Avoidance of false PCR results with the integron–retron junction in multiple antibiotic resistant Salmonella enterica serotype Typhimurium

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Received 17 April 2003; accepted for publication 30 May 2003

Abstract

Salmonella infections continue to cause gastrointestinal and systemic disease throughout the world. Another concern with this pathogen is the ability to acquire integrons that confer resistance to multiple antibiotics. For multiresistant Salmonella enterica serotype Typhimurium, the most common multiresistant Salmonella serotype, an integron structure can be found between thdF and a retron. Our objective was to investigate the utility of a 450 bp thdF-retron amplicon as an indicator of an insertless thdF-retron junction thus indicating an integron-free strain. Surprisingly, we found that the 450 bp thdF-retron amplicon was present, and thus incorrectly suggesting an integron-free status, in some multiresistant S. enterica isolates. However, this phenomenon was not observed if the isolate was enriched in the presence of two antibiotics. This demonstrates that, within some individual clinical isolates of multiresistant S. enterica serotype Typhimurium, there exists a small subpopulation of integron-free bacteria. Consequently, it appears that the thdF-retron amplicon is an inaccurate predictor of integron status in S. enterica serotype Typhimurium unless multiresistance is used as a selection tool during enrichment.

Published by Elsevier Ltd.

Keywords: Integron; Retron; Salmonella; Antibiotic resistance; PCR

1. Introduction

Salmonella infections continue to be a problem in both industrialized and developing countries [1]. This problem is exacerbated by the ability of Salmonella to become resistant to multiple antibiotics. The current paradigm for this problem is Salmonella enterica serotype Typhimurium DT104 (DT104), a pathogen with a wide range of hosts and a wide range of antibiotic resistances (ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline; ACSSuT antibiogram).

For DT104, the ACSSuT antibiogram is determined by a genomic collection of adjacent genes [2–4]. This arrangement is composed largely of two integrons containing the genes pse-1, floR, aadA2, sul1, and tetA that, respectively, encode resistance to ampicillin, chloramphenicol/florfenicol, streptomycin/spectinomycin, sulfonamides, and tetracycline. Insertion of the integrons into DT104 occurs between thdF and a retron in the genome [5]. The former is a gene involved in furan oxidation in E. coli [6] and the latter is a Typhimurium-specific segment of DNA [5] introduced as a result of an infection with an RNA bacteriophage [7]. Thus the thdF-retron junction may serve as a preferred site for DNA insertion.

Our original intent was to exploit the thdF-retron interface in order to ascertain the integron status of isolates of S. enterica serotype Typhimurium. Instead, we identified the existence of an integron-free subpopulation within individual isolates of DT104. This subpopulation was not evident when the isolate was enriched in the presence of two antibiotics.
2. Materials and methods

**Bacterial strains.** Multiresistant strains of *S. enterica* serotype Typhimurium (phagetypes DT104, U302, DT120, DT193 and DT208) were obtained from the National Veterinary Services Laboratories (Ames, IA). Multiresistance was defined as resistance to at least five antibiotics. Strains were obtained from clinical and environmental sources in 1997, 1998 and 2001. A synopsis of the 1997–1998 strain collection can be found in Frana et al. [8]. A similar collection was obtained in the year 2001.

**DNA isolation.** Purified DNA was isolated from Lennox L broth (GIBCO-BRL) broth using the GNome kit (Bio101, Vista, CA) as per manufacturer’s protocol. DNA concentration was determined using the spectrophotometric analysis of Kalb and Bernlohr [9].

**PCR.** Sequences and locations of oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) are described in Table 1. PCR was performed in an automated thermocycler (Hybaid, Teddington, UK) with a hot bonnet. PCR reactions were performed in 0.2 ml tubes with 20 μl containing 300 μM dATP, dTTP, dCTP and dGTP, 2.5 mM magnesium chloride, 4 μmoles of each primer, 10 mM Tris–HCl, 50 mM KCl, 0.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA) and 50 ng of template (or water as a negative control). Thermocycling entailed 95°C for 5 min, then 40 cycles of 95°C for 1 min, 48°C for 30 s and 72°C for 30 s.

