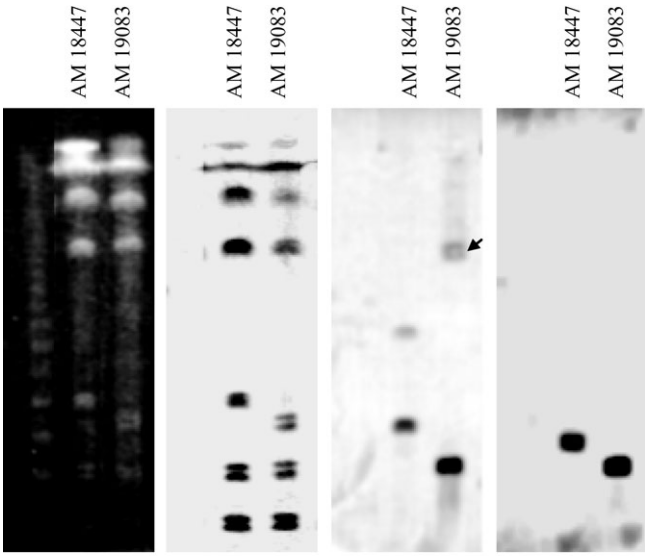

Figure 1. PFGE of I-*Ceu*I-digested DNA from *Salmonella* serovar Typhimurium isolates. (a) Isolates AM19083 and AM18447, (b) hybridized with a 16S rDNA-specific probe, (c) a bla_{CMY-2}-specific probe and (d) an IncI1-specific probe. Molecular weight markers (λ PFGE, New England Biolabs) are in the first lane. Arrow in (c) indicates the 500 kb chromosomal fragment from AM19083, which was positive for bla_{CMY-2}. Hybridization signals in (d) represent the bla_{CMY-2}-positive, IncI1 plasmids carried by both isolates.

the drugs, exceeded the range of dilutions tested (ampicillin, amoxicillin/clavulanate, ceftiofur, cefoxitin and cephalothin). Additionally, a comparison of total cephalosporinase activities by UV spectrophotometry using cell extracts and cefalotin as a reporter substrate did not indicate any significant influence of the extra chromosomal copy (data not shown). Therefore, the role of the chromosomal bla_{CMY-2} in determining resistance levels in this isolate is unclear. Nevertheless, *Salmonella* strains with chromosomally located bla_{CMY-2} may act as reservoirs for *de novo* acquisition of resistance by completely different plasmid backbones and other mobile structures. To the best of our knowledge, this is the first study providing indications as to the simultaneous chromosomal and plasmid location of bla_{CMY-2} in *Salmonella*.

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Transparency declarations

None to declare.

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Reduced susceptibility to tetracyclines is associated *in vitro* with the presence of 16S rRNA mutations in *Mycoplasma hominis* and *Mycoplasma pneumoniae*

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Sir,

Mycoplasma pneumoniae and *Mycoplasma hominis* are aetiological agents of respiratory and genitourinary tract infections, respectively, for which tetracyclines present potential for empirical treatment.[1] As mycoplasmas possess a small number of *rrn* operons, one for *M. pneumoniae* and two for *M. hominis*, the target-related mechanism of resistance to tetracyclines caused by 16S rRNA mutations could be expected as previously described for *Helicobacter pylori*.[2] The purpose of this study was to identify such a mechanism in the reference strains *M. hominis* PG21 and *M. pneumoniae* FH by selecting *in vitro* for tetracycline-resistant mutants and sequencing the 16S rRNA genes of the obtained mutants.

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Growth conditions and antibiotic susceptibility testing of the mycoplasma strains have been described previously.[3] Two selection methods, with either broth or agar medium, were used for *M. hominis* PG21 (ATCC 23114), although only the broth-based selection was done for *M. pneumoniae* FH (ATCC 15531).[4] Broth-selected mutants were obtained by serial transfers of *M. hominis* PG21 and *M. pneumoniae* FH in appropriate Hayflick-modified broth medium containing increasing subinhibitory concentrations of doxycycline. Stepwise selection of doxycycline-resistant mutants was performed onto Hayflick-modified agar medium containing increasing inhibitory concentrations of doxycycline, as described previously.[4] Two steps were performed with doxycycline concentrations at 2× and 8× MIC for the respective parent strain.

