

ANTIMICROBIAL RESISTANCE

## Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains

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Following characterisation by phenotypic tests and amplified ribosomal DNA restriction analysis (ARDRA), 50 tetracycline-resistant (MIC  $\geq$  16 mg/L) *Acinetobacter* strains from clinical (n = 35) and aquatic (n = 15) samples were analysed by PCR for tetracycline resistance (Tet) determinants of classes A–E. All the clinical strains were *A. baumannii*; most (33 of 35) had Tet A (n = 16) or B (n = 17) determinants, and only two did not yield amplicons with primers for any of the five tetracycline resistance determinants. The aquatic strains belonged to genomic species other than *A. baumannii*, and most (12 of 15) did not contain determinants Tet A–E. Strains negative for Tet A–E were also negative for Tet G and M; further analysis of two aquatic strains with specific primers for Tet O and Tet Y and degenerate primers for Tet M-S-O-P(B)-Q also showed negative results. Transfer of tetracycline resistance was tested for 20 strains with three aquatic *Acinetobacter* strains and *Escherichia coli* K-12 as recipients. Transfer of resistance was demonstrated between aquatic strains from distinct ecological niches, but not from clinical to aquatic strains, nor from any *Acinetobacter* strain to *E. coli* K-12. Most transconjugants acquired multiple relatively small plasmids (<36 kb). Transfer did not occur when DNA from the donor strains was added to the recipient cultures and was not affected by deoxyribonuclease I, suggesting a conjugative mechanism. It is concluded that Tet A and B are widespread among tetracycline-resistant *A. baumannii* strains of clinical origin, but unknown genetic determinants are responsible for most tetracycline resistance among aquatic *Acinetobacter* spp. These differences, together with the inability of clinical strains to transfer tetracycline resistance *in vitro* to aquatic strains, contra-indicate any important flow of tetracycline resistance genes between clinical and aquatic acinetobacter populations.

### Introduction

Tetracycline antibiotics inhibit bacterial growth by interfering with protein synthesis at the ribosomal level [1]. Although their use in human medicine has been reduced by the emergence of bacterial resistance, tetracyclines remain among the most commonly used antibiotics world-wide [2]. Different tetracycline resistance determinant classes have been recognised and classified [3], with classes A–E being the most frequently detected among gram-negative bacteria [4].

As most tetracycline resistance genes have been found on plasmids or transposons, acquisition of resistance is generally assumed to be mainly mediated by gene transfer [4].

*Acinetobacter* spp. are gram-negative, non-motile, non-fermentative coccobacilli easily isolated from soil, water, sewage, human skin and many foodstuffs [5]. At least 18 different DNA groups (genomic species) are defined by DNA–DNA hybridisation and seven of these have species names [5]. *A. calcoaceticus*, *A. baumannii*, and the unnamed genomic species 3 and 13 described by Tjernberg and Ursing are almost indistinguishable phenotypically, and it is proposed that the term *A. calcoaceticus*-*A. baumannii* complex (*Acb* complex) should be used for these genomic species [6].

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In the last decade, *Acinetobacter* spp. have increased considerably in importance as opportunist human pathogens, causing nosocomial infections that are often refractory to antimicrobial treatment [7]. Little is known about the mechanisms responsible for this resistance. Based on the high transformation frequencies for reference strain *Acinetobacter* BD4 [8, 9], it was suggested that natural transformation might play an important role in the acquisition of antibiotic resistance in *Acinetobacter* spp. [5]. A study on indigenous plasmids of *Acinetobacter* showed that most (70%) were <23 Kb in size [10], and, therefore, probably lacked conjugative functions. Nevertheless, plasmid-mediated transfer of antibiotic resistance in *Acinetobacter* does occur and is usually associated with transfer of broad host-range plasmids [11].

