ANTIMICROBIAL RESISTANCE

Evolution of chloramphenicol resistance, with emergence of cross-resistance to florfenicol, in bovine Salmonella Typhimurium strains implicates definitive phage type (DT) 104

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The prevalence of resistance to florfenicol, a phenicol drug newly introduced in veterinary therapy, was determined in 86 chloramphenicol-resistant Salmonella Typhimurium isolates from cattle collected during 1985–1995. All were highly resistant to chloramphenicol (MICs >128 mg/L) and 38 were simultaneously resistant to florfenicol (MICs >16 mg/L) and to β-lactam agents, spectinomycin, streptomycin, sulphonamides and tetracyclines. The isolates susceptible to florfenicol harboured the chloramphenicol acetyl transferase gene, cat of type I. All the florfenicol-resistant isolates harboured the floR resistance gene and the characteristic multiple resistance genetic locus, previously characterised in a S. Typhimurium DT104 strain and identified by a multiplex PCR. Plasmid profiles and ribotype patterns were determined for all the isolates. The florfenicol-resistant isolates were grouped into the same ribotyping pattern and presented similar plasmid profiles, whereas the florfenicol-susceptible isolates showed a wider genetic diversity that is usual for S. Typhimurium. Thus, the florfenicol-resistant isolates could represent a clonal cluster, closely related to, if not of DT104 phage type, which appeared in 1989 and is now predominant within chloramphenicol-resistant S. Typhimurium. The multiplex PCR provided a useful tool to survey further evolution of multiresistant S. Typhimurium strains.

Introduction

Salmonella enterica is one of the major causes of food poisoning, with a high health and cost penalty. During the last decade, S. enterica serotype Typhimurium (S. Typhimurium) became the most important serotype identified in Occidental countries [1–4]. During the 1980s, this serotype became the predominant one in cattle and replaced S. enterica serotype Dublin [5]. In the 1990s, it also became the most antibiotic-resistant serotype in cattle [6, 7]. In 1995, the French permanent nationwide network of surveillance of antibiotic resistance in bovine pathogens (RESABO) demonstrated that >50% of S. Typhimurium isolates were multiresistant [8], mostly to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines (ApCmSmSuTc). This antibiotic resistance pattern is now of particular concern with the emergence of the multiple drug-resistant S. Typhimurium strains of DT104 [2, 9–11]. Detection of the ApCmSmSuTc resistance pattern has often been considered the first step for the identification of these strains, before phage typing, although other resistance patterns have been described in this phage type [12, 13] and other phage types have also been described with the same dominant antimicrobial resistance pattern [14]. The clonality of these strains of S. Typhimurium DT104 had been suggested after studies that demonstrated the limits of their genetic characterisation [4, 15]. Only plasmid profile analysis and, to a lesser extent ribotyping, have
been suggested to permit subdivisions between these strains [12, 16–19].

For a long time chloramphenicol was considered the drug of choice to treat salmonellosis in animals. In August 1994, chloramphenicol was prohibited in Europe for veterinary use in farm animals. In January 1995, a new drug of the same family, florfenicol, was licensed in France for therapeutic use with a specific indication for bovine pasteurellosis. Soon after its introduction, isolates of S. Typhimurium were detected which appeared to be cross-resistant to the two phenicol drugs.

Resistance to chloramphenicol is mainly due to the production of inactivating enzymes, the chloramphenicol acetyl transferases (CAT). Three types of CAT (types I–III) have been described in gram-negative bacteria, but resistance due to the production of CAT of type I has been encountered most frequently in Enterobacteriaceae and is generally plasmid-encoded [20, 21]. The florfenicol drug remains active against strains harbouring one of the three types of cat genes [22]. Genes involving non-enzymic resistance mechanisms, active against both chloramphenicol as well as florfenicol, have also been described: cmlA from transposon Tn1696 of Pseudomonas aeruginosa [23], pp-flo from Pasteurella piscicida [24], and more recently floR from a bovine S. Typhimurium DT104 strain BN9181 [1]. The nucleotide sequence of these genes demonstrated a strong identity with the family of multidrug efflux pumps. In that study of the S. Typhimurium DT104 strain BN9181, the links between the genes responsible for the ApCmSmSuTc resistance phenotype were also determined. These are located on the chromosome and organised in a multiresistance genetic locus with implication of an integron structure (Fig. 1).