Amplifications of *M. hominis* and *M. pneumoniae* 16S rRNA genes were performed with primers described previously.[5] For operon *rrnA*, inverse PCR (IPCR) was carried out on self-ligated genomic DNA of *M. hominis* with two divergent primers H2pu1 (5′-GGTGCATGGTTGTCGTCAGC-3′) and Mh16S-6 (5′-GCC AGCGTTCATCCTGAGCC-3′), located at the 5′ and 3′ ends of the 16S rRNA gene, respectively. PCR products were cloned into the pGEM-T cloning vector (Promega). For operon *rrnB*, libraries of *Hin*dIII-digested *M. hominis* total DNA were constructed with the pGEM7ZF(+) cloning vector (Promega). Recombinant clones were selected by colony hybridization with a labelled PCR product, obtained with primers Mh16S-1 and Mh16S-3.[5] Plasmids and the purified PCR products were directly sequenced by using an ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems). The nucleotide sequences of the fragments encompassing the 16S rRNA of operons *rrnA* and *rrnB* of *M. hominis* have been deposited in GenBank under accession nos AF443616 and AF443617, respectively.

The molecular organization in the vicinity of both 16S rRNA copies was determined to differentiate them in *M. hominis*. IPCR allowed us to amplify a fragment of 1447 bp named X4 encompassing the region directly upstream of the 16S rRNA gene of operon *rrnA*. The screening of a representative library of *Hin*dIII-digested total DNA, with the 16S-specific probe amplified with primers Mh16S-1 and Mh16S-3, led to the identification of a 4472 bp genomic fragment encompassing the region directly upstream of the 16S rRNA of operon *rrnB*. Subsequently, two specific primers, Mh16S-A (5′-CCAAGCATGTGAAAACTG CGG-3′) and Mh16S-B (5′-GCTAGCTAAATTTAAAGCAGG-3′), were designed to amplify, in association with Mh16S-7,[5] the 16S rRNA genes of operons *rrnA* and *rrnB*, respectively.

For both species, after serial passages in subinhibitory concentrations of doxycycline, we were able to select mutants having reduced susceptibility to tetracyclines and harbouring a variety of mutations in 16S rRNA. No resistant strains could be obtained *in vitro* (tetracycline MICs >8 mg/L), as previously described for *H. pylori*.[2] Most of the mutations were located in the primary tetracycline binding site[6] formed by the 16S rRNA residues 1054–1056 and 1196–1200 of helix 34 and residues 964–967 of helix 31.

Table 1 shows the MICs of tetracyclines for each mutant studied and the mutations observed in the 16S rRNA genes. According to the 'Comité de l'Antibiogramme de la Société Française de Microbiologie', all *M. hominis* and *M. pneumoniae* selected mutants remained categorized as susceptible to the three tetracyclines (MICs ≤ 2 mg/L), except the *M. hominis* mutant DH12A which was categorized as intermediate to tetracycline (MIC 8 mg/L, Table 1).

For *M. hominis*, single (at positions 966, 967 or 1054), double (positions 346 and 965) and triple mutants (positions 346, 965 and 966) were obtained and showed various levels of tetracycline

Table 1. Characteristics of doxycycline-selected mutants of *M. hominis* and *M. pneumoniae*

Mycoplasma species, selection method or selected mutants[a]	MIC (mg/L)[b]			Acquired mutations[c] in the 16S rRNA (heterozygosity *rrsA*:*rrsB* for *M. hominis*)
	DOX	TET	MIN	
M. hominis PG21	0.06	0.25	0.06	none
mutants selected in broth				
DH10A	1	4	0.5	G966T (1G:1T)[d]
DH10B	1	4	0.25	G346A (1G:1A), A965T (1A:1T)
DH12A	2	8	1	G346A (1G:1A), A965T (1A:1T), G966T (1G:1T)
mutants selected on agar				
IDA	0.25	0.5	0.12	C1054T (1C:1T)
IIDB	0.5	2	0.25	A967T (1A:1T)
M. pneumoniae FH	0.06	0.25	0.12	none
DP9A	0.5	1	0.25	G1193A
DP18A	1	2	0.5	G1193A, T968C

