Molecular screening of antibiotic-resistant determinants among multidrug-resistant clinical isolates of *Proteus mirabilis* from SouthWest Nigeria.

Olumuyiwa Samuel Alabi¹, Nuno Mendonça², Olufemi Ezekiel Adeleke¹, Gabriela Jorge da Silva²,³

1. Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria, West Africa.
2. Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal
3. Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

Abstract

**Background:** Globally, and particularly in developing countries, the menace of anti-microbial resistance is an accelerating problem. In Nigeria, increase in bacterial resistance has been phenotypically established but due to high cost, few molecular studies have been reported.

**Objectives:** This study screened for presence of transferable resistance genes and mobile genetic elements (MGEs) such as integron among multi-drug resistant (MDR) *P. mirabilis*.

**Methods:** A total of 108 *P. mirabilis* strains collected from five tertiary hospitals in SouthWest Nigeria were subjected to antibi-otic susceptibility study using disc-diffusion method. Transferable resistance genes and MGEs were amplified using Polymerase chain reaction (PCR) analysis and amplicons sequenced.

**Results:** Varied resistance was observed against all the antibiotics tested. About 56% of the isolates were MDR including those from 0-12 years old children. PCR analysis revealed the presence of *aac(6')-Ib* (33.3%), plasmid mediated quinolone resistance (PMQR) genes [qnrA (36.7%), *ace(6')-Ib-cr* (5%)], TEM (48.3%), CTX-M (6.7%) and integrons class 1 (58.3%) and class 2 (26.7%). Sequencing analysis revealed *bla*<sub>TEM-1</sub>, *bla*<sub>CTX-M-15</sub> associated with ISEcp1 and eight different arrays of gene cassettes: *aadA1*, *aadA1-qacH*, *aadA2*, *aadA5*, *dfrA7*, *dfrA15*, *dfrA17*, *dfrA17-aadA5*.

**Conclusion:** Transferable resistance genes in association with MGEs are present in Nigerian *P. mirabilis* thus their potential in disseminating resistance.

**Keywords:** Multidrug resistance, resistance determinants, integrase, gene cassettes, *Proteus mirabilis*.

DOI: https://dx.doi.org/10.4314/ahs.v17i2.9

Cite as: Alabi OS, Mendonça N, Adeleke OE, Jorge da Silva G. Molecular screening of antibiotic-resistant determinants among multidrug-resistant clinical isolates of *Proteus mirabilis* from SouthWest Nigeria. Afri Health Sci. 2017;17(2): 356-365. https://dx.doi.org/10.4314/ahs.v17i2.9

Introduction

Anti-microbial drug resistance has become a global concern recognized by the World Health Organization¹. *Proteus* species, common inhabitants of the soil and part of the normal flora of the enteric region of man and animals, have been found to cause opportunistic infections in several anatomical sites.²,³ Among the various species of these genera, *Proteus mirabilis* is of medical importance and usually responsible for most of the common nosocomial opportunistic infections such as urinary tract infections, wounds, ear and other infections.³ Their possession of a number of virulence factors including antibiotic resistance genes, have placed the organism on the list of medically important nosocomial agents.⁴,⁵,⁶,⁷,⁸ The micro-organism has been implicated in several nosocomial infection outbreaks and community acquired infections in different parts of the world, including Nigeria.⁹,¹⁰,¹¹,¹² Among other antibiotics used in the treatment of nosocomial infections in Nigeria, the commonest classes of antibiotics usually employed for life threatening cases are the third generation cephalosporins, fluoroquinolones and aminoglycosides. However, several authors have reported resistance of *Enterobacteriaceae* including *Proteus mirabilis* to these classes of antibiotics.¹³,¹⁴,¹⁵,¹⁶

Corresponding author:
Gabriela Jorge da Silva,
Faculty of Pharmacy and Center for Neurosciences and Cell Biology,
University of Coimbra, Health Sciences Campus, Azinhaga de Santa Comba,
3000-548 Coimbra, Portugal.
Tel: + 351 239488460.
Email: gjsilva@ci.uc.pt;
silva.gj@gmail.com
Resistance to third generation cephalosporins has been attributed to the presence of certain resistance determinants called extended-spectrum beta-lactamase enzymes, ESBLs. Several variants of these enzymes which were believed to have evolved from the wild-type beta-lactamasases (TEM-1 and SHV-1) via mutation at one or more point in the gene coding for their amino acid sequence exist and are spread among bacterial isolates worldwide. Another widely spread ESBL group are the CTX-M-type discovered in the 1980s in Munich, Germany from clinical isolate of Escherichia coli and currently five phylogroups exist. They are known to have high hydrolytic property against cefotaxime.