Resistance to florfenicol has also been described: cmlA from transposon Tn1696 of Pseudomonas aeruginosa [23], pp-flo from Pasteurella piscicida [24], and more recently floR from a bovine S. Typhimurium DT104 strain BN9181 [1]. The nucleotide sequence of these genes demonstrated a strong identity with the family of multidrug efflux pumps. In that study of the S. Typhimurium DT104 strain BN9181, the links between the genes responsible for the ApCmSmSuTc resistance phenotype were also determined. These are located on the chromosome and organised in a multiresistance genetic locus with implication of an integron structure (Fig. 1).

Fig. 1. Structure of the multiresistance locus of S. Typhimurium DT104, strain BN9181 [1]. The genes of the class I integrons are represented by hatched arrows and extra-integron elements by black arrows. The lines A, B, C and D represent the amplification products obtained by the multiplex PCR. Line E represents the amplification obtained with CmI01 and cmI15 degenerate primers for detection of the non-enzymic chloramphenicol resistance genes and amplification of the floR gene detection probe.

Materials and methods

Clinical strains and plasmids

Eighty-six S. Typhimurium strains collected by the RESABO network were studied. They were isolated between 1985 and 1995 from clinical cases of bovine salmonellosis and selected for their resistance to chloramphenicol. Before 1989, no chloramphenicol-resistant strains had been isolated from adult cows; all the chloramphenicol-resistant strains originated from calves. After 1989 the chloramphenicol-resistant strains were isolated from adult cows. The S. Typhimurium DT104 strain BN9181, harbouring the floR gene [1], belonged to the strain collection. One strain, BN9264, susceptible to all the antibiotics tested, was also included as the negative control. Escherichia coli strains harbouring R55 (catI), Rsa (catI) and a recombinant plasmid (catIII) were used as controls for detection of cat genes [25]. E. coli harbouring plasmids pLQ821 from Tn1696 of Pseudomonas aeruginosa [23] and pFF from Pasteurella piscicida [24] were also used as controls for suspected efflux resistance genes cmlA and pp-flo, respectively.

Susceptibility testing

The strains were tested for their antibiotic susceptibility on Mueller-Hinton agar by the disk diffusion method. Resistance to the following antibiotics was tested with disks containing: ampicillin (Ap) 10 μg, amoxycillin + clavulanic acid (AmC) 20 + 10 μg, respectively, cefalothin (Cf) 30 μg, chloramphenicol (Cm) 30 μg, florfenicol (Ffc) 30 μg, gentamicin (Gm) 15 μg, neomycin (Nm) 30 IU, spectinomycin (Sp) 100 μg, streptomycin (Sm) 10 IU, sulphonamides (Su) 200 μg, tetracyclines (Tc) 30 IU, trimethoprim (Tp) 5 μg, nalidixic acid (Na) 30 μg and enrofloxacine (Enr) 5 μg. The media and disks were from Sanofi Diagnostics Pasteur (France), except for disks with
florfenicol and enrofloxacin, which were purchased from Schering-Plough Santé Animale (Ségé, France) and Bayer Pharma (Puteaux, France), respectively. MICs of chloramphenicol (Boehringer-Mannheim, Germany) and florfenicol (Schering-Plough) were determined according to the recommendations of the Comité de l’Antibiogramme de la Société Française de Microbiologie (CA-SFM) [26].

Plasmid isolation and transfer of resistance
Plasmid extractions were performed by the method of Takahashi and Nagano [27]. The supercoiled DNA ladder (Gibco-BRL, MD, USA) and plasmids R55, R112 and RP4 of c. 150, 100 and 54 kb, respectively, were used for mol. wt estimation.

Transfer of chloramphenicol and florfenicol resistances by conjugation was performed with representative donor strains. E. coli BM21 (prototroph, gyrA) or E. coli K-12 BM14 (pro met azl) were used as recipients in conjugation experiments with S. Typhimurium strains susceptible or resistant to quinolones, respectively. Agar plates for the selection of transconjugants were supplemented with nalidixic acid (Sigma) 50 mg/L or sodium azide (Sigma) 500 mg/L, and either chloramphenicol 30 mg/L or florfenicol 15 mg/L.

