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Reduced intracellular drug accumulation augments fluoroquinolone and β-lactam drugs resistance in clinical Gram negative bacteria from Nigeria

D. Olusoga Ogbolu[1,2]*, O. A. Daini[3], A. O. Terry Alli[1] and M. A. Webber[2]

[1]Department of Biomedical Sciences, Ladoke Akintola University of Technology, Ogbomoso, Osogbo Campus, Osun State, Nigeria.
[2]Antimicrobial Research Group, Immunity and Infection, Institute of Biomedical Research, University of Birmingham, Birmingham, UK.
[3]Biochemistry Department, Olabisi Onabanjo University, Remo Campus, Ago-Iwoye, Ogun State, Nigeria.

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In Nigeria, quinolones and β-lactam antibiotics are widely used as broad-spectrum antibiotics to treat infections caused by various Gram-negative pathogens. The outer membrane is the major permeability barrier limiting target access to quinolones and other drugs in Gram-negative bacteria. This study aimed to identify the role of outer membrane porins (OMPs) and uptake in fluoroquinolone (FQ) and β-lactam drugs accumulation. In total, 134 non-duplicate, Gram-negative bacilli isolates of 13 species from different hospitals were investigated for susceptibility to a panel of antibiotics, including loss of outer membrane porins and measuring active efflux. The minimum inhibitory concentrations (MIC) results showed level of resistance to many antibiotics was extremely high having MIC_{90} value of 256 µg/ml or higher for all drugs, most importantly fluorquinolones, ciprofloxacin; sparfloxacin or third generation cephalosporin, ceftazidime; ceftriaxone. SDS-PAGE revealed different outer membrane porin (OMP) profiles on the basis of relative mobility among the strains. The majority of the isolates lack OMPs. The steady-state concentration of drug taken up by the isolates was measured; most of the strains accumulate less bis-benzimidine than the control strain, *Salmonella enterica* L354. The isolates from University College Hospital, Ibadan accumulate fewer drugs and they are more resistant with high minimum inhibitory concentrations when compared with the rests of the hospitals. Active efflux either singly or in tandem with OMPs alterations could be responsible for the low accumulation of fluoroquinolone and β-lactam antibiotics seen in this study and their increased resistance to both important classes of antibiotics.

Key words: Fluoroquinolone, β-lactam, accumulation, resistance and Gram negative, bacteria.

INTRODUCTION

Chromosomal resistance to fluoroquinolones (FQs) due to amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (GyrA) and/or topoisomerase IV (ParC) has been reported

*Corresponding author. E-mail: olusogadave@yahoo.com. Tel: +2347055776547.

(Jacoby, 2005), and has also been found in Gram negative bacteria from Nigeria (Ogbolu et al., 2011). Plasmid-mediated mechanisms of resistance to FQs mediated by qnr alleles (qnrB, qnrS, qnrC and qnrD), the aac(6')-Ib-cr variant and qepA have been identified from many countries (Robicsek et al., 2006). Decreased activity of FQ against *Escherichia coli* has also been related to a reduced intracellular drug accumulation due to lipopoly-saccharide or porin alterations impairing uptake or because of enhanced efflux (Hirai et al., 1986; Hooper et al., 1986). The outer membrane is the major permeability barrier limiting target access to quinolones and other drugs in Gram-negative bacteria. Quinolones can penetrate the outer membrane of *E. coli* not only by diffusion through the OmpF and OmpC porin channels (Hirai et al., 1986) but also by diffusion through the phospholipids layer (Bedard et al., 1987; Chapman and Georgopapadakou, 1988).

Therefore, several mutations that modify outer membrane structural components can lead to quinolone resistance. These types of mutations are associated with low-level resistance to quinolones and cross resistance to other groups of antibiotics that use the same pathways across the outer membrane (Wolfson and Hooper, 1985). Also, association of multiple antibiotic resistance genes on mobile genetic elements has been an important mechanism of dissemination of multidrug resistance and may explain in addition the frequent association between FQ resistance and resistance to extended-spectrum β-lactams in *Enterobacteriaceae*. In addition, the presence of multiple resistance genes on a plasmid expands the subset of drugs that may select for dissemination of multidrug resistance plasmids.