Resistance to fluoroquinolones was initially thought to be majorly due to chromosomal mutation involving gyrA and topoisomerase genes until plasmid mediated quinolone resistance genes (PMQR) such as the qnr, qepA and aac(6′)-Ib-cr was later reported in 1998. Since then PMQR genes have been described in several bacterial isolates particularly members of the Enterobacteriaceae family worldwide. The qnr genes are known to produce proteins (QNR proteins) that protect the quinolone targets from inhibition. Studies have shown that they produce a low level resistance, but facilitate a higher level resistance in association with chromosomal mutations in bacteria harbouring them. The aac(6′)-Ib-cr, which encode a variant of aminoglycoside transferase that confers reduced susceptibility to ciprofloxacin and norfloxacin by N-acetylation of their piperazinyl amine, causes an increase in their MIC two to four fold and qepA gene, which encodes quinolone-specific efflux pump initiate decreased susceptibility to quinolones by up to 64-fold. Resistance to aminoglycosides such as gentamicin, amikacin and tobramycin, have been attributed to the aminoglycoside-modifying enzymes. Among the different classes of these aminoglycoside-modifying enzyme that have been reported, the one encoded by the aac(6′)-Ib gene is the most commonly isolated in many clinical isolates particularly among the Enterobacteriaceae worldwide.

Common resistance determinant genes that have been detected among isolates belonging to the Enterobacteriaceae family in Nigeria are ESBLs (TEM, CTX-M, SHV and OXA), AmpC Beta-Lactamase (CMY, DHA and ACT), plasmid-mediated quinolone resistance (qnrA, qnrB, qnrD, aac(6′)-Ib-cr and qepA) and aminoglycoside resistance (aac(6′)-Ib) genes. Most of these resistant genes are acquired from other bacterial isolates through mobile genetic elements such as integrons.

Integrons are composed of three key elements: a gene encoding an integrase, a primary recombination site, and a promoter that directs the transcription of captured genes. Integrase is involved in capturing of resistance genes and incorporating them by site-specific recombination within the integrons conserved segments. The aim of this study was to screen for the presence of common transferable resistance genes and mobile genetic elements such as integron and their associated gene cassettes among MDR clinical isolates of P. mirabilis from SouthWest Nigeria.

Methods
Clinical bacterial isolates
A total of 108 non-duplicated clinical isolates of P. mirabilis were randomly collected during a 12 months period (January to December, 2011) from Microbiology units of five selected tertiary hospitals in SouthWest Nigeria, namely University Teaching Hospital (UCH) Ibadan (n = 43), Olabisi Onabanjo University Teaching Hospital (OOUTH) Sagamu (n = 10), Federal Medical Centre (FMC) Abeokuta (n = 13), Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife (n = 28) and Lagos state University Teaching Hospital (LUTH) Lagos (n = 14). The isolates were obtained from different clinical samples (wound, ear, eye, vaginal swabs, urine, sputum, peritoneal effluents, finger abscesses, pus and stool). They were identified by using Gram stain, MacConkey and Chocolate agar, swarming activity and confirmed with Microbact 12E Identification kit (Oxoid Ltd. Basingstoke, Hants, UK). Information on the patient’s data such as age, sex and specimen types was also recorded.

Antibiotic susceptibility test
The clinical isolates were subjected to antibiotic susceptibility testing against twelve antibiotics (Oxoid Ltd. Basingstoke, Hants, UK): amoxicillin (10µg), amoxicillin-clavulanic acid (20/10µg), cefoxitin (30µg), ceftazidime (30µg), cefotaxime (30µg), aztreonam (30µg), imipenem (10µg), gentamicin (10µg), amikacin (30µg), nalidixic acid (30µg), ciprofloxacin (5µg), and trimethoprim-sulfamethoxazole (25µg), using the disc-diffusion method. Minimum Inhibitory Concentrations (MICs) of selected
antibiotics (cefotaxime, ceftazidime, amoxicillin-clavulanic acid, ciprofloxacin and gentamicin) against selected MDR strains was done by broth micro-dilution using microtiter plates to further ascertain their level of resistance. Interpretation was done by comparing results with Clinical and Laboratory Standards Institute guidelines.

Amplification of common resistance genes
Clinical isolates that showed resistance to at least three classes of antibiotics were regarded as MDR isolates and were selected for amplification of resistance genes using polymerase chain reaction (PCR) technique.

Beta-Lactamase gene detection
MDR strains that showed resistance to third generation cephalosporins (cefotaxime and ceftazidime), aztreonam but were susceptible to cefoxitin were screened by simplex PCR for \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{CTX-M}} \) gene. The CTX-M-positive strains were screened by simplex PCR for commonly associated insertion sequences (\( IS_{\text{Ep}}1, IS_{26} \) and \( IS_{903} \)) using them and \( \text{bla}_{\text{CTX-M}} \) primer as forward and reverse respectively. Those MDR isolates that showed resistance to the third generation cephalosporins, aztreonam and cefoxitin but were susceptible to imipenem were screened for common variants of AmpC (\( \text{bla}_{\text{CMY}}, \text{bla}_{\text{DHA}}, \text{bla}_{\text{FOX}} \)) and \( \text{bla}_{\text{OXA}} \) that are usually found in \( \text{Enterobacteriaceae} \) using a multiplex PCR.

Detection of genes encoding plasmid mediated quinolone resistance (PMQR) and aminoglycoside-modifying enzyme (AAC).
MDR isolates with reduced susceptibility or resistance to quinolones and aminoglycosides were screened for the presence of \( qnrA, qnrB, qnrS, qepA \) and \( aac(6^1)'-Ib \) genes using multiplex PCR. The \( aac(6^1)'-Ib \) amplicons were further digested with BstF5I (New England Biolabs, Ipswich, MA) and electrophoresed on 1% agarose gel to identify the \( aac(6^1)'-Ib-cr \) which lacks the BstF5I restriction site present in the wild-type \( aac(6^1)'-Ib \) gene.

