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Short Communication

Complete genome arrangement revealed the emergence of a poultry origin superbug *Citrobacter portucalensis* strain NR-12

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ABSTRACT

Objectives: *Citrobacter* spp. are part of normal human and animal intestinal flora. *Citrobacter portucalensis* (*C. portucalensis*) is closely related to *Citrobacter freundii*, which is an emerging opportunistic nosocomial pathogen. The aim of this study was to retrieve colistin-resistant *Citrobacter* spp. from poultry in Bangladesh.

Methods: The *C. portucalensis* strain NR-12 was isolated from poultry droppings and subjected to antibiotic susceptibility testing. Complete genome analysis of NR-12 was performed followed by bioinformatics. It is believed that this is one of first reports of its kind of complete genome sequence of multidrug-resistant (MDR) *C. portucalensis* isolated from veterinary samples.

Results: The *C. portucalensis* strain NR-12 showed resistance to polymyxin, sulfonamide, tetracycline, fluoroquinolone, and macrolide. Its complete genome revealed 13 acquired antimicrobial resistance gene markers (AMRs) conferring resistance to eight different antibiotic groups: *dfr*A12 (trimethoprim); *sul*1 and *sul*2 (sulfonamide); *mph* (A) (macrolide); *tet* (A) (tetracycline); *qnr*S1 and *qnr*B13 (fluoroquinolone); bla_{CMY-39} (extended-spectrum β-lactamase (ESBL)), $bla_{TEM-176}$ (non-ESBL) and *aad*A2, *aph* (3′)-Ia, *aph* (3″)-Ib, *aph* (3′)-Ic, *aph* (3′)-Id, *str*A, *str*B) (aminoglycoside). The genome possessed a class 1 integron (IntI1) gene cassette harbouring four different antibiotic resistance genes (*dfr*A12, *aad*A2, *sul*1, *mph* (A)). The organisation of class 1 integron (IntI1) carrying MDR determinants in *C. portucalensis* strain NR-12 was also first reported here. Colistin-resistant genes such as *mgr*B, *pho*P, *pho*Q, *pmr*A, *pmr*B, *ept*B and *arn*B were also present within NR-12.

Conclusion: *C. portucalensis* NR-12 was resistant to eight different antibiotics from six antimicrobial groups. To formulate a control strategy, it is important to understand this resistant mechanism.

1. Introduction

The emergence of multidrug-resistance (MDR) in Gram-negative bacteria, especially Enterobacteriaceae, and a paucity of new antibiotics is now a prime global concern. *Citrobacter portucalensis* is a Gram-negative facultative anaerobic bacterium of the Enterobacteriaceae family and two studies have recently identified it as a new species [1,2].

Transmission of *Citrobacter* spp. through the faecal-oral route is common like other Enterobacteriaceae. As a zoonotic pathogen, *Citrobacter* spp. can be transmitted to humans from poultry sources during handling of eggs, cooked or uncooked meat processing, and carcasses in the slaughterhouse [3]. As a low virulent bacterium, *Citrobacter* spp. can persist for long periods of time in host populations and can accumulate antimicrobial resistance (AMR) determinants, which can make them more virulent [4]. Multidrug-resistant and extended spectrum β-lactamase (ESBL)-producing *Citrobacter* spp. were reported by Shrestha et al. at a prevalence of 26.1% whereas Kanamori et al. reported 19.3% prevalence of ESBL-producing *Citrobacter* spp. [5,6].

The poultry industry is an integral part of the farming system in Bangladesh and provides the largest source of meat and eggs [7,8]. The lack of hygiene knowledge in cases of raw poultry processing and easy accessibility of all antibiotics, along with excessive misuse of antibiotics against bacterial, fungal, viral and parasitic infections in the poultry industry have increased the risk of antibiotic resistance in Bangladesh. To date, there has been no conclusive global report on MDR *C. portucalensis* in poultry and nothing is

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known about its virulence. The present study used a systematic approach to explore the antimicrobial resistant patterns of retrieved *Citrobacter* spp. in poultry and conclusive complete genome data of *C. portucalensis* strain NR-12 (GenBank accession number MZZE00000000) of poultry origin in Bangladesh. A complete genome sequence of the isolate was performed to detect integron and transposable elements inherited with AMR. Although the presence of class 1 integron has already been reported in clinical *Citrobacter freundii* isolates [9], The current study presents the first report of the organisation of class 1 integron with other transposable elements carrying AMR determinants in the *C. portucalensis* strain NR-12 of poultry droppings.

