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Short communication

Multidrug-resistant *tet(X)*-containing hospital isolates in Sierra Leone

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ABSTRACT

The *tet(X)* gene encodes a flavin-dependent monooxygenase that confers resistance to all clinically relevant tetracycline antibiotics including tigecycline. It has only previously been identified in environmental and non-human pathogenic bacteria. To investigate levels of multidrug resistance in Bo, Sierra Leone, a molecular epidemiological study was conducted using an antimicrobial resistance determinant microarray (ARDM), PCR and DNA sequencing. The study found that 21% of isolates from Mercy Hospital (Bo, Sierra Leone) were *tet(X)*-positive, all of which originated from urinary specimens. Use of molecular epidemiological surveillance tools has provided the first evidence of *tet(X)*-containing multidrug-resistant Gram-negative hospital isolates in a hospital in Sierra Leone.

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1. Introduction

Tetracyclines are useful antibiotics owing to their clinical efficacy, low cost, oral availability, broad-spectrum antibacterial activity and relative ease of synthesis [1]. However, extensive use over the past 60 years or more for treating bacterial infections in humans, agricultural applications, aquaculture and non-antibiotic use has been accompanied by a commensurate increase in the number and spread of tetracycline resistance genes [1,2]. To date, there are three known mechanisms of tetracycline resistance (active efflux, ribosomal protection and enzymatic modification) and ca. 43 established tetracycline resistance genes [2]. Whilst the vast majority of these genes encode efflux ($n = 27$) and ribosomal protection proteins ($n = 12$), three [*tet(X)*, *tet(34)* and *tet(37)*] are unique in that they encode enzymes that inactivate tetracycline antibiotics [2]. Chemical modification of tetracycline was first described in *tet(X)*-containing bacteria and has since become the most studied and only model for this specific mechanism of action [3–7].

The *tet(X)* gene encodes a flavin-dependent monooxygenase (TetX) that requires NADPH and oxygen for enzymatic activity [4,5,7]. In vitro and in vivo assays have demonstrated that TetX can catalyze the inactivation of a variety of tetracyclines (e.g. oxytetracycline, chlortetracycline, demeclocycline, doxycycline, minocycline, tetracycline) [7] with perhaps the most important being tigecycline, a novel third-generation glycylcycline derivative of tetracycline that was designed to circumvent the most commonly encountered efflux and ribosomal protection resistance mechanisms [8]. Although presence of the *tet(X)* gene in a bacterial pathogen could herald the clinical failure of all tetracycline antibiotics, as yet there has been no observed clinical impact as the *tet(X)* gene is seemingly rarely found. It has only been previously documented in obligate anaerobes such as *Bacteroides* [3,5,9,10], an agricultural soil bacterium (*Sphingobacterium* sp.) [11], piggery manure-treated soil [12] and within activated sludge from sewage treatment plants [13]. Thus, whilst the absence of *tet(X)* from aerobic human pathogens is notable, it is also viewed as a temporary condition as it has been suggested that tetracycline resistance genes 'unique' to environmental bacteria will eventually be discovered in human pathogens [2].

In this study, a novel molecular epidemiological screening tool, the antimicrobial resistance determinant microarray (ARDM), as well as additional confirmatory assays were used to provide the first description of *tet(X)*-containing multidrug-resistant (MDR) hospital isolates.

2. Materials and methods

2.1. Collection and identification of bacterial isolates

A total of 52 clinical stool, semen, blood and urine samples (collected from November 2010 to September 2011 at Mercy

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Hospital, Bo, Sierra Leone) were used to inoculate agar medium (Biotec, Lab21 Healthcare Ltd., Bridport, UK). Single colony isolates were harvested from each and were subjected to total genomic DNA extraction using a MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Bacterial isolates were then identified based on PCR amplification and sequencing of a 16S rDNA amplicon that spanned the V3 and V4 variable regions [14] and the *rpoB* or *gyrA* gene as previously described [15,16]. For the preliminary identifications, the obtained 16S rDNA sequences were used to classify each isolate using the Naïve Bayesian Classifier available through Ribosomal Database Project (release 10) (http://rdp.cme.msu.edu/classifier/classifier.jsp) [17].