Detection of classes 1, 2 and 3 Integrons and associated gene cassettes
All the MDR isolates were screened for the presence of classes 1, 2 and 3 integron-associated integrase genes using specific primers. The variable region of the integrase positive strains were then amplified by PCR using specific primers to know if they carry gene cassettes.

Purification and sequencing of amplified PCR products
The resulting PCR amplification products of the detected beta-lactamase genes and the amplified variable region of the integrons were purified using GF-1 PCR cleanup kit according to the manufacturer’s guidelines (Qiagen, Izasa, Portugal) and then sequenced in both forward and reverse nucleotide chains (Stabvida, Portugal) to identify the genes.

Conjugation assay for ESBL positive strains
The conjugation assay was performed using a modified method described by Kim et al. (2004). The ESBL positive donor strains were conjugated with the standard strain \( E. coli \) J53 (resistant to sodium azide) as a recipient cell. MacConkey agar containing 4 \( \mu \)g/mL of cefotaxime and 200 \( \mu \)g/mL of sodium azide was used as the selection medium. The growth of the sodium azide resistant \( E. coli \) J53 in the selection plate was interpreted as an indication of an ESBL embedded plasmid transfer. The transconjugants were confirmed by determining the antimicrobial susceptibility profile and PCR amplification.

Mutagenic treatment of the isolates harbouring ESBL genes
Isolates harbouring ESBL genes were subjected to R-plasmid curing experiment using the modified method described by Adeleke et al. (2002). Briefly, the overnight culture of each ESBL-positive \( P. mirabilis \) strains was exposed to 200 and 100 \( \mu \)g/ml of ethidium bromide and incubated for 24 hours at 37°C. Pure colonies of the ethidium bromide treated and untreated isolates were first selected on MacConkey agar plates and then on Nutrient agar, after which they were then diluted to 0.5 MacFarland standard for susceptibility testing against selected antibiotics (cefotaxime, ceftazidime, aztreonam, ciprofloxacin, gentamicin and trimethoprim-sulphamethoxazole) by disc-diffusion method, and zones of inhibition interpreted by comparing with the CLSI guideline.

Results
Source of bacterial isolates and antimicrobial susceptibility testing
Out of 108 clinical isolates of \( P. mirabilis \), 47 (43.5%) were from infected wounds, 24 (22.2%) from infected ears, 23 (21.3%) from urine of patients suffering from urinary
tract infections, 4 (3.7%) from infected vaginal swabs, 3 (2.8%) from stool of diarrheic patients, 2 (1.9%) from infected eye exudate, 1 (0.9%) from peritoneal effluent of patient with kidney disease, 1 (0.9%) from sputum of patient with infected respiratory tract, and 1 (0.9%) from pus of finger abscess. Twenty nine (26.9%) of the isolates were obtained from children within the ages of 0 to 17 years, 67 (62%) from patients within the ages 18 to 59 years and 12 (11.1%) from patients above 60 years.

The result of the anti-microbial susceptibility test showed that 80 (74.1%) of the isolates were resistant to trimethoprim-sulfamethoxazole, 70 (64.8%) to amoxicillin, 58 (53.7%) to nalidixic acid, 36 (33.3%) to cefotaxime, 29 (26.9%) to gentamicin, 26 (24.1%) to ceftazidime, 17 (15.7%) to amoxicillin-clavulanic acid, 16 (14.8%) to aztreonam, 15 (13.9%) to ciprofloxacin, 7 (6.5%) to cefoxitin, 5 (4.6%) to amikacin and 2 (1.9%) to imipenem.

Sixty (55.6%) of the isolates were classified as MDR since they were resistant to at least three classes of antibiotics. Twelve (20%) of these MDR isolates were from children within 0 – 12 years of age suffering from various infections such as otitis media (50%), conjunctivitis (8.3%), burn wounds (25%), abscess (8.3%) and UTI (8.3%). The MICs of the selected antibiotics against some of the MDR isolates are presented in Table 1.

| Isolate ID | Clinical Sample | Patient Demography | Antibiotic/MICs (μg/mL) | Resistant genes detected | Gene cassette arrays |
|------------|----------------|-------------------|-------------------------|-------------------------|---------------------|
|            |                | Age (yrs) | Sex | NA | CIP | GN | AMC | CTX | CAZ | dfrA15 | qnrA | aac(6')-Ib-l |
| Pm 02      | Urine          | 32 yrs    | M   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 03      | Urine          | 50 yrs    | F   | 4   | 64 | 8  | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 04      | Urine          | 21 yrs    | M   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 06      | Ear pus        | 44 yrs    | F   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 07      | Ear swabs      | 30 yrs    | M   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 10      | Urine          | 26 yrs    | M   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 12      | Wound swabs    | 51 yrs    | M   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 14      | Wound swabs    | 28 yrs    | F   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 16      | Wound swabs    | 28 yrs    | M   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 18      | Ear swabs      | 112 yrs   | M   | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |
| Pm 20      | Peritoneal effluent | 36 yrs | M | 64 | 64 | 64 | 256 | +   | +   | +     | +    | +             |