2. Materials and methods

2.1. Sample collection and preparation

Samples were collected from three different poultry farms located in the Narayanganj district (Latitude: 23° 47′ 35.16″ N; Longitude: 90° 31′ 0.12″ E) of Bangladesh. A total of 23 different poultry samples – such as swabs from cloacae, droppings, handlers, feed, and eggs – were collected in buffered peptone water (Oxoid Limited, England) and immediately stored in insulated ice-boxes at 4 °C until transported to the laboratory. Although a number of isolates were retrieved on MacConkey agar, Xylose Lysine Deoxycholate agar (XLD agar) and Nutrient agar plates, the present study focused on retrieval of colistin-resistant *Citrobacter* spp.

2.2. Susceptibility testing

All of the colistin-resistant isolates from poultry sources were subjected to antibiotic susceptibility testing by the Kirby-Bauer agar disc diffusion method to determine their MDR pattern. This included colistin using 11 antimicrobial drugs belonging to eight antibiotic groups: polymyxin (polymyxin B (PB), colistin sulfate (CS)); sulfonamide (trimethoprim (TM)); cephalosporin (cephalexin (CL), cefotaxime (CTX)); carbapenem (imipenem (IMP)); tetracycline (oxytetracycline (OT), doxytetracycline (DXT)); aminoglycoside (gentamicin (CN)); fluoroquinolone (levofloxacin (LEV)); and macrolide (azithromycin (AZM), which are widely used in poultry farms.

The microdilution method was used to determine the level of minimum inhibitory concentrations (MIC) of colistin sulfate (Potency: 21954.11 U/mg; Lot No: STB1505002; Shangdong Lukang Phramaceutical Co. Ltd, China). The MIC breakpoint of colistin sulfate for Enterobacteriaceae is $R > 2\,\mu g/mL$, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (version 6.0, 2016). *Escherichia coli* (*E. coli*) DH5α ATCC 53868 was used as the control strain in antimicrobial susceptibility testing.

2.3. Selection of C. portucalensis *strain NR-12 for whole genome sequencing*

One of the poultry MDR *Citrobacter* isolates – *C. portucalensis* strain NR-12 – was chosen for whole genome sequencing to reveal the antimicrobial resistance markers (AMRs). Whole genomic DNA extraction was performed using QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Plasmid DNA was extracted by using PureYield™ Plasmid Miniprep (Promega, USA) according to the manual instructions. *E. coli* V517 was used as extraction and plasmid control. Sequencing of *C. portucalensis* strain NR-12 based on RAPD typing as well as plasmid typing and 16S rRNA sequencing was performed using Ion Torrent: Proton/PGM sequencing system (ThermoFisher Scientific, India). The generated data were transferred to Ion Torrent server where the data were run through signal processing and base calling algorithms to produce individual reads mate pair sequencing.

2.4. Data analysis

Raw read pre-processing and quality control (QC) were performed with FastQC [10], which allowed assessment of diverse quality metrics of the sequencing data. Trimmomatic was used to remove the adapters and low-quality reads, and reads were assembled into contigs using the SPAdes v. 3.1.0 de novo assembler tool's pipeline and using k-mer value 21. In silico genome-to-genome comparison was assessed by average nucleotide identity (ANI) calculated by using jSpecies [11]. The assembled genome of *C. portucalensis* strain NR-12 was annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and Rapid Annotation using Subsystem Technology (RAST) [12].

To reveal the presence of all AMRs in *C. portucalensis* strain NR-12, high throughput sequence data were analysed by CARD 3.0.0 (The Comprehensive Antibiotic Resistance Database), which is a rigorously curated collection of known resistance determinants and associated antibiotics. ResFinder 3.0, a Center for Genomic Epidemiology tool, was used to identify the acquired AMRs in the genome.

2.5. GenBank accession numbers

The 16S rRNA gene sequences of the isolates were submitted under the accession numbers MH429835-MH429838. The draft genome sequence of strain NR-12 is available under the accession number MZZE00000000.