Ribotyping and analysis
DNA extraction, electrophoresis and transfer of digested DNA were done as described previously [28]. The restriction endonucleases HindIII, PvuII and Smal (Boehringer-Mannheim) were used as recommended by the supplier. The ribosomal probe was obtained from the E. coli RNA 16-23S, as described previously [29] and cDNA was then labelled by digoxigenin (Dig High Prime; Boehringer-Mannheim). The hybridised probe was detected immunologically with CSPD® (Dig Luminescent detection kit, Boehringer). Cluster analysis was derived from the distance matrix by the UPGMA method and conducted by the software program StatITCF (Institut Technique des Céréales et des Fourrages, Paris, France) [28].

Detection of chloramphenicol resistance mechanism and genes
The production of a CAT enzyme was studied by the method of Azemun et al., with some modifications [25]. The presence of the cat genes was determined by PCR amplification with primers specific for type I, II or III as described previously [25].

The presence of a chloramphenicol efflux resistance gene was also assessed by PCR amplification with degenerate primers designed by comparison between nucleotide sequences of floR, pp-fio and cmlA, respectively, from S. Typhimurium, P. piscicida and Ps. aeruginosa [1]. The strains with positive amplification were then examined by colony hybridisation to confirm the presence of the floR gene. The probe used was the amplification product of S. Typhimurium strain BN9181 labelled with digoxigenin (Dig High Prime; Boehringer-Mannheim) and revealed by CSPD® [1].

Multiplex PCR
A multiplex PCR was designed to assess the presence of the multidrug resistance genetic locus organised around the floR gene in the BN9181 strain of S. Typhimurium DT104 [1]. It was then used to examine the 40 florfenicol-resistant S. Typhimurium strains. Two florfenicol-susceptible chloramphenicol-resistant strains (BN3937 and BN9315) were used as negative controls, as well as one more strain (BN9264) that was susceptible to all the antibiotics tested. The amplification primer pairs used are shown in Table 1. The amplification reactions were performed on boiled extracts in 25 μl with 2 mM MgCl2, 0.4 mM dNTP and 6 IU of taq DNA polymerase (Promega, Charbonnières, France). The primers were added at 10 pmol for tnp10, aadB and sulTER, 20 pmol for F4 and tetAR, and 1 pmol for tetRB. PCR amplification was performed with the following programme: 3 min at 94°C,
25 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min and 30 s, and elongation at 72°C for 3 min, with a final extension of 10 min at 72°C.

Results

Antimicrobial resistance

All the 86 strains studied possessed multiple antibiotic resistance (Table 2). All were resistant to sulphonamides, 85 to tetracyclines, 84 to streptomycin and 82 to ampicillin. Resistance to spectinomycin, trimethoprim and quinolones was frequent, (51, 45 and 30 strains, respectively). Resistance to cefalothin or enrofloxacin was not detected. Growth inhibition diameters were always > 20 mm.

All the strains were highly resistant to chloramphenicol (MICs > 128 mg/L), 38 were simultaneously resistant to florfenicol (MICs > 32 mg/L) and 46 were susceptible to florfenicol (MICs < 4 mg/L). Two strains possessed intermediate florfenicol MICs (8 and 16 mg/L for strains BN6111 and BN8098, respectively). The 38 strains simultaneously resistant to chloramphenicol and florfenicol showed a decreased susceptibility to amoxyillin + clavulanic acid, and could be classified as intermediate according to the recommendations of the CA-SFM [26]. A similar modification was observed in two more strains. One was susceptible to florfenicol, the other (BN6111), expressed intermediate resistance to florfenicol. The dominant pattern of resistance was ampicillin, chloramphenicol, florfenicol, streptomycin, spectinomycin, sulphonamides, tetracyclines (ApCmFtcSmSpSuTc) - represented by 31 strains. Eight strains had further acquired resistance to nalidixic acid.

