Detection and characterization of tet(M) in tetracycline-resistant Listeria strains from human and food-processing origins in Belgium and France

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In the present study, three Listeria monocytogenes strains and one Listeria innocua strain out of a collection of 241 Listeria isolates from human and food-processing sources were found to display resistance to tetracycline (TC) due to the presence of the tet(M) gene. Through sequence analysis, it was shown that tet(M) genes in two of the isolates belong to sequence homology group (SHG) II, a group comprising chromosomally encoded tet(M) genes previously found in Staphylococcus aureus and in lactobacilli. The tet(M) genes of the two other L. monocytogenes strains were associated with a member of the Tn916–Tn1545 family of conjugative transposons and were closely related to SHG III, which harbours enterococcal tet(M) genes associated with Tn916. One of these transposon-containing strains was able to transfer the tet(M) gene to Enterococcus faecalis recipient strain JH2-2. Collectively, these sequence and conjugation data indicate that the acquisition of tet(M) by Listeria strains may be triggered by successive transfers between other Gram-positive organisms.

INTRODUCTION

Bacteria belonging to the genus Listeria are widely distributed in the environment. Within this genus, Listeria monocytogenes is the only species that can cause serious animal and human infections, including abortion and septicemia (Rocourt, 1996; Rocourt & Cossart, 1997).

Consumption of contaminated foods and/or feedstuffs is recognized as the main route of acquisition of epidemic and sporadic human listeriosis. This disease has been associated with mortality rates of up to 30% in infants and in patients with underlying diseases (Hof et al., 1997). Standard antibiotic therapy for the effective treatment of listeriosis consists of the administration of either ampicillin or penicillin G whereas, for established infections, aminoglycosides can be used in combination with penicillins (Safdar & Armstrong, 2003).

Since the first description of L. monocytogenes strains with acquired antibiotic resistance(s) in 1988 (Poyart-Salmeron et al., 1990), an increasing number of Listeria isolates exhibiting resistance(s) from foodstuffs, animals and humans have been reported (Poyart-Salmeron et al., 1990; Facinelli et al., 1993). The emergence of such resistant strains has highlighted the need for surveillance programmes to monitor temporal and geographical shifts in resistance patterns and the associated phenotypes and genotypes (Safdar & Armstrong, 2003). Emergence of resistance is not only the case for L. monocytogenes but also for other Listeria species such as Listeria innocua that can occur in similar habitats (e.g. pathological samples or food products) (Margolles & de los Reyes-Gavilan, 1998) and that may represent reservoirs of antimicrobial resistances for L. monocytogenes.

Although still relatively rare, tetracycline (TC) resistance is the most frequently reported resistance phenotype in Listeria species from various origins (Poyart-Salmeron et al., 1990; Charpentier & Courvalin, 1999).

Resistance to TC compounds in many commensal and pathogenic bacteria is due to the acquisition of tet genes via self-transferable plasmids or conjugative transposons (Facinelli et al., 1993; Charpentier et al., 1995). The different tet genes confer resistance by two main mechanisms involving...
either (i) a protein that protects the ribosome from the action of TC and minocycline (MC) (Connell et al., 2003) or (ii) an efflux protein which actively exports TC out of the bacterial cell (Guillaume et al., 2004). Up to now, 38 different determinants encoding resistance to TC compounds are known but relatively little information is available on their distribution in Listeria strains (Clewel et al., 1995; Soussy, 2005).

The purpose of the present study was to unravel the genetic basis of TC resistance in four Listeria strains isolated from distinctly different origins (environment, time and geography).

METHODS

Epidemiological and phenotypic resistance data for bacterial strains from various origins. In the period 1999–2002, a total of 180 human L. monocytogenes isolates were collected during a Belgian surveillance programme. All strains were serotyped and tested for antimicrobial susceptibility to 11 antibiotics including ampicillin, amoxicillin, streptomycin, gentamicin, vancomycin, erythromycin, TC, MC, ciprofloxacin, chloramphenicol and trimethoprim/sulfa-methaxazole. MIC values were determined with E-test (AB-Biodisk) on Mueller–Hinton agar (Oxoid) incubated at 37°C. MIC values were determined with E-test (AB-Biodisk) on Mueller–Hinton agar (Oxoid) incubated at 37°C. MIC values were determined with E-test (AB-Biodisk) on Mueller–Hinton agar (Oxoid) incubated at 37°C. MIC values were determined with E-test (AB-Biodisk) on Mueller–Hinton agar (Oxoid) incubated at 37°C. MIC values were determined with E-test (AB-Biodisk) on Mueller–Hinton agar (Oxoid) incubated at 37°C. MIC values were determined with E-test (AB-Biodisk) on Mueller–Hinton agar (Oxoid) incubated at 37°C.

