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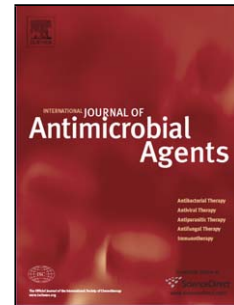
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**Resistance to macrolides by ribosomal mutation in clinical isolates
of *Turicella otitidis***

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Abstract

The genetic basis of erythromycin resistance in *Turicella otitidis*, a coryneform bacteria associated with otitis, was studied in five macrolide-resistant clinical isolates. Macrolide resistance genes were searched for by polymerase chain reaction (PCR). Genes for domain V of 23S rRNA (*rrl*) as well as *rpID* (L4 protein) and *rpIV* (L22 protein) genes were characterised, amplified by PCR from total genomic DNA and sequenced. In the resistant isolates, cross-resistance to macrolides and clindamycin was associated with mutations at positions 2058 and/or 2059 (*Escherichia coli* numbering). Three isolates displayed A2058 mutations, one isolate had an A2059G mutation whereas another one contained mutations at positions 2058 and 2059. Southern blot experiments revealed that *T. otitidis* had three copies of the *rrl* gene. In conclusion, resistance to macrolides in *T. otitidis* is due, at least in part, to mutations in the *rrl* gene.

1. Introduction

Coryneform bacteria represent a heterogeneous group of microorganisms. Several new species have been described as a result of the increasing number of taxonomic investigations within this group. *Turicella otitidis* was originally described in 1994 by Funke et al. [1] and is characterised by long, unbranching, Gram-positive bacilli that form 2-mm, non-haemolytic colonies after incubation for 24 h on blood agar, with a creamy appearance. The habitat of *T. otitidis* appears to be confined almost exclusively to the ear or adjoining areas [1].

The literature contains a number of reports of middle ear pathology and concomitant isolation of *T. otitidis*. Most cases involve children aged <3 years and the organism is isolated in either pure or mixed culture. However, the role of *T. otitidis* in chronic or acute otitis media remains controversial [2]. Data in the literature regarding the susceptibility of *T. otitidis* to different antimicrobial agents are scarce. The available data suggest susceptibility to most β -lactams, ciprofloxacin, rifampicin, tetracyclines and gentamicin. However, significant resistance to macrolides and lincosamides has been reported [2].

Macrolides, lincosamides and streptogramins B (MLS_B) inhibit bacterial protein biosynthesis by interaction with the 50S subunit. These antibiotics bind to the 2058–2062 (*Escherichia coli* numbering) region within domain V of the 23S rRNA and stimulate the dissociation of peptidyl-tRNA from the ribosome during the translocation process. MLS_B resistance has been reported in *Corynebacterium diphtheriae*, *Corynebacterium jeikeium* and *Corynebacterium xerosis*. In these organisms, resistance was conferred by genes belonging to the *erm*(X) class [3–5]. The efflux

gene *mef(A)* has been detected in a strain of *C. jeikeium*, whereas another efflux gene, *ImrB*, conferring resistance to lincomycin has been reported in a strain of *Corynebacterium glutamicum* [6]. In the coryneform bacterium *Arcanobacterium pyogenes*, erythromycin resistance has been related to the presence of *erm(X)* or *erm(B)* genes and recently to mutations of the 23S rRNA [7,8]. In contrast to the abovementioned organisms, *Propionibacterium acnes* is nearly always resistant to erythromycin by mutations in 23S rRNA [9].

In this study, we investigated the mechanism of macrolide resistance in clinical isolates of *T. otitidis*.

2. Materials and methods

2.1. Bacterial strains

Clinical isolates of *T. otitidis* UCN52, UCN53, UCN54, UCN55 and UCN56, resistant to macrolides and isolated at the hospitals of Caen and Aix from the ears of children suffering from otitis, were studied. Clinical isolate *T. otitidis* UCN57 and four other clinical isolates susceptible to macrolides were used as controls. Isolates were routinely identified by the API CORYNE panel (bioMérieux, La-Balme-les-Grottes, France) and identification was confirmed by sequencing in part the *rrs* gene (16S rRNA gene). *Staphylococcus aureus* strains HM290 [*erm(A)*] and HM1054R [*erm(C)*], *Enterococcus faecium* HM1032 [*erm(B)*], *Streptococcus pyogenes* UCN1 [*erm(A)*] and *C. jeikeium* UCN58 [*erm(X)*] were used as controls for polymerase chain reaction (PCR) of *erm* genes.

2.2. Antibiotic susceptibility

Minimum inhibitory concentrations (MICs) of antibiotics were determined by agar dilution using Mueller–Hinton medium according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (<http://www.sfm.asso.fr>; last release January 2008).

