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Emergence of *bla*$_{CTX-M-15}$, *qnrB1*, and the *aac(6')-Ib-cr* resistance genes in *Pantoea agglomerans* and *Enterobacter cloacae* from Nigeria (sub-Saharan Africa)

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Abstract

Besides hyper-production of chromosomal AmpC β-lactamases, the expression of plasmid-encoded extended-spectrum β-lactamases (ESBL) in *Enterobacter* spp has increased in recent years. In this study, we characterized 10 clinical isolates of *Enterobacter* spp and 1 isolate of *Pantoea agglomerans*, with respect to the occurrence of ESBL- and plasmid-mediated quinolone resistance (PQMR) genes. Species identification and antimicrobial susceptibility testing were performed by the Vitek 2 system, broth microdilution, agar diffusion and Etests methods. ESBL-, PQMR- and other resistance genes were detected using PCR and sequencing. Strain typing was done by ERIC-2 PCR. The *P. agglomerans* and an *Enterobacter cloacae* isolate were found to harbour ESBL gene $bla_{\text{CTX-M-15,}}$ PQMR genes *qnrB* and *aac-(6')-Ib-cr,* trimethoprim/sulfamethoxazole resistance genes *dfrA14/Sul1* and tetracycline resistance genes (*tet*). In addition, class 1 and 2 integrons were found in these 2 isolates. The result of the ERIC-2 PCR showed distinct patterns indicating heterogeneity of all 10 isolates. This report is the first description of CTX-M-15 production and the emergence of PMQR in *P. agglomerans* and *E. cloacae* isolates from Nigeria. Transfer of resistance genes by conjugation and the presence of mobile elements demonstrate the risk of further dissemination into other *Enterobacteriaceae* which may result in limited treatment options.

CORRESPONDENCE

Resistance of *Enterobacter* spp. to expanded-spectrum cephalosporins is known to be mediated by the hyperproduction of chromosomal AmpC β-lactamases. However, the additional expression of a plasmid-encoded extended-spectrum beta-lactamase (ESBL) has become more prevalent worldwide in recent years (Ko *et al*., 2008). In Nigeria, ESBL-production in *Enterobacter* spp has been associated with TEM- and SHV-type ESBL *(*Aibinu *et al., 2003;* Kasap *et al., 2010).* Other β-lactamase resistance determinants, conferring resistance to extended spectrum cephalosporins, such as bla_{VEB}, bla_{OXA} and bla_{CMY} have just recently been reported in Nigerian *Providencia* spp strains. (Aibinu *et al*., 2011). In addition, the worldwide report of the spread of CTX-M-15 (Canto´n and Coque, 2006), has emerged in Nigeria, having being identified in only *Klebsiella* spp and *E coli* (Soge *et al*., 2006; Olowe *et al*., 2010). There is no documented report yet on ESBL-production mediated by $bla_{CTX\text{-}M\text{-}15}$ or the association of the spread of plasmid-mediated quinolone resistance (PMQR) determinants in *Enterobacter* spp from Nigeria. This study reports the phenotypic and genotypic characteristics of 10 clinical isolates of *Enterobacter* spp and 1 isolate of *Pantoea agglomerans* with respect to the occurrence of CTX-M ESBL and other different resistance genes. The *Enterobacter* spp consisted of *Enterobacter asburiae* (n=1), *Enterobacter aerogenes* (n=1), *Enterobacter cloacae* (n=8) and one isolate of *Pantoea agglomerans,* representing 9.5% of all *Enterobacteriaceae* isolated within a period of 6 months from October 2008 to March 2009 at Lagos University Teaching Hospital (LUTH), a tertiary hospital, in Nigeria. *Enterobacter agglomerans* had previously been renamed *Pantoea agglomerans* to reflect its genetic distance from the genus *Enterobacter* (Sanders and Sanders, 1997).

Bacterial species identification was performed using VITEK 2 system (VITEK2 GN-card; bioMérieux, France). Antimicrobial susceptibility testing was determined according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2010) by broth microdilution method and VITEK2 AST-N13 card. Quality control strain used was *Escherichia coli* ATCC 25922 (Oxoid UK). Etest strips containing cefotaxime in combination with clavulanic acid; and the double disk synergy tests (ESBL/AmpC ID D68C, Mast Group) were used for phenotypic detection and differentiation of both ESBL and AmpC-production. Broth mate conjugation assays were performed as described by Pfeifer *et al.* (2009). Different ESBL genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}), plasmid-mediated quinolone resistance (PMQR) genes (*qnr*, *aac(6')-Ib-cr)*, class 1 and 2 integron with tetracycline and trimethoprim resistance genes were detected by PCR as previously described (Ng *et al*., 2001; Frech *et al*., 2003; Boualle`gue-Godet *et al*., 2005; Cano *et al*., 2009; Jin and Ling, 2009). All positive PCR products were sequenced using the ABI Prism 3100 genetic analyser (Applied Biosystems). Additionally, sequence analysis of the quinolone resistance determining region (QRDR) of genes *gyr*A and *par*C were performed (Cano *et al*. (2009). Epidemiological relationship between the 11 isolates was analysed by ERIC-2 PCR (Versalovic *et al.*, 1999).