In Nigeria, quinolones and β-lactam antibiotics are widely used as broad-spectrum antibiotics to treat infections caused by various Gram-negative pathogens. Ogbolu et al. (2011) reported an extremely high level of resistance to multiple antibiotics, including FQs and cephalosporins, detected amongst a diverse panel of Gram-negative isolates from various hospitals in Nigeria. This resistance was underpinned by the carriage of a wide variety of plasmid-borne quinolone resistance alleles and ESBL genes, including the first identification of the qnrD allele outside of China and CTX-M group of enzymes. In this study, we identified a reduced FQ and β-lactam drugs accumulation in clinical Gram negative bacteria caused by loss of outer membrane porins and decreased uptake.

MATERIALS AND METHODS

Bacterial isolates and their properties

One hundred and thirty-four clinical Gram-negative bacterial isolates of 13 species were obtained from 585 non-duplicate clinical specimens, including aspirates, ear swab, wound swab, throat swab, high vaginal swab, eye swab, sputum, urine, catheter tip, cerebrospinal fluid and blood culture, for the period, 2005-2007. Single isolates from each specimen were retained. Isolates were from four teaching hospitals in South-Western Nigeria, namely University College Hospital (Ibadan), Obafemi Awolowo University Teaching Hospital (Ile-Ife), Ladoke Akintola University of Technology Teaching Hospital (Osogbo) and Olabisi Onabanjo University Teaching Hospital (Sagamu). All isolates were identified using API 20E strips (bioMérieux, Marcy l'Etoile, France).

Our previous study showed that mutations were found within gyrA and additional mutations within parC for quinolones resistance isolates. This included carriage of a variety of transferable quinolone resistance alleles *qnrA1, qnrB, qnrD*, *aac(6')-Ib-cr* and *qepA*. For β-lactam resistance, *CTX-M-15* was found predominantly, only 1 was *CTX-M-3*. The presence of *ampC* genes was indicated for a number of strains phenotypically and was confirmed in six isolates by PCR. Sequencing identified these genes as *ACT-1, DHA-1* and *CMY-2*. These genes were found in isolates co-producing other extended-spectrum β-lactamase (ESBL) genes such as *CTX-M, TEM, SHV* and *OXA* genes (Table 1) (Ogbolu et al., 2011).

Determination of antibiotic susceptibility

Antimicrobial disc susceptibility tests were carried out on the isolates using freshly prepared Mueller-Hinton agar (Oxoid, England), 0.5 Macfarland of inoculum was used and standardised by the method of Clinical and Laboratory Standard Institute (CLSI, 2012). The following antibiotic discs were used; pefloxacin, 30 µg; sparfloxacin, 30 µg; ciprofloxacin, 5 µg; ceftriaxone, 30 µg; ceftazidime, 30 µg; amoxicillin, 25 µg; amoxicillin/clavulanic acid, 20/10 µg; gentamicin, 10 µg; tetracycline, 30 µg; nalidixic acid, 30 µg. All susceptibility testing runs included the control organisms *E. coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662. Plates with antibiotic discs were incubated for 24 h at 37°C and sensitivity pattern was compared with that of the control culture.

Minimum inhibitory concentrations (MICs) of a panel of antibiotics were determined and interpreted using the agar dilution method according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC) (http://www.bsac.org.uk/susceptibility testing/guide to antimicrobial susceptibility testing.cfm). The antibiotics tested were gentamicin, tetracycline, amoxicillin, amoxicillin/clavulanic acid (AMC), nalidixic acid, ciprofloxacin, pefloxacin, ofloxacin, sparfloxacin, ceftazidime and ceftriaxone. MIC breakpoints for defining ciprofloxacin susceptibility used were: susceptible, 1 µg/ml; intermediate susceptible, 2 µg/ml; and resistant, 4 µg/ml. All susceptibility testing runs included the control organisms as stated above.

Analysis of outer membrane proteins

Bacterial cells were grown in Mueller-Hinton broth to the logarithmic phase, and were lysed by sonication. Outer membrane proteins (OMPs) were extracted with sodium lauryl sarcosinate (Sigma, UK) and recovered by ultracentrifugation, as described previously (Filip et al., 1973). Protein concentrations were determined with the Bradford protein assay kit (Sigma, UK) as described by the manufacturer. The OMP profiles were determined by SDS-PAGE using 12% SDS gels followed by Coomassie blue staining (Carlsbad, CA). The presence and intensity of bands were visualised when compared with wild type isolates of *E. coli*, I364; *K. pneumoniae*, H43; *P. aeruginosa*, GI using Syngene Image analyser software.

Measuring the activity of active efflux using Hoescht 33342 (bis-benzimide)

The efflux activity of bacteria; test isolates or control, L354 (a wild

Table 1. Properties of bacterial isolates used in the study (Ogbolu et al., 2011).

