A novel Salmonella genomic island 1 and rare integron types in Salmonella Typhimurium isolates from horses in The Netherlands

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Received 27 September 2006; returned 3 November 2006; revised 8 December 2006; accepted 10 December 2006

Objectives: To investigate the genotypic resistance of integron-carrying Salmonella Typhimurium isolates from horses and their genetic relationship.

Methods: Sixty-one Salmonella isolates were screened for the presence of class 1 integrons by PCR. The gene cassettes of integron-positive isolates were detected by PCR, restriction fragment length polymorphism typing, and sequencing. The potential for the transfer of resistance determinants was investigated by conjugation experiments. The presence of Salmonella genomic island 1 (SGI1) or its variants was studied by PCR and nucleotide sequencing. PFGE was used to genotype the isolates.

Results: Eight distinct XbaI-PFGE profiles and seven integron types were observed among 26 integron-carrying Salmonella Typhimurium isolates. The gene cassettes detected were dfrA1, dfrA7, dfrA14, aadA1, aadA2, aadB and blaPSE. A rare type of integron found in nine isolates carried the dfrA14 and aadA1 gene cassettes. Twelve Salmonella Typhimurium DT104 isolates contained SGI1 or one of its variants (SGI1, SGI1-B and SGI1-C). A novel variant of SGI1, designated SGI1-M, was identified in one isolate in which the aadA2 gene of SGI1 was replaced by the aadB gene. Transfer of integrons and antimicrobial resistance determinants to Escherichia coli K12 via conjugation was possible with nine isolates. Resistance to fluoroquinolones in nine isolates was caused by mutations in the gyrA gene leading to the amino acid changes Ser-83 → Ala and Asp-87 → Asn.

Conclusions: The integron-positive clinical Salmonella Typhimurium isolates from horses belong to distinct strains. The data demonstrate the capability of Salmonella Typhimurium to acquire additional antibiotic resistance determinants and underline the need for the prudent use of antimicrobials.

Keywords: Salmonella spp., genotyping, SGI1, multidrug resistance, conjugation

Introduction

Over the last decade, a large increase in multidrug-resistant (MDR) Salmonella enterica isolates has been documented.1 In humans, Salmonella Typhimurium is a major aetiological agent of food-borne salmonellosis. In The Netherlands, Salmonella Typhimurium is the predominant serovar causing salmonellosis in horses, and this serovar was more often resistant to antimicrobial agents when compared with other Salmonella serovars.2 MDR Salmonella isolates from horses can be transferred to humans by direct contact or indirectly through the food chain.3

Multidrug resistance is strongly linked to the presence of class 1 integrons.4 Integrons are genetic elements that recognize and capture mobile gene cassettes (often encoding antibiotic resistance) by site-specific recombination.5 Three classes of integrons have been described. Class 1 integrons are the most common integrons found in clinical Salmonella isolates.6 Salmonella genomic island 1 (SGI1) is a 43 kb genomic island, which contains a complex integron.7 Variants of SGI1 (A–L) have been found in several Salmonella serovars including Salmonella Typhimurium.8–10 SGI1 can be transferred to other bacteria like Escherichia coli in the presence of a helper plasmid.11 Van Duijkeren et al.12 described that a particular type of integron, which was identified by restriction fragment length polymorphism (1600 bp, type XIII), was exclusively detected in equine Salmonella Typhimurium isolates. This observation prompted the current investigation.
Class 1 integrons in equine Salmonella Typhimurium

In the present study we investigated (i) the genetic relationship of 26 clinical isolates of equine integron-carrying MDR Salmonella Typhimurium, (ii) the characteristics of the antimicrobial resistance determinants among the isolates, and (iii) the potential to transfer these antibiotic resistance determinants to other bacterial species.