Phenotypical analysis of the 11 isolates of this study revealed that 2 isolates (*E. cloacae* 213K *and P. agglomerans* 69K) were ESBL-producers. The ESBL gene $bla_{CTX-M-15}$ gene was identified in both isolates. Isolate *P. agglomerans* 69K was isolated from the blood culture of an adult male patient admitted for sepsis and diagnosed HIV type-1 positive on admission. The patient was treated empirically with ceftriaxone and was referred to another clinic for follow-up on HIV treatment. Several weeks later, the patient was rushed back to the emergency unit of LUTH and examination showed the patient was

brought in dead.

Isolate *E. cloacae* 213K, was recovered from the urine sample of an adult female patient attending the outpatient clinic of LUTH. She was diagnosed with a urinary tract infection and treated empirically with ceftriaxone. Two different urine cultures yielded each time, 2 isolates (*E. coli* and *E. cloacae*) with both isolate harbouring the gene $bla_{\text{CTX-M-15}}$. The 2 ESBL study isolates (*P. agglomerans* 69K and *E. cloacae* 213K) were multiply resistant to different antibiotics including ampicillin, cefepime, cefoxitin, aztreonam, ceftazidime, cefotaxime, gentamicin, tobramycin, levofloxacin, ciprofloxacin, tetracycline and sulfmethoxazole/trimethoprim. Both isolates harboured the class 1 and 2 integrons. The identified gene cassettes within the class 1 integrons included aminoglycoside resistance genes (*aadA1*, *aph* and *aac-(6')-Ib*), sulphonamide resistance genes (*sul1*) and the chloramphenicol resistance gene (*cat1*) in the *P. agglomerans* isolate (Table 1). The presence of the insertion sequence IS*Ecp1* upstream of the $bla_{\text{CTX-M-15}}$ gene was confirmed by PCR (Baraniak *et al*., 2002). Additionally, both isolates harboured the PQMR gene *qnrB1* and *aac-(6')-lb-cr*. The tetracycline resistance gene *tet*(K), encoding an efflux pump, was identified in *P. agglomerans* 69K while *E. cloacae* 213K harboured *tet*(A) and *tet*(E) resistance determinants. By conjugation experiments, plasmids of >90kbp size were successfully transferred into *E. coli* J53 recipients. The *E. coli J53* transconjugants had resistance pattern similar to that of the donor strain but remained susceptible to cefoxitin and showed MIC reduction for ciprofloxacin from 8 to 2 µg/ml (69K) and from 4 to 2µg/ml (213K), respectively. The transconjugants displayed co-resistance to gentamicin with MIC of 8µg/ml for both strains and their transconjugants. PCR and sequence analysis, showed the *E. coli J53* transconjugants harbored $bla_{\text{CTX-M-15}}$, *dfrA14, qnrB1, the aac-(6`)-Ib* (encoding aminoglycoside modifying enzyme) and the *aac-(6')-lb-cr* variant. QRDR analysis revealed that the *P.*

agglomerans 69K isolate had a mutation at codon 87 but no mutation at codon 80 of the topoisomerase IV gene *parC* (nalidixic acid MIC= 32µg/ml). In the QRDR of the *E. cloacae* 213K isolate, no *gyr*A or *par*C mutation was observed (nalidixic acid MIC=32µg/ml).

The other nine *Enterobacter* spp isolates in the present study were susceptible to many antibiotics and were non-ESBL-producers. They all harboured the class 1 integron. The Class 2 integron was additionally found in 45% (n=4) of the isolates. Resistance to trimethoprim/sulfamethoxazole was associated with the presence of *sul1* (100%) and either a *dfrA1* (72.7%), or *dfrA14* (54.6%) or both genes (36.4%) (Table 1). The *tet*(A) and *tet*(E) *genes* were the predominant *tet* gene occurring. The strain typing by ERIC-2 PCR revealed distinct patterns indicating heterogeneity of all *Enterobacter* spp isolates.

