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First report on the detection and whole genome sequencing analysis of virulent and multidrug resistant *Enterobacter cloacae* ST84 strain harboring *qacE*-like disinfectant resistance gene isolated from bovine mastitis
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First report on the detection and whole genome sequencing analysis of virulent and multidrug resistant *Enterobacter cloacae* ST84 strain harboring *qacE*-like disinfectant resistance gene isolated from bovine mastitis

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Abstract

Enterobacter cloacae is infrequently reported in milk samples and bovine mastitis. Here we report for the first time, the detection and phylogenomic analysis of an *Enterobacter cloacae* strain isolated from a raw milk sample obtained from a clinical mastitis-infected cow in Bangladesh using whole genome sequence analyses. *Enterobacter cloacae* strain BR-MHR107Ecl belonged to sequence type ST84, belonging to a rarely reported ST in the *Enterobacter* genus. Phylogenomic analyses revealed that the strain is closely related to a strain previously isolated in Bangladesh. The closest relative to BR-MHR107Ecl was a strain isolated in 2016 from a human rectal swab sample in Nigeria. The BR-MHR107Ecl strain co-harbored beta-lactamase genes including *bla*_{DHA-1} and *bla*_{CMH-3-like}. Other antimicrobial resistance genes were detected and included aminoglycoside resistance genes (*aph*(3'')-Ib-like, *aph*(6)-Id), fluoroquinolone resistance gene (*qnr*B4) and sulphonamide resistance gene (*sul*1). The genome harbored an additional disinfectant resistance gene (*qacE*-like). Several

plasmid replicons, including Col(pHAD28), IncFIA(HI1), IncFIB(K), IncR, and repB(R1701) were detected. Virulence genes encoding for heat-stable enterotoxin EAST-1 (*astA*) and lipoprotein NlpI precursor (*nlpI*) were detected. The multidrug resistance and virulence genes repertoire of BR-MHR107Ecl and its isolation from bovine mastitis milk render it a potentially significant public health concern, highlighting its emerging threat to dairy herds and underscoring the importance of genomic surveillance in food animals and non-clinical environment to detect emerging pathogens. This study will contribute to understanding the transmission of *E. cloacae* from farm-to-fork which requires collaborative and comprehensive One health approaches. The findings will help monitor the global dissemination of antimicrobial resistance in livestock-associated *Enterobacter cloacae* in the One Health context.

Key words: antimicrobial resistance, disinfectant, *Enterobacter cloacae*, genome sequence, bovine mastitis, multidrug, raw milk, ST84, *bla*_{DHA-1}, *bla*_{CMH-3}, *qacE*-like, *qnrB4*

Bovine mastitis, the inflammation of the mammary gland in cattle, is a commonly reported serious disease affecting the dairy industry worldwide (Zadoks and Fitzpatrick, 2009; Reshi et al., 2015; Tommasoni et al., 2023). Furthermore, it is of increasing importance for public health due to the devastating impacts on dairy farms with costly treatments and severe economic losses (Gröhn et al., 2005; Bar et al., 2008; Hertl et al., 2010; Cheng and Han, 2020). The presence of various virulent and drug-resistant Gram-positive and Gram-negative pathogens in milk samples of bovine mastitis dairy cattle may lead to potential public health risks associated with zoonotic transmission to humans (Jiménez-Guerra et al., 2020). Bovine mastitis is a complex disease caused by multiple etiological agents, including both Gram-positive and Gram-negative pathogens (Oikonomou et al., 2014; Derakhshani et al., 2018; Hoque et al., 2020; Pascu et al., 2022). Among the emerging opportunistic mastitis-causing pathogens, the prevalence of *Enterobacter* species have notably increased in bovine clinical mastitis cases, including *Enterobacter cloacae* (12.6%) and *Enterobacter sakazakii* (1.1%) (Abegewi et al., 2022). Recent advances and application of genomic surveillance tools have contributed in the accurate and fast detection and monitoring of various mastitis-causing and foodborne pathogens.