The distribution of the chloramphenicol-resistant strains according to their resistance or susceptibility to florfenicol is shown in Fig. 2. The first strain resistant to florfenicol was isolated in 1989. In 1992 and 1993, resistance to florfenicol was present in almost 50% of the strains. In 1994, the phenotype expressing cross-resistance to chloramphenicol and

Table 2. Results of ribotyping, antibiotic resistance, plasmid profiling and phage typing in chloramphenicol-resistant S. Typhimurium from cattle

<table>
<thead>
<tr>
<th>Ribotype*</th>
<th>Antibiotic resistance†</th>
<th>Plasmid sizes (kb)</th>
<th>Strain number</th>
<th>Phage type‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (H1 P1 S1)</td>
<td>ApCmNmSmSuTcTp</td>
<td>180 - 95 - 6.6 - 4</td>
<td>1</td>
<td>1 DT175</td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa (Gm)</td>
<td>180 - 95 - 6.6 - 4</td>
<td>10</td>
<td>ND</td>
<td></td>
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<tr>
<td>ApCmNmSmSuTcTpNa</td>
<td>180 - 95 - 4</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa</td>
<td>180 - 6.6 - 4</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa</td>
<td>180 - 105 - 95 - 6.6</td>
<td>2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa</td>
<td>105 - 95 - 6.6</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa</td>
<td>180 - 95 - 70</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa</td>
<td>95 - 70 - 6.6 - 5.4</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa (Gm)</td>
<td>180 - 95 - 70 - 6.6</td>
<td>2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApAmCcGmSmSpSuTcTpNa</td>
<td>180 - 150 - 70 - 7.5</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCcSmSpSuTc</td>
<td>180 - 95</td>
<td>2</td>
<td>1 untypable</td>
<td></td>
</tr>
<tr>
<td>ApCcSmSuTc</td>
<td>105 - 95</td>
<td>1</td>
<td>Untypable§1</td>
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<tr>
<td>ApCcSmSuTc</td>
<td>180 - 95</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>B (H2 P2 S2)</td>
<td>ApCmNmSmSpSuTcTpNa</td>
<td>180 - 150</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>ApCmNmSmSuTcTpNa</td>
<td>180 - 70 - 95 - 6.6</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>C (H2 P2 S4)</td>
<td>ApCcSmSuTc</td>
<td>180 - 95 - 2.7</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>D (H3 P3 S2)</td>
<td>CmNmSmSpSuTc</td>
<td>180 - 95 - 70 - 3</td>
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<td>ND</td>
</tr>
<tr>
<td>E (H4 P4 S1)</td>
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<td>105 - 95</td>
<td>1</td>
<td>ND</td>
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<tr>
<td>F (H5 P5 S3)</td>
<td>CmSmSpSuTc</td>
<td>180 - 3.5</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>G (H6 P5 S3)</td>
<td>CmSmSuTc</td>
<td>180 - 3.5</td>
<td>1</td>
<td>ND</td>
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<tr>
<td>H (H7 P1 S5)</td>
<td>ApCcSmSuTc</td>
<td>180 - 4.8</td>
<td>7</td>
<td>DT193</td>
</tr>
<tr>
<td>ApCcSmSuTc</td>
<td>180 - 70 - 4.8</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApCcSmSuTc</td>
<td>180 - 70 - 6.6 - 4.8</td>
<td>1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>I (H8 P1 S1)</td>
<td>ApCcSmSuTc</td>
<td>105 - 95 - 4.8 - 3.5</td>
<td>1</td>
<td>DT193</td>
</tr>
<tr>
<td>J (H9 P6 S1)</td>
<td>ApCcNmSmTc</td>
<td>180</td>
<td>1</td>
<td>DT193</td>
</tr>
<tr>
<td>K (H10 P1 S1)</td>
<td>ApAmCcFmCcSmSpSuTc</td>
<td>95</td>
<td>11</td>
<td>IDT104</td>
</tr>
<tr>
<td>ApAmCcFmCcSmSpSuTc</td>
<td>95 - 2.1</td>
<td>11</td>
<td>IDT104</td>
<td></td>
</tr>
<tr>
<td>ApAmCcFmCcSmSpSuTc</td>
<td>95 - 3.3 - 2.1</td>
<td>7</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ApAmCcFmCcSmSpSuTc</td>
<td>95 - 70</td>
<td>1</td>
<td>IDT104</td>
<td></td>
</tr>
<tr>
<td>ApAmCcFmCcSmSpSuTcNa</td>
<td>95 - 7.2</td>
<td>1</td>
<td>ND</td>
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<tr>
<td>ApAmCcFmCcSmSpSuTc(Na)</td>
<td>95 - 7.2</td>
<td>1</td>
<td>ND</td>
<td></td>
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<tr>
<td>ApAmCcFmCcSmSpSuTc(Na)</td>
<td>95 - 7.2 - 2.1</td>
<td>1</td>
<td>ND</td>
<td></td>
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<tr>
<td>ApAmCcFmCcSmSpSuTc(Na)</td>
<td>95 - 2.1</td>
<td>5</td>
<td>2DT104</td>
<td></td>
</tr>
<tr>
<td>CmMsSpSuTc</td>
<td>105 - 95 - 4.8 - 3.5</td>
<td>1</td>
<td>DT193</td>
<td></td>
</tr>
<tr>
<td>L (H11 P1 S1)</td>
<td>AcmFtcGmSmSpSuTcTcNa</td>
<td>120</td>
<td>1</td>
<td>DT120 or DT104</td>
</tr>
</tbody>
</table>