We report in this study, the first description of ESBL-type CTX-M-15 in *P. agglomerans* and *E. cloacae* isolates from Nigeria. This study showed a low occurrence of *Enterobacter* spp in clinical infection during this study period (9.5%) and the rate of prevalence of ESBL-production was 18.2% (n=2). Unfortunately, it was not possible to determine whether the ESBL- and PMQR genes in the isolates were hospital- or community-acquired because clinical data showed no record of previous hospital admission for the patients. The result of this study furthermore suggests, that the association of CTX-M-15, PQMR determinants *qnrB1, aac-(6')-lb-cr* and other resistance genes in addition to mobile elements (IS*Ecp1*, class 1 and 2 integrons) may facilitate the rapid dissemination of antimicrobial resistances into other Gram-negative bacteria in Nigeria limiting the choice of antibiotic therapy.

The nucleotide sequences of resistance genes in *P. agglomerans* 69K have been deposited in the GenBank nucleotide sequence database under accession numbers GU990082-GU990087.

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References

1. **Aibinu, I., Pfeifer, Y., Ogunsola, F., Odugbemi, T., Koenig, W., & Ghebremedhin, B. (2011).** Emergence of Beta-Lactamases OXA-10, VEB-1 and CMY in *Providencia* spp from Nigeria. *Journal of Antimicrobial Chemotherapy doi:10.1093/jac/dkr197.*
2. **Aibinu, I., Ohaegbulam, V., Adenipekun, E., Ogunsola, F., Odugbemi, T. & Mee, B. (2003).** Extended-spectrum β-lactamase enzymes in clinical isolates of *Enterobacter* species from Lagos, Nigeria. *J. Clin. Microbiol* **41**, 2197-2200.
3. **Baraniak, A., Fiett, J., Hryniewicz, W., Nordmann, P. & Gniadkowski, M. (2002)**. Ceftazidime-hydrolysing CTX-M-15 extended-spectrum β-lactamase (ESBL) in Poland. *Journal of Antimicrobial Chemotherapy* **50**, 393–396.
4. **Boualle`gue-Godet, O., Salem, Y.B., Fabre, L., Demartin, M., Grimont, P.A., Mzhougi, R. & Weill, F-X (2005)**. Nosocomial Outbreak Caused by *Salmonella enterica* Serotype Livingstone Producing CTX-M-27 Extended-Spectrum β-Lactamase in a Neonatal Unit in Sousse, Tunisia. *J Clin Microbiol* **43**, 1037-1044
5. **Cano, M.E., Rodríguez-Martínez, J.M., Agüero, J., Pascal, A., Calvo, J.,** Garcı´a-Lobo, **J.M., Velasco, C., Francia, M.V. &** Martı´nez-Martı´nez, **L. (2009).** Detection of Plasmid-Mediated Quinolone Resistance Genes in Clinical Isolates of *Enterobacter* spp. in Spain *J. Clin. Microbiol* **47,** 2033-2039.
6. **Canto´n, R. & Coque. T.M. (2006).** The CTX-M β-lactamase pandemic. *Current Opinion in Microbiology* **9**, 466–475.

7. **Clinical and Laboratory Standards Institute (2010)**. Performance standards for antimicrobial antimicrobial susceptibility testing: twentieth informational supplement M100-S20U. CLSI, Wayne, PA, USA,
8. **Frech, G., Kehrenberg, C. & Schwarz, S. (2003).** Resistance phenotypes and genotypes of multiresistant *Salmonella enterica* subsp. Enterica serovar Typhimurium var. Copenhagen isolates from animal sources. *J. Antimicrob. Chemother* **51,** 180-2.
9. **Jacobs, L .& Chenia, H.Y. (2007).** Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems, *Int J Food Microbiol* **114,** 295-306.
10. **Jin, Y. & Ling, J.M. (2009).** Prevalence of Integrons in Antibiotic-Resistant *Salmonella* spp in Hong Kong. *Jpn. J. Infect. Dis*. **62,** 432-439.
11. **Kasap, M., Fashae, K., Torol, S., Kolayli, F., Budak, F. & Vahaboglu, H. (2010).** Characterization of ESBL (SHV-12) producing clinical isolate of *Enterobacter aerogenes* from a tertiary care hospital in Nigeria. *Ann Clin Microbiol Antimicrob* **9,**1.
12. **Ko, K.S., Lee, M.Y., Song, J.H., Lee, H., Jung, D.S., Jung, S.I., Kim, S.W., Chang, H.H., Yeom, J.S., Kim, Y.S., Ki, H.K., Chung, D.R., Kwon,K.T., Peck, K.R. & Lee, N.Y. (2008).** Prevalence and characterization of extended-spectrum beta-lactamase-producing Enterobacteriaceae isolated in Korean hospitals. *Diagn. Microbiol. Infect. Dis.* **61,** 453–459.
13. **Ng, L.-K., Martin, I., Alfa, M. & Mulvey, M. (2001).** Multiplex PCR for the detection of tetracycline resistant genes. *Mol Cell Probes* **15,** 209-215.
14. **Olowe, O., Grobbel, M., Buchter, B., Lubke-Becker, A., Fruth, A. & Wieler, L. (2010).**

Detection of bla$_{CTX\text{-}M\text{-}15}$ extended-spectrum beta-lactamase genes in *E. coli* from Hospitals in Nigeria. *Intenational Journal of Antimicrobial Agents* **35,** 200-209.