Among Gram-negative bacteria, *Enterobacter* can be found in the farm environment (e.g. the soil, manure, and animal bedding) and cause bovine mastitis. They are also recognized as opportunistic human pathogens, causing nosocomial infections in clinical settings (Leister and Hügler, 2022; Schukken et al., 2012; Davin-Regli and Pagès, 2015). Members of the *Enterobacter cloacae* complex (ECC) are often multidrug-resistant, and therefore, they are considered of high importance to public health. Antimicrobial agents are commonly used in dairy production systems worldwide. The over-usage, non-prudent application of antimicrobial agents in dairy farms, and the indiscriminate application of antibiotics in the treatment of bovine mastitis drive and contribute to the emergence of multidrug-resistant bacteria (Kovačević et al., 2022; Naranjo-Lucena and Slowey, 2023; Santos et al., 2023). In Bangladesh, *Escherichia coli* strain of a previously unreported sequence type (ST-13054) from raw milk samples collected from cows with mastitis was characterized using whole genome sequencing (Rahman et al., 2023). In addition, we recently reported and characterized methicillin-resistant *Staphylococcus aureus* (Rahman et al., 2024 a), *E. faecalis* and *E. faecium* including multidrug resistant isolates from bovine mastitis using whole genome sequencing-based approach (Rahman et al., 2024 b).

It was previously reported that coliforms such as *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, and *Klebsiella* spp. are responsible for approximately 50% of acute clinical mastitis cases (Hogan and Smith, 2003). *Enterobacter* are rod-shaped facultative anaerobes which are usually motile (flagellated), oxidase negative, non-spore forming Gram-negative bacteria. The genus *Enterobacter* includes a heterogeneous group of bacteria in the order *Enterobacterales*, *Enterobacteriaceae* family within the class *Gammaproteobacteria*. The *Enterobacter* genus is diverse, being composed of 22 species (Davin-Regli et al., 2019). These have been isolated from a wide range of environments such as soil, water, vegetation, as well as vertebrates and invertebrates (Davin-Regli et al., 2019; Moxley, 2022), and from food production facilities as previously reported (Shaker et al., 2007; Mladenović et al., 2021).

The capacity of *Enterobacter* spp. to produce extended spectrum β -lactamases (ESBLs) and carbapenemases conferring resistance to multiple antimicrobial agents (Bush, 2010) is a major challenge in managing *Enterobacter* infections (Yaikhan et al., 2025). *Enterobacter cloacae* (*E. cloacae*) is a clinically significant pathogen ranking the third pathogen among all *Enterobacteriaceae* in causing healthcare-associated infections due to its multidrug antimicrobial resistance, presenting a considerable challenge in the treatment of infections (Davin-Regli and Pagès, 2015).

As opportunistic pathogens, they can cause clinically highly significant nosocomial infections such as pneumonia, urinary tract infections and septicemia in humans (Mezzatesta et al., 2012; Ganbold et al., 2023). Members of the ECC belong to the ESKAPE group of pathogens and have been classified as high priority pathogens by the WHO (Tacconelli et al., 2018; De Oliveira et al., 2020).

The detection of coliform-associated, antimicrobial resistant and mastitis-causing *E. cloacae* isolated from livestock and dairy farm environments has been reported worldwide (Bradley et al., 2007; Nam et al., 2009; Botrel et al., 2010; Abegewi et al., 2022). However, scarce data are available on the prevalence and genomic characterization of *E. cloacae* and coliform mastitis-causing pathogens in dairy cows in Bangladesh. In the present study, we report the first detection, isolation, antimicrobial resistance, and genomic characterization of an *E. cloacae* ST84 strain isolated from raw milk sample obtained from a mastitis dairy cow in Bangladesh.

Material and methods

Ethics statement

The study was approved by the Animal Welfare and Experimentation Ethics Committee, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh under reference number AWEEC/BAU/2020(44), protocol entitled “Development of polyvalent mastitis vaccine and probiotics for prevention of mastitis in cows” under the project entitled “Polyvalent Vaccine Development for Mastitis in Dairy Cow”.

Sample collection

In the current study, raw milk samples were collected from female cows (32 months old, Red Chittagong breed) from different locations in Mymensingh, Bangladesh in April 2021. Samples were tested using the California mastitis test and Lactoscan Combo's SCC (Milkotronic Ltd, Zagora, Bulgaria) according to the manufacturer's protocol as previously reported (Rahman et al., 2024 a, b).