[a]Broth-selected mutants are designated by the initial of the selector doxycycline (D) followed by the initial of the mycoplasmal species (H for *M. hominis* or P for *M. pneumoniae*) and the passage number. Fifteen and 18 passages were performed for *M. hominis* and *M. pneumoniae*, respectively. Only passages with a significant MIC increase (at least 4-fold) and a 16S rRNA mutation are presented. For these passages, two of the five clones, named A and B, subcultured were studied. When both clones from one passage were identical, only one clone is represented in this table. *M. hominis* agar-selected mutants are designated by a prefix corresponding to the selection step (I or II), followed by the initial of the selector doxycycline (D). Only clones with significant increased MICs or 16S rRNA mutations are shown.
[b]TET, tetracycline; DOX, doxycycline; MIN, minocycline.
[c]*Escherichia coli* numbering.
[d]Letters in parentheses indicate the nucleotides found in both *rrs* genes of *M. hominis*. *M. pneumoniae* has only one copy of the *rrs* gene.

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MIC increases. Positions 965–967 correspond to the triple-base-pair mutation involved in the high-level tetracycline resistance in *H. pylori*,[2] whereas nucleotide 1054 contacts the A-site tRNA.[6] In contrast, the G346A mutation is located in a 16S rRNA region not closely associated with tetracycline binding.

For *M. pneumoniae*, two mutations were found in the broth-selected mutants and were associated with 2- to 16-fold increases in MICs of the three tetracyclines studied, in comparison with those of the parental strain FH. The two mutations described for this species, G1193A and T968C, are located very close to or contact the primary tetracycline binding site, respectively.

In summary, this is the first description of mutations in 16S rRNA associated with decreased susceptibility to tetracyclines in human mycoplasmas. However, what real effect these new mutations have on the tetracycline susceptibility of both mycoplasmas has yet to be determined.

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Multidrug-resistant *Providencia stuartii* expressing extended-spectrum β-lactamase PER-1, originating in Kosovo

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Sir,

The current emergence and dissemination of clavulanic acid-inhibited, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae represent a global threat, as they are difficult to trace and eradicate, and cause both nosocomial and community-acquired infections.[1] Although the unexplained worldwide escalation of CTX-M-type enzymes is of major concern, other types of ESBLs may be prevalent in more restricted geographical areas. The ESBL PER-1 has been identified in several Gram-negative species including *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[2] This ESBL determinant has been identified in several countries such as France, Italy, Poland and South Korea and is particularly widespread in Turkey.[2]

We report here a multidrug-resistant *Providencia stuartii* isolate, which was recovered in 2004 from a 60-year-old patient who had a femur head prosthesis implantation at the University Hospital Pristina, Kosovo. He subsequently developed a chronic infection, fistulations occurred and he was transferred to the University Medical Clinic of Orthopaedic Surgery, Liestal, Switzerland, where his femur head was removed. Cultures made from intra-operative biopsies yielded oxacillin-susceptible *Staphylococcus aureus*, *Enterococcus faecium* and *P. stuartii* isolates; the latter isolate was identified with the API32GN system (bioMérieux, Marcy l'Étoile, France). Disc diffusion and broth microdilution methods were used to determine its antibiotic susceptibility and results were interpreted according to CLSI criteria. *P. stuartii* isolate 166 was resistant to multiple antibiotics including oxyimino-cephalosporins, fluoroquinolones, chloramphenicol, tetracycline, trimethoprim, aminoglycosides (except amikacin) and colistin, but it remained susceptible to cephamycins and carbapenems. Synergy tests performed with discs containing ticarcillin/clavulanic acid and either ceftazidime or cefepime indicated production of an ESBL. PCR with primers specific for known ESBL genes[2] identified bla_{PER-1}, and PCR mapping showed this to be part of a Tn*1213* composite transposon.[3] Plasmid-mediated Qnr-type determinants have been associated with ESBL genes, but isolate 166 lacked known Qnr-encoding genes.[4] Conjugation studies, with ceftazidime selection and *Escherichia coli* J53 (azide-resistant) as the recipient, showed that the bla_{PER-1} gene was located on a 150 kb plasmid, which also conferred resistance to chloramphenicol, trimethoprim, aminoglycosides (except amikacin) and sulphonamides.

The patient was treated for 6 weeks in Switzerland with imipenem and vancomycin and recovered, but further follow-up was not possible because the patient returned to Kosovo.

There are no epidemiological data regarding ESBLs available for Kosovo. A survey recently performed in Bosnia and

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