15. **Pfeifer, Y., Matten, J. & Rabsch, W. (2009).** *Salmonella enterica* serovar Typhi with CTX-M β-lactamase, Germany [letter]. *Emerg Infect Dis* **15,** 1534.
16. **Sanders, W. E., Jr. & Sanders, C.C. (1997).** *Enterobacter* spp.: pathogens poised to flourish at the turn of the century. *Clin. Microbiol. Rev* **10,** 220–241.
17. **Soge, O., Adeniyi, B. & Robert, M. (2006).** New antibiotic resistance genes associated with CTX-M plasmids from Uropathogenic Nigerian *Klebsiella Pneumoniae*. *Journal of Antimicrobial Chemotherapy* **58,**1048-1053.
18. **Versalovic, J., Koeuth, T. & Lupski, J. R. (1991).** Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19,** 6823–31.

186 Table 1: *Pantoea agglomerans* and *Enterobacter* spp Antibiotic Resistance Phenotypes and

187 Determinants

Species	Specimen (clinical condition)	Antibiotic Resistance Phenotype	Trimethoprim and Tetracycline Genes	ESBL, PMQR, Integrons and Resistance Gene Cassettes
E. aerogenes (28K)	High Vaginal Swab (copious vaginal discharge)	amp, ams, cet, cfz, tet	*tet*(A)	Class 1 integron, *qac*Δ*E, sul1*
E. asburiae (85K)	Urine (urinary tract infection)	Amp, ams, cfz, sxt, tet	dfrA1, dfrA14, *tet*(A)	Class 1 and 2 integron, *qac*Δ*E, sul1*
E. cloacae (91b)	Catheter-tip	Amp, ams, cfz, sxt, tet	dfrA1, dfrA14, *tet*(E)	Class 1 integron, *qac*Δ*E, sul1*
E. cloacae (97K)	Urethral discharge	Amp, ams, cfz, sxt, tet	dfrA1, dfrA14, *tet*(E)	Class 1 and 2 integron, *qac*Δ*E, sul1*
E. cloacae (60K)	Semen	Amp, ams, cfz, sxt, tet	dfrA1, dfrA14, *tet*(E)	Class 1 integron, *qac*Δ*E, sul1*
E. cloacae (54K)	Blood (sepsis)	Amp, ams, cfz, sxt, tet	dfrA1, *tet*(E)	Class 1 integron, *sul1*
E. cloacae (56K)	Blood (Neonatal sepsis)	Amp, ams, cfz, sxt, tet	dfrA1, *tet*(E)	Class 1 integron, *qac*Δ*E, sul1*
E. cloacae (59K)	Catheter-tip	Amp, ams, cfz, sxt, tet	dfrA1, *tet*(E)	Class 1 integron, *sul1*
E. cloacae (64K)	Blood (Neonatal	Amp, ams, cfz, sxt,	dfrA1, *tet*(A)	Class 1 and 2

	sepsis)	tet		integron, *qac*Δ*E, sul1*
E. cloacae (213K)	Urine (urinary tract infection)	amp, ams, azt, cfz, fep, cet, caz, cip, gen, lev, tob, sxt, tet, ctx, fox	dfrA14, *tet*(A), *tet*(E)	CTX-M-15, *qnrB1*, *aac-(6')-lb-cr*, Class 1 and 2 integron, *aph*, *aadA1*, *qac*Δ*E, sul1*
Pantoea agglomerans (69K)	Blood (sepsis)	amp, ams, azt, cfz, fep, caz, cip, gen, pt, tob, sxt, tet, ctx, fox	dfrA14, *tet*(K)	CTX-M-15, TEM-1, *qnrB1*, *aac-(6')-lb-cr*, Class 1 and 2 integron, *aph*, *aadA1*, *cat1, qac*Δ*E, sul1*

188 Key: amp=ampicillin, ams=ampicillin/sulbactam, azt=aztreonam, cet=cephalothin, cfz=cefazolin, fep=cefepime,
189 caz=ceftazidime, cip=ciprofloxacin, gen=gentamicin, fox=cefoxitin, pt=piperacillin/tazobactam, tobramycin,
190 sxt=trimethoprim/sulfamethoxazole, lev=levofloxacin, tet=tetracycline, ctx=cefotaxime