**Key:** ID = Identity, M = Male, F = Female, ECS = Endocervical swabs, PE = Peritoneal effluent, NA = Nalidixic acid, CIP = Ciprofloxacin, GN = Gentamycin, AMC = Amoxicillin-clavulanic acid, CTX = Cefotaxime, CAZ = Ceftazidime, MICs = Minimum Inhibitory Concentrations, + = presence of gene, − = absence of gene, yrs = Years, Pm = Proteus mirabilis.
Antimicrobial resistance genes

Amplification and sequencing of the beta-lactamase genes from the MDR strains resistant to cefotaxime or/and ceftazidime revealed that 29 (48.3%) harbored *bla*~TEM-1~ and 4 (6.7%) harboured *bla*~CTX-M-15~. The *bla*~SHV, bla*C-MY, bla*OXA, *bla*~DHA-1~ and *bla*~FOX~ genes were not found. Two of the *bla*~CTX-M-15~ genes were associated with ISEcp1. One of the *bla*~CTX-M-15~ genes was detected from an MDR isolate collected from a six years old male child with an infected burn wound (Table 1).

Twenty (33.3%) of the MDR isolates carried the *aac(6')-Ib* gene, including two isolated from a 2½ and 10 year old female and male patients, respectively, both suffering from ear infections (Table 1). The *aac(6')-Ib-cr* and *qnrA* genes, associated with quinolone resistance, were present in 3 (5%) and 22 (36.7%) of the MDR isolates, respectively, while none was positive for *qepA*.

All (100%) MDR isolates harboured integrase genes, either *IntI1, IntI2* or both, among which 96.7% harboured *IntI1*, 53.3% *IntI2*, 51.7% carried both *IntI1* and *IntI2* while none harboured *IntI3*. Amplification of the integrons variable region produced positive amplicons in 76.7%, which included 58.3% and 26.7% of the *IntI1* and *IntI2* positive strains respectively. Nine of the children within the ages 0 and 12 years were infected with integron-positive MDR strains. Sequencing analysis revealed eight different gene cassette arrays (*aadA1, aadA1-qacH, aadB-aad-A2, aad-A5, dfr-A7, dfr-A15, dfr-A17 and dfr-A17-aa-d-A5*) spread among the integron positive MDR isolates. The gene cassette array *dfr-A17-aa-d-A5* (47.1%) was the most common among the MDR isolates.

**Conjugation and curing assays**

The conjugation experiment performed with four CTX-M-15 strains did not yield any noticeable growth of transconjugants in the selection plates. Moreover, the curing experiment did not alter the susceptibility profile of the CTX-M-15 positive isolates to the cephalosporins and aztreonam significantly but rather increased the susceptibility of the isolates to the effect of the sulphamethoxazole-trimethoprim, gentamicin and to ciprofloxacin, as shown in Table 2.

### Table 2: Antibiotic susceptibility profiles of the mutagen treated and untreated CTX-M-15 positive clinical isolates of MDR *P. mirabilis*

| Isolate ID | CTX | CAZ | AT | CIP | GN | SXT |
|------------|-----|-----|----|-----|----|-----|
|             | Zones of growth inhibitions (mm) | Untreated Clinical isolates of *P. mirabilis* |   |     |     |     |
| Pm 044 | R | R | S | R | R | R |
| Pm 073 | R | R | S | I | R | R |
| Pm 100 | R | R | S | R | R | R |
| Pm 107 | R | R | S | R | R | R |
|           | Clinical isolates of *P. mirabilis* treated with 100µg/mL Ethidium bromide |   |     |     |     |     |
| Pm 044 | R | R | S | S | S | S |
| Pm 073 | R | R | S | I | S | S |
| Pm 100 | R | R | S | S | S | S |
| Pm 107 | R | R | S | S | S | S |
|           | Clinical isolates of *P. mirabilis* treated with 200µg/mL Ethidium bromide |   |     |     |     |     |
| Pm 044 | R | R | S | S | S | S |
| Pm 073 | R | R | S | R | S | S |
| Pm 100 | R | R | S | S | S | S |
| Pm 107 | R | R | S | R | S | S |

Pm – *Proteus mirabilis, S – Sensitive, I – Intermediate, R – Resistant, CTX – Cefotaxime, CAZ – Ceftazidime, ATM – Aztreonam, CIP – Ciprofloxacin, GN – Gentamicin, SXT – Sulphamethoxazole-trimethoprim.
All the primers used in the amplification of the genes detected are presented in Table 3.

