Epidemiol. Infect. (1994), **112**. 33–43

Polymerase chain reaction for salmonella virulence-associated plasmid genes detection: a new tool in salmonella epidemiology

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(*Accepted 22 June 1993*)

SUMMARY

The important role of plasmid genes in assessing virulence for BALB/c mice in salmonella, and the difficulty of using standard techniques to detect them, led us to develop a detection method by gene amplification.

One hundred and forty-three strains (71 serovars) of salmonella and 35 strains of other species were tested using specific oligonucleotide primers. The amplification products were identified by a specific oligonucleotide probe. Forty-nine salmonella strains from ten serovars (*S. abortus ovis*, *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum/pullorum*, *S. hessarek*, *S. typhimurium*, *S. IIIa* 48:z_4, z_{23}, *S. IV* 43:z_4, z_{23}:-, *S. V* 28:a:-) produced a positive and specific response.

Because of various origins of the strains possessing the gene sought and the diversity of the responses, both from one serovar to another and in the same serovar, this search has its place among the epidemiological markers in general use. This method appears well suited to the research and detection of plasmid genes associated with mouse virulence in salmonella.

INTRODUCTION

Animals are known to constitute a vast reservoir of salmonella. The general problem of environmental contamination by organic waste in regard of human and animal salmonellosis [1] has acquired a new importance for the developed countries, due to extended livestock farming. Thus any information which can be obtained on the potential virulence of salmonella is of great interest.

For some years, various publications have illustrated the role of plasmids as virulence-related factors in various serovars of salmonella [2–9]. The genetic determinants of invasiveness are chromosomal [10]. Chromosomal DNA is an important factor in the expression of virulence, especially in the capacity of strains to survive and multiply in reticulo-endothelial system cells [11]. Nevertheless, the virulence of salmonella strains is linked to a combination of chromosomal and plasmid factors [6, 10–13]. So far, no role other than that of their connection with virulence [7, 14] has been attributed to these plasmids, yet for *S. enteritidis*, increased production of long chains of lipopolysaccharides has been noted [15]. In

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1982 Jones [6] showed that these plasmids were linked to adhesion and invasion of HeLa cells in the mouse. In 1985 Chikami [12] showed that, without plasmids, salmonella could invade intestinal mucous membranes, but were unable to propagate infections, at given rates of contamination. Since then, in 1986, Hackett [10] and Pardon [13] have shown that the plasmids were necessary for colonization of the spleen and liver in the mouse. Also, Hackett [10] has demonstrated that strains possessing the plasmids were not phagocytosed by macrophages and thus remained in the organism. Other studies have shown that among various serovars of salmonella there exists a high degree of structural homology in all or some of the plasmids linked with virulence [11, 16–18], to such an extent that cross-immunization has been achieved [19–20].

In the light of the above, it appeared interesting, for salmonella strains, to propose a method for demonstrating the genetic virulence potential for mice linked to the presence of this type of plasmid. Use of a polymerase chain reaction (PCR) seemed a quick, simple and reliable method capable of achieving the objectives set.

MATERIALS AND METHODS

Bacterial strains

One hundred and forty-three strains of salmonella, i.e. 71 serovars and 35 strains of other species of bacteria, were used in this study (Table 1). They were all isolated either from animals (51 strains) or from food products (73 strains) or from the environment (53 strains); one strain was isolated from a human faecal culture following salmonellosis. They were all subjected to biochemical and serological analysis. Bacteria cells were grown in Trypticase Soy Broth (Difco) overnight at 37 °C.

Boiling DNA extraction

A simple and fast technique was adopted to obtain target DNA for PCR analysis. Overnight bacterial culture (1 ml) was heated to 100 °C for 10 min to inactivate any protease and DNase which may have been present. Then the crude broth culture was cooled to 0 °C for 10 min and 5 μl portions were submitted to PCR, as described below.

Primers and probe

The computer analysis of the *S. typhimurium mkf*A gene [21–24] and the *S. choleraesuis* virulence plasmid *mba* region [25] allowed us to find a primers set and an internal probe, after sequence alignment. Primers (VIR 113, VIR 561) and the internal probe (VIR 334) were chosen according to the following criteria: no cross-hybridization with other known sequences, high G+C content at 3′ extremity, minimum of dimer formation and self-complementarity. The primers set generates a 472 bp amplified DNA fragment. Positions and sequences of the oligonucleotides are shown in Table 2.