Based on their ubiquity and remarkable ability to develop resistance, *Acinetobacter* spp. were used as bacterial indicators for monitoring antibiotic resistance in waste water, freshwater aquacultural habitats and other aquatic sources [12–14]. Tetracycline-resistant *Acinetobacter* strains were detected in the sewers receiving waste effluent from a hospital and pharmaceutical plant [12] and in a stream receiving the effluent from a trout farm using oxolinic acid (quinolone)-medicated feed [14]. The objective of this study was to determine whether the same genetic determinants cause tetracycline resistance in clinical and aquatic *Acinetobacter* spp.

## Materials and methods

### Bacterial strains

Eighty-six clinical *Acinetobacter* strains from different specimens, hospitals and European countries were obtained from the strain collection at the Leiden University Medical Centre, The Netherlands. After testing by a breakpoint method [15], 35 tetracycline-resistant strains (MIC  $\geq$  16 mg/L) were examined for Tet A–E. A further 15 unrelated tetracycline-resistant *Acinetobacter* strains (MIC values  $\geq$  16  $\mu$ g/ml) from different aquatic habitats [12–14] were also studied. Source and details for the total of 50 tetracycline-resistant *Acinetobacter* strains are shown in Table 1 [16–19]. *E. coli* strains with different tetracycline resistance determinants [20] were used as positive controls in PCR.

Rifampicin-resistant mutants were obtained, by the gradient plate method [21], from three tetracycline-sensitive *Acinetobacter* strains isolated from an unpolluted stream (recipient A) and waste water (recipients B and C). These were used as recipients for the transfer experiments. Based on phenotypic and ARDRA identification, recipients A and B were ungrouped (not identified to any described genomic species) and recipient C belonged to the species *A.*

*calcoaceticus*. The rifampicin-resistant strain *E. coli* mutant K-12 J53-2 was used as an additional recipient.

### Phenotypic tests

Materials and methods used for phenotypic characterisation at the (genomic) species level were described previously [13]. Briefly, the following phenotypic characters were tested: growth at 37°C in Brain Heart Infusion broth, acidification of glucose, haemolysis of sheep blood, utilisation of azelate, citrate, DL-lactate, glutarate, L-arginine, L-histidine, L-leucine and L-phenylalanine.

### Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA was performed, essentially as described previously [22–23]. Briefly, crude DNA extracts of heated cell suspensions were used for amplification of 16S rDNA fragments by PCR. Amplified fragments were digested with *Cfo*I, *Alu*I, *Mbo*I, and/or *Rsa*I, *Msp*I, *Bfa*I, and *Bsm*AI, and the digestion products were separated by agarose gel electrophoresis. For identification of genomic species, the restriction patterns obtained with the different enzymes were compared with reference data [22].

### PCR reagents and conditions

Tetracycline resistance determinants of classes A–E were detected by multiplex PCR [24] with the primers listed in Table 2 [25–31]. Based on the melting temperatures of the primers and the molecular size of the amplification products, three separate PCR reactions were used, one for Tet A and E, one for Tet B and D, and one for Tet C. Crude lysates were obtained by heating 100  $\mu$ l of bacterial suspensions for 10 min at 95°C. After centrifugation at 13 000 *g* for 20 s, 2  $\mu$ l of the supernate were used as template DNA for PCR. PCR reactions were performed with Ready-To-Go RAPD Analysis Beads, prepared according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, USA). The conditions were as follows: (i) Tet A and E, 95°C for 5 min, followed by 23 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s, followed by a final extension at 72°C for 7 min; (ii) Tet B and D, 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 20 s, followed by a final extension at 72°C for 7 min; and (iii) Tet C, 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. PCR products were detected by electrophoresis on agarose 2% gels using a 100-bp ladder (Boehringer Mannheim, Mannheim, Germany) as a size marker.