Materials and methods

Bacterial isolates

During the period 1993–2005, a total of 406 clinical equine Salmonella isolates were collected by the Veterinary Microbiological Diagnostic Center (VMDC) of Utrecht University, The Netherlands. The isolates were identified as Salmonella by biochemical testing and further characterized by serotyping. Based on the susceptibility testing data which were recorded by the VMDC, 61 Salmonella group B isolates from this collection were selected, based on their resistance to at least one antimicrobial drug. All isolates were cultured from different horses that belonged to different owners living throughout the country. The 61 isolates were screened for the presence of class 1 integrons using PCR amplification of the class 1 integrase gene. The 26 integron-carrying Salmonella Typhimurium isolates were phase-typed using the Dutch phage-typing system. Phage type 506 in this system corresponds to phage type DT104 in the English phage-typing system. The 26 isolates (Table 1), including 8 isolates described in a previous study, were tested for their antimicrobial susceptibility by the disc diffusion assay using Neo-Sensitab discs (Rosco, Denmark) based on the procedure recommended by the Dutch Committee on Guidelines for Susceptibility Testing CRG.

The antimicrobials tested were ampicillin (30 μg), amoxicillin/clavulanic acid (30/15 μg), cefalexin (30 μg), cefotiofur (30 μg), flumequine (30 μg), enrofloxacin (10 μg), streptomycin (100 μg), gentamicin (40 μg), kanamycin (100 μg), chloramphenicol (60 μg), tetracycline (80 μg), and trimethoprim/sulfamethoxazole (5.2/240 μg). In addition, the isolate H37 was tested for its susceptibility to tobramycin (40 μg) since its integron contained the aadB gene encoding resistance to aminoglycosides.

Gene cassette characterization

The gene cassettes inserted in the integrons of the isolates were determined by PCR with primers for the conserved segment regions (CS-PCR). CS-PCR amplicons of the same size were subjected to detection by PCR amplification of the class 1 integron. The phage types, resistance phenotypes, and integrons were determined as described above. In addition, plasmid analysis was performed using the phenol–chloroform extraction procedure for both the Salmonella donors and the E. coli transconjugants. The reference strain was a Salmonella Typhimurium phage type 13 strain containing five plasmids ranging in size between 4.4 and 180 kb.

Pulsed field gel electrophoresis

To determine the genetic relationship among the 26 integron-carrying Salmonella Typhimurium isolates, PFGE analysis was performed as described previously. The reference isolates were PulseNet Salmonella Braenderup and Salmonella Senftenberg. PFGE profiles were defined as different when their PFGE patterns had at least one band difference.

Southern-blot hybridization

To determine whether integrons were located at the same position of the Salmonella Typhimurium genome for all isolates, Southern blot hybridization was performed by the capillary blot procedure using the nine enrofloxacin-resistant isolates. A luminescent DIG labelling and detection kit (Roche, Mannheim, Germany) was used according to the manufacturer’s instructions.

Results

Twenty-six (43%) of the Salmonella Typhimurium isolates carried at least one integron. The phage types, resistance phenotypes, characteristics of inserted gene cassettes, SG1 types, XbaI-PFGE profiles, and the results of the conjugation experiments are summarized in Table 1. The integron-carrying isolates belonged to 8 different phage types, had 9 different phenotypic resistance profiles and were resistant to 1–9 antimicrobial agents. Eight PFGE profiles were defined (Table 1 and Figure 2). Seven integron types were found. Nine Salmonella Typhimurium isolates of different...
Table 1. Antimicrobial resistance characteristics of clinical equine *Salmonella* Typhimurium isolates in The Netherlands