PCR amplifications. PCRs contained 1·5 μM of each primer (Table 2), 1 × PCR buffer II (Applied Biosystems), 1·5 mM MgCl₂, each of the four dNTPs at a concentration of 200 μM and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The specificity of tet primer pairs was tested for each class of tet gene using a number of purified plasmids harbouring the different determinants Tet K-M, Tet O and Tet S-T as references (Table 2). All primer pairs tested resulted in PCR products of the predicted size only, demonstrating their high specificity (data not shown).

Amplifications were performed in a final volume of 25 μl on an iCycler (Bio-Rad) using the following temperature programmes. For the amplification of tet genes: initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension step at 72°C for 10 min. For the 16S rRNA gene amplification: initial denaturation at 94°C for 10 min, 25 cycles of 97°C for 1 min, 59°C for 1 min and 72°C for 1 min 30 s and a final extension step at 72°C for 15 min. For the amplification of the integrase gene int of the Tn916–Tn1545 family: initial denaturation at 94°C for 10 min, 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min 30 s and a final extension step at 72°C for 10 min. PCR products (10 μl) were separated by electrophoresis on a 1% agarose gel and visualized under UV light after staining in a 1 μg ethidium bromide ml⁻¹ solution.

DNA sequencing of the tet(M) PCR fragment. Purified tet(M) amplimers were sequenced directly with the DI, DII and TetM-R primers (Gevers et al., 2003; Haes et al., 2004) in order to obtain a 1420 bp partial sequence (74% of the 1920 bp open reading frame) of the tet(M) gene. Sequencing was performed with a BigDye Terminator version 2 Ready Reaction cycle sequencing kit (Applied Biosystems) on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Reference sequences of tet(M) were retrieved from the EMBL database (http://www.ebi.ac.uk) and compared with the new sequences using BioNumerics version 3.5 software (Applied Maths).

Table 1. Characteristics of the Listeria strains analysed in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin (country)</th>
<th>Species</th>
<th>MIC (TC) (μg ml⁻¹)</th>
<th>MIC (MC) (μg ml⁻¹)</th>
<th>tet(M)</th>
<th>int gene of Tn916–Tn1545</th>
<th>Resistance transfer to JH2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 22251</td>
<td>Human (Belgium)</td>
<td>L. monocytogenes</td>
<td>48</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>AFSSA 12410</td>
<td>Food (France)</td>
<td>L. innocua</td>
<td>≥32</td>
<td>2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AFSSA 12446</td>
<td>Food (France)</td>
<td>L. monocytogenes</td>
<td>≥32</td>
<td>4</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AFSSA 12468</td>
<td>Food (France)</td>
<td>L. monocytogenes</td>
<td>≥32</td>
<td>4</td>
<td>+</td>
<td>+ (tet(M), int)</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 2. Primers for PCR amplification of tet and int genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference DNA</th>
<th>Reference or source of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP-tet type</td>
<td>DI</td>
<td>GAYACNCNNCGNCAYRNTGAYTT</td>
<td>1083</td>
<td>pJI3 (Morse et al., 1986)</td>
<td>Clermont et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>DII</td>
<td>GCCCARWANGGRTNGNGNHYCAG</td>
<td>382</td>
<td>pAT102 (P. Courvalin, Collection of Institut Pasteur)</td>
<td>Wellington (2000)</td>
</tr>
<tr>
<td>tet(K)</td>
<td>TetKfw1</td>
<td>TTATGTTTAGTGACTAGAAA</td>
<td>717</td>
<td>pBluescriptII SK+ tet(L) (Schwarz et al., 1992)</td>
<td>Wellington (2000)</td>
</tr>
<tr>
<td></td>
<td>TetKrv1</td>
<td>AAAGGGTTAGAAACTCTTGGAAA</td>
<td>633</td>
<td>pJ13 (Morse et al., 1986)</td>
<td>Guardabassi et al. (2000)</td>
</tr>
<tr>
<td>tet(L)</td>
<td>TetLfw3</td>
<td>GTMGGTGGCGCTATATCC</td>
<td>589</td>
<td>pVP2 (Perreten et al., 1997b)</td>
<td>This study</td>
</tr>
<tr>
<td>tet(M)</td>
<td>TetMfw2</td>
<td>GTRAYGAACCTCAGGAATC</td>
<td>362</td>
<td>pLP1697 (Clermont et al., 1997)</td>
<td>Wellington (2000)</td>
</tr>
<tr>
<td>tet(S)</td>
<td>TetSfw1</td>
<td>ATGGGAACCTTGAAAGGA</td>
<td>1028</td>
<td>Tn1545 (P. Courvalin)</td>
<td>Doherty et al. (2000)</td>
</tr>
<tr>
<td>tet(T)</td>
<td>TetTfw2</td>
<td>GTATTTCTGTTGGTCGCTT</td>
<td>7</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>int</td>
<td>Intfw</td>
<td>GCATGTATGGTTACTCCT</td>
<td>509</td>
<td>This study</td>
<td></td>
</tr>
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</table>