Strains that gave negative results for Tet A–E were analysed for the presence of Tet G and Tet M. The amplification reaction mixture, in 50 mM Tris-HCl

**Table 1.** Origin, identification and tetracycline resistance determinants of the 50 *Acinetobacter* strains used

| Strain no.           | Other designations | Geography      | Source      | Year | Identification     |                        | Tet class |
|----------------------|--------------------|----------------|-------------|------|--------------------|------------------------|-----------|
|                      |                    |                |             |      | Phenotypic*        | Genotypic              |           |
| LUH5605 <sup>†</sup> | F2SEP36            | Jutland (DK)   | Fish farm   | 1997 | 5                  | Ungrouped <sup>‡</sup> | –         |
| LUH5606 <sup>†</sup> | F4AUG7             | Jutland (DK)   | Fish farm   | 1997 | Ungrouped          | 11 <sup>‡</sup>        | –         |
| LUH5607 <sup>†</sup> | F4SEP66            | Jutland (DK)   | Fish farm   | 1997 | 7-8/9-15TU         | Ungrouped <sup>‡</sup> | –         |
| LUH5608 <sup>†</sup> | F4S4-30            | Jutland (DK)   | Fish farm   | 1998 | 7-8/9-15TU         | Ungrouped <sup>‡</sup> | –         |
| LUH5609 <sup>†</sup> | F4S4-37            | Jutland (DK)   | Fish farm   | 1998 | 7-8/9-15TU         | Ungrouped <sup>‡</sup> | –         |
| LUH5610 <sup>†</sup> | R1-35              | Taastrup (DK)  | Sewage      | 1997 | 16BJ               | 17 BJ                  | B         |
| LUH5611 <sup>†</sup> | R1-37              | Taastrup (DK)  | Sewage      | 1997 | 5                  | 5 <sup>‡</sup>         | B         |
| LUH5612 <sup>†</sup> | R1-47              | Taastrup (DK)  | Sewage      | 1997 | 7-8/9-15TU         | 7 <sup>‡</sup>         | B         |
| LUH5613 <sup>†</sup> | R1-73              | Taastrup (DK)  | Sewage      | 1997 | 7-8/9-15TU         | Ungrouped <sup>‡</sup> | –         |
| LUH5614 <sup>†</sup> | R3-112             | Taastrup (DK)  | Sewage      | 1997 | 7-8/9-15TU         | Ungrouped <sup>‡</sup> | –         |
| LUH5615 <sup>†</sup> | G1-31              | Glostrup (DK)  | Sewage      | 1997 | 7-8/9-15TU         | 11 <sup>‡</sup>        | –         |
| LUH5616 <sup>†</sup> | G1-34              | Glostrup (DK)  | Sewage      | 1997 | 7-8/9-15TU         | Ungrouped <sup>‡</sup> | –         |
| LUH5617 <sup>†</sup> | G2-76              | Glostrup (DK)  | Sewage      | 1997 | Ungrouped          | Ungrouped <sup>‡</sup> | –         |
| LUH5618 <sup>†</sup> | G2-79              | Glostrup (DK)  | Sewage      | 1997 | 4/6                | Ungrouped <sup>‡</sup> | –         |
| LUH5619 <sup>†</sup> | G3-80              | Glostrup (DK)  | Sewage      | 1997 | 5                  | Ungrouped <sup>‡</sup> | –         |
| RUH3305 <sup>†</sup> | H1                 | Odense (DK)    | Wound       | 1984 | <i>Acb</i> complex | 2 <sup>§</sup>         | B         |
| RUH3245 <sup>†</sup> | H2                 | Salisbury (UK) | Urine       | 1989 | <i>Acb</i> complex | 2 <sup>§</sup>         | B         |
| RUH3242 <sup>†</sup> | H3                 | Basildon (UK)  | Wound       | 1989 | <i>Acb</i> complex | 2 <sup>§</sup>         | A         |
| RUH510 <sup>†</sup>  | H4                 | Nijmegen (NL)  | Bronchus    | 1984 | <i>Acb</i> complex | 2 <sup>§</sup>         | A         |
| RUH2158 <sup>†</sup> | H5                 | Rotterdam (NL) | Blood       | 1987 | <i>Acb</i> complex | 2 <sup>§</sup>         | A         |
| RUH1835              | H6                 | Enschede (NL)  | Pus         | 1986 | <i>Acb</i> complex | 2 <sup>§</sup>         | A         |