PCR conditions and electrophoresis

Bacterial DNA was amplified with a PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA). *Taq* polymerase (2 units, Boehringer Mannheim) was added to 50 μl of a solution of Tris-HCl pH 8·3 (10 mM), KCl (50 mM), $MgCl_2$ (1·5 mM), gelatin (0·1 mg/ml), deoxynucleotide

https://doi.org/10.1017/S0950268800057393 Published online by Cambridge University Press

ıble 1. *Ability of PCR assay to distinguish the virulence-associated gene among salmonella serovars and other bacterial strains*

Strains	Origins	Results
abortus ovis	Ovine abortion product	+
abortus ovis	Ovine abortion product	−
agona	Breeding environment	−
agona	Guinea fowl viscera	−
alachua	fish meal	−
anatum	Pork butchery	−
banana	Foodstuffs	−
blockley	Hen viscera	−
bovis morbificans	Bovine faecal sample	−
bovis morbificans	Pork butchery	−
bovis morbificans	Sea sediment	−
braenderup (×2)	Bovine faecal samples	−
braenderup (×2)	Breeding environment	−
braenderup	Chicken meat	−
braenderup	Dog faecal sample	−
braenderup	Surface water	−
braenderup	Raw milk	−
bredeney	Pork butchery	−
bredeney	Sea water	−
bredeney	Sea sediment	−
broughton	Beef meat	−
cerro	Dog faecal sample	−
choleraesuis	Young wild boar	+
cubana	Foodstuffs	−
derby	Calf offals	−
derby	Pork butchery	−
derby	Sea sediment	−
dublin	Beef meat	−
dublin (×2)	Bovine abortion product	+
dublin (×2)	Bovine faecal sample	+
eboko	Bovine abortion product	−
enteritidis	Bovine faecal sample	+
enteritidis (×3)	Breeding environment	+
enteritidis	Cacao	+
enteritidis	Cooked food	+
enteritidis (×2)	Duck viscera	+
enteritidis (×2)	Egg product	+
enteritidis	Hen viscera	−
enteritidis (×2)	Hen viscera	+
enteritidis	Pastry	+
enteritidis	Pastry	−
enteritidis	Raw milk	+
enteritidis	River water	+
enteritidis	Turkey meat	+
gallinarum/pullorum (×4)	Hen viscera	+
gloucester	Turnsol meal	−
goldcoast	Beef meat	−
hadar	Chicken meat	−
heidelberg	Hen viscera	−
hessarek	Surface water	+
idikan	Fish meal	−
indiana	Beef meat	−
infantis	Beef meat	−

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Table 1 (*cont.*)

Strains	Origins	Results
infantis	River water	−
isangi	Shellfish	−
kedougou	Hen viscera	−
kottbus	Breeding environment	−
llandoff	Breeding environment	−
lille	Foodstuffs	−
london	Pork butchery	−
mbandaka	Soya meal	−
meleagridis	Beef meat	−
montevideo	Fish meal	−
montevideo	Hen viscera	−
münchen	Processing plant	−
münster	Horse meat	−
newport	Pastry	−
newport	Turkey viscera	−
nima	Poultry faecal sample	−
panama	Beef meat	−
paratyphi B	Beef meat	−
paratyphi B	Bovine viscera	−
paratyphi B	Fish	−
paratyphi B	Ice cream	−
paratyphi B	Treatment plant	−
reading	Turkey faecal sample	−
regent	Breeding environment	−
rissen	Turnsol meal	−
rubislaw	Fish viscera	−
saintpaul (×2)	Poultry meat	−
sandiego	Turkey viscera	−
schwarzengrund	Hen viscera	−
senftenberg	Hen viscera	−
senftenberg	Manioc	−
stanley	Bovine faecal sample	−
tennessee	Beef meat	−
thompson	Poultry meat	−
typhimurium	Bovine faecal sample	+
typhimurium (×4)	Breeding environment	+
typhimurium	Chicken meat	+
typhimurium	Dog faecal sample	+
typhimurium (×2)	Duck viscera	+
typhimurium	Egg product	+
typhimurium	Foodstuffs	+
typhimurium	Foodstuffs	−
typhimurium	Human faecal sample	+
typhimurium (×2)	Pork butchery	+
typhimurium	Pork butchery	−
typhimurium	Pork meat	+
typhimurium	Poultry meat	+
typhimurium	River water	+
typhimurium	Treatment plant	+
virchow	Hen viscera	−
virchow	Poultry meat	−
wien	Pork butchery	−
worthington	Pork butchery	−
zanzibar	Turkey viscera	−
S. I 4:-:-	Quail viscera	−
S. I 6,7:-:-	Processing Plant	−

