the presence of heterogeneous plasmid sizes. Inserts were recovered by digestion with *Sac*I and *Kpn*I. The presence of IS*200* was detected by Southern hybridization against the standard probes of plasmids pIZ46 and pIZ47 (9, 25). A plas-

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TABLE 1. *Salmonella* strain list

Serovar	Strain	Reference or source[a]
Salmonella abortusovis	SS 35	5
	SS 44	5
	27K IP	A
	INRA Rv6	B
	A93–A99	C
	460R	D
	SSM 110	E
Salmonella typhimurium	LT2	Lab stock
Salmonella typhi	SVT001	F
Salmonella havana	SL5479	G
Salmonella bovismorbificans	SL5747	G
Salmonella kentucky	SL197	G
Salmonella memphis	SL5311	G
Salmonella montgomery	SL457	G
Salmonella choleraesuis	BS2181	H
Salmonella enteritidis	27	Lab stock
	28	Lab stock
Salmonella abortusequi	ATCC 4842	I

[a] A, M. Popoff, Institut Pasteur, Paris, France; B, BioMérieux (vaccine strain); C, G. Zoraqi, University of Tirana, Tirana, Albania; D, B. Poglazov, Russian Academy of Sciences, Moscow, Russia; E, C. Wray, Central Veterinary Laboratory, Weybridge, England; F, Swiss Serum and Vaccine Institute, Bern, Switzerland; G, Bruce Stocker, Stanford University, Stanford, Calif.; H, Istituto Zooprofilattico Sperimentale della Lombardia, Brescia, Italy; I, American Type Culture Collection, Rockville, Md.

mid carrying an ~1-kb insert (pIZ74) was chosen for DNA sequencing.

For sequencing, pIZ74 DNA was prepared by the alkaline lysis method, without phenol extraction (29). DNA sequencing was performed by the dideoxy chain termination procedure (23), using the T3 and T7 sequencing primers of pBluescript. Plasmid pIZ74 was found to contain one IS*200* element of 708 bp flanked by host DNA sequences of 79 and 252 bp. These flanking sequences correspond to an unknown region of the *S. abortusovis* chromosome; no homology with known loci was found in data bank searches.

Two primers, both 20 nucleotides long, were designed for PCR amplification: (i) 5′-CGATGAAAGCGTAAATAAGG-3′, whose 3′ end is 160 bp from one IS*200* end, and (ii) 5′-TTCTCTTGTCAGTCTCAAAC-3′, whose 3′ end is 24 nucleotides from the other end of IS*200*. The rationale of the identification test is that the amplification of DNA carrying an IS*200* element can be expected to yield a fragment of 932 bp, while DNAs lacking IS*200* in that position will yield a fragment ~220 nucleotides long; both can be easily distinguished by gel electrophoresis. Thus, *S. abortusovis* DNA can be expected to yield an ~900-bp fragment. In turn, DNAs from other salmonellae will yield ~200-bp fragments, while DNA from other sources will not be amplified.

Genomic DNA preparations for PCR assays were obtained by the "boiling" method (11); the starting material was a colony resuspended in 300 μl of distilled water. The final volume of all PCR amplification mixtures was 25 μl, and the final concentration of $MgCl_2$ was 1.5 mM. Reagents were used at the following concentrations: deoxynucleoside triphosphates, 200 μM; primers, 1 μM; and *Taq* polymerase, 1 U per reaction. The thermal program included the following steps: (i) initial denaturation for 3 min at 94°C; (ii) 30 cycles of denaturation at 94°C for 30 s, annealing at 51.6°C for 30 s, and extension at 72°C for 1 min; and (iii) final incubation at 72°C for 5 min, to complete extension. Amplification products were separated by electrophoresis on 1% agarose gels stained with ethidium bromide (final concentration, 0.5 μg/ml).