| RUH1947              | H7                 | Haag (NL)      | Urine       | 1986 | <i>Acb</i> complex | 2 <sup>§</sup>         | B         |
| RUH2032              | H8                 | Venlo (NL)     | Sputum      | 1986 | <i>Acb</i> complex | 2 <sup>§</sup>         | –         |
| RUH3413 <sup>†</sup> | H9                 | London (UK)    | Skin        | 1981 | <i>Acb</i> complex | 2 <sup>§</sup>         | –         |
| RUH193               | H10                | Rotterdam (NL) | Blood       | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| LUH2319              | H11                | Rotterdam (NL) | Blood       | 1994 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| LUH4932              | H12                | Rotterdam (NL) | Leg         | 1998 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| LUH5466              | H13                | Freiburg (G)   | Trachea     | 1994 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH2158              | H14                | Rotterdam (NL) | Blood       | 1987 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH3403              | H15                | Gwent (UK)     | Skin        | 1991 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH47                | H16                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH83                | H17                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH94                | H18                | Rotterdam (NL) | CAPD        | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH95                | H19                | Rotterdam (NL) | Wound       | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH98                | H20                | Rotterdam (NL) | Wound       | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH99                | H21                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH100               | H22                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH163               | H23                | Rotterdam (NL) | Line tip    | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH169               | H24                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH176               | H25                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH179               | H26                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH180               | H27                | Rotterdam (NL) | Sputum      | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | B         |
| RUH181               | H28                | Rotterdam (NL) | Blood       | 1983 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH1445              | H29                | Rotterdam (NL) | Drainage    | 1985 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH1449              | H30                | Nijmegen (NL)  | Tongue      | 1985 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH1452              | H31                | Nijmegen (NL)  | Bronchus    | 1985 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH1453              | H32                | Nijmegen (NL)  | Urine       | 1985 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH1619              | H33                | Nijmegen (NL)  | Urine       | 1985 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH1620              | H34                | Nijmegen (NL)  | Wound       | 1985 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |
| RUH1623              | H35                | Nijmegen (NL)  | Drain fluid | 1985 | <i>Acb</i> complex | 2 <sup>‡</sup>         | A         |