<table>
<thead>
<tr>
<th>ID no.</th>
<th>Year</th>
<th>Phage type</th>
<th>Resistance phenotype</th>
<th>Cassette size (in bp)</th>
<th>Gene cassette</th>
<th>SGI1 type</th>
<th>XbaI-PFGE</th>
<th>GyrA change</th>
<th>Conj.</th>
<th>int</th>
<th>resistance type</th>
<th>plasmid (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>95</td>
<td>RDNC</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>I</td>
<td>+a</td>
<td>+</td>
<td>+</td>
<td>ACSTSuRif</td>
<td>not detected</td>
</tr>
<tr>
<td>H2</td>
<td>95</td>
<td>RDNC</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>I</td>
<td>+a</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>95</td>
<td>351</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>II</td>
<td>+</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>95</td>
<td>UT</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>I</td>
<td>+a</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5</td>
<td>95</td>
<td>508</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>II</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ACSTSuRif</td>
<td>not detected</td>
</tr>
<tr>
<td>H6</td>
<td>96</td>
<td>353</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>I</td>
<td>+</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H7</td>
<td>96</td>
<td>150</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ACSTSuRif</td>
<td>45</td>
</tr>
<tr>
<td>H8</td>
<td>96</td>
<td>150</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>I</td>
<td>+</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9</td>
<td>97</td>
<td>508</td>
<td>ACSSuTEFGK</td>
<td>1600</td>
<td>dfrA14, aadA1</td>
<td>–</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ASTSuRif</td>
<td>90; 3.7</td>
</tr>
<tr>
<td>H36</td>
<td>97</td>
<td>RDNC</td>
<td>ACGSuT</td>
<td>1600</td>
<td>dfrA1, aadA1</td>
<td>–</td>
<td>V</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>ASTSuRif</td>
<td>90</td>
</tr>
<tr>
<td>H27</td>
<td>03</td>
<td>RDNC</td>
<td>AAcSuT</td>
<td>1600</td>
<td>dfrA1, aadA1</td>
<td>–</td>
<td>VI</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>ASSuRif</td>
<td>90; 6.6</td>
</tr>
<tr>
<td>H35</td>
<td>05</td>
<td>DT104</td>
<td>AAcCSuGKTo</td>
<td>800; 1200</td>
<td>aadB, blapSE-1</td>
<td>SGII-M</td>
<td>III</td>
<td>NT</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H39</td>
<td>93</td>
<td>RDNC</td>
<td>ACSSuTKF</td>
<td>800</td>
<td>dfrA7</td>
<td>–</td>
<td>VIII</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>ACSTSuRif</td>
<td>not detected</td>
</tr>
<tr>
<td>H40</td>
<td>93</td>
<td>204</td>
<td>ACSSuTKF</td>
<td>800</td>
<td>dfrA7</td>
<td>–</td>
<td>VII</td>
<td>NT</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H18</td>
<td>01</td>
<td>DT104</td>
<td>ASSu</td>
<td>1000</td>
<td>aadA2</td>
<td>SGII-C</td>
<td>III</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>ASuRif</td>
<td>90</td>
</tr>
<tr>
<td>H29</td>
<td>03</td>
<td>DT104</td>
<td>A</td>
<td>1200</td>
<td>blapSE-1</td>
<td>SGII-B</td>
<td>IV</td>
<td>NT</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conj., conjugation; *int*, integrase gene; RDNC, reaction does not correspond to any recognized phage types; UT, untypeable; NT, not tested; A, ampicillin; Ac, amoxicillin/clavulanic acid; C, chloramphenicol; S, streptomycin; G, gentamicin; K, kanamycin; T, tetracycline; Su, trimethoprim/sulfamethoxazole; E, enrofloxacin, F, flumequine; Rif, rifampicin; To, tobramycin.

*a* Amino acid changes: Ser-83 → Ala and Asp-87 → Asn.
phage types with resistance pattern ACSSuTEFGK and integron type XVI (dfrA14, aadA1), were grouped in PFGE profile I or II. The 13 Salmonella Typhimurium DT104 isolates were grouped in PFGE profiles III or IV. Ten of these isolates had the resistance phenotype ACSSuT and contained a type I integron (aadA2, blaPSE-1). The two isolates that were non-typeable by phages and carried a type VII integron (dfrA1, aadA1) were classified into PFGE profiles V or VI. The two isolates of phage type 204 and RDNC, respectively, both with integron type XVIII (dfrA7), were classified into profile VII or VIII.