3. Results and discussion

3.1. Multidrug resistant profile of colistin-resistant isolates

Sixty-two isolates from the Enterobacteriaceae group (*Salmonella* spp., *E. coli*, *Citrobacter* spp., *Proteus* spp.) were retrieved from 23 different poultry samples, and 16 poultry isolates out of these 62 (26%) were found to be resistant to colistin sulfate. Among the 16 isolates, five were identified as presumptive *Citrobacter* spp. (NR-12, NR-26, NR-27, NR-28, NR-46) through a series of biochemical tests such as the indole test, methyl red test, Voges-Proskauer test, nitrate reduction test, catalase test, oxidase test and urease test (Bergey's Manual of Determinative Bacteriology, 2012) [13]. The MDR pattern of the presumptive *Citrobacter* spp. was determined according to Clinical Laboratory Standards Institute (2016) and EUCAST (2016) guidelines. All of these isolates showed resistance to polymyxin (CS, PB); sulfonamide (TM); tetracycline (OT, DXT); aminoglycoside (CN); fluoroquinolone (LEV); and macrolide (AZM) antimicrobial groups. Minimum inhibitory concentrations (MIC) of colistin sulfate was determined ranging from 2 to 512 μg/mL and the isolates showed variation in their MIC pattern. Two of the isolates (NR-12 and NR-46) showed moderate levels of MIC at 16 μg/mL and three of the isolates (NR-26, NR-27 and NR-28) showed low levels of MIC at 8 μg/mL.

3.2. Genotyping and selection of C. portucalensis *strain NR-12 for whole genome sequencing*

Presumptive *Citrobacter* spp. isolates were genotypically divided into two genotypes using Random Amplified Polymorphic DNA (RAPD) polymerase chain reaction (PCR). The 16S rRNA gene sequence analysis of the representatives of the RAPD genotypes identified the isolates as *C. portucalensis* (NR-12 and NR-46) and *Citrobacter werkmanii* (NR-26, NR-27 and NR-28).

Colistin resistance with MDR potent *Citrobacter* is not well reported. *C. portucalensis* strain NR-12 was resistant to eight antibiotics of six different antimicrobial groups and showed moderately high levels of MIC at 16 μg/mL to CS and contained a single large plasmid sized 54.2 Kb. Therefore, *C. portucalensis* strain NR-12 was chosen for complete genome sequencing for genome-wide analysis of AMRs as well as transposable genetic elements.

4. Acquired antimicrobial resistance gene marker analysis in *C. portucalensis* strain NR-12

Complete genome analysis of *C. portucalensis* strain NR-12 showed 13 AMR gene markers conferring resistance to eight different groups of antibiotics such as aminoglycoside (*aad*A2, *aph* (3′)-Ia, *aph* (3″)-Ib, *aph*(3′)-Ic, *aph*(3′)-Id *str*A, *str*B); the plasmidic cephalosporinase $bla_{\text{CMY-39}}$; the non-ESBL gene $bla_{\text{TEM-176}}$; trimethoprim (*dfr*A12); tetracycline (*tet* (A)); fluoroquinolone (*qnr*S1, *qnr*B13); sulfonamide (*sul*1, *sul*2); and macrolide (*mph* (A)) (Supplementary Table 1). The efflux pump genes *emr*A and *emr*B, encoding fluoroquinolone resistance, were also detected. Antimicrobial resistance was analysed using RGI 4.2.0 (Resistance Gene Identifier), CARD 3.0.0 (The Comprehensive Antibiotic Resistance Database) and Resfinder 3.0 (Fig. 1).

A classical class 1 integron, containing a *qac*EΔ1-*sul*1 element in the conserved 3′ region, was retrieved within the contig 27 (GenBank: MZZE01000027.1) of the complete genome of *C. portucalensis* strain NR-12. The variable region of this integron harboured *dfr*A12 and *aad*A2 gene cassettes (Fig. 2(A)). Presence of these genes in class 1 integrons along with other genes has already been reported in several bacteria such as *C. freundii* [9], *E. coli* [14] and *Salmonella enterica* [15]. On the other 3′ region of this contig, the insertion sequences IS110 and IS6 were found in inverse orientation forming a transposon-like region. Finally, downstream of IS6 and in inverse orientation, the genes encoding macrolide 2′-phosphotransferase, MFS transporter, and Mph(A) family occurred respectively.

Extended-spectrum β-lactamases (ESBL) are a group of plasmid-mediated, diverse and rapidly evolving enzymes that confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and the monobactam aztreonam, which is presently a noteworthy challenge in the treatment of hospitalised and community-based patients [16]. An ESBL $bla_{\text{CMY-39}}$ was detected in contig number 8 along with a non-ESBL gene $bla_{\text{TEM-176}}$ responsible for ampicillin resistance, which is flanked by *est*P gene encoded Esterase EstP protein catalysing the hydrolysis of p-nitrophenyl esters of tween detergents and five transposase genes in the upstream of contig number 18 (Fig. 2(B)) in *C. portucalensis* strain NR-12. The sulfonamide-resistance genes *sul*1 and *sul*2 were located in contigs 27 and 30, respectively [17]. However, *tet* (A), *qnrS1 and qnrB13* were located in the contigs 18, 35 and 2, respectively. This isolate also contained integrase and insertion sequences (IS) in various contigs. Some *Citrobacter* spp. have chromosomal antibiotic resistance genes, which can be transferred via different mobile genetic elements [1,18]. The presence of plasmid class 1 integron with other integrase and transposes is responsible for harbouring various resistant genes within this isolate.