RESULTS AND DISCUSSION

Epidemiological and phenotypic resistance data

From a screening survey of 180 human and 61 food-processing-associated Listeria isolates, four strains were found to display the TC-resistance phenotype. The clinical L. monocytogenes strain LMG 22251 was isolated from a haemoculture of a 76-year-old woman, whereas the three food-environment strains [two L. monocytogenes (AFSSA 12446 and 12468) strains and one L. innocua (AFSSA 12410) strain] were isolated in pork- and poultry-processing facilities.

Among these 241 isolates, no resistance against the other tested antibiotics (ampicillin, amoxicillin, streptomycin, gentamicin, vancomycin, erythromycin, ciprofloxacin, chloramphenicol and trimethoprim/sulfamethoxazole) was observed.

The finding that only four out of the 241 screened isolates (1·6 %) were TC resistant indicates that the TC-resistance phenotype is relatively rare in Listeria isolates from human (one of 180 pathogenic strains) or, to a lesser extent, from food-associated origins (3 of 61 isolates). This observation corroborates the very low incidence of multiresistant Listeria isolates reported previously (Charpentier & Courvalin, 1999).

It has been argued that the presence of TC resistance in Listeria strains could result from an extensive use of TC compounds, particularly in feed additives and in veterinary therapy (Antunes et al., 2002), and/or from the acquisition, under natural conditions, of transferable elements originating from enterococci and streptococci (Poyart-Salmeron et al., 1990). Nevertheless, tetracyclines were banned as feed additives in the 1970s as a consequence of the report of Swann (1969). Facinelli et al. (1993) also suggested that L. innocua could act as a reservoir of TC-resistance genes for other species, including L. monocytogenes. Transfer of resistance between L. innocua and L. monocytogenes may occur in the gastrointestinal tract of domestic animals or in food environments where members of both species can coincide (Facinelli et al., 1993).

Molecular detection and characterization of resistance genes

Using the degenerate primer set DI–DII, all four resistant Listeria strains were found to contain a tet gene of the RP (ribosomal protection) group. According to the updated distribution of TC-resistance genes among Gram-positive bacteria published on the internet by Professor M. Roberts (Roberts, 2004), only tet(K), (L), (M) and (S) have been detected in Listeria. We therefore decided to test in priority the presence of these determinants in the four Listeria strains. The use of specific primers indicated that these strains all harboured the tet(M) gene, whereas none of the other tested tet genes (Table 1) were detected (Roberts, 2004). The presence of RP-type gene tet(M) also explains the MC-resistance phenotype in the human isolate LMG 22251 (MIC of 8 μg ml⁻¹) and the reduced susceptibility to this agent observed in the three isolates from food-processing environments (MIC range, 2–4 μg ml⁻¹; Table 1) according to the criteria developed by the antibiogram committee of the Société Française de Microbiologie (Soussy, 2005). MIC values for the transconjugant were 24 μg TC ml⁻¹ and 4 μg MC ml⁻¹. TC resistance mediated by efflux is determined by proteins belonging to the major facilitator superfamily. Most of these proteins confer resistance to TC, but not to MC (a semi-synthetic tetracycline derivative). Only tet(B) (found in Gram-negatives) and naturally occurring Salmonella mutant isolates of tet(A) confer resistance to both TC and MC (but at a low level). In contrast, RP proteins confer resistance to both TC and MC.
Because the tet(M) gene is often associated with large conjugative transposons such as Tn1545 (Clewell et al., 1995; Marra et al., 1999), the possible presence of the integrase gene int of the Tn1545 transposon family was investigated by PCR-based detection. Only L. monocytogenes strains LMG 22251 and AFSSA 12468 were shown to contain the int gene, which may indicate that the tet(M) gene in these two strains is integrated in a conjugative element of the Tn1545 family (Table 1).