Species	N	PCR-positive for PMQR genes [n(%)]						PCR-positive for β-lactamase genes [n(%)]			
		qnrA	*qnrB*	*qnrS*	*QnrD*	*qepA*	*aac(6')-Ib-cr*	*TEM*	*SHV*	*OXA*	*CTX-M*
Klebsiella pneumoniae	63	0(0)	0(0)	0(0)	0(0)	1(1.6)	5(21.7)	47(74.6)	9(14.3)	1(1.6)	4(6.3)
Escherichia coli	28	3(10.7)	1(3.6)	0(0)	0(0)	2(7.1)	3(10.7)	24(85.7)	10(35.7)	5(17.9)	9(32.1)
Pseudomonas aeruginosa	13	0(0)	0(0)	0(0)	1(7.7)	0(0)	2(15.4)	12(92.3)	4(30.8)	2(15.4)	3(23.1)
Proteus mirabilis	11	1(9.1)	0(0)	0(0)	2(18.2)	0(0)	7(63.6)	10(90.9)	3(27.3)	3(27.3)	3(27.3)
Pseudomonas oryzihabitans	6	1(16.7)	0(0)	0(0)	0(0)	1(16.7)	3(50)	6(100)	3(50)	0(0)	1(16.7)
Burkholderia cepacia	2	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(100)	1(50)	0(0)	1(50)
Aeromonas hydrophilia	1	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)
Enterobacter cloacae	2	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Morganella morganii	3	0(0)	0(0)	0(0)	0(0)	1(33.3)	1(33.3)	2(66.7)	1(33.3)	1(33.3)	1(33.3)
Pseudomonas luteola	1	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)	0(0)
Serratia adorifera	1	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)
Stenotrophomonas maltophilia	2	0(0)	1(50)	0(0)	0(0)	0(0)	0(0)	2(100)	1(50)	1(50)	1(50)
Citrobacter freundii	1	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	1(100)	1(100)	1(100)

PMQR, plasmid-mediated quinolone resistance.

type *Salmonella enterica* subsp *enterica* serovar Typhimurium) was determined by measuring the accumulation of the fluorescent dye Hoechst 33342 (bis-benzimide; 2.5 µM) + known EPIs (40 mg/L PAßN). Measurements were taken at excitation and emission wavelengths of 350 and 460 nm, respectively, over 30 min using a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK), as previously described (Webber et al., 2008).

The data were recorded and analysed by averaging each set of triplicate repeats and subtracting the appropriate average blank values e.g. for test isolate 'A' average the values from each well for each time point and subtract the average value from the three PBS with bis- benzimide blank wells for each time point to give a corrected value. Standard deviation for each sample at each data point was also calculated. The data was presented by plotting graphs to include standard deviation.

RESULTS

Susceptibility testing

The *in vitro* susceptibility pattern of all isolates to 10 antibiotics was determined by disc diffusion and data is presented in Table 2. All the strains examined showed resistance to one or more of the ten antibiotics used for the study. The results depict a pattern of multiple and high level resistance; more than 60% of isolates were resistant for each antibiotic. Overall, fluoroquinolones showed slightly lower level of resistance than the rest of the antibiotics including the third generation cephalosporins except nalidixic acid and tetracycline with only 0 - 4% sensitivity to *E. coli, K. pneumoniae* and *P. aeruginosa*. Table 2 also shows the minimum inhibitory concentrations of 10 antibiotics using the agar dilution method. Determination of precise MIC values confirmed the numbers of strains resistant to clinical breakpoint concentrations for all antibiotics. The MIC results also showed that the level of resistance to many antibiotics was extremely high having MIC_{90} value of 256 µg/ml or higher for all drugs.

Loss of outer membrane porins in quinolone resistance

Outer membranes were prepared from cells grown overnight in a medium to induce expression of the outer membrane porins OmpF and OmpC. SDS-PAGE revealed several different OMP profiles among the strains (Figure 1). OMPs were identified on the basis of relative mobility; the majority of the isolates lack both OmpF and OmpC in *E. coli*. The highest MICs of fluoroquinolones and β-lactam were usually, although not always associated with those isolates lacking Omps.

Reduced accumulation of fluoroquinolone was responsible for resistance

The steady-state concentration of drug taken up by the isolates was measured; representatives of the results of experiments are presented in Figure 2. Overall, most of the strains accumulate less bis-benzimidine than L354 wild-type control strain (Figure 2A). Majority of the isolates are multiply drug resistant (MDR) with quinolone

Table 2. Antimicrobial susceptibility of some of the isolates.