The cassettes present in the integrons carried the aadA1, aadA2 and aadB genes encoding resistance to aminoglycosides; the dfrA1, dfrA7 and dfrA14 genes conferring resistance to trimethoprim, and the blaPSE-1 gene encoding resistance to ampicillin. Nine Salmonella Typhimurium isolates carried an integron with the dfrA14–aadA1 gene cassettes. Southern blot hybridization with an integrase-specific probe showed that the integrons in eight of nine isolates tested hybridized to similar sized fragments suggesting that these integrons have a similar position on the chromosome.

Ten Salmonella Typhimurium DT104 isolates contained SG1H, one isolate carried SG1H-B and one isolate contained SG1H-C (Table 1). Based on the nucleotide sequencing results, the antibiotic resistance cluster in isolate H37 (Figure 1) is part of a new SG1 variant for which we propose the name SG1H-M. The aadA2 gene encoding resistance against spectinomycin and streptomycin of SG1H and other variants (SG1H-A, -C, -D, -E, -I) was replaced in this variant by the aadB gene encoding kanamycin, gentamicin and tobramycin resistance. The isolate H37 indeed showed phenotypic resistance to kanamycin, gentamicin and tobramycin.

The nine enrofloxacin-resistant Salmonella Typhimurium isolates had mutations in the two codons for amino acids at positions 83 and 87 of the gyrA gene as shown by AS-PCR-RFLP. Nucleotide sequence analysis confirmed that mutations were present at codons 83 and 87 where nucleotides TCC and GAC were replaced by GCC and AAC, respectively, leading to amino acid substitutions Ser-83 → Ala and Asp-87 → Asn. No qnrA1-carrying isolate was found.

Integrase amplification using genomic DNA of the transconjugants indicated that nine Salmonella Typhimurium isolates belonging to different phage types could transfer integrons to E. coli (Table 1). The antibiotic resistance phenotype of the transconjugants and the sizes of the plasmids detected are shown in Table 1. It should be noted that a large 90 kb plasmid was found in half of the transconjugants. However, no plasmid was detected in some transconjugants, although they had a resistance phenotype (ACSSuTrif) similar to that of the donor.

The isolates H16 and H18, which carried SG1H and SG1H-C, were tested for their ability to transfer SG1H to E. coli. The transconjugants had the resistance patterns ASSuRif (transconjugant from H16 and E. coli) and ASuRif (transconjugant from H18 and E. coli) (Table 1). Integrons were detected in these transconjugants.

**Discussion**

This study describes the antibiotic resistance phenotypes and resistance genes of 26 integron-carrying MDR Salmonella.
Typhimurium isolates from horses, the ability of these isolates to transfer their antimicrobial resistance determinants to E. coli, and their genetic relationship. These data indicate that equine Salmonella Typhimurium isolates may be potential risk factors for both animal and human health because they can easily spread their resistance determinants and because of the close contact between horses and humans.

An interesting finding in our study was that isolates of different phage types can have the same PFGE profile, carry the same integron type and show a similar resistance phenotype. Vice versa, isolates of the same phage type can have different PFGE profiles, contain distinct integrons in various genomic islands (SGI1, SGI1-C or SGI1-B) and have different resistance phenotypes. The combination of phage typing, PFGE analysis and the analysis of the integrons indicated that the equine integron-carrying Salmonella Typhimurium isolates are not clonal but belong to a number of different strains. These data and the great potential of horizontal transfer indicate that the multidrug resistance is due to acquired resistance rather than to the spreading a single clone.

Apart from resistance to sulphonamides, resistance to ampicillin, chloramphenicol, streptomycin and tetracycline was commonly observed regardless of the phage types of the isolates. Phenotypic resistance to these antimicrobials in the Salmonella Typhimurium DT104 isolates may be caused by the presence of the resistance genes [aadA2, blatase1, floR, tet(G) and sul1] associated with SGI1. In isolates of phage types other than DT104, integron-associated resistance genes (aadA1, dfrA1, dfrA7, dfrA14) were responsible for part of the resistance phenotype detected. In these non-DT104 isolates resistance to gentamicin, kanamycin and enrofloxacin was frequently observed, but integron-associated gene cassettes encoding these resistances were not found. The trimethoprim resistance gene cassettes (dfrA1, dfrA7 and dfrA14) were frequently detected in integrons in the present study. This is in accordance with a previous report on high percentages of phenotypic resistance to sulphonamides and trimethoprim in equine salmonellae in The Netherlands. It seems that resistance to trimethoprim and sulphonamides is due to the frequent use of these antimicrobials for the treatment of horses. In The Netherlands, trimethoprim/sulphonamide combinations are the first choice in the treatment of equine salmonellosis. All horses in the present study were clinically ill and were probably treated with trimethoprim/sulphonamides or other antimicrobials before the samples for culturing were taken. However, the exact data on the usage of antimicrobials in the horses were not available.