2.2. Antibiotic susceptibility testing

The sensitivity of a subset of the hospital isolates to azithromycin, tetracycline, gentamicin, kanamycin, ofloxacin, ampicillin/sulbactam, ampicillin, sulfisoxazole, penicillin, erythromycin, ciprofloxacin and doxycycline was determined using a disk diffusion method according to Mercy Hospital and Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, single isolated colonies grown on agar medium (Biotec, Lab21 Healthcare Ltd.) were transferred into tubes containing 0.9% sterile saline solution and were emulsified. This inoculum was then used to streak Mueller–Hinton agar plates (Biotec, Lab21 Healthcare Ltd.), and antibiotic-containing disks (Hardy Diagnostics, Santa Maria, CA) were dispensed onto the agar surface. All plates were incubated for 18 h at 37 °C and the diameters of inhibition zones were then measured and interpreted.

2.3. Antimicrobial resistance determinant microarray hybridisation and data analysis

The ARDM v.1 is a custom-designed CustomArray microarray (4 × 2K chips) that contains 2240 oligonucleotide probes targeting 239 unique genes that confer resistance to 12 classes of antimicrobial compounds (CustomArray, Bothell, WA) [18]. To begin processing samples for ARDM hybridisation, 10 ng of each genomic DNA extract was subjected to whole-genome amplification using a GenomiPhi V2 Reagent Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions, with the reaction mixture incubated for 90 min at 30 °C and subsequently at 65 °C for 10 min to stop the reaction. Then, 2 μg of the resulting high-molecular-weight amplicons were fragmented for 1 min at 37 °C using Fragmentation Reagent (DNase I, 0.045 U/μg DNA) and Fragmentation Buffer (GeneChip® Resequencing Assay Kit; Affymetrix, Santa Clara, CA) in a total reaction volume of 60 μL. The enzyme was subsequently inactivated by incubation at 95 °C for 10 min. The amplified and fragmented DNA was then purified using DNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA) and labelled using the ULS Platinum Bright™ Biotin Nucleic Acid Labeling Kit (Kreatech Diagnostics, Durham, NC) according to the manufacturer's instructions (total reaction volume of 10 μL). Following purification of the labelled fragments using the kit's KREApure columns, the samples were hybridised to the ARDM according to the manufacturer's instructions, with the following changes to the standard protocol: (i) both prehybridisation and hybridisation temperatures were increased to 60 °C; and (ii) a highly polymerised streptavidin–peroxidase conjugate S104PHRP (Fitzgerald Industries, North Acton, MA) was used in place of the CustomArray Biotin Labeling Solution. All microarrays were processed and interrogated electrochemically using an ElectraSense® reader (CustomArray) and the data were analysed with custom-developed Perl scripts using the following criteria for positive/negative signal determinations: individual probes were designated positive when their signal exceeded a threshold defined as the average signal of all microarray probes (excluding the top 5%) plus three standard deviations. Each gene was considered present if ≥40% of its probes provided positive signal determinations.

2.4. Confirmatory PCR and DNA sequencing

Confirmatory PCR and screening of the hospital isolates for the *tet(X)*, *tet(X1)* and *tet(X2)* genes was performed by PCR amplification of internal segments of each gene using previously published primers and cycling conditions [9,14]. All PCR amplicons were size-confirmed by electrophoresis using 1.2% FlashGel™ DNA Cassettes (Lonza, Walkersville, MD) and the identities of the detected amplicons were further verified by DNA sequencing (Eurofins MWG Operon, Huntsville, AL). Full-length *tet(X)* and *tet(X2)* gene sequences from isolates SL-1 and SL-20 were determined using the following additional primers that were designed based on publicly available sequences: tetX-III-F (5′-CCTGATGAATGGAAAAACCAAACGC-3′)/tetX-III-R (5′-ACAATTGCTGAAACGTAAAGTCGGG-3′); tetX-V-F (5′-GCTTGAACCTGGTAAGAAGAAGTGG-3′)/tetX-V-R (5′-GCGTTCGTCCCAATCGGAAA-3′); tetX-7-F (5′-TGGGCCATTTTTGAATGACAATGCG-3′)/tetX-7-R (5′-TGTGTAGGTCAAGGGTTCCACCA-3′); and tetX-8-F (5′-TGGGTTGGTGGATGCCTTGATATTG-3′)/tetX-8-R (5′-CGGCTGGCAAATTGGCGATGG-3′). Confirmation of the terminal open reading frame sequences and sequencing of the *tet(X)* flanking regions was performed using a DNA Walking SpeedUp™ Premix Kit (Seegene, Seoul, South Korea) according to the manufacturer's instructions.