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Table 1 (*cont.*)

Strains	Origins	Results
S. I 9,12:-:-	Bovine viscera	−
S. I 1,3,19:-:-	Hen viscera	−
S. I 1,3,19:i:-	Fish meal	−
S. I 1,3,19:z_{27}	Colza meal	−
S. I 1,3,19:z_{27}	Foodstuffs	−
S. II 42:b:enz$_{15}$	Frog leg	−
S. IIIa 48:z_4, z_{23}	Foodstuffs	+
S. IIIb 38:r:z	Treatment plant	−
S. IIIb 61:i:z_{53}	Equine viscera	−
S. IIIb 61:i:z_{53}	Pork butchery	−
S. IIIb 61:k:1,5,7	Ovine viscera	−
S. IV 43:z_4, z_{23}:-	Wild boar meat	+
S. V 28:*a*:-	Gelatine	+
S. rough	Pork butchery	−
S. rough (×2)	Sea water	−
Aeromonas sp. (×2)	Estuary water	−
Citrobacter freundii	Poultry breeding	−
Citrobacter freundii	Poultry meat	−
Citrobacter freundii	Surface water	−
Enterobacter aerogenes	Cooked food	−
Enterobacter cloacae	Estuary water	−
Enterobacter cloacae	Poultry breeding	−
Enterobacter hafniae	Bovine faecal sample	−
Enterobacter hafniae	Estuary water	−
Escherichia coli	Bovine breeding	−
Escherichia coli (×2)	Estuary water	−
Escherichia coli (×2)	Poultry breeding	−
Klebsiella pneumoniae	Poultry breeding	−
Moraxella sp.	Estuary water	−
Listeria innocua	Cheese	−
Listeria ivanovii	Cheese	−
Listeria monocytogenes	Cheese	−
Proteus mirabilis	Horse meat	−
Proteus morganii	Pig offals	−
Proteus rettgeri	Bovine viscera	−
Providencia sp.	Estuary water	−
Providencia sp.	Poultry breeding	−
Pseudomonas sp. (×4)	Estuary water	−
Sarcina lutea	Bovine breeding	−
Shigella sonnei	Slaughterhouse	−
Staphylococcus aureus	Cheese	−
Staphylococcus epidermidis	Milk	−
Vibrio vulnificus	Estuary water	−
Yersinia enterocolitica	Poultry breeding	−

(200 μM each, Boehringer), primers (0·5 μM each) and target DNA. The reaction mixture was heated for 5 min at 95 °C and overlaid with mineral oil to prevent evaporation. PCR reaction was performed with 30 cycles as follows: 5 sec primer annealing at 60 °C, +1 °C/3 sec up to 95 °C, 5 sec denaturation at 95 °C, −1 °C/sec down to 60 °C. After the 30th cycle, the extension reaction was continued for another 10 min at 72 °C. Samples of the reaction mixture (10 μl) were loaded on to a 2% agarose gel containing ethidium bromide (0·5 μl/ml) for electrophoresis. After 30 min under 100 V, the gel was examined under u.v. light

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Table 2. *Positions and sequences of oligonucleotides*

Oligonucleotides	Sequences (5′–3′)	Corresponding position in virulence plasmid gene [22]
VIR 113	TTGTAGCTGCTTATGATGGGGCGG	113–136
VIR 561	TGGAGAAACGACGCACTGTACTGC	561–583*
VIR 334	CGAGAATCACCTCAGTCTCAGGGC	334–357

* Complementary DNA strand.

(312 nm) and the amplification product size was compared to DNA fragments size of a molecular weight marker (Marker VI, Boehringer). Negative controls containing all reagents except template DNA were performed in each amplification set. To avoid contamination, sample preparation, PCR amplification and electrophoresis were performed in three different rooms.

