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Citation for final published version:

Farzana, Refath, Jones, Lim S., Rahman, Md Anisur, Andrey, Diego O., Sands, Kirsty, Portal, Edward, Watkins, W. John , Pervin, Monira, Banerjee, Manisha and Walsh, Timothy R. 2019. Outbreak of hypervirulent multi-drug resistant *Klebsiella variicola* causing high mortality in neonates in Bangladesh. *Clinical Infectious Diseases* 7 , pp. 1225-1227. 10.1093/cid/ciy778

Publishers page: <http://dx.doi.org/10.1093/cid/ciy778>

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Title: Outbreak of Hypervirulent Multi-Drug Resistant *Klebsiella variicola* causing high mortality in neonates in Bangladesh

Authors: Refath Farzana,¹ Lim S. Jones,² Md. Anisur Rahman,⁴ Diego Andrey,¹ Kirsty Sands,¹ Edward Portal,¹ W. John Watkins,³ Monira Pervin,⁴ Manisha Banerjee,⁵ Timothy R. Walsh¹

Author affiliations: ¹Department of Medical Microbiology, Institute of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, UK, ²Public Health Wales Microbiology, University Hospital of Wales, Cardiff, UK, ³Institute of Infection and Immunity, School of Medicine, Cardiff University, Cardiff, UK, ⁴Department of Virology, Dhaka Medical College, Dhaka, Bangladesh, ⁵Neonatology, Dhaka Medical College, Dhaka, Bangladesh

Correspondence: R. Farzana, Department of Medical Microbiology, Institute of Infection and Immunity, School of Medicine, Cardiff University, UK (FarzanaR@cardiff.ac.uk, refath_farzana@yahoo.com)

Running title. MDR *K. variicola* outbreak in Bangladeshi NICU

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Abstract

We report an outbreak by MDR *K. variicola* clone (ST771) in a Bangladeshi neonatal unit from October, 2016 to January, 2017, associated with high mortality (54.5%). During the outbreak, *K. variicola* ST771 acquired MDR plasmid harbouring *bla*_{NDM-1}, linked to high exposure to ceftriaxone and amikacin in the NICU of DMCH.

Keywords. *Klebsiella variicola*, outbreak, NICU, Bangladesh

Introduction

Neonatal sepsis is a major challenge in health care settings of low and middle income countries (LMICs), a substantial proportion of sepsis leads to neonatal death which is further complicated by the emergence of multi-drug resistance (MDR) [1, 2]. Healthcare resources in Bangladesh allow limited surveillance of healthcare associated infections [3]. Given the paucity of data on MDR epidemics in Bangladesh, we investigated patients referred to Dhaka Medical College Hospital (DMCH). Blood cultures are not routinely taken at DMCH neonatal intensive care unit (NICU) and neonates are only followed up when empirical antibiotics fail with minimal antibiotic stewardship management or infection control. Herein we undertook a pilot study examining neonatal sepsis outcomes and report a MDR *Klebsiella variicola* outbreak on a NICU illustrated by an ideogram detailing the course of infection, phenotypic and genomic characterization and clinical outcome.

Methods

DMCH is a 2300-bed public teaching hospital, with approx. 3,500 neonatal admissions annually. Blood cultures were taken from neonates (<30 days old) from Oct 2016 to March 2017 presenting with clinical sepsis (study outline is represented in Supplementary Figure 1). During this period, a sepsis outbreak of *K. variicola* in the NICU was suspected during October, 2016 to January, 2017. Clinical history, prenatal risk associations [low birth weight (LBW), pre-term, premature rupture of membrane (PROM)], outcomes and demographic information were collected. Ethical approval (Ethical Review Committee; Dhaka Medical College [MEU-DMC/ECC/2017122]) was granted prior to the start of the study.

Blood stream infections were detected using BacT/ALERT 3D (bioMerieux, North Carolina, USA). All blood culture isolates were referred to Cardiff University, subcultured onto

chromogenic UTI agar (Liofilchem, Roseto, Italy) and identified by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Antimicrobial minimum inhibitory concentrations (MIC) were determined by agar dilution and interpreted according to EUCAST breakpoints (Figure 1) [4]. Whole genome sequencing (WGS) was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA). Multi-locus sequence type (MLST) loci, resistance genes and plasmid replicons were retrieved from online databases (<http://www.genomicepidemiology.org>). Single nucleotide polymorphism (SNPs) calling to WGS reads was performed by Snippy (3.2). Putative virulence factors were determined from publicly available reference genomes. Annotation of genes with 75% identity of reference sequences was performed by Geneious (10.2; Biomatters Ltd.). Capsular (*K*) loci were evaluated using Kaptive (<https://github.com/katholt/Kaptive>).