Organisms (no of strains)	Antimicrobial agents (µg)	Disc sensitive (%)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)
Escherichia coli N = 28	Ciprofloxacin (5)	32	256	256	0.015-256
	Pefloxacin (5)	25	256	256	0.25-256
	Sparfloxacin (5)	29	256	256	0.25-256
	Ceftazidime (30)	24	32	256	0.25-256
	Ceftriazone (30)	25	8	256	0.25-256
	Augmentin (30)	25	256	256	0.25-256
	Amoxycillin (25)	21	256	256	0.25-256
	Gentamicin (10)	36	256	256	0.25-256
	Tetracycline (30)	4	256	256	0.25-256
	Nalidixic acid (30)	4	>256	>256	1-256
K. pneumoniae ssp *pneumoniae* N = 63	Ciprofloxacin (5)	17	256	256	0.015-256
	Pefloxacin (5)	14	256	256	0.25-256
	Sparfloxacin (5)	29	256	256	0.25-256
	Ceftazidime (30)	10	256	256	0.25-256
	Ceftriazone (30)	8	256	256	0.25-256
	Augmentin (30)	9	256	256	0.25-256
	Amoxycillin (25)	21	256	256	0.25-256
	Gentamicin (10)	10	256	256	0.25-256
	Tetracycline (30)	0	256	256	0.25-256
	Nalidixic acid (30)	0	>256	>256	1-256
Pseudomonas aeruginosa N = 13	Ciprofloxacin (5)	31	256	256	0.015-256
	Pefloxacin (5)	23	256	256	0.25-256
	Sparfloxacin (5)	23	256	256	0.25-256
	Ceftazidime (30)	18	32	256	0.25-256
	Ceftriazone (30)	0	16	256	0.25-256
	Augmentin (30)	0	256	256	0.25-256
	Amoxycillin (25)	0	256	256	0.25-256
	Gentamicin (10)	8	256	256	0.25-256
	Tetracycline (30)	0	256	256	0.25-256
	Nalidixic acid (30)	0	>256	>256	1-256

MIC, Minimum inhibitory concentration; MIC50/90, MIC for 50 and 90% of the organisms, respectively; ( ), content of the disc for diffusion test.

and β-lactam antibiotics inclusive. Figure 2B shows quinolone and β-lactam susceptible strains accumulate more bis-benzimidine (I8, I9, IK, SGF) while quinolone and β-lactam resistant strains which are not different from MDR accumulate less. The isolates from University College Hospital, Ibadan accumulate less drugs correlating to the fact that they are more resistant with high minimum inhibitory concentrations of quinolone compared to the rests of the hospital.

DISCUSSION

The presence of topoisomerase- (specifically mutations in *gyrA* and *parC* genes) and plasmid-mediated quinolone resistance and β-lactam genes had been used to explain in part the high level of MICs of the strains under study in Nigeria (Ogbolu et al., 2011), loss of OMPs can lower the permeability of drug into the cell and cause antimicrobial resistance (Ardanuy et al., 1998; Martinez-Martinez et al., 2000). OMPs were identified on the basis of relative mobility, the majority of the isolates lack both OmpF and OmpC. The highest MICs of fluoroquinolones and β-lactam were usually, although not always associated with those isolates lacking OMPs. The defect of the expression of OmpC and OmpF is responsible for reduced-susceptible phenotype of these strains. This result suggests that besides mutations in *gyrA*, *parC* and presence of plasmid mediated resistance loss of outer membrane, porins can also serve as the first-step mutation for developing