Bacterial isolation and identification

E. cloacae were isolated and identified as previously reported (Rahman et al., 2023, 2024 a, b). In brief, raw milk sample (500 µl) was used to inoculate nutrient broth (10 ml) and tubes were incubated for 18 h at 37°C. Subsequently, cultures were streaked on brain heart infusion agar (HiMedia, Maharashtra, India), MacConkey and Edwards blood agar media. The inoculated plates were incubated overnight at 37°C and sub-cultured to isolate pure colonies which were identified using Gram staining and were subjected to further analysis as previously reported (Rahman et al., 2023, 2024 a, b). Pure single colonies were identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry using MALDI Biotyper (Bruker Daltonics, Bremen, Germany), and the isolates with a log score ≥ 2 indicate species identification as previously reported (Nonnemann et al., 2019).

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed using the Kirby-Bauer disk diffusion method (Oxoid Ltd., Hampshire, UK) as previously reported (Rahman et al., 2024 a) against different antibiotics across different classes including penicillins – ampicillin (10 µg), amoxicillin-clavulanate (30 µg), meropenem (10 µg), ceftazidime (10 µg), penicillin G (10 IU); cephamycins – cefoxitin (30 µg); macrolides – azithromycin (15 µg); quinolones – ciprofloxacin (5 µg); phenicols – chloramphenicol (10 µg); aminoglycosides – amikacin (30 µg), gentamicin (10 µg); phosphonic acid antibiotics – fosfomycin (50 µg); sulfonamides – sulfamethoxazole-trimethoprim (1.25/23.75 µg); and tetracyclines – tetracycline (30 µg), and doxycycline (30 µg). *E. coli* ATCC 25922 was used as a negative control strain and the results were interpreted according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2020; Humphries et al., 2018). A multidrug-resistant isolate was defined as an isolate which was non-susceptible to at least one agent in three or more different antimicrobial categories as previously reported (Magiorakos et al., 2012).

Genomic DNA extraction and whole genome sequencing analysis

The identified *E. cloacae* isolate was subjected to whole genome sequencing as previously described (Rahman et al., 2023, 2024 a, b). One isolate was submitted to Invent Technology Ltd. (Banani, Dhaka, Bangladesh) for whole genome sequencing analysis as previously reported (Rahman et al., 2023 a, 2024 b, c). In brief, DNA was extracted from overnight cultures grown in nutrient broth at 37°C using the genomic DNA Purification Kit (Promega, WI, USA). Sequencing libraries were prepared using the Nextera XT library preparation kit (Illumina Inc., CA, USA) and sequencing was performed using the Illumina NextSeq 550 platform (Illumina Inc., CA, USA) using the NextSeq 500/550 High Output Kit v2.5 (300 cycles).

Genome assembly, annotation, MLST, and detection of virulence and antimicrobial resistance genes

Default parameters were used for all software unless otherwise specified. Quality control such as trimming and filtering was performed using trimmomatic while sequence assembly was performed using SPAdes v3.13.0 integrated in the ASA³P pipeline (v1.4.0) (Schwengers et al., 2020). Contigs, excluding those smaller than 200 bp, were uploaded to NCBI and annotated using the NCBI Prokaryotic Genome Annotation Pipeline v6.0 (Tatusova et al., 2016). Multilocus sequence type (MLST) was determined using PubMLST (Jolley et al.,

2018) and antimicrobial resistance genes were detected using ResFinder (v4.0) (Bortolaia et al., 2020) database hosted by the Centre for Genomic Epidemiology (CGE) (<http://cge.cbs.dtu.dk/services/ResFinder-4.0/>). Plasmid replicon types were identified using PlasmidFinder 2.1 on the CGE website (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>). The assembled genomes were further analyzed for mobile genetic elements (MGE) including insertion sequences using ISFinder (<https://isfinder.biotoul.fr/>). Virulence genes were analyzed using VirulenceFinder 2.0 available at the CGE website (<https://cge.cbs.dtu.dk/services/VirulenceFinder/>).

Phylogenetic comparison of BR-MHR107Ecl with *Enterobacter cloacae* ST84 isolates

All *E. cloacae* ST84 isolates (Table 1) available at the PubMLST database (as of 24th of September 2024) were downloaded as contigs. Core genome-based phylogeny was determined using the ParSNP tool included in the HARVEST package. Core-Single nucleotide polymorphisms (SNPs) were exported from the ParSNP output and analyzed using the snp-dists tool v. 0.8.2 included in the online version of Galaxy (https://usegalaxy.org/root?tool_id=toolshed.g2.bx.psu.edu/repos/iuc/snp_dists/snp_dists/0.8.2+galaxy0) to determine the number of SNPs. The core genome phylogenetic tree was annotated using the tool iTOL v. 1.8 and amended using Inkscape v. 0.9.1 (<https://inkscape.org/release/inkscape-0.91/?latest=1>).