Amplification tests were carried out on DNAs from 63 isolates of *S. abortusovis* obtained from Sardinian flocks and on DNAs from 13 collection strains of *S. abortusovis* (Table 1). Flock isolates were from three sources: vaginal discharges, fetal tissues (stomach, spleen, liver, and brain), and milk from mastitis-affected sheep that had undergone abortion. The flocks were located in the Sardinian towns of Bolotana (7 isolates), Nulvi (7 isolates), Mara (4 isolates), Suni (2 isolates), Bultei (19 isolates), Bonassai (2 isolates), Bonorva (19 isolates), Osilo (2 isolates), and Sindia (1 isolate). Strains SSM 817 and SSM 818 (see Fig. 1) are the two isolates from the Bonassai flocks (one from milk and the other from fetal tissue). The isolation and preservation of these isolates were as previously described (5, 25). Eleven *Salmonella* strains belonging to serovars different from *S. abortusovis* (Table 1) were also analyzed. The results were as follows. (i) Seventy-four of the 76 *S. abortusovis* samples tested gave rise to an amplification DNA fragment of 900 bp. Relevant examples are shown in Fig. 1 (top, lanes A through G). Two strains (the French strains 27K IP and INRA Rv6) yielded a larger amplification fragment, of about 2 kb (Fig. 1, top, lanes H and I). (ii) *Salmonella* strains from other serovars gave rise to amplification bands of 200 bp (Fig. 1, top, lanes J through L), except *Salmonella memphis* SL5311 and *Salmonella montgomery* SL457, which did not yield amplified DNA (Fig. 1, top, lanes M and N).

Southern hybridization against an IS*200* probe showed that IS*200* was present in both the 900-bp and 2-kb fragments, but not in the 200-bp fragments (Fig. 1, bottom). These results

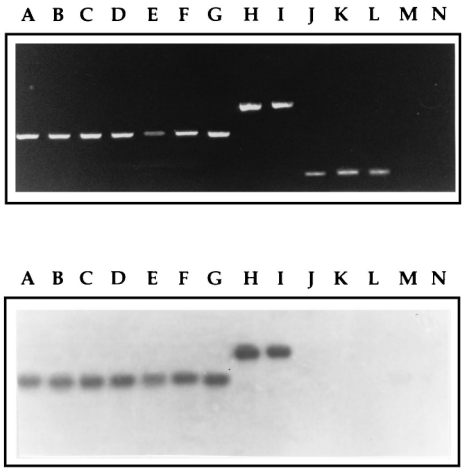

FIG. 1. Amplification of *Salmonella* target DNAs by PCR with primers that amplify a chromosomal region which contains the *S. abortusovis*-specific IS*200* insertion. (Top) Amplification products, as separated by gel electrophoresis; (bottom) DNA hybridization of the same fragments against an IS*200* probe. Lanes: A, *S. abortusovis* SS 44; B, *S. abortusovis* SS 35; C, *S. abortusovis* SSM 817; D, *S. abortusovis* SSM 818; E, *S. abortusovis* SSM 110; F, *S. abortusovis* 460R; G, *S. abortusovis* A97; H, *S. abortusovis* 27K IP; I, *S. abortusovis* INRA Rv6; J, *Salmonella enteritidis* 27; K, *S. typhimurium* LT2; L, *S. typhi* SVT001; M, *S. memphis* SL5311; N, *S. montgomery* SL457.

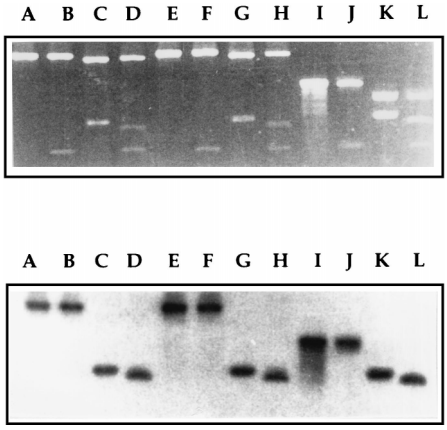

FIG. 2. Restriction analysis (top) and Southern hybridization (bottom) of PCR fragments obtained upon amplification of *S. abortusovis* target DNAs. Lanes: A, 27K 1P, undigested; B, 27K 1P, digested with *Hin*dIII; C, 27K 1P, digested with *Eco*RI; D, 27K IP, digested with *Hin*dIII and *Eco*RI; E, INRA Rv6, undigested; F, INRA Rv6, digested with *Hin*dIII; G, INRA Rv6, digested with *Eco*RI; H, INRA Rv6, digested with *Hin*dIII and *Eco*RI; I, SS 44, undigested; J, SS 44, digested with *Hin*dIII; K, SS 44, digested with *Eco*RI; L, SS 44, digested with *Hin*dIII and *Eco*RI.

fulfill the predictions made about the PCR test, with the caveat that bands larger than 900 bp, like those of the two *S. abortusovis* strains of French origin, must also be taken as a positive result (see below).