**Agarose gel electrophoresis.** PCR products (10 μl of the reaction) were electrophoresed in 2.0% agarose (Perkin Elmer) gels for 1.5 h at 150 V with Tris (40 mM)–acetate (20 mM)–EDTA (1 mM) as the running buffer. Ethidium bromide-stained amplicons were visualized on a UV transilluminator using the GelDoc system (BioRad, Richmond, CA).

**DNA sequence analysis.** The thdF-retron segment was amplified by PCR using the forward and reverse primers described in Table 1. PCR was performed as described previously [10]. PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) as per manufacturer’s protocol. Plasmids containing the appropriate size inserts were sequenced by Iowa State University DNA Sequencing and Synthesis Facility using the vector-specific primers T7 and M13.

**Antibiotic resistance assays.** Minimum inhibitory concentration (MIC) values were determined by inoculating 10^6 bacteria into 1 ml aliquots of Mueller–Hinton broth (DIFCO, Detroit, MI) containing two-fold serial dilutions of antibiotics as per the National Committee on Clinical Laboratory Standards guidelines [11]. Bacteria were grown aerobically and MIC values were based on the lowest concentration of antibiotic that inhibited growth. Resistance breakpoints, obtained from the National Committee on Clinical Laboratory Standards are: 32 μg/ml for ampicillin; 32 μg/ml for chloramphenicol; 64 μg/ml for streptomycin; 512 μg/ml for sulfamethoxazole; 16 μg/ml for tetracycline. All antibiotics were obtained from Sigma (St Louis, MO).

3. Results

**PCR for detecting the integron in *S. enterica* serotype Typhimurium.** During an assessment of the thdF-retron as an indicator of integron insertion in *S. enterica* serotype Typhimurium, we noticed that a strain of multiresistant DT104 exhibited an apparent amplicon in a PCR using primers spanning the thdF-retron junction. We assumed that the thdF-retron PCR product would be too large to be observed since this DT104 strain possessed the integron structure based on the presence of a *floR-tetR* amplicon [10]. Sequencing of the thdF-retron amplicon revealed the absence of an integron structure between *thdF* and the retron. As depicted in Fig. 1 and Table 2, several multiple antibiotic resistant isolates of DT104 also appear to have a subpopulation that lacks the integron. Specifically, these isolates have an amplicon from both the thdF-retron and floR-tetR PCRs. This genotype was found in 10 of 838 isolates from 1997 to 2001 and is represented by strain 418 in Fig. 1. Nine of the 10 isolates were obtained in 2001 while one isolate (strain 773) was obtained in 1997. All 10 isolates belong to the DT104 phagetype, i.e. this phenomenon was not observed in the DT120, DT193, U302 phagetypes that are related to DT104 and possess the same integron structure as DT104.

As shown in the parentheses in Table 2, the thdF-retron/floR-tetR genotype was observed if these isolates were grown in the absence of antibiotics or in the presence of ampicillin, chloramphenicol, streptomycin, sulfamethoxazole or tetracycline (latter four not shown). However, only the floR-tetR genotype was observed if the isolate was grown in a combination of ampicillin and chloramphenicol. Similar results were obtained when the isolates were grown in a combination of streptomycin and tetracycline (data not shown). The inability to detect the thdF-retron amplicon

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>thdF-Retron</td>
<td>5’ACACCTTGAGCAGGGCAAAG</td>
<td>5’AGCAAGTGTGCAGTCATTTGG</td>
<td>450 or 40 kb^*</td>
<td>[5]</td>
</tr>
<tr>
<td>floR-tetR</td>
<td>5’GGGTCCCTTGAGGTTCCCGT</td>
<td>5’GCTGGGTTTACGACGAGAT</td>
<td>275 bp</td>
<td>[10]</td>
</tr>
</tbody>
</table>

^* 450 bp indicates absence of the integron while 40 kb (i.e. no product) indicates the presence of the integron structure; no product also suggests the absence of the retron.
was not related to alterations in thdF or the retron since both of these sequences were found to be intact when amplified with gene-specific primers (data not shown).