Probe labelling

The probe labelling was performed with the 'DNA Tailing Kit' (Boehringer). According to the manufacturer's instructions, tailing buffer, $CoCl_2$, dTTP, digoxygenin-11-dUTP, sterile distilled water, terminal desoxynucleotidyl transferase and the oligonucleotide to be labelled (100 p.m.) were mixed and incubated for 15 min at 37 °C. To end the reaction, a stop solution (Glycogen, EDTA), was added. The labelled probe was purified on a G 50 Sephadex column (Pharmacia, Uppsala, Sweden) with a Tris-HCl pH 8 (10 mM), EDTA (1 mM) elution buffer.

Southern blot and hybridization

After agarose gel electrophoresis, DNA denaturation was performed for 15 min with NaOH (0·4 N), followed by neutralization with Tris-HCl pH 7·2 (0·5 M), NaCl (1·5 M) for 45 min to 1 h 30 min. Then amplified DNA fragments were transferred to a positively charged nylon membrane (Boehringer) using saline sodium citrate buffer (SSC, 20 ×) in a vacuum blotter (10 mm mercury pressure) for 45 min at room temperature, and fixed on to the membrane by heating at 120 °C for 30 min. The filter was pre-hybridized in SSC (5 ×) buffer, *N*-laurylsarcosine (0·1 %), sodium dodecyl sulphate (SDS, 0·02 %) and blocking reagent (1 %, Boehringer) at 65 °C for 1 h. For hybridization, labelled probe VIR 334 (25 ng/ml) was added and the filter was incubated at 55 °C for 2 h 30 min. After SSC (2 ×), SDS (0·1 %) washing, the filter was then washed to a final stringency of 55 °C in SSC (0·1 ×), SDS (0·1 %). Detection was performed with alkaline phosphate-labelled anti-digoxygenin antibody (Boehringer), nitro blue tetrazolium (NBT) and bromo-4-chloro-3-indolyl phosphate (BCIP) according to the manufacturer's instructions (Boehringer).

RESULTS

Optimization of the amplification conditions

In this study it was not necessary to perform three thermal steps. A very good amplification yield was obtained by using only two temperatures with a ramping time of +1 °C/3 sec between annealing and denaturation temperatures. This allowed reduction of the reaction time in comparison with standard PCR. The global reaction time was thus reduced to 1 h 30 min, with results equivalent to

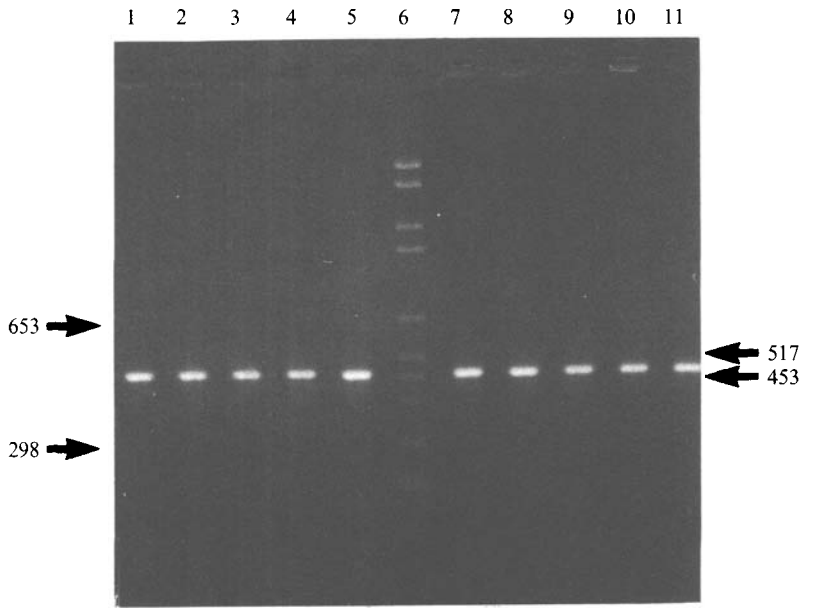

Fig. 1. Salmonella PCR results. Lanes: 1, *S. typhimurium*; 2, *S. enteritidis*; 3, *S. hessarek*; 4, *S. choleraesuis*; 5. *S. dublin*; 6, molecular weight marker VI (Boehringer); 7, *S. gallinarum/pullorum*; 8. *S. abortus ovis*; 9, *S. IIIa* 48:z_4, z_{23}; 10, *S. IV* 43:z_4, z_{23}:-: 11. *S. V* 28:*a*:-. (Numbers on figure are molecular weights in base pair.)

those obtained using conventional amplifications, which incorporate a temperature hold time during each step (data not shown).