DK, Denmark; G, Germany; NL, Netherlands; UK, United Kingdom.

\*Phenotypic identification of genomic species according to Dijkshoorn [16]; TU, genomic species 15 designated by Kämpfer *et al.* [17]; BJ, genomic species 16 designated by Bouvet and Jeanjean [18].

<sup>†</sup>Strains used as donors in the mating experiments.

<sup>‡</sup>Identification of genomic species based on amplified ribosomal DNA restriction analysis (ARDRA).

<sup>§</sup>Identification by DNA-DNA hybridisation performed in a previous study [19].

buffer pH 8.3, contained 50 mM KCl; bovine serum albumin (Sigma Cohn fraction V, Sigma-Aldrich, St. Louis, USA) 0.1 mg/ml; 3 mM MgCl<sub>2</sub>; 200 μM of each dNTP (Amersham Pharmacia Biotech AB, Uppsala, Sweden); 20 pmoles of each amplimer (Table 2), 1 unit of Taq polymerase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, USA), 2 μl of the supernate from crude lysates and autoclaved MilliQ water up to 50 μl final volume. After denaturation at 94°C for 1 min, the samples

were submitted to 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min.

For those strains negative for all tetracycline determinants tested, PCR with universal primers for 16S rDNA [32] was used with crude lysates as DNA templates. Furthermore, the total DNA from the two strains LUH5609 and LUH5613 was extracted according to

**Table 2.** PCR primer sets used for the detection of Tet classes A, B, C, D, E, G and M

| Class | Primer set (5' → 3')   | References  |
|-------|--|---|
| A     | FW: GTA ATT CTG AGC ACT<br>GTC GC<br>RV: CTG CCT GGA CAA CAT<br>TGC TT | [25]  |
| B     | FW: CTC AGT ATT CCA AGC<br>CTT TG<br>RV: CTA AGC ACT TGT CTC<br>CTG TT | D. Sandvang<br>(unpublished data)                       |
| C     | FW: TCT AAC AAT GCG CTC<br>ATC GT<br>RV: GGT TGA AGG CTC TCA<br>AGG GC | [26–27]<br>D. Sandvang<br>(unpublished data)            |
| D     | FW: ATT ACA CTG CTG GAC<br>GCG AT<br>RV: CTG ATC AGC AGA CAG<br>ATT GC | [28–29]   |
| E     | FW: GTG ATG ATG GCA CTG<br>GTC AT<br>RV: CTC TGC TGT ACA TCG<br>CTC TT | [30, 31]  |
| G     | FW: TTT CGG ATT CTT ACG<br>GTC<br>RV: TCC TGC GAT AGA GCT<br>TAG A     | Guillaume G. and<br>J.-M. Collard<br>(unpublished data) |
| M     | FW: GTR AYG AAC TTT ACC<br>GAA TC<br>RV: ATC GYA GAA GCG GRT<br>CAC T  | Guillaume G. and<br>J.-M. Collard<br>(unpublished data) |

the method described by Chen and Kuo [33] and further analysed using specific primers for Tet A, B, C, D, E, G, M, O, Y and degenerate primers for Tet M-S-O-P(B)-Q [34].

#### Transfer experiments

The ability to transfer tetracycline resistance was tested by plate mating for 20 unrelated strains selected by source and genomic species. These included 15 aquatic and five clinical strains (Table 1). Briefly, 0.5 ml volumes of overnight donor and recipient cultures were inoculated on Luria Bertani Agar (LBA) and streaked uniformly on the agar surface by a sterile cotton swab. After incubation for 24 h at 30°C, the numbers of recipient, donor and transconjugant colonies were determined on LBA plates containing rifampicin (25 mg/L), tetracycline (8 mg/L) and both antimicrobial agents, respectively. When possible, the acquisition of tetracycline resistance was confirmed by phenotypic tests differentiating between donor and recipient strains. Mating experiments in which transfer of tetracycline resistance was demonstrated were repeated with deoxyribonuclease I (Amersham Pharmacia Biotech) 10 mg/L in the medium. The possibility of natural transformation was tested by the method of Juni [35].

#### Plasmid profiles

Plasmid isolation was performed by a method based on the hot alkaline technique of Olsen [36]. Cells from overnight cultures were resuspended in 200 µl of SET buffer (1 M Tris, 0.5 M EDTA, saccharose 20%) con-

taining lysozyme (Boehringer Mannheim) 0.01 mg/L and RNAase (Boehringer Mannheim) 5 mg/L and incubated 20 min at 37°C before alkaline lysis at 56°C. Plasmids were detected by electrophoresis in agarose 0.8% gels, with *E. coli* 39R 861 and V517 as sources of reference markers [37, 38].

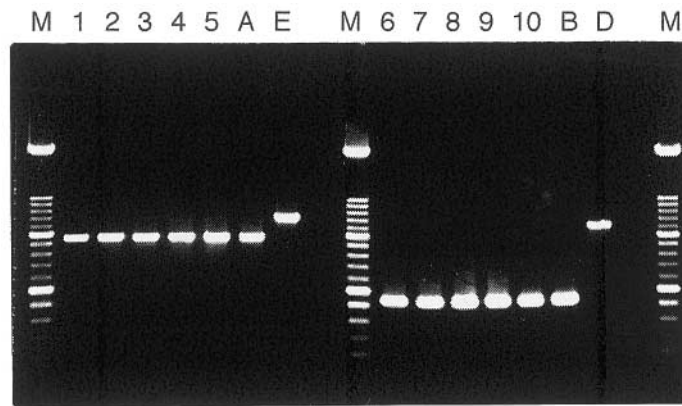
## Results

All the clinical strains were identified as members of the *Acb* complex according to the phenotypic method proposed by Dijkshoorn [16] and as *A. baumannii* according to genotypic identification. Aquatic strains were characterised phenotypically as members of the group of genomic species 7-8/9-15TU (n = 8), genomic species 5 (n = 3), 4/6 (n = 1) and 16BJ (n = 1); two had atypical phenotypic traits precluding speciation. Most aquatic strains (10 of 15) remained ungrouped by ARDRA (Table 1).