3. Results and discussion

As a part of a larger study on the distribution of antimicrobial resistance determinants and level of multidrug resistance in West Africa, we began testing bacterial isolates collected from Mercy Hospital in Bo, Sierra Leone, using the ARDM as a screening tool. ARDM is capable of detecting 43 known tetracycline resistance genes and an assortment of other determinants covering 12 classes of antibiotics. One of the first isolates tested (urine isolate SL-4) revealed that 100% of the ARDM probes specific for the tetracycline resistance genes *tet(X)* and *tet(39)* (which encodes a tetracycline efflux pump) were found to be hybridisation-positive. In addition, the analysis under stringent threshold settings identified 17 other putative resistance genes under stringent threshold settings known to confer resistance to trimethoprim (*dfrA14*), sulfamethoxazole (*sulI*, *sulII*), quaternary ammonium compounds (*qacEΔ1*), macrolides (*macB*), streptomycin (*strA*, *strB*), aminoglycosides [*aac(3)-III*, *aac(6)-Ib* family, *aadB*/*ant(2″)-1a*, *aadA1*/*A2* family, *aphAI*] and β-lactams (bla_{TEM}, bla_{OXA-10} family) strongly suggesting the likely MDR nature of isolate SL-4.

As a response to this finding, we chose to screen all clinical isolates using two previously described *tet(X)*-specific PCR assays [9,19] and subsequent DNA amplicon sequencing to determine the prevalence of *tet(X)* in this environment. The results demonstrated that 11 (21%) of the 52 isolates were *tet(X)*-positive. All of the *tet(X)*-positive strains had been isolated from clinical urine specimens. DNA sequence analyses also revealed that 9 (82%) of the 11 *tet(X)*-positive isolates were indeed *tet(X)*, whilst 2 (18%) harboured the *tet(X2)* orthologue [20]. None of the isolates tested harboured the *tet(X1)* orthologue [20].

The identities of the 11 *tet(X)*-positive isolates were determined using PCR amplification and sequencing of the 16S rRNA and *rpoB* or *gyrA* genes using previously published primers and methods [15,16]. Species-level identification was obtained for 8 (73%) of the isolates. Of the three remaining isolates, SL-10 was identified to the genus-level, and SL-8 and SL-22 did not yield *rpoB* and *gyrA* amplicons. *Enterobacter cloacae*, *Comamonas*

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Table 1
Summary of *tet(X)*-containing hospital isolates in Bo, Sierra Leone.