PCR amplification was also performed on control DNA templates from the following sources: (i) microorganisms that do not cause abortion but are frequently found in sheep (two strains of *Enterococcus faecium*, one of *E. coli*, and one of *Bacillus thuringiensis*); (ii) potentially abortive agents that may coexist with *Salmonella* in the flocks (*Mycoplasma agalactiae*, *Chlamydia psittaci*, *Listeria ivanovii*, and *Brucella melitensis*); and (iii) sheep DNA, kindly provided by A. Alberti (University of Sassari). All gave negative results (data not shown).

To investigate the origin of the 2-kb bands produced by the two French strains of *S. abortusovis*, the 2-kb PCR amplification fragments from strains 27K IP and INRA Rv6 were subjected to single and double digestions with *Eco*RI and *Hin*dIII. As a control, the same restriction analysis was performed on a 0.9-kb DNA fragment generated by amplification of DNA from *S. abortusovis* SS 44. The results, shown at the top of Fig. 2, revealed the presence of single *Eco*RI and *Hin*dIII restriction sites in both the 2-kb and the 0.9-kb fragments. Because IS*200* contains single *Eco*RI and *Hin*dIII sites (9), the results suggest that only one IS*200* element is present in the ~2-kb fragments (and, of course, in the ~0.9-kb fragments). Furthermore, double digestion with *Eco*RI and *Hin*dIII yielded a three-fragment pattern, with a constant band of ~300 bp (Fig. 2, top). Southern hybridization analysis confirmed the presence of a single IS*200* copy in both types of fragments (Fig. 2, bottom). Thus, the French strains 27K IP and INRA Rv6 carry a DNA rearrangement in the region where the *S. abortusovis*-specific IS*200* element is found. The presence of >1 kb of extra DNA generates a larger DNA fragment upon PCR amplification.

The nature of the DNA rearrangement in the region under study was investigated in additional PCR amplification experiments with one of the external primers described above and one internal IS*200* primer. The internal IS*200* primers used were 5′-TTCGCCGTGTTCTTACCCACCGT-3′ and 5′-GCC GAAGATGAGTGTGTCCAGTT-3′. The results can be summarized as follows. (i) One primer combination yielded a fragment of 540 bp with target DNA from all *S. abortusovis* strains. (ii) The second primer combination yielded a fragment of 1,600 bp with target DNA from the French strains, while amplification of SS 44 DNA yielded a fragment of 550 bp. Thus, we conclude that the extra DNA is located at one side of IS*200*. These results confirm that the French strains of *S. abortusovis* contain extra DNA in the region where the serovar-specific IS*200* element is located. Thus, the positive result of the PCR identification test for *S. abortusovis* is the amplification of a fragment of 0.9 kb or larger.

Nucleotide sequence accession number. The sequence of pIZ74 has been deposited in the EMBL database under accession no. Y08755.