2.3. PCR and sequencing

The *erm*(A), *erm*(TR) [subclass of *erm*(A)], *erm*(B), *erm*(C) and *erm*(X) genes encoding ribosomal methylases, the *ere*(A) and *ere*(B) genes encoding esterases, the *mph*(A) and *mph*(B) genes encoding phosphotransferases, and the efflux genes *mef*(A) and *msr*(A) were searched for by PCR amplification as described previously [10].

To detect mutations in the target of macrolides, portions of the *rrl* gene for domain V of 23S rRNA were amplified by PCR and sequenced. Since the sequences of the *rrl* alleles of *T. otitidis* are not available in databanks, oligonucleotides 23SCPU8 to 23SCPU10 and 23SCPL8 to 23SCPL10 previously designed for *S. pyogenes* were used [10]. The PCR reaction consisted of 30 cycles of denaturation (95 °C, 30 s), annealing (50 °C, 30 s) and extension (72 °C, 30 s to 3 min).

Sequences of the *rpID* (L4 ribosomal protein) and *rpIV* (L22 ribosomal protein) genes involved in macrolide resistance in various organisms were not available for *T. otitidis* in the databanks. Sets of degenerate primers directed toward conserved regions of *C. diphtheriae*, *Corynebacterium efficiens*, *C. glutamicum* and *C. jeikeium* proteins L4 and L22 were used to amplify by PCR overlapping fragments of *T. otitidis* UCN57

rplD and *rplV* genes. Specific primers were then used to amplify *rplD* and *rplV* genes from the other *T. otitidis* isolates. Nucleotide sequences of the *rplD* and *rplV* genes of *T. otitidis* UCN57 and of the *rplV* gene of *T. otitidis* UCN53 have been deposited in the GenBank data library under accession nos. FJ392606, FJ392607 and FJ392608.

2.4. Southern blot hybridisation

Chromosomal DNA from *T. otitidis* isolates was digested with restriction endonucleases *Sma*I or *Eco*RI for 2 h at 25 °C or 37 °C in the buffer recommended by the manufacturer (Amersham International, Orsay, France). Digested DNA was submitted to electrophoresis at 40 V for 18 h in a 0.8% agarose gel.

Restricted DNA was analysed by Southern blotting. A 471-bp fragment internal to the *rrl* gene was amplified by PCR from *T. otitidis* UCN57 DNA and labelled with digoxigenin-dUTP (DIG DNA labelling kit; Roche, Meylan, France) to be used as a biotin-labelled cDNA probe.

DNA in the gel was denatured and transferred to a Hybond-N membrane (Amersham International) by capillary action for 2 h under vacuum. The nylon membrane was hybridised at 42 °C for 18 h and washed twice in 0.1% sodium dodecyl sulphate (SDS)–2× standard sodium citrate (SSC) for 5 min at room temperature and twice in 0.1% SDS–0.1× SSC for 15 min at 42 °C. Hybridisation reactions were visualised colorimetrically with the NBT/BCIP nucleic acid detection system (Roche), as recommended by the manufacturer.

3. Results and discussion

3.1. Antibiotic resistance patterns

MICs of erythromycin, clarithromycin (14-membered ring macrolides), azithromycin (15-membered ring macrolide) and spiramycin (16-membered ring macrolide) were increased by 64- to 2000-fold compared with those for the wild-type strains (Table 1). All erythromycin-resistant isolates were also resistant to clindamycin. By contrast, pristinamycin MICs remained unchanged.

This phenotype of cross-resistance between erythromycin and clindamycin appears to be frequent in *T. otitidis*, as reported by Funke et al. [11]. According to these authors, ca. 25% of *T. otitidis* isolates were resistant to erythromycin (MIC \geq 32 mg/L) and in nearly all cases were also resistant to clindamycin.

3.2. Mutations in domain V of 23S rRNA relate to resistance to macrolides/lincosamides

Since the phenotype of cross-resistance between erythromycin and clindamycin evokes ribosomal methylation by Erm methylases, we searched for the presence of *erm* genes in the isolates. No sequence for *erm* genes was detected by PCR. PCR was also negative for the other tested resistance genes.

Entire sequences of the *rpID* (protein L4) and *rpIV* (L22 protein) genes were obtained as described in Section 2.3 for the five resistant isolates and the susceptible control. The genes putatively encoded 217- and 120-amino acid proteins, respectively. L4 and L22 deduced amino acid sequences displayed 77% and 84% identity with the

corresponding sequences of *C. glutamicum* ATCC 13032 (NP_599749.1) and *C. diphtheriae* (NP_938857.1), respectively. Alignment of the proteins of macrolide-resistant and -susceptible *T. otitidis* did not reveal any difference, except for an S/G substitution at position 73 for *T. otitidis* UCN53. However, this substitution was located in a variable central portion of the L22 protein whereas only insertions or deletions in the C-terminus of the protein have been reported to confer resistance to macrolides [12,13].

Portions of the *rrl* gene encoding domain V of 23S rRNA and containing bases critical for erythromycin resistance (A2058, A2059, G2505 and C2611) were sequenced. Since *rrl* genes were amplified as a whole, the sequence traces for each channel (i.e. A, C, G and T) were visually analysed to identify possible combinations of mutated nucleotides at the same position.