PCR/DNA hybridization assay specificity

After PCR assay, no cross-reaction was observed with the 35 non-salmonella strains derived from 15 species (Table 1).

No amplification products, specific or not, could be observed for 94 salmonella strains from 66 serovars (Table 1), irrespective of the source under consideration.

An amplification product of expected molecular size (Fig. 1) has been obtained for 49 salmonella strains, from 10 serovars (Table 1). The 10 serovars' amplification products have all hybridized with the digoxygenin-labelled probe VIR 334 (Fig. 2).

DISCUSSION

We have developed a PCR assay for the detection of salmonella virulence-associated plasmid gene. A primers set, derived from the virulence gene, allowed us to detect 10 serovars among 71 tested serovars. No amplification product was observed with 35 strains from 15 other bacterial species, and an internal probe ensured the specificity of the salmonella amplification products using a DNA/DNA hybridization test. Thus the 472 bp amplification product revealed the presence of a plasmid gene linked to virulence in salmonella.

As shown in Table 3, the strains responding to amplification were of different origins: human, 1; animal pathology, 19; food hygiene, 18; environment, 11; illustrating that strains with increased virulence are present in all biotopes.

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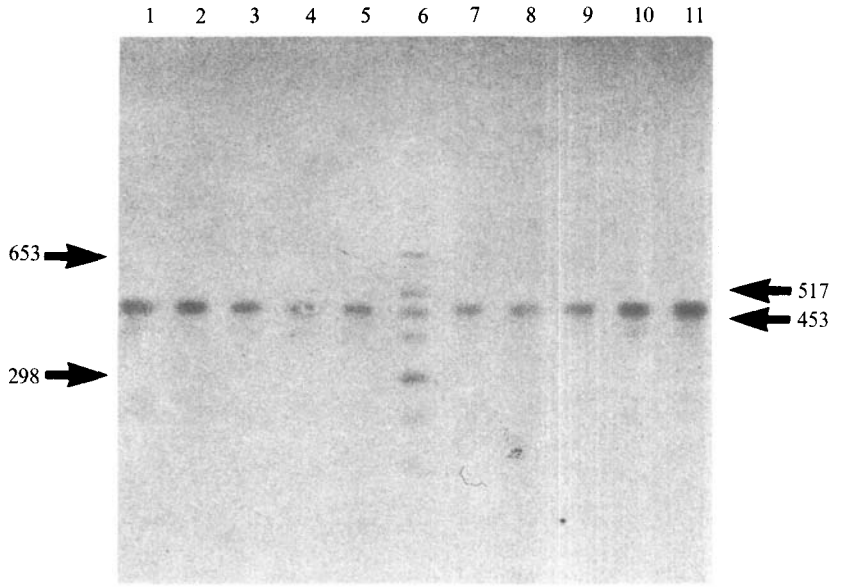

Fig. 2. Vir 334 probe hybridization results, after Southern blot of the electrophoresis gel shown in Fig. 1. Lanes: 1, *S. typhimurium*; 2, *S. enteritidis*; 3, *S. hessarek*; 4. *S. choleraesuis*; 5, *S. dublin*; 6, molecular weight marker VI (Boehringer); 7, *S. gallinarum pullorum*; 8, *S. abortus ovis*; 9, *S. IIIa* 48:z_4, z_{23}; 10, *S. IV 43*:z_4, z_{23}:-; 11, *S. V 28*:*a*:-. (Numbers on figure are molecular weights in base pair.)

Seven of the ten serovars producing an amplified DNA fragment were mentioned in the literature as possessing virulence-related plasmids: *S. abortus ovis* [7, 17], *S. choleraesuis* [3, 16, 19, 25], *S. dublin* [12, 16–18], *S. enteritidis* [4, 8, 15, 26], *S. gallinarum/pullorum* [2, 3, 11], *S. hessarek* [18], *S. typhimurium* [5, 14, 19, 21]. The three other serovars were *Salmonella* subspecies IIIa, IV and V, which were encountered less frequently (Table 1).