*H, P and S, correspond to the ribotype patterns obtained with Hincll, PvuII and SmaI enzymes, respectively.
†Antibiotic resistance abbreviations: Ap, ampicillin; AmC, amoxycillin + clavulanic acid; Cm, chloramphenicol; Ftc, florfenicol; Gm, gentamicin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin; Su, sulphonamides; Tc, tetracyclines; Tp, trimethoprim; Na, nalidixic acid. Gm in brackets indicates that the resistance is represented only by one strain.
‡Phage typing was determined by the Laboratoire de Typage des Entérobactéries (F. Grimont), Institut Pasteur, Paris. ND, not done.
§Particular strains, respectively: 1BN9315, 2BN6111, 3BN9181, 4BN3937, 5BN8098.
florfenicol was predominant. In 1995, because special attention was paid to florfenicol resistance in the surveillance network, a preferential selection of corresponding resistant strains resulted, and it was considered that a bias was introduced in the constitution of the collection. Therefore, these strains have not been included in Fig. 2.

Plasmid profiles and transfer of resistance

Twenty-three plasmid profiles were identified within the florfenicol-susceptible chloramphenicol-resistant strains, with plasmid sizes ranging from 180 to 2.1 kb. The largest plasmid present in the florfenicol-resistant strains was c. 95 kb, and six different plasmid profiles were observed. Two of them represented the majority of the strains (Table 2).

The ability to transfer phenicol resistance was tested with strains representative of the different plasmid profiles. In all cases, transconjugants were obtained from the florfenicol-susceptible strains, with transfer of the largest plasmid, except for strain BN3937 and two other strains without a large plasmid, which did not transfer their resistance to chloramphenicol. Each conjugation experiment resulted in a single transconjugant antibiotic resistance profile with transfer of resistance to chloramphenicol, streptomycin, spectinomycin, tetracyclines, sulphonamides and trimethoprim. No transconjugant from florfenicol-resistant strains could be obtained despite repeated conjugation experiments with chloramphenicol or with florfenicol selection.

Ribotyping

Ribotypes of the florfenicol-susceptible chloramphenicol-resistant strains were studied after the digestion of the total DNA by HincII, PvuII and Smal, which resulted in 10, 6 and 5 patterns respectively.

Ribotyping grouped all the florfenicol-resistant strains in only one pattern whichever enzyme was used, with the exception of strain BN8098, which had an intermediate florfenicol MIC. The ribotype pattern of the florfenicol-resistant group differed from the patterns obtained with the florfenicol-susceptible strains (Fig. 3).

Fig. 2. Distribution of florfenicol MICs in chloramphenicol-resistant strains of S. Typhimurium isolated between 1985 and 1994. (□), 1985; (■), 1987; (□), 1989; (□), 1990; (□), 1991; (■), 1992; (■), 1993; (■■), 1994.