Isolate	Isolation date	Symptoms	Isolation agar	*tet(X)* allele	Identification	ARDM profile[a]	MDR phenotype[b]
SL-1	Nov. 2010	Abdominal pain and painful urination	Nutrient	*tet(X)*[c]	*Enterobacter cloacae* (100)[d]	bla_{OXA-58}, bla_{TEM-1}, *aac(3)-III*, *aadA1/A2* family, *strA*, *strB*, *tet(39)*, *tet(B)*, *tet(D)*, *qacEΔ1*, *sat2*, *sulII*, *dfrA1*, *dfrA14*, *folA*[e]	n.d.
SL-4	Dec. 2010	n.d.	Nutrient	*tet(X)*	*Comamonas testosteroni* (100)[f]	bla_{OXA-1}, $bla_{OXA-10/PSE-2}$, bla_{TEM-1}, *aac(3)-III*, *aac(6′)-Ib*, *aac(6)-Ib*, *aadA1*, *aadA1b*, *aadB*, *ant(2″)-Ia*, *aphAI*, *strA*, *strB*, *macB*, *tet(39)*, *qacEΔ1*, *sulI*, *sulII*, *dfrA1*[g]	n.d.
SL-5	Dec. 2010	n.d.	Sensitivity	*tet(X2)*	*Escherichia coli* (100)[d]	$bla_{CTX-M-3}$, $bla_{TEM-10/MGH-1}$, bla_{TEM-1}, *penA*, *aadA1*, *strA*, *strB*, *macA*, *macB*, *mphA*, *mphK*, *tet(39)*, *tet(B)*, *catA1*, *cmr*, *qacEΔ1*, *sulI*, *sulII*, *dfrA14*, *folA*[e,g]	n.d.
SL-7	Dec. 2010	Frequent and painful urination and slight discharge of pus	Nutrient	*tet(X)*	*E. cloacae* (98)[d]	$bla_{TEM-10/MGH-1}$, bla_{TEM-1}, *aadB*, *ant(2″)-Ia*, *aphAI*, *strA*, *strB*, *tet(39)*, *tet(A)*, *qacEΔ1*, *sulII*, *dfrA2*[g]	n.d.
SL-8	Dec. 2010	Lower abdominal pain, menstrual irregularities	Sensitivity	*tet(X)*	Enterobacteriaceae (100)[h]	n.t.	n.d.
SL-9	Dec. 2010	Frequent urine, lower abdominal pain, vaginal discharge	Sensitivity	*tet(X)*	*E. cloacae* (100)[d]	*ant(2″)-Ia*, *strA*, *strB*, *tet(39)*, *tetS*, *sulII*[g]	n.d.
SL-10	Dec. 2010	Painful urination	CLED	*tet(X)*	*Enterobacter* sp. (90)[d]	bla_{OXA-1}, bla_{TEM-1}, *aac(6′)-Ib*, *strA*, *strB*, *tet(39)*, *sulII*[g]	n.d.
SL-13	March 2011	Fever, frequent urination, high blood pressure	Nutrient	*tet(X)*	*Klebsiella pneumoniae* (99)[d]	n.t.	Yes
SL-15	April 2011	General body pain, painful urination	MacConkey	*tet(X)*	*E. cloacae* (97)[d]	n.t.	Yes
SL-20	April 2011	Fever, abdominal pain	Nutrient	*tet(X2)*[c]	*Delftia acidovorans* (99)[f]	*aadA1b*, *strA*, *strB*, *macA*, *macB*, *tet(39)*, *tet(A)*, *tet(B)*, *catA1*, *cmlA*, *cmr*, *qacEΔ1*, *sulI*, *sulII*[e]	Yes
SL-22	April 2011	Chest pain, vaginal discharge and itching, swollen foot	Nutrient	*tet(X)*	Pseudomonadaceae (100)[h]	n.t.	Yes

MDR, multidrug-resistant; n.d., not documented; n.t., not tested; CLED, cysteine–lactose–electrolyte-deficient agar.
[a] Additional drug resistance determinants identified using the antimicrobial resistance determinant microarray (ARDM).
[b] Tested isolates were found to be resistant to azithromycin, tetracycline, gentamicin, kanamycin, ofloxacin, ampicillin/sulbactam, ampicillin, sulfisoxazole, penicillin, erythromycin, ciprofloxacin and doxycycline via susceptibility disk measurements. Tigecycline resistance was not tested at the time of isolation.
[c] Full gene sequences deposited in GenBank under accession nos. JQ990987 and JQ990988.
[d] Derived from *rpoB* gene sequencing using the methods of Mollet et al. [15]. All identifications are genus- and species-level identifications. Numbers in parentheses indicate the percent sequence identity to reference sequences.
[e] Strain tested using ARDM v.2.0.
[f] Derived from *gyrA* gene sequencing using the methods of Tayeb et al. [16]. All identifications are species-level identifications. Numbers in parentheses indicate the percent sequence identity to reference sequences.
[g] Strain tested using ARDM v.1.0.
[h] Derived from 16S rDNA sequencing. Number in parenthesis indicates the identification confidence threshold. Both identifications are family-level identifications.