Among the 50 tetracycline-resistant *Acinetobacter* strains tested, Tet A and B were detected in 16 and 20 strains, respectively. Most clinical strains (33 of 35) had one of these two classes of tetracycline resistance determinants but, only three aquatic strains were positive for Tet B and none for Tet A (Table 1). The other 12 aquatic strains were also negative for other Tet determinants, including Tet E, Tet G and M. Negative results were also obtained when the total genomic DNA was extracted from strains LUH5609 and LUH5613 and tested for Tet A, B, C, D, E, G, M, O and Y; amplicons were not detected by the use of degenerate primers for Tet M,-S-O-P(B)-Q.

The sizes of the PCR products for Tet A and B corresponded to those obtained with the reference strains included as positive controls (Fig. 1). Only in one case (strain RUH3413) was a non-specific reaction observed in tests for Tet B and D, giving a product of a different size to the reference strains. Consequently, this strain was considered negative for all five classes. Strains negative for Tet A, B, C, D, E, G and M remained positive in the PCR with universal primers for 16S rDNA, confirming that adequate DNA and methods were being used.

Among the 20 donor strains tested, transfer of tetracycline resistance did not occur from any *Acinetobacter* strain to *E. coli* K-12 nor from any clinical strain to any of the aquatic strains. Transfer was detected from four aquatic strains (LUH5605, LUH5609, LUH5613 and LUH5618) to both recipients A and B, and with strain LUH 5618 also to recipient C. Transfer frequencies are reported in Table 3. Small (< 36 Kb) multiple plasmids were acquired by recipients A and B after mating with the donor strains LUH5605, LUH5609 and LUH5613 (Fig. 2), whereas transfer of tetracycline resistance from the donor strain LUH5618 to recipients A, B and C was not associated



**Fig. 1.** Example of results obtained by multiplex PCR for detection of Tet classes A to E determinants. Lane **M**, 100-bp ladder; lanes **1–5**, strains positive for Tet A: **1**, strain RUH181; **2**, strain LUH4932; **3**, strain LUH5466; **4**, strain RUH2158; **5**, strain RUH1445; **A**, positive control for Tet A; **E**, positive control for Tet E; **6–10**, strains positive for Tet B: **6**, strain RUH3305; **7**, strain RUH3245; **8**, strain LUH5610; **9**, strain LUH5611; **10**, strain LUH5612; **B**, positive control for Tet B; **D**, positive control for Tet D.

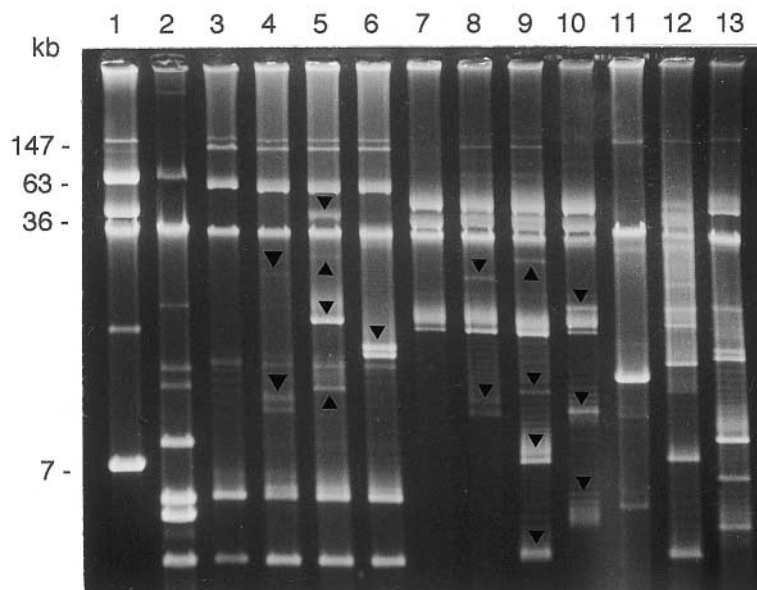
**Table 3.** Transfer frequencies after incubation for 24 h at 30°C