The results of the conjugation experiments showed that only strain AFSSA 12468 was able to transfer its tet(M) gene to Enterococcus faecalis JH2-2 (but not to S. aureus 80CR5), yielding TC-resistant transconjugants at a frequency of 4.7 × 10⁻⁶ (data not shown).

The finding that this strain does not possess detectable plasmids (data not shown) but harbours a member of the Tn1545 family suggests that tet(M) transfer to recipient JH2-2 involved movement of a conjugative transposon element. In line with the conclusions of a recent Italian study reporting on the presence of transferable tet(M) genes in food isolates of L. monocytogenes (Pourshaban et al., 2002), our data indicate that this species could act as a reservoir of mobile tet genes along the human food chain. Previously, the transfer of tet(M) has also been demonstrated in the reverse direction from Enterococcus faecalis to Listeria sp. both in vitro and in the digestive tract of gnotobiotic mice via the Tn916-like element TnFO1 (Perreten et al., 1997a) and via Tn1545 (Doucet-Populaire et al., 1991), respectively. Although transfer could not be detected for the three other isolates, it can not be excluded that the tet(M) gene could also be transferred at frequencies below the detection limit (1.6 × 10⁻⁶).

**Genetic diversity of tet(M) genes in Listeria**

In previous studies, it has been shown that the tet(M) gene can display several mosaic structures, leading to the recognition of at least five different sequence homology groups (SHGs I–V) (Gevers et al., 2000; Huys et al., 2004). In order to investigate whether any of these SHGs are also represented in the four tet(M)-containing Listeria strains of this current study, the open reading frame of this gene was partially sequenced and aligned with a selection of tet(M) reference sequences (Fig. 1). Based on an internal sequence similarity level of ≥99.6 % for the delineation of tet(M) SHGs (Huys et al., 2004), the unrooted maximum-parsimony tree revealed that the tet(M) genes of strains AFSSA 12410 and AFSSA 12446 belonged to SHG II (internal sequence similarity level of 99.79 %). This SHG comprises chromosomally encoded tet(M) genes previously found in S. aureus strain MRSA 101 and in several Lactobacillus species associated with fermented dry sausage (Schwarz et al., 1992), as exemplified by Lactobacillus curvatus strain LMG 21681 (Fig. 1). The tet(M) gene of Listeria strains AFSSA 12410 and AFSSA 12446 was not found to be transferable to neighbouring Gram-positive taxa and does not seem to be associated with the int gene of the Tn916–Tn1545 family. In contrast, the tet(M) sequences of strains LMG 22251 and AFSSA 12468 that were both associated with a member of the Tn916–Tn1545 family were genetically positioned close to but somewhat separated from SHG III (99.42 % sequence similarity between the two alleles), which harbours enterococcal tet(M) genes located on Tn916 or related elements (Fig. 1) (Morse et al., 1986).

These findings may indicate that the two tet(M) sequences represent a new allelic variation of SHG III that has evolved from site-specific recombination with members of SHG II. However, the inclusion of new tet(M) sequences from

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**Fig. 1.** Unrooted maximum-parsimony tree of multiple aligned partial tet(M) sequences of Listeria strains (indicated in bold) and reference tet(M) sequences retrieved from the EMBL database. SHGs showing ≥99.6 % internal sequence similarity are indicated. The number of base conversions over the tree is indicated along the phylogenetic distance lines, and bootstrap percentages for analysis of 100 replicates are in parentheses. If available, the designation of the tet(M)-carrying strain or transposon is indicated followed by the EMBL accession number in parentheses.
L. and other species has to be awaited before this allelic variation can be defined as a new SHG of tet(M) (Huys et al., 2004).

In conclusion, the results of the current study indicate that the incidence of TC resistance remains very low in listeria from human or, to a lesser extent, food-associated origins. Possibly, the extensive use of TC in veterinary therapy or in animal foodstuffs may have favoured the dissemination of TC determinants among a multitude of species. In addition, this is the first study to report on the phylogenetic classification of listerial tet(M) genes from human and food-environment strains. Sequence analysis suggests that the acquisition of tet(M) by L. strains may be the result of successive transfers between other Gram-positive organisms, possibly followed by site-specific recombination events.

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