**Identification, characterization and quantification of individual colonies that lack the integron structure.** Individual isolates were plated on antibiotic-free media and then evaluated for colony morphology, ampicillin resistance, integron presence and phagetype. One hundred colonies were examined for each of the 10 isolates displaying the thdF-retron/floR-tetR genotype. As shown in Table 2, 26 of 1000 (or 2.6%) of these colonies lacked the integron structure and thus were sensitive to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline. These colonies had a similar morphology to their cohorts and they all belong to the DT104 phagetype like the rest of the population.

### 4. Discussion and Conclusions

Since the thdF-retron junction appears to be a site for receiving large fragments of exogenous DNA in *S. enterica* serotype Typhimurium, we attempted to develop a PCR protocol that would reveal if an isolate possessed an insertion at this junction. Instead, we found that this strategy was not reliable due to integron inconsistencies in multi-resistant isolates from a collection of strains obtained in 1997–2001. This is in contrast to a recent study by Boyd...
et al. [5] in which the thdF-retron amplicon was used as a marker for the integron structure. Integron inconsistency was based on the presence of a thdF-retron amplicon in some isolates of multiresistant _S. enterica_ serotype Typhimurium (especially phage type DT104). The thdF gene lies on the ‘left’ side of the retron, a Typhimurium-specific gene segment [5] that is a result of a previous RNA bacteriophage infection [7]. For _S. enterica_ serotype Typhimurium DT104, an integron structure is inserted between thdF and the retron [5]. Thus the presence of this structure would generate a 40-kb product which would not be observed using the PCR conditions employed.

In this study we identified two subpopulations within certain isolates. One subpopulation is positive for the thdF-retron amplicon and negative for the integron amplicon ( _floR-tetR_ ) while the other subpopulation is negative for thdF-retron amplicon and positive for the integron amplicon. For the former subpopulation it appears that the integron structure is absent or inserted at an alternative site or that the retron has been translocated. It is possible for the integron structure to be inserted independent of the retron given the presence of the integron structure in multiresistant _S. enterica_ serotype Agona [12], a strain that lacks the retron but possesses the integron structure adjacent to thdF. Retron translocation does not appear to be valid since the thdF-retron PCR indicates that the retron is adjacent to thdF. Retron-independent insertion of the integrons also appears invalid since the integron-specific PCR is negative and the colonies are antibiotic-sensitive. Thus it appears that some colonies do not possess the integron. Interestingly, this ‘integron inconsistency’ phenomenon was not observed in multiresistant _S. enterica_ serotype Agona although only one isolate was examined.

The existence of the integron-free subpopulation can be eliminated by the use of multiple (e.g. ampicillin and chloramphenicol), but not single, antibiotics during the enrichment phase. Evidently the antibiotic-sensitive colonies are able to co-exist with antibiotic resistant colonies unless enrichment is performed with multiple selection pressures.

In summary, this study is the first to document an occasional inconsistency in the integron status in multiresistant _S. enterica_ serotype Typhimurium. The implications are two-fold. First, it now appears that it will be inappropriate to use the thdF-retron amplicon as a diagnostic tool unless two or more antibiotics are used during the enrichment phase. That is, the presence of this amplicon should not be used as a tool for examining thdF-retron insertion events in _S. enterica_ serotype Typhimurium. Second, it appears that either the integrons are not stable or integron-free subpopulations co-exist with colonies possessing the integron structure. Thus these studies could provide the basis for studying the genetic drift and population dynamics of multiresistant _S. enterica_ serotype Typhimurium.

**Acknowledgements**

The authors wish to thank Ruth Willson for technical assistance, Sandy Johnson for secretarial assistance, Dr David Alt for DNA sequencing, Kathy Ferris for contributing strains, and Drs Vijay Sharma and Irene Wesley for reading the manuscript.

**References**