The RAST analysis disclosed the presence of several genes such as *mgr*B, *pho*P, *pho*Q, *pmr*A, *pmr*B, *ept*B and *arn*B, which are responsible for colistin resistance in *C. portucalensis* strain NR-12. The *arn*B gene that can covalently modify the lipid A moiety of bacteria Lipopolysaccharide (LPS) through catalysing the incorporation of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the LPSs, was located in the contig 5 (GenBank: OPX50993.1) of strain NR-12. The *ept*B gene located within the contig 1 (GenBank: OPX53284.1) can further modify the lipid A through the addition of phosphoethanolamine (PEtN). Both of these positively charged groups (PEtN and L-Ara4N) reduce the binding affinity of colistin to the LPS through neutralisation of the negative charges of LPS [19].

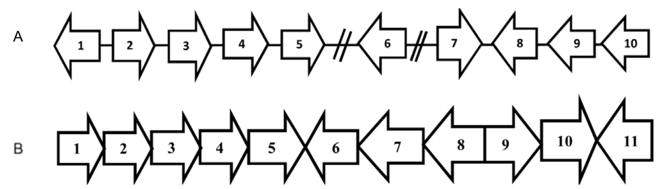

Fig. 2. (A) Genetic makeup of the IntI1 cassette in contig 27 (1. IntI1; 2. DfrA12; 3. AadA2; 4. QacE delta 1; 5. Sul1; 6. IS110 family transposase; 7. IS6 family transposase; 8. macrolide 2′-phosphotransferase; 9. MFS transporter; 10. Mph(A) family macrolide 2′-phosphotransferase). (B) Genetic makeup of contig 18. $bla_{\text{TEM-176}}$ is flanked by five transposase genes in the upstream and a resolvase gene in the downstream (1. IS3 family transposase; 2. Transposase; 3. IS110 family transposase; 4. Restriction endonuclease; 5. EstP; 6. Tn3 family transposase; 7. Hypothetical protein; 8. Tn3 family transposase; 9. Transposase; 10. $bla_{\text{TEM-176}}$; 11. Resolvase).

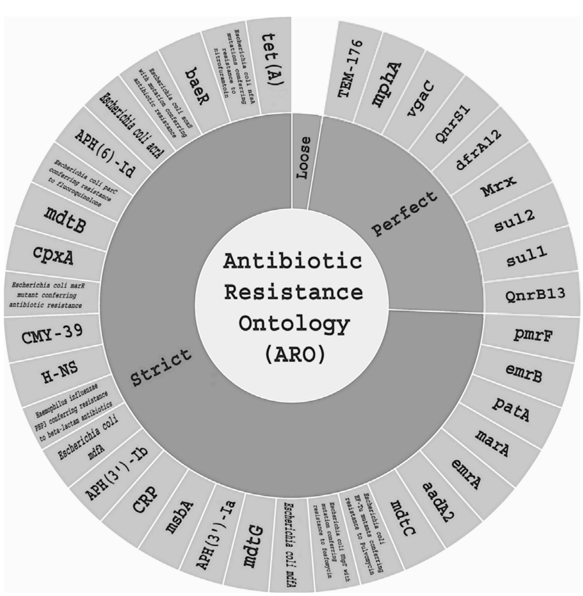

Fig. 1. Antibiotic Resistance Ontology (ARO) of *Citrobacter portucalensis* strain NR-12 based on RGI (resistance gene identifier) criteria (perfect, strict, complete genes only) using RGI 4.2.0 (https://card.mcmaster.ca/analyze/rgi).

5. Conclusion

The poultry industry in Bangladesh has recently been struggling with antibiotic resistome development due to overuse of antibiotics in animal food such as for poultry. The problem of AMR is a worrying issue to developing countries like Bangladesh. To date there is no conclusive data on MDR *C. portucalensis* of poultry origin in Bangladesh. Being an opportunistic pathogen and expressing the colistin resistance trait along with MDR potentiality, the *C. portucalensis* strain NR-12 isolated from droppings was subjected to genome-wide analyses. Both the results of in silico analysis of whole genomic data and experimental assay indicated extensive AMR harbourage along with the presence of class 1 integron and different classes of transposases, representing *C. portucalensis* strain NR-12 as an emerging superbug.

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Competing interests

The authors declare that they have no competing or conflict of interests.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2019.05.031.

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