| Target gene | Primers | Primer Sequence (5'-3') | Amplicon size (bp) | References |
|-------------|---------|-------------------------|--------------------|------------|
| **OXA-1 variants** | OXA-F | GGCACCGATTCACTTTTCAAG | 564 | Caroline et al., 2010 |
| | OXA-R | GACCCCAAGTTCTCAGAAGTG | | |
| | DHA-1-F | TGATGGCAAGGATATTC | | |
| **DHAl-1 variants** | DHA-1-R | GCTTTCGACTTTGCTGATTCG | 997 | Caroline et al., 2010 |
| | CMY-F | CGAAAGGCAAATGACAC | | |
| **Multiple CMY (2,7,12,18,21-23)** | CMY-R | ACGGACAGGGTTAGATAGY | 538 | Caroline et al., 2010 |
| **Multiple FOX** | FOX-F | CTACAGTGCGGTGGTTT | 162 | Caroline et al., 2010 |
| | FOX-R | CTATTTCGCGCGATGTGA | | |
| **blaCTX-M** | CTX-M-F | TTTTGGATGCTGACTCACTGTA | 543 | Edelstein et al., 2003 |
| | CTX-M-R | CGATATCGTTGTGGTGCCATA | | |
| **blaESX1** | TEM-F | TACGATACGGGAGGGCTTAC | | |
| | TEM-R | TCTCCTTTGGCTCACA | 716 | Belanouaj et al., 1994 |
| **blaA09** | SHV-F | TCACCGAAAAACACCTTG | 471 | M’Zali et al., 1996 |
| | SH-R | TCCCCGATATAAATCACCA | | |
| **qnrA** | qnrA-F | ATT TCT CAC GCC AGG ATT TG | | |
| | qnrA-R | GAT CGG CAA AGG TTA GGT CA | 516 | Kim et al., 2009 |
| **qnrB** | qnrB-F | GAT GTG GAA AGC CAG AAA GG | | |
| | qnrB-R | ATG AGC AAC GAT GGC TGG TA | 476 | Kim et al., 2009 |
| **qnrS** | qnrS-F | GCA AGT TCA TGG AAC AGG GT | | |
| | qnrS-R | TCT AAA CCG TCG AGT TCG GCG | 428 | Kim et al., 2009 |
| **aac(6')-Ib** | aacIb-F | TTG CGA TGC TCT ATG AGT GCC TA | 482 | Kim et al., 2009 |
| | aacIb-R | CTC GAA TGC CTG GCG TGT TT | | |
| **qepA** | qepA-F | AAC TGC TCG AGC CCG TAG AT | | |
| | qepA-R | GTC TAC GCC ATG GAC CTC AC | 596 | Kim et al., 2009 |
| **IS853** | IS853L | AAAAAATGATGAGAGGTGTGG | Variable | Eckert et al., 2004 |
| | IS26 | AGCGGTAAATCTGAGATGGA | Variable | Eckert et al., 2004 |
| | IS903 | CCGTGAATCTGACGTGCA | Variable | Eckert et al., 2004 |
| **IntI1** | Int 1-F | CAG TGG ACA TAA GCC TGT TC | 160 | Dillon et al., 2005 |
| | Int 1-R | CCC GAG GCA TAG ACT GTA | | |
| **IntI2** | Int 2-F | CAC GCA TAT GCG ACA AAA AGG T | 788 | Dillon et al., 2005 |
| | Int 2-R | GTA GCA AAC GAG TGA CGA AAT G | | |
| **IntI3** | Int 3-F | GCC TCC GGC AGC TAC TTT CAG | 979 | Dillon et al., 2005 |
| | Int 3-R | AGC GAT CGT CCA AAG CTC ACT | | |
| **Class 1 array** | hsp58 | TCA TGG CTT GTG ATG ACT GT | Variable | Dillon et al., 2005 |
| | hsp59 | GTA GGG CTT ATT ATG CAC GC | | |
| **Class 2 array** | hsp74 | CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA | Variable | Dillon et al., 2005 |
| | hsp51 | GAT GCC ATC GCA AGT ACG AG | | |

African Health Sciences Vol 17 Issue 2, June, 2017
Discussion

*P. mirabilis* is becoming a common opportunistic pathogen particularly within the Nigerian hospital setting.\(^{15}\) *P. mirabilis* is often associated with urinary tract infections especially among patients with long-term indwelling catheters or under frequent antibiotic therapy.\(^{42,43}\) However, in this study, high frequency of MDR phenotype observed among *P. mirabilis* in wounds than in urine.\(^{3,15}\) The reason for the observed increased cases of wounds infected with *P. mirabilis* in previous and current studies cannot be explained but calls for serious caution in infection control program in these hospitals. Though the majority of the patients (62%) were between the age of 18 and 59 years, about 30% of the isolates were recovered from infected children less than 17 years (including newborn and children that were a few months old). This should be a worrisome development to healthcare professionals, highlighting that control of nosocomial infection must be drastically improved in Nigerian tertiary hospitals.

The high frequency of MDR phenotype observed among the *P. mirabilis* isolates in this study (55.6%) confirms earlier reports that MDR is increasing in Nigeria among members of the Enterobacteriaceae family.\(^{15}\) It is a concern to see that 20% of the MDR isolates detected in this study are from children between the ages 0 and 12 years. This may not be surprising since various reports mention that self-medication of children is rampant among nursing mothers, who usually take antibiotics irrationally even during breastfeeding.\(^{44,45,46,47}\)