Fig. 3. Schematic representation of selected HincII ribotype patterns obtained after digestion of the Salmonella genomic DNA. H1, H2, H7, H10 and H11 refer to the ribotype patterns as numbered in Table 2; St, mol. wt standard (Raoul®, Appliedgen, revealed with Dig-labelled pUC18).
In total, the ribotyping scheme permitted the differentiation of 12 patterns (A-L, Table 2), with one of them (K) containing all the florfenicol-resistant strains, together with one florfenicol-susceptible chloramphenicol-resistant strain, BN3937.

Chloramphenicol resistance mechanism and gene identification

The 46 florfenicol-susceptible strains, and their transconjugants, as well as the three positive reference strains, expressed CAT activity without prior induction by chloramphenicol. No CAT activity was observed with the 40 florfenicol-resistant strains, including the two strains with intermediate florfenicol MICs. PCR amplification tests with cat primers were positive with the 46 florfenicol-susceptible strains and with the strains harbouring the reference plasmids, but not with the 40 florfenicol-resistant strains whatever the specific primer pair used. The size of the amplification product always corresponded to that of the catI gene.

PCR amplification of the florfenicol- and chloramphenicol-resistant strains gave rise to a 496-bp product, as for the two control strains, when the degenerate primers were used for non-enzymic chloramphenicol resistance gene detection. Colony hybridisation with the floR gene-specific probe was positive with all but two strains: S. Typhimurium BN8098 and E. coli strain harbouring the pLQ821 plasmid containing the cmlA gene.

Multiresistance locus

The multiplex PCR was first evaluated with S. Typhimurium DT104 strain BN9181, and gave rise to four amplification fragments of 600, 900, 1450 and 2300 bp (Table 1). They corresponded to the expected sizes for floR-tetR, truncated sul1-floR, integrase-aadA and floR-tetA amplifications, respectively (Fig. 1).

The florfenicol-resistant strains, the florfenicol-susceptible and chloramphenicol-resistant S. Typhimurium strains BN3937 and BN9315, and the susceptible strain BN9264 were tested likewise. All 39 strains that harboured the floR gene, including the florfenicol intermediate strain BN6111, gave the same amplification pattern with the four expected bands. Strain BN8098, like the two florfenicol-susceptible chloramphenicol-resistant strains, gave only one band corresponding to the integrase-aadA amplification. The susceptible strain gave no amplification at all (Fig. 4).

Discussion

The S. Typhimurium strains selected in this study were isolated over a 10-year period. They were retained on the basis of their resistance to chloramphenicol, at the time it was withdrawn and when florfenicol was licensed for veterinary use. At the same time, the number of adult clinical cases of S. Typhimurium infections that involved chloramphenicol-resistant isolates increased. Most of these strains were resistant to ampicillin, spectinomycin, streptomycin, sulphonamides and tetracyclines, confirming that this multi-resistance pattern was frequently encountered in S. Typhimurium strains of non-human sources, namely those of bovine origin [6, 8, 14], and not only those belonging to the DT104 phage type. During that period, a significant change was observed with the emergence of resistance to florfenicol in 1989, and a decreasing susceptibility to amoxycilllin + clavulanic acid. This change affected c. 50% of the strains in the collection. The florfenicol MICs were in a bimodal distribution (<4 or ≥32 mg/L, respectively). In such conditions, it was concluded that for an MIC =4 mg/L, the strains were susceptible, and for an MIC >16 mg/L, the strains were resistant to florfenicol (more than 2 two-fold step increase). Two strains only had intermediate MICs of 8 and 16 mg/L. These data were in agreement with those obtained by Syriopoulou et al. [30].