An important finding was that the nine MDR Salmonella Typhimurium isolates carrying a rare integron type with the dfrA14 and aadA1 gene cassettes, belong to distinct strains because different phage types and two distinct PFGE patterns were observed. Four of these isolates were able to transfer their integron and the resistance determinants encoding for ampicillin, chloramphenicol and tetracycline resistance to E. coli. This clearly indicates the potential of these strains for gene transfer to other members of the Enterobacteriaceae. These nine isolates also showed resistance to fluoroquinolones and enrofloxacin. This resistance is caused by mutations leading to the amino acid changes Ser-83 → Ala and Asp-87 → Asn in GyrA. In Salmonella, a single mutation in gyrA can be sufficient to cause high-level resistance to nalidixic acid but additional mutations are required to attain high-level resistance to fluoroquinolones. In previous studies, single amino acid changes at Ser-83 → Phe and Asp-87 → Asn/Gly were most commonly observed. The mutations at both codons mentioned above were previously detected in in vitro experiments, and they were also described in six Salmonella Typhimurium isolates obtained from humans and cattle in Germany. The presence of enrofloxacin-resistant Salmonella isolates in Dutch horses is unexpected because quinolones are not licensed for use in horses in The Netherlands. A possible explanation is that these isolates originate from other animal species or humans.

A 90 kb plasmid can be transferred from Salmonella Typhimurium to E. coli, including the antimicrobial resistance genes that are present on it. In most transconjugants at least one plasmid was present, but in some cases, no plasmid could be observed although the transconjugants had obtained a resistance phenotype similar to that of the donor. A possible explanation for this phenomenon is the presence of a low-copy plasmid, which could not be detected with the procedure used. Another explanation may be that the resistance determinants were present on a conjugative transposon and may be integrated into the chromosome of the recipients. Another interesting feature of the present study is the presence of a conjugal plasmids-associated integron and a chromosomally located integron in the same Salmonella Typhimurium DT104 strain. Evidence for the presence of a conjugal plasmid-associated integron includes the presence of integrons in the E. coli transconjugants obtained after mating between Salmonella Typhimurium H16 or H18 and E. coli K12; and the phenotypic resistance to ampicillin, streptomycin, sulphonamide and rifampicin observed in the transconjugants. Salmonella isolates H16 and H18 contained SGII and SGII-C. However, these genomic islands appear not to be transferred because neither resistance to chloramphenicol or tetracycline nor structures of SGII in the E. coli chromosome were detected in the transconjugants.

A novel variant of SGII was found in a Salmonella Typhimurium DT104 isolate. In this isolate the aadA2 gene, the resistance gene in the first integron of SGII or its variants (SGII-A, -C, -D, E, and I) is replaced by the aadB gene. The presence of this new type of SGII with the resistance gene cluster aadB-sul1-floR-tet(G)-blaPSE-1 coincided with the resistance to aminoglycosides, sulphonamides, tetracycline, chloramphenicol/ florfenicol and ampicillin observed in this isolate. It is proposed to name this variant SGII-M. The resistance to streptomycin and trimethoprim is probably not encoded by gene cassettes integrated in integrons in this isolate.

Acknowledgements

We thank Anjo Verbruggen, Henny Maas and Max Heck of the Dutch National Institute of Public Health and the Environment and Marc Wüst of the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University for their help. This work was supported by a grant from the Vietnamese Government.

Transparency declarations

None to declare.
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