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Emergence of Diarrhoeagenic *Klebsiella pneumoniae* Carrying *ast*A and *sen*B genes in Nigeria

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***Klebsiella pneumoniae* is increasingly being isolated from the stool of Nigerian patients. *K. pneumoniae* was isolated from stool samples submitted to two clinical laboratories in Nigeria from patients presenting with various symptoms of gastrointestinal tract infections, including diarrhoea. The authors characterized the virulence and antimicrobial resistance genes of these *K. pneumoniae* strains. Sixteen pure cultures of heavy growth of *K. pneumoniae* isolated from two facilities in Nigeria were subjected to susceptibility testing using a panel of antibiotic, with agar dilution method. Paired-end Illumina whole genome sequencing was completed using a NextSeq instrument. Virulence genes including *ast*A, *sen*B, and *gad* were found in 5 isolates. Multiple plasmid replicons were present; IncF and Col were common plasmids while others were IncR and IncY. Four different STs were found; ST914, ST1962, ST494, and novel ST. These isolates carried various important resistance genes to cephalosporins, fluoroquinolones, aminoglycosides, and so on, including blaCTX-M-15 in one of the isolates. Diarrhoeagenic *K. pneumoniae* is present, which is caused by plasmid-mediated virulence genes such as *ast*A, *sen*B, and *gad*. Fluoroquinolone and third generation cephalosporin resistance were discovered.**

Key words: *Klebsiella*, diarrhoea, virulence genes, antibiotics resistance, genomics, Nigeria.

INTRODUCTION

The common diarrhoea-inducing pathogens include, rotavirus, norovirus, diarrhoeagenic *Escherichia coli*, *Salmonella spp*., *Shigella spp*., and *Yersinia* species (Operario and Houpt 2011; Sjoling et al., 2015). *Klebsiella pneumoniae* can also cause diarrhoea, but most studies focus on extra intestinal infections. Although, *K. pneumoniae* occurs as a commensal in the intestine, it may induce diarrhoea through the production of toxins

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(Guarino et al., 1989; Panigrahi et al., 1991). Some of these diarrhoeagenic strains encode thermostable toxins similar to enterotoxigenic or enteroaggregative toxins of *E. coli*. Enteroaggregative heat stable toxin 1 (EAST-1) is encoded by *ast*A gene on a 60-MDa pAA plasmid (Telli et al., 2010). *ast*A produces a toxin that stimulates the production of high levels of cyclic guanosine monophosphate (cGMP) in cells such that sodium/ chloride co-transport is inhibited and absorption of water and electrolytes from the intestine at villus tips is reduced, resulting in diarrhoea (Telli et al., 2010). Similarly, *sen*B a plasmid-mediated gene has been described to produce the TieB enterotoxin in enteroinvasive and uropathogenic *E. coli* strains (Nataro et al., 1995; Touchon et al., 2009). *K. pneumoniae* isolation from stool is increasing in Nigerian hospitals, and they are usually ignored as native intestinal flora even when isolated as a pure culture from cases of diarrhoea in children and adults. The authors therefore investigated the presence of *K. pneumoniae* and characterized their virulence and antimicrobial resistance genes in Nigeria.

METHODS

Bacterial isolates

Sixteen pure cultures of heavy growth of *K. pneumoniae* isolated from stool of different patients from two clinical laboratory facilities in Nigeria (diagnosed with various diseases) were identified using cultural morphology, Gram reaction (Bartholomew and Mittwer, 1952), standard biochemical tests; indole, citrate, motility, methyl red, voges proskauer and sugars (Ewing, 1986; Barrows and Feltham, 1993), and API 20E strips (BioMérieux, Basingstoke, UK). Ethical approval was obtained for the study from Federal Capital Territory, Health Research Ethics Committee with approval number FHREC/2018/01/95/14-08-18, including informed written consent from the participants.