In France, florfenicol was introduced in 1995 for treatment of pasteurellosis in cattle. No florfenicol-resistant Pasteurella strains have been detected by the RESABO network. Surprisingly, the first resistant strain of S. Typhimurium was isolated in 1989, 6 years before the licensing of florfenicol for veterinary use. Moreover, from that year onwards, this process has developed rapidly, and in 1994, the year before the introduction of florfenicol, it appeared that the florfenicol-resistant strains outnumbered the florfenicol-susceptible chloramphenicol-resistant strains (Fig. 2). Resistance genes and mechanisms separated the strains into two groups: the florfenicol-susceptible
strains producing the type I CAT inactivating enzyme usually encountered within the Enterobacteriaceae [21], and the florfenicol-resistant strains harbouring the floR gene which presented homology with the multidrug resistance efflux pumps [1]. This gene was first detected in an S. Typhimurium isolate in 1989, when the emergence of S. Typhimurium DT104 strains was observed [2, 19, 31]. In all the florfenicol-resistant strains, the presence of the multiresistance genetic locus associated with the floR gene was detected. This result indicated a common organisation in these strains as in S. Typhimurium DT104 strain BN9181 [1]. Phage typing was performed on 13 strains: seven florfenicol-susceptible chloramphenicol-resistant strains and six florfenicol resistant strains, including the florfenicol intermediate strains BN6111 and BN8098. The seven florfenicol-susceptible strains were of phage types 175, 193 or untypable (Table 2). Among the florfenicol-resistant strains, five were of DT104 phage-type, including BN6111. The phage type of the sixth strain, BN8098, was difficult to establish, as it was successively phage typed as DT120 and DT104. For this strain, although a PCR amplification with cml primers was obtained, no further hybridisation with the probe was revealed, which indicated that a closely related gene was involved in its chloramphenicol/ florfenicol resistance. Studies on its chloramphenicol and florfenicol resistance mechanism are in progress.

All the resistance determinants were transferred from all the florfenicol-susceptible strains which were tested. The florfenicol-resistant strains harboured only the floR gene which was non-transferable. This result, together with the multiplex PCR, suggested the chromosomal integration of all the resistance genes, as already described in S. Typhimurium DT104 strain BN9181 [1]. No transfer of resistance genes was described for the S. Typhimurium DT104 strains [32], except for apramycin resistance [12]. Plasmid analysis of S. Typhimurium DT104 strains had already been done. It revealed that all the strains of this phage type harboured the 95-kb serotype-specific plasmid (SSP) as largest plasmid [12, 18, 19]. This feature was also noted among the florfenicol-resistant strains, where six close plasmid profiles were defined, all harbouring the SSP as largest plasmid. The predominant patterns were similar to those already described in DT104 strains [12, 18, 19].

The relationships between the strains was investigated further by ribotyping. The enzymes were chosen for their discriminatory power with S. Typhimurium strains [28, 33, 34] that included DT104 strains [16]. The group of florfenicol-resistant strains differed from the rest of the sample population, with the florfenicol-susceptible strains presenting a large ribotyping pattern diversity as usually described for S. Typhimurium [16, 28, 34]. All the florfenicol-resistant strains were grouped in a single ribotype, together with strain BN3937 which, interestingly, was the unique florfenicol-susceptible strain that harboured a large but non-transferable plasmid. This ribotyping pattern has already been described for S. Typhimurium DT104 strains with two of the enzymes used [16]. However, the study by Guerra et al. [16], like the more recent one from Hilton and Penn [17], could differentiate two more patterns amongst the S. Typhimurium DT104 strains. The results of the present study reflected a stronger homogeneity of the French bovine strains, suggesting that they were grouped in a clone of at least national distribution within the strains of bovine origin [15].

In conclusion, this study of chloramphenicol-resistant strains from cattle suggested that the floR gene for florfenicol resistance was associated with ApCmSm-SuTc multiresistance as previously described for S. Typhimurium DT104 strain BN9181 [1]. Moreover, the present results highlighted four features in common between the florfenicol-resistant strains and S. Typhimurium DT104: (i) the dominant antimicrobial resistance phenotype is ApCmSmSpSmSuTc; (ii) the florfenicol resistance was not transferable, despite the presence of large plasmids, which suggested its chromosomal location, as in strain BN9181 [1]; (iii) the plasmid profiles were close, with the SSP as largest plasmid [12, 16, 19]; (iv) ribotyping could not discriminate between the florfenicol-resistant strains, suggesting the clonality of the strains, [4, 15–17]. Thus, emergence and diffusion of florfenicol resistance might be due to the dissemination of chloramphenicol-resistant S. Typhimurium DT104 strains. The multiplex PCR, which highlighted the particular situation of the floR gene in a chromosomal multiresistance locus, could be a useful tool to survey further evolution of multiresistant S. Typhimurium strains.

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