| Donor   | Number of transconjugants/recipient |                      |                      |
|---------|-------------------------------------|----------------------|----------------------|
|         | A                                   | B                    | C                    |
| LUH5605 | $3.4 \times 10^{-7}$                | $2.1 \times 10^{-6}$ | ...                  |
| LUH5611 | $3.3 \times 10^{-8}$                | $1.1 \times 10^{-7}$ | ...                  |
| LUH5623 | $9.6 \times 10^{-7}$                | $2.0 \times 10^{-6}$ | ...                  |
| LUH5616 | $3.4 \times 10^{-4}$                | $1.0 \times 10^{-2}$ | $1.6 \times 10^{-8}$ |

..., transfer not detected.

with a detectable plasmid. Transfer did not occur when naked DNA from the four donor strains was added to the recipient cultures and was not affected by deoxyribonuclease I.

The two recipients and two of the four donor strains (LUH5609 and LUH5613) were identified phenotypically as belonging to the group including *A. johnsonii*, *A. lwoffii* and genomic species 15TU, whereas the two remaining donor strains were assigned to *A. junii*



**Fig. 2.** Plasmid profiles with plasmid DNA detected in each transconjugant but not in recipients indicated by arrows. Lane **1**, reference strain *E. coli* 39R 861; **2**, reference strain *E. coli* V 517; **3**, recipient A; **4**, transconjugant A/LUH5605; **5**, transconjugant A/LUH5609; **6**, transconjugant A/LUH5613; **7**, recipient B; **8**, transconjugant B/LUH5605; **9**, transconjugant B/LUH5609; **10**, transconjugant B/LUH5613; **11**, strain LUH5605; **12**, strain LUH5609; **13**, strain LUH5613.

(LUH5605) and *A. haemolyticus* (LUH5618) (Table 1). Based on ARDRA, all the strains showed atypical profiles. Nevertheless, recipient B and the two donor strains LUH5605 and LUH5609 were assigned to the same group, whereas recipient A and the other two donor strains were assigned to three different groups (not shown).

## Discussion

To our knowledge, this is the first study of tetracycline resistance determinants in *Acinetobacter* spp. The results demonstrated that Tet A and B are widespread among tetracycline-resistant *A. baumannii* from clinical sources. Although tetracyclines are not usually employed for the treatment of acinetobacter infections, resistance was detected in *A. baumannii* strains from different countries and hospitals. Tetracycline-resistant *A. baumannii* clones might be selected by the use of tetracyclines for other bacterial infections, as these organisms can colonise the intestinal tract of hospital patients [39]. Furthermore, the intestinal tract is a suitable habitat for the acquisition of Tet A and B by horizontal gene transfer, as these tetracycline determinants are common in Enterobacteriaceae [4].

Unlike the clinical strains, aquatic *Acinetobacter* strains generally contained tetracycline resistance determinants other than Tet A and B, although three strains did harbour Tet B. These strains belonged to different genomic species (Table 1) but originated from the same sewers receiving waste effluent from a pharmaceutical plant [12]. The occurrence of Tet B among acinetobacters in this habitat was likely to be enhanced by antibiotic selective pressure. In fact, products containing tetracyclines were manufactured at the pharmaceutical plant and the waste effluent was found to increase significantly the levels of tetracycline resistance in the sewers [12].