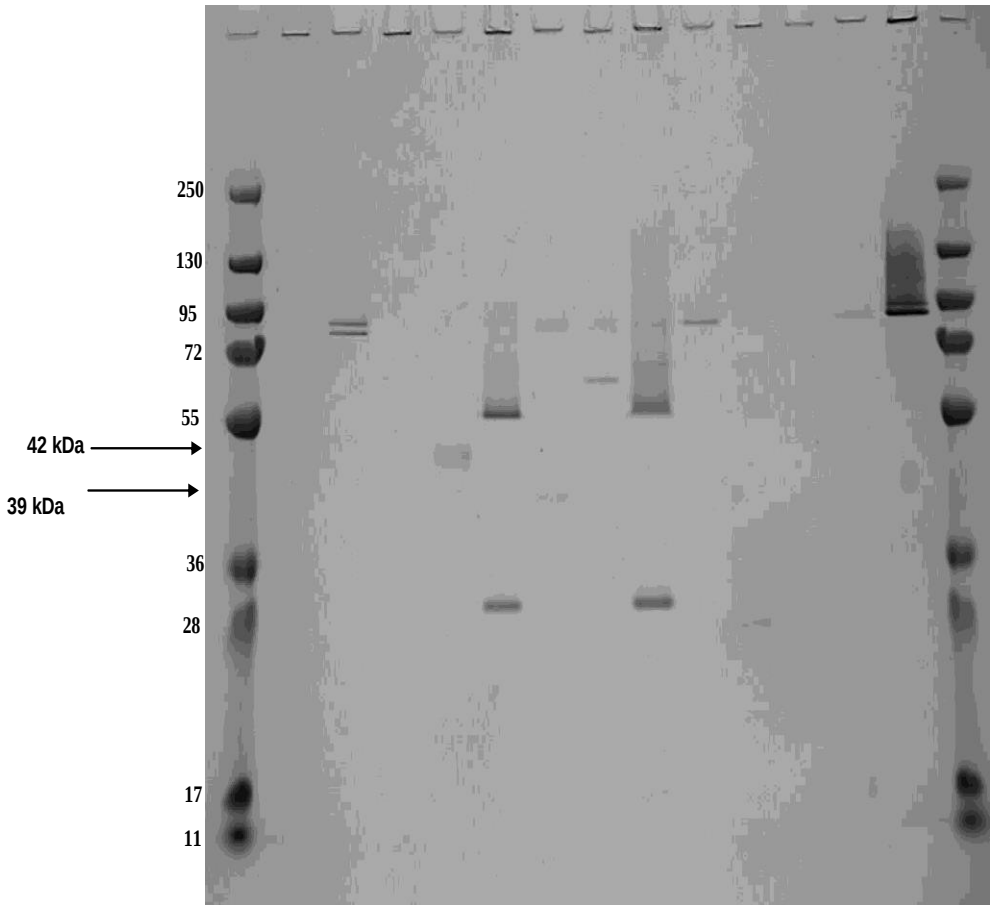

Figure 1. Example of OMP profiles of bacterial strains by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis expressing OmpC or OmpF alone or in combination. The molecular mass marker (M) and OmpC and OmpF with their corresponding sizes, 42 and 39 kDa respectively are indicated.

resistance to fluoroquinolones or β-lactam in the strains of Gram negative bacilli.

The loss of porin has been described as an important cause in the resistance between fluoroquinolones and other forms of antibiotics (Chen et al., 2003). Overall, most of the strains accumulate less bis-benzimidine than L354 wild-type control strain. This is expected since majority of the isolates are multiply drug resistant (MDR) with quinolone and β-lactam antibiotics inclusive. Quinolone susceptible strains accumulate more bis-benzimidine while quinolone resistant strains which are not different from MDR accumulate less. The isolates from University College Hospital, Ibadan accumulated fewer drugs since they were more resistant with high minimum inhibitory concentrations of quinolone compared to the rest of the hospitals. Fluoroquinolones are not subject to enzymatic degradation, so the accumulation of antibiotics within cells is determined by the relative rates of influx and efflux across the cell envelope (Everett et al., 1996).

However, drug accumulation is reduced by an active efflux system which is especially prominent in quinolone-resistant strains (Tran and Jacoby, 2002; Usui et al., 2011). Nevertheless, permeability plays a secondary role in quinolone susceptibility and resistance, in sharp contrast to the situation with β-lactam antibiotics (Nikaido, 1989). The steady-state concentration of drug taken up by the isolates was measured after 10 min of exposure to determine whether decreased accumulation may contribute to the fluoroquinolone and β-lactam resistance phenotype. Decreased activity of drugs against *E. coli* has been related to a reduced intracellular drug accumulation due to lipopolysaccharide or porin alterations impairing uptake or because of enhanced efflux (Hooper et al., 1986). Decreases in the amount of OmpF were found to be associated with decreased accumulation of fluoroquinolones in *E. coli* (Karczmarczyk et al., 2011). Similarly, Garcia-Fernandez et al. (2010) in their study, also implicated loss of porins in increased resistance to β-lactams in comparison with their respective strains, and did not discountenance the importance of the efflux mechanism as a contributor to β-lactam resistance in *K. pneumoniae.* This has also been previously shown for