Pulsed field gel electrophoresis (PFGE) analysis was performed using *SpeI* or *S1* nuclease DNA digests. We performed in gel hybridization of *S1* treated gels with *bla*_{CTX-M} or *bla*_{NDM} probes [5]. Conjugation experiments were undertaken as previously described [6] with a NDM positive *K. variicola* donor and *Escherichia coli* J53 recipient (azide resistant). Transconjugants were investigated by in gel hybridization of *S1* digest with *bla*_{NDM} and Illumina MiSeq sequencing (Figure 1). In vivo pathogenicity of *K. variicola* was compared to hypervirulent *K. pneumoniae* (K1 ST23 liver abscess strain A58300) [7] in a *Galleria mellonella* model [5] (Live Foods UK Ltd.) (See Supplementary Material).

All statistics [Chi-square, independent t-test, Kaplan-Meier survival curves, log rank, odd ratios (ORs) and 95% confidence interval (CI)] were calculated using SPSS 23, with a cut-off *p* value of <0.01 for statistical significance.

Results

In total, 406 blood samples were taken from neonates (<30 days old) from the beginning of Oct 2016 to March 2017 of which 148 samples were sent for culture and sensitivity to DMCH Clinical Microbiology Laboratory. The percentage of positive cultures recovered at DMCH laboratory was 24.32% (36/148). Of 36 neonates [outbreak (n=14) and non-outbreak (n=22)], 83.3% were male; the mean (SD) age was 5.4 days (± 3.6) and 10 (27.8%) were lost to follow up. Non-outbreak strains are listed in Supplementary Table 1. The overall mortality for *K. variicola* sepsis was 54.5% compared to 33.3% for non-outbreak sepsis cases excluding discharged against medical advice (DAMA) [$p > 0.01$ (non-significant); OR=2.40; CI= 0.48-11.89] (Supplementary Table 2). *K. variicola* outbreak strain ST771 was first recovered from a premature (31 weeks), LBW baby admitted to DMCH on 21st of October, 2016 and was DAMA (Supplementary Table 3; Figure 1). This first isolate was negative for *bla*_{CTX-M-15} and *bla*_{NDM-1}. Subsequently two *K. variicola* from twins were identified on 27th October and had acquired a *bla*_{CTX-M-15} plasmid. One twin died and the other was DAMA (case 2 & 3; Supplementary Table 3). Five *K. variicola* (from cases four to eight; Supplementary Table 3) isolated from 7th November to 4th December had an increased antibiotic resistance profiles, with the acquisition of an additional plasmid carrying *bla*_{CTX-M-15} and *bla*_{NDM-1}. Interestingly, this plasmid was also shown to be positive for the resistance genes, *aph(3')-VIa*, *aadA1*, *qnrS1*, *bla*_{OXA-9}, *aac(6')-Ib* and *aac(6')-Ib-cr* when transferred to *Escherichia coli* J53 (Figure 1). WGS confirmed the presence of these genes in transconjugants (Figure 1). One death was recorded among these five cases; three recovered and one DAMA (mortality is 25%, excluding DAMA). During this period (14th to 24th of November), the same clone but with a different resistance genotype (*bla*_{CTX-M-15} positive, *bla*_{NDM-1} negative) was isolated for cases 9 to 14. The outbreak cases admitted after 7th December (cases 10 to 13; Supplementary Table 3) were infected with *bla*_{NDM-1}-negative *K. variicola*. The mortality for neonates infected with *bla*_{CTX-M} positive, *bla*_{NDM-1} negative *K.*

variicola was 71.42% (5/7) (not including patients who were DAMA). Data from the *G. mellonella* model shows that *K. variicola* ST771 was significantly more virulent than the *K. pneumoniae* comparator at 10^5 and 10^6 cfu/ml ($p < 0.0001$). Similar to the clinical outcome, NDM-1-positive were significantly less pathogenic than NDM-1-negative *K. variicola* isolates ($p < 0.000001$) (Supplementary Figure 2).

All neonates received multiple antibiotics during NICU admission, either in combination or consecutively, with overall antibiotic usage being: 62% ceftriaxone, 53% amikacin, 31% vancomycin, 16% gentamicin, 15% carbapenem, 9% azithromycin, 6% colistin, 6% metronidazole and 3% ciprofloxacin.

K. variicola isolates were clonal by PFGE. Polymorphism variants (SNPs from WGS on *K. variicola* isolated from case 2 to 14) ranged from 3 to 27 compared to *K. variicola* from case 1 (Supplementary Table 4) and sequence type of the clone was ST771. *K. variicola* antimicrobial susceptibility profiles and genomic data (resistance genes, virulence genes and plasmid replicons) are summarized in Figure 1. A total of 21 antimicrobial resistance genes were identified, demonstrating concurrence between antimicrobial MICs and presence of known resistance genes (*fosA* did not result in MICs above the resistance break point but this is expected in *Klebsiella* species) (Figure 1) [8]. WGS identified identical putative virulence factors (adhesins, siderophores, invasins etc.) [9] and a unique capsule locus in *K. variicola* ST771 (Figure 1, Supplementary Figure 3). *K. variicola* strains were deposited in NCBI under the accession no of PJOO00000000, PJQN00000000, PJQO00000000, QTZQ00000000, QTZR00000000, QTZS00000000, QTZT00000000, QTZU00000000, QTZV00000000, QTZW00000000, QTZX00000000, QTZY00000000, QTZZ00000000 and QUAA00000000.