Results

In the present study, randomly selected lactating cows were tested for the presence of *Enterobacter* spp. in raw milk samples (n=423). Results revealed that 189 cows (44.68%) were mastitis-positive, of which 74 cases (17.49%) were classified as clinical mastitis and 115 cows (27.19%) were classified as sub-clinical mastitis as previously reported (Rahman et al., 2023, 2024 a, b). The detection and isolation of *Enterobacter* spp. was performed using conventional microbiological methods and presumptive isolates were subsequently confirmed using MALDI-TOF mass spectrometry as previously reported (Rahman et al., 2024 b, c). The prevalence rate of *E. cloacae* was 9.52% (18 out of 189 samples) in the tested milk samples in the present study. One *E. cloacae* isolate was chosen for whole genome sequencing analysis and genetic characterization.

Phenotypic antimicrobial resistance profiling revealed that BR-MHR107Ecl strain was resistant to amoxicillin-clavulanate, ceftazidime, ampicillin, penicillin G, cefoxitin, and azithromycin. BR-MHR107Ecl strain was sensitive to chloramphenicol, sulfamethoxazole-trimethoprim, amikacin, gentamicin, fosfomycin and tetracycline. Additionally, BR-MHR107Ecl strain was intermediate-susceptible to ciprofloxacin, doxycycline and meropenem when tested using the disk diffusion method rather than broth microdilution method to confirm the intermediate-susceptibility to ciprofloxacin, doxycycline and meropenem. The findings revealed that BR-MHR107Ecl strain was non-susceptible to three different antimicrobial categories (extended-spectrum cephalosporins, carbapenems, and fluoroquinolones), and therefore BR-MHR107Ecl was designated as multidrug-resistant strain as previously reported (Magiorakos et al., 2012).

The identification and differentiation of members in the ECC remains difficult when using conventional microbiological and phenotypic methods. Here we report the first whole genome sequence of an *E. cloacae* strain BR-MHR107Ecl isolated from a cow with bovine mastitis. The assembled draft genome of the BR-MHR107Ecl strain spans approximately 4.7 Mb in size with a guanine (G) and cytosine (C) content of 55.02%, comprising 176 contigs and 458× sequencing

coverage with contig L_{50} and N_{50} values of 20 and 79,623 bp, respectively.

In the current study, strain BR-MHR107Ecl was isolated from a domestic animal and the resistome analysis indicated the presence of several antibiotic resistance genes including the beta-lactam resistance genes *bla*_{DHA-1} and *bla*_{CMH-3-like}, aminoglycosides resistance genes *aph*(3'')- and *Ib-like*, *aph*(6)-*Id*, and *sul1* gene which encodes only sulphonamide resistance. The genome of the BR-MHR107Ecl strain co-harbored an additional quaternary ammonium compound-resistance gene (*qacE*-like) which results in resistance to antiseptics and disinfecting agents.

The PlasmidFinder analysis revealed five different plasmid replicons Col(pHAD28), IncFIA(HI1), IncFIB(K), IncR, and repB(R1701) which are frequently detected in *Klebsiella* spp. In the current study, different virulence genes such as *astA* and *nlpI* encoding for the heat-stable enterotoxin EAST-1, and the lipoprotein NlpI precursor, respectively were detected. *In silico* multilocus sequence typing (MLST) analysis revealed that BR-MHR107Ecl strain belongs to the multilocus sequence type ST84.

Discussion

Antimicrobial resistance is recognized as silent pandemic and growing dilemma for both animal and human health, demanding the implementation of One Health principles. Close and continuous programmed surveillance efforts including the application of whole genomic characterization of *Enterobacter* species associated with mastitis in dairy animals and food production settings and environment will help understand its pathogenesis and zoonotic potential. Furthermore, monitoring of mastitis pathogens will help contain and mitigate the health risks associated with the spread and transmission of antimicrobial resistance among different hosts. Despite the global significance of ECC infections, there is limited genomic data on ECC strains, particularly in certain regions such as Bangladesh. This study is the first to report the detection and characterization of mastitis-associated *E. cloacae* in dairy herds in Bangladesh using the whole genome sequencing-based approach.