A low prevalence of *bla*\(_{\text{CTX-M-15}}\) was found, (6.7%) contrary to the 27.3% prevalence of *bla*\(_{\text{SHV}}\) and *bla*\(_{\text{CTX-M-15}}\) reported by Ogbolu et al.\(^ {15}\) among 11 clinical *P. mirabilis* isolates from SouthWest Nigeria. Although *bla*\(_{\text{CTX-M-15}}\) has been previously described in clinical *P. mirabilis* isolates in Southwest Nigeria,\(^ {15}\) this study reported ISE\(_{Ep1}\) associated *bla*\(_{\text{CTX-M-15}}\) usually involved in the transposition of the gene and its dissemination.\(^ {48}\) The association of *bla*\(_{\text{CTX-M-15}}\) with mobile genetic elements such as insertion sequences and conjugative plasmids usually explain its worldwide dissemination. However, the conjugation assays performed in this study with all the CTX-M-15 positive strains did not produce any transconjugants suggesting that it is not likely to be associated with conjugative plasmid. Furthermore, the R-plasmid curing experiments with ethidium bromide against these strains retained the resistance profile conferred by the *bla*\(_{\text{CTX-M-15}}\); this still suggest a non-plasmid based gene. Although *bla*\(_{\text{CTX-M-15}}\) was already found within a chromosomal location associated with ISE\(_{Ep1}\) in *P. mirabilis* strains isolated in Tunisia\(^ {49}\) and Korea,\(^ {50}\) the two phenotypic experiment done in this study are inadequate to conclude a chromosomally based *bla*\(_{\text{CTX-M-15}}\). A molecular analysis, which was not the objective of this study, is required to finally confirm the actual genetic location of the *bla*\(_{\text{CTX-M-15}}\) detected in this study.

Plasmid mediated quinolone resistance (PMQR) genes are known to confer resistance to nalidixic acid and to increase the minimum inhibitory concentrations of fluoroquinolones up to 32-fold or more.\(^ {26}\) In this study, the detection of *qnrA* and *aac(6')-Ib-cr* associated with the reduced susceptibility to fluoroquinolones showed that these genes are spreading among clinical isolates in Nigeria. Previously, these genes have been reported in some Enterobacteriaceae in Nigeria, together with the *qnrD* gene variant which was not detected in this study.\(^ {15}\)

The *aac(6')-Ib* gene is the most prevalent aminoglycoside modifying enzyme, conferring resistance to tobramycin, kanamycin and amikacin.\(^ {51}\) The gene was first identified in *Klebsiella pneumoniae* isolates in 1986 and since then several variants of this enzyme have been described.\(^ {26,51}\) However, in this study, 3 (5%) of the MDR isolates harboured *aac(6')-Ib-cr*, a variant of the *aac(6')-Ib* gene.

Of relevance is the high prevalence of MDR *P. mirabilis* strains with class 1 and 2 integrase genes, *IntI1* and *IntI2*, and the occurrence of eight different gene cassette arrays (*aadA1, aadA1-qacH1, aadB-aadA2, aadA5, dfrA7, dfrA15, dfrA17 and dfrA17-aadA5*) within the variable region of the integrons in some of the isolates. The size of the amplicons (variable region) ranged from 300bp to 600bp depending on the type and number of gene cassettes present. This result shows the potential of these opportunistic micro-organisms to acquire resistance genes, which is a threat for under-resourced countries, where more potent antibiotics against MDR strains are usually expensive or inexistent.

Overall, accumulation of resistant determinants through the activities of mobile genetic elements is associated with the observed multidrug phenotype of *P. mirabilis*.
clinical isolates from SouthWest Nigeria. Our findings showed that these strains have the tools to acquire resistance genes as much as possible. Therefore, strict surveillance of anti-microbial resistance, rational prescription of antibiotics and strict laws to be enforced against over-the-counter sales of antibiotics must be implemented in Nigeria, to reduce to a minimum antibiotic resistance cases in the country. The frequent isolation of *P. mirabilis* in infected wounds in tertiary hospitals in Nigeria suggests the urgent need to review the existing infection control measures in hospital settings and to educate the clinical staff on this scenario.

**Acknowledgements**

We appreciate all the staff members of the Department of Microbiology, Faculty of Pharmacy and Center of Neurosciences and Cell Biology, University of Coimbra for their support in providing all the necessary assistance to the success of this research. We also sincerely appreciate Mrs Gbadeyan O Felicia, Adegoke OA and Ejilude O for their assistance in the isolates collection and Dr Olusegun O Soge for helpful discussions and his role in the initial review of the manuscript.

Part of this research work was presented as abstract in poster session (P1306) at the 23rd European Congress of Clinical Microbiology and Infectious Diseases (EC-ICMID) Berlin, Germany (27 – 30 April, 2013).

**Financial support**

This research was partly funded by the Center of Pharmaceutical Studies (CEF), Faculty of Pharmacy, University of Coimbra, Portugal.

**Conflict of interest**

None to declare.

**Ethical approval**

Not required.