Antibiotic susceptibility testing

Susceptibility of all isolates to a panel of antibiotics classes in common clinical use in these hospitals were determined by the agar dilution method on Mueller–Hinton agar according to the recommendations of CLSI breakpoints (Wayne, 2018). All runs included the control organism *E. coli* (ATCC 25922).

Whole genome sequencing and bioinformatics

DNA was extracted using a QIAamp1 DNA Mini Kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. Paired-end Illumina whole genome sequencing was completed using a NextSeq instrument at the Quadram Institute Bioscience. Bioinformatics used an in-house pipeline hosted on an IRIDA instance; sequences were assembled with shovill and annotated with prokka and core snps identified with snippy. Furthermore, assemblies were used to search for plasmid content using the 'PlasmidFinder' tool hosted at the Centre for Genomic Epidemiology(https://cge.cbs.dtu.dk/services/PlasmidFinder), and for isolates likely to carry significant resistance genes in plasmids, 'plasmidSPAdes' was used to assemble likely plasmid contigs from the trimmed reads.

Comparative genomics

Strains belonging to sequence types implicated in globally disseminated disease were compared against completed genomes were available to determine relationships between sources of strains, Nigerian strains and others in global circulation. Reads were also mapped against reference strain HS11286, SNPs identified and phylogenetic relationships determined to determine whether clones in Nigeria are divergent or highly similar from those seen globally.

RESULTS AND DISCUSSION

In the 16 isolates of *K. pneumoniae,* 5 encoded virulence genes namely, *ast*A, *sen*B, and *gad*. All of these 5 isolates had the gad gene while only 2 had all the 3 genes (Figure 1). *ast*A encodes enteroaggregative *E. coli* heat-stable enterotoxin (EAST 1), *sen*B encodes TieB enterotoxin, while gad is a glutamate decarboxylase protein. The contribution to pathogenicity of gad has been debatable; this enzyme has been reported to be essential in the survival of enteric pathogens in the acidic conditions of the mammalian stomach (Lin et al., 1996). Two isoforms of glutamate decarboxylase (GAD) encoded by *gad*A and *gad*B are the most effective *E. coli* acid resistance system (De Biase et al., 1999). The major role of this system might be to facilitate the colonization of the intestines by commensal strains of *E. coli*. This is a housekeeping gene in *E. coli*, but not part of the *K. pneumoniae* core genome and here it was 31.3%.

EAST 1 has been identified as a plasmid-mediated enterotoxin of low molecular weight and associated with enteroaggregative *E. coli*. It shares about 50% protein identity with heat-stable enterotoxin (STa), and the gene has also been found in many enterotoxigenic *E. coli* strains (Contreras et al., 2011) and other members of Enterobacteriaceae such as *Salmonella* (Paiva de Sousa et al., 2001). The authors demonstrated the presence of the gene in different strains of *K. pneumoniae* (ST914 and ST1962) from two different geographic regions of Nigeria, and these patients presented with diarrhoea episodes. There are reports debating whether astA is sufficient to cause diarrhoea without other virulence factors in *E. coli,* but Soto et al. (2009) and Mirzarazi et al. (2015) reported that *E. coli* acquired this toxin to become a diarrhoea-causing agent, which may be the situation in these *K. pneumoniae* strains.

The two *K. pneumoniae* that encoded *ast*A also encoded *sen*B, a plasmid-mediated gene associated with enterotoxicity of enteroinvasive *E. coli* (EIEC) that codes the TieB protein. It has also been described to have some role in uropathogenic *E. coli*. Multiple plasmid replicons were present in all strains; however IncF and Col plasmids play a major role in the dissemination of these genes (Figure 1).