Two of the 18 *S. enteritidis* strains studied showed no reaction (Table 1), proving that for a given serovar known to contain virulence-related plasmids [4, 8, 26–27], there is diversity. This was also confirmed for *S. abortus ovis*, since only one of the two tested strains possessed the plasmid gene associated with virulence (Table 1) and for *S. typhimurium*, since only 2 of the 20 tested strains did not respond to amplification (Table 1). Moreover, one serovar (two strains), described as possessing virulence-associated plasmid, i.e. *S. newport* [3, 17], did not produce a positive response when analysed by PCR during this study.

Several authors [2, 3, 17, 18, 28–29] have described serovars containing plasmids of various molecular size which did not contain the genes associated with virulence. The search for the plasmid content of strains was often used in the study of epidemiological markers [8, 29, 30–32]. While remaining of some interest, this approach does not provide any information as to the potential virulence of strains, since on the one hand the presence of plasmids does not necessarily imply the presence of the plasmid gene associated with mouse virulence [15, 17, 28], and on the other hand the plasmid/virulence combination is not inevitably linked to the molecular weights of the plasmids observed [4, 28, 32].

These facts illustrate the advantages of searching for genetic factors associated with virulence. The PCR technique used is perfectly suited to this search and has

Table 3. *PCR salmonella tested strains distribution according to the origin*

Origins	Tested strains number (serovars number)	PCR positive reaction strains number and involved serovars (number)
Human pathology	1	1 *typhimurium*
Animal pathology		
Bovine	13 (9)	6 *dublin* (4), *enteritidis*, *typhimurium*
Dog	3 (3)	1 *typhimurium*
Equine	1	0
Fish	2 (2)	0
Ovine	3 (2)	1 *abortus ovis*
Poultry	26 (18)	10 *enteritidis* (4), *gallinarum/pullorum* (4), *typhimurium* (2)
Young wild boar	1	1 *choleraesuis*
Food hygiene		
Beef meat	10 (10)	1 *dublin*
Cacao	1	1 *enteritidis*
Colza meal	1	0
Cooked food	1	1 *enteritidis*
Egg product	3 (2)	3 *enteritidis* (2), *typhimurium*
Feedstuffs	7 (6)	2 *typhimurium*, *S. IIIa* 48:z_4, z_{23}
Fish meal	4 (4)	0
Frog leg	1	0
Gelatine	1	1 *S. V* 28:a:-
Horse meat	1	0
Ice cream	1	0
Manioc	1	0
Milk	2 (2)	1 *enteritidis*
Pastry	3 (2)	1 *enteritidis*
Pork butchery	12 (10)	2 *typhimurium*
Pork meat	1	1 *typhimurium*
Poultry meat	9 (7)	3 *enteritidis*, *typhimurium* (2)
Shellfish	1	0
Soya meal	1	0
Turnsol meal	2 (2)	0
Wild boar meat	1	1 *S. IV* 43:z_4, z_{23}:-
Breeding environment	29 (17)	11 *enteritidis* (4), *hessarek*, *typhimurium* (6)

the advantage of being quick, reliable and easy to use, in comparison with conventional techniques. The standard methods used to investigate the relationship between the presence of a plasmid and increased virulence call for preliminary extraction of the plasmids followed by the study, in mice, of the effect of strains with or without their plasmids, or the use of DNA/DNA hybridization techniques [7, 9, 10, 11, 13, 16, 21, 26]. In comparison with the PCR assay, these techniques are time-consuming and difficult to apply in a systematic way when investigating the presence of these genes.

The ubiquitous presence of the strains (Table 3), the diversity of the serovars involved and the preservation of plasmid genes associated with virulence during evolution [33] merit a proper place for this research among the ranks of epidemiological markers currently in use. The PCR method proposed gives the information sought directly and quickly, either by comparing strains from the same biotope or by the knowledge of the strains' capacity to develop in a given organism [6, 10, 12–13].

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All these findings show that the search for plasmid genes associated with mouse virulence is of proven epidemiological interest and the method described above is perfectly suited to this tasks, in terms of its ease of use in comparison to tests on mice or DNA/DNA hybridization techniques, its reliability, reproducibility, rapidity (amplification; 1 h 30 min; detection, 30 min) and the possibility of investigating a large number of strains.

ACKNOWLEDGEMENTS

The authors thank J. P. Guillou (CNEVA/LCRV) for his invaluable help in the computer analysis for choosing the primers set and the probe. They are grateful also to S. Fremy, M. Goncalves, C. Oudart, C. Piquet and C. Pires Gomes (CNEVA/LCHA) for biochemical and serological analysis of salmonella isolates.

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