**References**

1. WHO Report on Resistance. Anti-microbial resistance: global report on surveillance. Geneva, World Health Organization (WHO) 2014. Available from http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf.
2. Prescott LM, Harley JP, Klein DA. Microbiology. 6th ed. McGraw-Hill, New York, 2005; pp.833-842.
3. Okesola AO, Adeniji TW. Pattern of extended-spectrum beta-lactamase production among clinical isolates of *Proteus* species in Western Nigeria. *World J. Med. Sci.* 2010; 5: 94-97
4. Al-Duliami AA, Nauman NG, Hasan ASH, Al-Azawi ZH. Virulence Factors of *Proteus mirabilis* isolated from patients otitis media in Baquba and its peripheries. *Diyala Journal of Medicine* 2011; 1: 69 – 75.
5. D’Andrea MM, Literacka E, Zioga A, Giani T, Baraniak A, Fiett J et al. Evolution and spread of a multidrug-resistant *Proteus mirabilis* clone with chromosomal AmpC-type cephalosporinases in Europe. *Antimicrob. Agents Chemother.* 2011; 55: 2735–2742.
6. Vinodkumar CS, Nitin B, Basavarajappa KG, Prabhakar PJ, Nagaraj P. Beta-lactamases mediated resistance amongst Gram-negative bacilli in burn infection. *Int. J. Biol. Med. Res.* 2011; 2: 766-770
7. Mohammed G, Wang Y, Hindi A. The effect of p-nitrophenylglycerol on swarming and the production of some virulence factors in *Proteus vulgaris*. *N. Y. Sci. J.* 2013;6: 8 – 14.
8. Cristiani B, Sergio PDR. Virulence factors of uropathogenic *Proteus mirabilis* - A Mini Review. *Int. J. Scient. Techn.* 2014; 3: 24 – 27.
9. Chukwu BF, Okafor HU, Ikefuna AN. Asyptomatic bacteriuria in children with sickle cell anemia at the University of Nigeria teaching hospital, Enugu, Southeast, Nigeria. *Ital. J. pediatr.* 2011; 37: 45.
10. Samuel SO, Kayode OO, Musa OI, Nwigwe GC, Aboderin AO, Salami TAT et al. Nosocomial infections and the challenges of control in developing countries. *Afr. J. Cln. Exp. Microbiol.* 2010; 11: 102-110.
11. Gebre-Sealsssie S. Antimicrobial resistance patterns of clinical bacterial isolates in SouthWestern Ethiopia. *Ethiop. Med. J.* 2007; 45: 363-370.
12. Kim S, Kim J, Kang Y, Park Y, Lee B. Occurrence of extended-spectrum beta-lactamases in members of the genus *Shigella* in the Republic of Korea. *J. Clin. Microbiol.* 2004; 42: 5264–5269.
13. Soge OO, Bolanle AA, Marilyn CR. New antibiotic resistance genes associated with CTX-M plasmids from uropathogenic Nigerian *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* 2006; 58: 1048–1053.
14. Okesola AO, Makajuola O. Resistance to third-generation cephalosporins and other antibiotics by *Enterobacteriaceae* in Western Nigeria. *Am J. Inf. Dis.* 2009; 5: 17–20.
15. Ogbolu DO, Daini OA, Ogunledun A, Alli AO, Web-
ber MA. High levels of multidrug resistance in clinical isolates of Gram-negative pathogens from Nigeria. Int. J. Antimicrob. Agents 2011; 37: 62-66.
16. Tijani J, Arzai AH, Sadiq NM. Antimicrobial susceptibility pattern of extended-spectrum beta-lactamase producers in Gram negative urogenital isolates in Kano, Nigeria. Bayero J. Pure Appl. Sci. 2012; 5: 20-25
17. Paterson DL, Bonomo RA. Extended-Spectrum \( \beta \)-Lactamases: A Clinical Update. Clin. Microbiol. Rev. 2005; 18: 657-686.
18. Canton R, Gonzalez-Alba JM, Galan JC. CTX-M enzymes: origin and diffusion. Front. Microbiol. 2012; 3:1–19.
19. Martínez-Martínez L, Pascual A, Jacoby GA. Quinolone resistance from a transposable plasmid. Lancet 1998; 351:797–799.
20. Enabulele IO, Yah SC, Yusuf EO, Eghafona NO. Emerging quinolones resistant transfer genes among gram-negative bacteria, isolated from faeces of HIV/AIDS patients attending some Clinics and Hospitals in the City of Benin, Edo State, Nigeria. Online J. Health Allied Sci. 2006; 5: 1-9.
21. Kim HB, Park CH, Kim CJ, Kim EC, Jacoby GA, Hooper DC. Prevalence of plasmid-mediated quinolone resistance determinants over a 9-year period. Antimicrob. Agents Chemother. 2009; 53: 639-645.
22. Chen X, Zhang W, Pan W, Yin J, Pan Z, Jiao X. Prevalence of \( qnr \), \( aac(6')-Ib-cr \), \( qepA \), and \( opq:AB \) in Escherichia coli isolates from humans, animals, and the environment. Antimicrob. Agents Chemother. 2012; 56: 3423-3427
23. Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proceedings of the National Academy of Science of the United States of America 2002; 99: 5638–5642.
24. Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein Qnr with Escherichia coli DNA gyrase. Antimicrob. Agents Chemother. 2005; 49: 118–125.
25. George AJ. Mechanisms of resistance to quinolones. Clin. Infect. Dis. 2005; 41: S120-126.
26. Robicsek A, Jacoby GA, Hooper DC (2006). The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. 6: 629–640.
27. Perichon B, Courvalin P, Galimand M. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in Escherichia coli. Antimicrob. Agents Chemother. 2007; 51: 2464–2469.
28. Yamane K, Wachino J, Suzuki S, Kimura K, Shibata N, Kato H et al. New plasmid mediated fluoroquinolone efflux pump, QepA, found in an Escherichia coli clinical isolate. Antimicrob. Agents Chemother. 2007; 51: 3354–3360.
29. Olowe OA, Oladipo GO, Makanjuola OA, Olaitan JO. Prevalence of extended spectrum beta-lactamases (ESBLs) carrying genes in Klebsiella spp. from clinical samples at Ile-Ife, South Western Nigeria. Int. J. Pharma Med. Biol. Sci. 2012; 1: 2278-5221
30. Boucher Y, Labbate M, Koenig JE, Stokes HW. Integrins: mobilizable platforms that promote genetic diversity in bacteria. Trends Microbiol. 2007; 15:301-309.
31. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Second Informational Supplement. CLSI document M100-S22, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA 2012.
32. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 2012; 18: 268–281.
33. Belaouaj A, Lapoumeroulie C, Canica MM, Vedel G, Névot P, Krishnamoorthy R et al. Nucleotide sequences of the genes coding for the TEM-like Beta-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol. Lett. 1994; 120:75–80.
34. M'Zali F, Gascoyne-Binzi DM, Heritage J, Hawkey PM. Detection of mutations conferring extended-spectrum activity on SHV Beta-lactamases using polymerase chain reaction single strand conformational polymorphism (PCR-SSCP). J. Antimicrob. Chemother. 1996; 37:797–802.
35. Edelstein M, Pimkin M, Edelstein I, Stratchounski L. Prevalence and molecular epidemiology of CTX-M extended-spectrum Beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in Russian hospitals. Antimicrob. Agents Chemother. 2003; 47:3724–3732.
36. Eckert C, Gautier V, Saladin-Allard M, Hidri H, Vertet C, Ould-Hocine Z et al. Dissemination of CTX-M-type Beta-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. Antimicrob. Agents Chemother. 2004; 48:1249–1255.
37. Dallenne C, Da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases.
es in *Enterobacteriaceae*. *J. Antimicrob. Chemother*. 2010; 65: 490–495.