Susceptibility testing identified all 5 isolates with

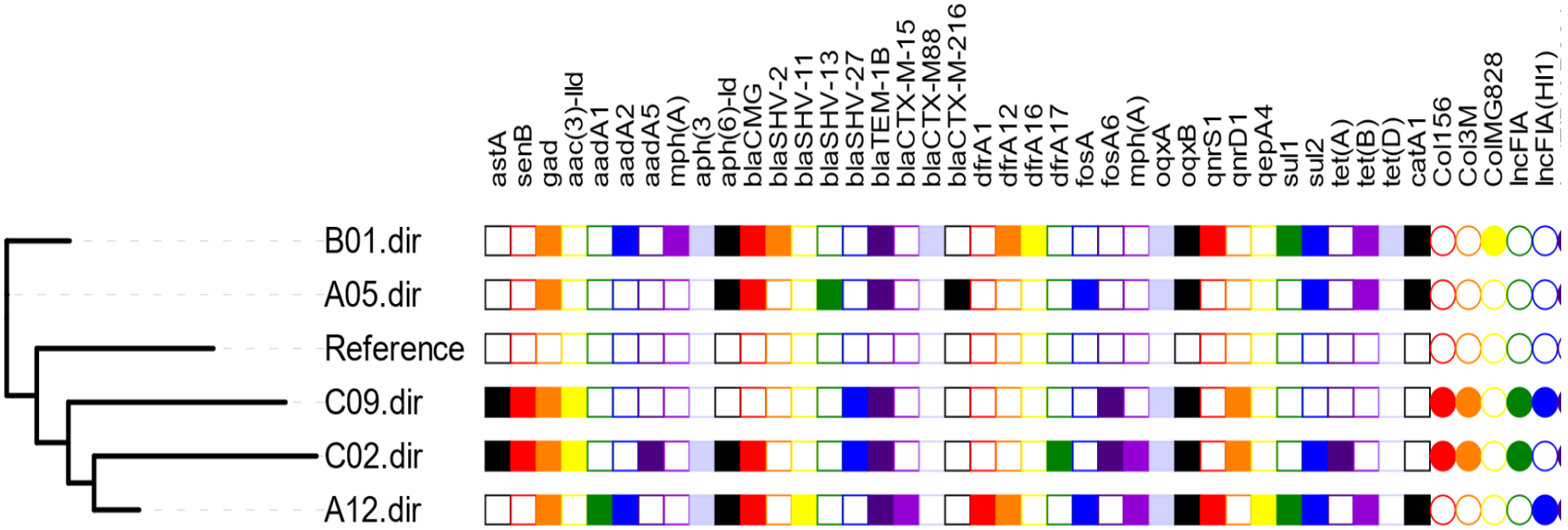

Figure 1. Relationship of the 5 *K. pneumoniae* strains carrying virulence genes (the first three filled boxes indicate presence of virulence genes). Filled boxes indicate antimicrobial resistance genes. Filled circles indicate plasmid replicons. Filled stars indicate antimicrobial resistance, MPM: Meropenem; CTX: Cefotaxime; CIP: Ciprofloxacin; GEN: Gentamycin; AK: Amikacin; COL: Colistin; AZM: Aztreonam.
Source: Authors

these virulence genes were sensitive to meropenem (MIC values of ≤ 0.03 µg/ml) while isolates with both *ast*A and *sen*B had low level resistance to ciprofloxacin and cefotaxime (MIC value of 8 µg/ml each), whereas other strains had high level resistance to ciprofloxacin (MIC value of >64 µg/ml). Resistance to colistin varied with MICs between 1 and >64 µg/ml. The isolates carried a range of important resistance genes to cephalosporins, fluoroquinolones, aminoglycosides, with the presence of various PMQR genes, aph(3'')-Ib, aac(3)-IId, variants of blaCTX-M including blaCTX-M-15 found in one of the isolates.

Conclusion

There is presence of diarrhoeagenic *K. pneumoniae* linked to carriage of plasmid mediated virulence genes such as *ast*A, *sen*B, and *gad* in diversity strains in Nigeria. This may represent a greater burden of diarrhoea caused by *K. pneumoniae* in future.

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CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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