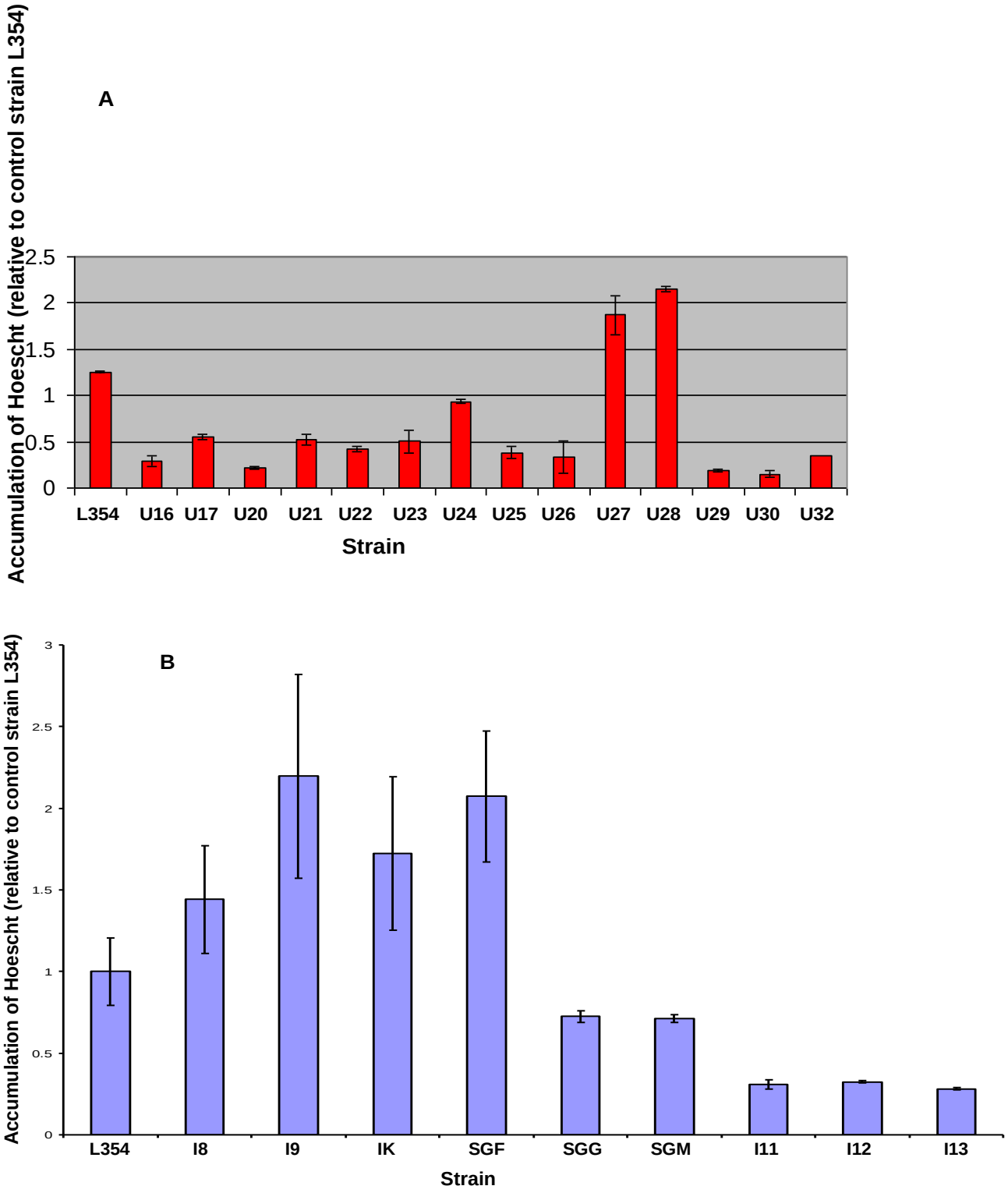

Figure 2. Accumulation of bis-benzimide by strains of Gram negative enteric bacilli. L354 is a wild type strain. (A) is for some of the strains from University College Hospital, Ibadan, while (B) is for some strains from Obafemi Awolowo University Teaching Hospital, Ile-Ife and Olabisi Onabisi Onabanjo University Teaching Hospital, Sagamu.

P. aeruginosa (Farra et al., 2008). Active efflux either singly or in tandem with OMP alterations would be responsible for the low accumulation of fluoroquinolone and β-lactam antibiotics seen in this study and their increased resistance

to both important classes of antibiotics.

Conflict of Interests

The authors did not declare any conflict of interest.

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