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REFERENCES

1. **Australian Salmonella Reference Center.** 1994. Salmonella annual report. Institute of Medical and Veterinary Science, Adelaide, Australia.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Greene Publishing Associates & Wiley Interscience, New York, N.Y.
3. **Blackburn, B. O., K. Sutch, and R. Harrington, Jr.** 1980. The changing distribution of *Salmonella dublin* in the United States. Proc. U. S. Anim. Health Assoc. **84:**445–451.
4. **Brenner, D. J.** 1984. Family I. *Enterobacteriaceae*, p. 408–516. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore, Md.
5. **Colombo, M. M., G. Leori, S. Rubino, A. Barbato, and P. Cappuccinelli.** 1992. Phenotypic features and molecular characterization of plasmids in *Salmonella abortusovis*. J. Gen. Microbiol. **138:**725–731.
6. **Dennis, S. M.** 1972. Infectious ovine abortion in Australia. Vet. Bull. **42:** 415–419.
7. **East, N. E.** 1983. Pregnancy, toxemia, abortions, and preparturient diseases. Vet. Clin. North Am. Large Anim. Pract. **5:**601–618.
8. **Favre, J.** 1980. Contribution à l'étude des avortements salmonelliques ovins dans le département de l'Haute-Vienne. Thesis. Ecole Nationale Véterinaire, Alfort, France.
9. **Gibert, I., J. Barbé, and J. Casadesús.** 1990. Distribution of insertion sequence IS*200* in *Salmonella* and *Shigella*. J. Gen. Microbiol. **136:**2555–2560.
10. **Gitter, M., and W. J. Sojka.** 1970. *Salmonella dublin* abortion in sheep. Vet. Res. **87:**775–778.
11. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
12. **Inoue, H., H. Nojima, and H. Okayama.** 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene **93:**26–28.
13. **Jack, K. J.** 1968. *Salmonella abortusovis*: an atypical Salmonella. Vet. Rec. **82:**1168–1174.
14. **Linklater, K. A., and D. A. Dyson.** 1979. Field studies on enzootic abortion of ewes in South East Scotland. Vet. Rec. **105:**387–389.
15. **Murray, C. J.** 1984. Isolates of *Salmonella* and *Escherichia coli* serotyped at the Salmonella Reference Laboratory in 1982 and 1983 from veterinary and human sources. Aust. Vet. J. **61:**273–274.
16. **Nicolas, J. A.** 1980. Les avortements infectieux de la brebis: de nombreux germes en cause, un diagnostique indispensable. Elevage **92:**41–44.
17. **Nicolas, J. A., and M. Lamachère.** 1984. Les avortements infectieux des petits ruminants: leur diagnostique, les résultats obtenus par un laboratoire de terrain. Rev. Med. Vet. **135:**211–215.
18. **Pardon, P., R. Sanchis, J. Marly, F. Lantier, M. Pepin, and M. Popoff.** 1988. Salmonellose ovine due à *Salmonella abortusovis*. Ann. Rech. Vet. **19:**221–235.
19. **Plagermann, O.** 1989. The differential diagnosis of *Salmonella abortusovis* and *Yersinia pseudotuberculosis* in sheep abortion. Zentralbl. Veterinaermed. **36:**509–514.
20. **Plommet, M.** 1977. Maladies abortives de la brebis, p. 78–79. *In* Journées de recherche ovine et caprine. INRA-ITOVIC, Paris, France.

21. **Rubino, S., P. Rizzu, G. Erre, M. M. Colombo, G. Leori, P. A. Gulig, T. J. Doyle, M. Pisano, S. Uzzau, and P. Cappuccinelli.** 1993. Mechanism of pathogenicity in *Salmonella abortusovis*, p. 141–147. *In* F. Cabello et al. (ed.), Biology of Salmonella. Plenum Press, New York, N.Y.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
24. **Saunders, C. N., D. A. Kinch, and R. D. Martin.** 1966. An outbreak of *Salmonella typhimurium* infection in sheep. Vet. Rec. **79:**554–557.
25. **Schiaffino, A., C. R. Beuzón, S. Uzzau, G. Leori, P. Cappuccinelli, J. Casadesús, and S. Rubino.** 1996. Strain typing with IS*200* fingerprints in *Salmonella abortusovis*. Appl. Environ. Microbiol. **62:**2375–2380.
26. **Smith, N. H., P. Beltran, and R. K. Selander.** 1990. Recombination of *Salmonella* phase 1 flagellin genes generates new serovars. J. Bacteriol. **172:** 2209–2216.
27. **Sojka, W. J., C. Wray, J. E. Schreeve, and J. C. Bell.** 1983. The incidence of Salmonella infection in sheep in England and Wales, 1975 to 1981. Br. Vet. J. **139:**386–392.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
29. **Stephen, D., C. Jones, and P. J. Schofield.** 1990. A rapid method for isolation of high quality plasmid DNA suitable for DNA sequencing. Nucleic Acids Res. **18:**7463–7464.
30. **Vodas, K., and S. Martinov.** 1986. Diagnostic value of serological and bacteriological procedures in sheep infected with *Salmonella abortusovis* and *Chlamydia psittaci* var. *ovis*. Vet. Med. Nauki **23:**14–22.
31. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
32. **Zinder, N. D., and J. Lederberg.** 1952. Genetic exchange in *Salmonella*. J. Bacteriol. **64:**679–699.