38. Park CH, Robicsek A, Jacoby GA, Sahm D, Hooper DC. Prevalence in the United States of *aac(6')-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob. Agents Chemother*. 2006; 50: 3953–3955.

39. Dillon B, Thomas L, Mohmand G, Zelynski A, Iredell J. Multiplex PCR for screening of integrons in bacterial lysates. *J. Microbiol. Methods*. 2005; 62: 221–232.

40. Adeleke OE, Odelola HA, Oluwolde FA. Curing of antibiotic resistance in clinical strains of *Staphylococcus aureus*. *Afr. J. Med. Pharm. Sci*. 2002; 6: 19 – 25.

41. Chikere CB, Omoni VT, Chikere BO. Distribution of Potential Nosocomial Pathogens in an Hospital Environment. *Afr. J. Biotechnol*. 2008; 7: 3535-3539.

42. O’Hara CM, Brenner FW, Andmiller JM. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clin. Microbiol. Rev*. 2000; 13: 534-546.

43. Coker C, Poore CA, Li X, Mobley HL. Pathogenesis of *Proteus mirabilis* in urinary tract infection. *Microbes and Infection / Institut Pasteur* 2000; 2: 1497-1505.

44. Oluvege AO, Ojo-Bola O, Oludada OE. Carriage of antibiotic resistant commensal *E. coli* in infants below 5 months in Ado- Ekiti. *Int. J. Curr. Microbiol. App. Sci*. 2015; 4: 1096-1102.

45. Ekwochi U, Chinawa JM, Osuorah CD, Odetunde OI, Obu HA, Agwu S. The use of unprescribed antibiotics in management of upper respiratory tract infection in children in Enugu, South East Nigeria. *J. Trop. Pediatr*. 2014; 60: 249-252.

46. Tamuno I, Mohammed SI. Self-medication with antibiotics amongst students of a Nigerian Tertiary Institution. *J. Basic Appl. Sci*. Res. 2011; 1: 1319-1326.

47. Mathew JL. Effect of maternal antibiotics on breast feeding infants. *Postgrad. Med. Journal* 2004; 80:196–200.

48. Poirel L, Lartigue MF, Decousser JW, Nordmann P. ISEcp1B-mediated transposition of *blaCTX-M* in *Escherichia coli*. *Antimicrob. Agents Chemother*. 2005; 49: 447– 450.

49. Mahrouki S, Belhadj O, Chihi H, Mohamed BM, Celenza G, Amicosante G et al. Chromosomal *blaCTX-M-15* associated with ISEcp1 in *Proteus mirabilis* and *Morganella morganii* isolated at Military Hospital of Tunis, Tunisia. *J. Med. Microbiol*. 2012; 61: 1286-1289.

50. Song W, Kim J, Bae IK, Jeong SH, Seo YH, Shin-Jong H et al. Chromosome-encoded AmpC and CTX-M extended-spectrum beta-lactamases in clinical isolates of *Proteus mirabilis* from Korea. *Antimicrob. Agents Chemother*. 2011; 55: 1414-1419.

51. Vakulenko SB, Mobashery S. Versatility of aminoglycosides and prospects for their future. *Clin. Microbiol. Rev*. 2003; 16: 430–450.