The determinants of tetracycline resistance in the other 12 aquatic strains remain unknown, and did not belong to classes C, D, E, G or M. Two strains (LUH5609 and LUH5613) were further analysed for other tetracycline resistance determinants and were negative for Tet O, Y, S, P(B) and Q. The genetic determinants of these strains may belong to rare classes previously described in gram-negative genera, e.g., Tet H, I, J, K and L [4], or to undescribed classes. The fact that two of these strains could transfer tetracycline resistance excludes resistance mediated by chromosomal mutation.

The distribution of Tet A–E has been previously studied in gram-negative bacteria from aquatic sources. Tet E (67.4%) and Tet D (17.2%) were prevalent in bacteria from marine sediment [20]; Tet A (46.4%) was reportedly the most common tetracycline resistance determinant among gram-negative bacteria from catfish ponds [40, 41], whereas Tet E was twice as prevalent as

Tet A in *Aeromonas hydrophila* from the intestinal contents of catfish [42]. Nevertheless, c. 16% of the strains analysed in these studies were negative for all five determinants, indicating that gram-negative bacteria of aquatic habitats often harbour tetracycline resistance determinants other than Tet A–E.

In-vitro transfer of tetracycline resistance was demonstrated between aquatic *Acinetobacter* strains from distinct ecological niches and belonging to different species. Multiple small plasmids appeared to be transferred. As the conjugation system requires a relatively large amount of DNA and generally occupies c. one-third of the plasmid genome [43], the smaller plasmids transferred were probably exchanged by mobilisation [44]. As multiple plasmids were transferred, it is impossible, without probing, to determine which encoded tetracycline resistance. In the case of the donor strain LUH5618, the transfer of tetracycline resistance was not associated with plasmid transfer and therefore was likely to be mediated by other genetic elements.

The authors are unaware of any previous study of conjugal transfer of antimicrobial resistance among aquatic *Acinetobacter* spp., but plasmids encoding heavy metal resistance in *Acinetobacter* strains from polluted aquatic environments has been documented [11]. Like the plasmids encoding tetracycline resistance, these are mostly of small molecular size [11].

Transfer was not affected by deoxyribonuclease I, and transformation tests were negative, suggesting conjugative transfer. Various studies have reported the ability of *A. calcoaceticus* to transform under natural conditions [45–48], but the importance of this process and the distribution of naturally transformable strains remain uncertain. It should be noted, in context, that studies on the transformability of *Acinetobacter* spp. have generally used the naturally transformable strain originally described by Juni [35].

None of the 20 *Acinetobacter* strains tested could transfer tetracycline resistance to *E. coli* K-12. Previous studies have indicated that this widely-used strain may not be suitable for detection of plasmid transfer in *Acinetobacter* spp. [49–51] and, as suggested by Towner [11], the failure of previous studies to detect transfer of indigenous plasmids could simply reflect the use of inappropriate recipients (e.g. *E. coli* K-12).

Phenotypic tests and ARDRA gave mutually consistent identification of clinical *Acinetobacter* strains but most (13 of 15) aquatic strains remained ungrouped by ARDRA. Previously [13], we found *A. johnsonii* and *A. lwoffii* to be the prevalent species among aquatic *Acinetobacter* isolates as identified by phenotypic methods but, in this study, most aquatic strains identified phenotypically as *A. johnsonii* and *A. lwoffii*

showed ARDRA patterns different from the reference strains of these species. As previously discussed by Dijkshoorn *et al.* [22], the taxonomy of the genus is largely based on studies of clinical strains and probably does not reflect the level of genetic diversity of environmental *Acinetobacter* spp.

Genotypic analysis can allow study of the epidemiology of antimicrobial resistance genes. The recovery of the same resistance gene in strains of different species or different habitats implies transfer of the gene [52]. *Acinetobacter* spp. are particularly suitable organisms for this type of study as they occur in both clinical and natural environments. Nevertheless, the present investigation found distinct tetracycline resistance determinants in clinical and aquatic strains, indicating limited exchange of tetracycline resistance genes between clinical and aquatic *Acinetobacter* populations. This conclusion was substantiated by the inability of clinical strains to transfer tetracycline resistance to aquatic strains under laboratory conditions.

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