No mutation was detected at positions G2505 and C2611. Sequences of the resistant isolates differed from the sequences of the susceptible isolates by nucleotide substitutions at positions 2058 and 2059 (*E. coli* numbering) (Table 1). Of the five resistant isolates, three displayed A2058 mutations (A2058U, A2058G or A2058C), one had an A2059G mutation, whereas another one contained mutations at positions 2058 and 2059. In *T. otitidis* UCN53 and UCN55, sequence analysis of the PCR amplification products revealed the presence of both C2058 and G2058 sequences. *Turicella otitidis* UCN55 also displayed an additional A2059C mutation.

Many independent lines of evidence indicate that adenosine 2058 is the key nucleotide involved in macrolide interaction on the ribosome [14]. Change of the

adenine at position 2058 for a guanine conferred the MLS_B phenotype, defined as high-level resistance to all drugs in this group. This phenotype is similar to that conferred by dimethylation of A2058 encoded by *erm* genes [14]. A2058C and A2058U mutations have also been reported in *Helicobacter pylori* where they confer a low level of resistance [15]. By contrast, in *Streptococcus pneumoniae* mutants the same level of macrolide resistance is conferred regardless of the nature of the substitution [12]. For *T. otitidis*, A2058U gave the lowest level of resistance to erythromycin, whereas A2059G/C mutations gave the highest level of resistance to spiramycin.

3.3. Copy number of the *rrl* gene in *Turicella otitidis*

Resistance to macrolides owing to ribosomal mutations has been described in a variety of microorganisms. Generally, these mutations are observed in pathogens (e.g. *Mycobacterium*, *Brachyspira*, *Helicobacter*, *Treponema*) with one or two copies of the 23S rRNA, often with each allele carrying the mutation [14]. Therefore, we hypothesised that *T. otitidis* might have a low copy number of rRNA. Southern blot experiments were performed using DNA digested with *EcoRI* that does not cut into the probe as well as with DNA digested with *SmaI* that cuts into the probe. Fig. 1 shows results for three strains. In lanes containing DNA digested with *EcoRI* or *SmaI*, three and six bands hybridised, respectively. Similar results were obtained with the seven remaining *T. otitidis* isolates (data not shown). These results showed that the studied *T. otitidis* possess only three *rrl* genes.

Generally, susceptibility to erythromycin decreases as the number of mutated gene copies increases. In *T. otitidis* UCN55 where at least one copy of the *rrl* gene was

wild-type, the level of resistance to erythromycin was high (MIC = 128 mg/L), whereas the activity of clindamycin appeared to be less affected, with an MIC of 4 mg/L vs. 8–64 mg/L for the other strains.

In conclusion, it is likely that exposure to macrolides during therapy of otitis has led to the selection of resistant mutants of *T. otitidis*. The relative prevalence of mutations as a mechanism of resistance to macrolides versus the acquisition of mobile elements remains to be determined by studying a large number of *T. otitidis* isolates. However, our study suggests that 23S rRNA mutation is an essential mechanism of resistance to macrolides in this organism.

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Table 1

Minimum inhibitory concentrations (MICs) of macrolides and related antibiotics for *Turicella otitidis* isolates

<i>T. otitidis</i> strain	Base at position ^a		MIC (mg/L)					
	2058	2059	ERY	CLR	AZM	SPI	CLI	PRI
UCN57 ^b	A2058	A2059	<0.06	<0.06	<0.06	0.25	0.12	<0.06
UCN52	U2058	A2059	16	128	128	32	8	<0.06
UCN53	C/G2058	A2059	128	128	128	16	8	<0.06
UCN54	G2058	A2059	128	128	128	16	64	<0.06
UCN55	C/G2058	C2059	128	128	128	128	4	<0.06
UCN56	A2058	G2059	128	128	128	128	16	<0.06

ERY, erythromycin; CLR, clarithromycin; AZM, azithromycin; SPI, spiramycin; CLI, clindamycin; PRI, pristinamycin.

^a Mutated nucleotides (*Escherichia coli* numbering) are indicated in bold characters.

^b MICs of macrolides and related antibiotics were similar for four other isolates of *T. otitidis* susceptible to erythromycin.

Fig. 1. Copy number of the *rrl* gene in *Turicella otitidis*. Chromosomal DNA was restricted with *EcoRI* or *SmaI* restriction enzymes and submitted to agarose gel electrophoresis, transferred to a nylon membrane and then hybridised with an *rrl* probe labelled with digoxigenin. Lanes 1–3, DNA from strains UCN52, UCN54 and UCN55, respectively, restricted with *EcoRI* enzyme; lanes 4–6, DNA from strains UCN52, UCN54 and UCN55, respectively, restricted with *SmaI* enzyme.

1 2 3 4 5 6