S1 PFGE analysis revealed that *bla*_{CTX-M-15} was carried on ~134 kb and ~158 kb plasmids (Supplementary Figure 4), and *bla*_{NDM-1} on an ~134 kb plasmid. Transconjugants were found to be positive for *bla*_{NDM-1} located on a plasmid of ~134 kb (Supplementary Figure 5). The resistance profiles of transconjugants are shown in Figure 1.

Discussion

K. variicola bacteraemia in adults has been associated with higher mortality than *K. pneumoniae* [10]. This is the first reported outbreak of neonatal sepsis by MDR *K. variicola* at DMCH, Bangladesh which was associated with higher mortality than non-outbreak sepsis cases, despite the small sample, the confidence interval for the OR was 0.48-11.89, suggesting a likely difference (Supplementary Table 2). Three outbreak cases were lost to follow up because the parents chose to DAMA and it is likely that mortality was high amongst these neonates. Presumably, as the families could not cover hospital costs (economic status is shown in Supplementary Table 3; Bangladeshi demography are stated in Supplementary Figure 6) opted for DAMA; a frequent occurrence in resource limited settings [11].

The high mortality attributable to the *K. variicola* outbreak strain was supported by *G. mellonella* models, demonstrating high larval death rates compared to the hypervirulent ST23 K1 *K. pneumoniae* strain, A58300. The outbreak clone was associated with iron-acquisition (*ent*, *kfu*), adhesins (*fim*, *mrk*) and complement resistance (*traT*), described previously as bacterial virulence determinants [7,9,12] and the ST771 *K. variicola* capsular operon suggests a novel *K* locus (Figure 1, Supplementary Figure 3), however, the clone lacked hypermucoid phenotype and *rmpA* and *magA*, indicators for the hypervirulent K1 capsular phenotypes of *K. pneumoniae* [7]. It is likely that there are other, as yet unidentified, determinants of virulence responsible for the ST771 *K. variicola*.

K. variicola ST771 acquired antimicrobial resistance genes including *bla*_{NDM-1} (Figure 1) horizontally. Exposure to ceftriaxone and/or ceftazidime and amikacin in the NICU may have favoured the *bla*_{CTX-M-15} and *aph(3')*-*Vla/bla*_{NDM-1} acquisition (Figure 1, Supplementary Table 1). Acquiring a resistance plasmid is often associated with bacterial fitness costs [5]. This could explain the lower mortality observed clinically and in the in vivo virulence model for *bla*_{NDM-1} positive vs *bla*_{NDM-1} negative *K. variicola*. However, the spread of MDR virulent strains, in under-resourced hospitals, where antibiotics usage is empirical and infection control programs are suboptimal, poses significant challenges. Therefore, the hospital environment including patients and healthcare providers could be the potential reservoirs of MDR infections within the hospital and the community [2].

Author contributions: R. F. and T. R. W contributed equally to the work, T. R. W contributed to study concept, R. F., L. S. J. and T. R. W. prepared the draft of the manuscript, All authors have significant role in data collection and analysis.

Acknowledgments: We thank to Prof. Ismail Khan, Former Principal, Dhaka Medical College for access to collecting clinical data and to provide laboratory support in Dhaka; to Dr. Jonathan Tyrrell for useful suggestions on *Galleria* infection model. The authors wish to acknowledge Prof. Ana Cristina Gales (Universidade Federal Paulista, Brazil), for providing *K. pneumoniae* A58300 strain.

Funding: Farzana is the recipient of a Commonwealth Scholarship. Andrey is the recipient of a Swiss National Science Foundation (APM P300PB_171601) Fellowship. Sequencing and molecular data was supported by Cardiff University (CU).

Conflict of interest disclosures: We have no conflict to declare.

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Figure Title

Figure 1. Antimicrobial resistance phenotypes and genetic profile of outbreak strains

Figure legend. Abbreviations: Amp, ampicillin; Aug, amoxicillin-clavulanate; PT, piperacillin-tazobactam; CAZ, ceftazidime; CXT, cefotaxime; CRO, ceftriaxone; Cef, cefepime; IMP, imipenem; MEM, meropenem; CIP, ciprofloxacin; LV, levofloxacin; AK, amikacin; GEN, gentamycin; ST, trimethoprim-sulfamethoxazole; FOS, fosfomycin; CL, colistin. Antimicrobial sensitivity and absence of genes are indicated by white cells. Blue and red shades indicate phenotypic resistance and presence of genes by the isolates. Seven capsular genes were identified (*galF*, *rmlA*, *gnd*, *ugd* were identical to KL34 and *cpsACP* matches to KL153, *rmlB* to KL36 and *wzi* to KL114); 12 expected genes did not match to known capsular (KL) type. Capsular operon indicates a novel *K* locus in this clone. Entire capsular loci are depicted in Supplementary Figure 3. Siderophores (yersiniabactin, aerobactin, salmochelin and colibactin) were absent in this clone.