testosteroni, *Escherichia coli*, *Klebsiella pneumoniae*, *Delftia acidovorans*, *Enterobacter* sp. and other members of Enterobacteriaceae and Pseudomonadaceae harboured the *tet(X)* gene. Thus, *tet(X)* has now been found in nosocomial pathogens known to cause urinary tract infections and associated complications. The *tet(X)*-containing isolate SL-22 (a pseudomonad) was of particular interest as this finding corroborated the previously unpublished observation of a minocycline-resistant *tet(X3)*-containing *Pseudomonas aeruginosa* clinical isolate from Japan (GenBank accession no. AB097942) and suggested that clinically relevant pseudomonads may also play a role in the dissemination of the *tet(X)* gene and its orthologues. Also important, ARDM-based genotypic evidence (strains SL-1, SL-5, SL-7, SL-9, SL-10, SL-20) and antibiotic susceptibility testing-based phenotypic evidence (strains SL-13, SL-15, SL-20, SL-22) demonstrated that 10 of the 11 *tet(X)*-containing isolates harboured multidrug resistance determinants or were indeed MDR (Table 1).

These results provide the first description of *tet(X)*- and *tet(X2)*-containing bacteria isolated from clinical specimens. As there were some procedural constraints regarding the collection of urine specimens in November and December of 2010, we cannot be certain whether the identified *tet(X)*-containing bacteria collected during this timeframe were assigned causative agents of disease, commensal organisms or hospital contaminants. However, (i) all of the *tet(X)*-containing isolates were from urine collections, (ii) the majority of isolates belonged to bacterial species and families known to harbour human urinary tract pathogens, (iii) no other organisms were isolated from these specimens on non-selective media and (iv) accepted human pathogens containing *tet(X)* were also isolated in March and April of 2011 (after procedural constraints had been ameliorated), suggesting that these were true clinical isolates containing *tet(X)*.

Despite having been found in only obligate anaerobes and environmental bacteria, demonstration of *tet(X)* and its orthologues in these hospital isolates is not surprising considering the size of the environmental reservoir for tetracycline resistance genes and the relative ease of lateral genetic transmission between bacterial species. For example, analyses of the DNA sequences (ca. 150 bp) flanking the 3′ end of the *tet(X)* alleles in strains SL-1 and

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SL-20 revealed that they were identical to sequences previously found in a number of conjugative transposons [e.g. Tn*4351* from *Bacteroides fragilis* (accession no. M37699) and CTnDOT from *Bacteroides thetaiotaomicron* (accession no. AJ311171)] that harbour the *tet(X)* gene. Thus, as seen previously [4,11,20], these findings also suggest that these *tet(X)* genes likely reside in mobilisable transposons or transposon-like elements.

Tigecycline resistance is not currently tested for at Mercy Hospital, and a survey of independent pharmacies and hospital dispensaries in Bo indicated that none had access to Tygacil® (tigecycline IV; Pfizer Inc.). However, 87% of pharmacies readily dispensed other tetracycline antibiotics without requiring a prescription. Thus, the positive selection pressure that results from the continuous application of tetracycline antibiotics in such settings may serve to maintain and spread the tetracycline resistance determinants identified in this study [*tet(X)*, *tet(X2)*, *tet(39)*, *tet(B)*, *tet(A)*, *tet(S)*].

The fact that 21% of the isolates tested in this study were shown to harbour *tet(X)* suggests that the presence of this resistance gene in hospital isolates is not a rare occurrence and that it is being readily circulated in Sierra Leone. The spread of this gene within nosocomial pathogens has potentially significant implications as it may portend eventual clinical failure of all tetracycline antibiotics in this region.

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Competing interests: None declared.

Ethical approval: Ethical approval was obtained from the Njala University Institutional Review Board (Bo, Sierra Leone). All clinical samples from Sierra Leone were obtained from Mercy Hospital (Bo, Sierra Leone) as pre-existing diagnostic specimens that had been stripped of all identifiers. All clinical protocols were approved by the Institutional Review Board at the Naval Research Laboratory (Washington, DC).

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