Enterobacter spp. are considered as very important pathogens involved in antibiotic-resistant nosocomial infections often resulting in life-threatening outcomes (Rada et al., 2020; Denissen et al., 2022). The presence of antimicrobial-resistant bacteria such as *E. cloacae* in the animal food production chain is an increasing concern to human and animal health (Silbergeld et al., 2008; Economou and Gousia, 2015; Robinson et al., 2016 a; de Mesquita Souza Saraiva et al., 2022), as these bacteria might spread from food to humans and lead to not well treatable infections (Marshall and Levy, 2011; Capita and Alonso-Calleja, 2013; Lekshmi et al., 2017). The emergence of multidrug resistance, including resistance to the last-resort antibiotics, such as colistin, has increased the interest in the ECC (Annavaiah et al., 2019). Given that antimicrobial resistance in animals used for food production is of increasing risk to human health, the detection of multidrug-resistant, virulent, and mastitis-associated *E. cloacae* in the present study is concerning.

Mastitis is a common and economically devastating disease affecting dairy herds worldwide. Altogether, the findings of the current study emphasize the significance of the prudent and controlled use of veterinary antibiotics to prevent the selective pressure which aggravates the emergence and dissemination of multidrug resistance among animal-origin bacteria such as *E. cloacae*.

In the present study, findings revealed that BR-MHR107Ecl strain belonged to ST84, which was also reported in 2018 from a clinical colistin-resistant *E. cloacae* strain from China. The latter strain co-harbored the colistin resistance gene *mcr*-4.3 and the carbapenemase resistance gene *bla*_{NDM-1} as previously reported (Chavda et al., 2018).

Phenotypically, BR-MHR107Ecl strain was resistant to amoxicillin-clavulanate, ceftazidime, ampicillin, penicillin G, cefoxitin and was intermediate-susceptible to imipenem. Genomic analysis showed that BR-MHR107Ecl harbored several antibiotic resistance genes including beta-lactam resistance genes *bla*_{DHA-1} and *bla*_{CMH-3-like}. It was previously reported that only in combination with the overexpression of these genes and mutations causing porin deficiency leads to a mainly low resistance to carbapenems (Mammeri et al., 2025).

The detection of *bla*_{CMH-3-like} in BR-MHR107Ecl genome in the present study is concerning since it had been reported that there is limited evidence about *bla*_{CMH} in animal-derived *E. cloacae* (Huang et al., 2024). It was previously reported that third-generation cephalosporin-resistant *E. cloacae* strains carrying *bla*_{CMH} gene were identified from migratory bird samples across human-bird-environment interfaces in China (Huang et al., 2024).

A comparison of the phenotypic antibiotic susceptibility profiles and the presence of antibiotic resistance determinants was performed as detailed in Table 2. BR-MHR107Ecl was phenotypically resistant to beta-lactams (amoxicillin-clavulanate, ceftazidime, ampicillin, penicillin G) which is explained by the genotypic detection of the beta-lactam resistance genes *bla*_{DHA-1} and *bla*_{CMH-3-like}. The intermediate resistance towards carbapenems may be due to overexpression of these genes and/or additional porin gene mutations leading to porin deficiency which results in a mainly low resistance to carbapenems (Mammeri et al., 2025).

BR-MHR107Ecl was phenotypically sensitive to amikacin, gentamicin, and sulfamethoxazole-trimethoprim. These results correlate with the presence of the particular genes. For example, *aph(3'')-Ib-like* (Hainrichson et al., 2007) and *aph(6)-Id* (Ashenafi et al., 2014) genes confer resistance to other aminoglycosides than amikacin and gentamicin. The gene *aph(3'')-Ib* confers resistance to kanamycin A, neomycin B, neamin, ribostamycin, but no resistance to amikacin, tobramycin and gentamicin (Hainrichson et al., 2007). The gene *aph(6)-Id* confers resistance to streptomycin, only, and not to the other aminoglycosides tested in this study. The intermediate ciprofloxacin resistance can be explained by the presence of the gene *qnrB4* that confers low-level resistance towards fluoroquinolones (Jacoby et al., 2014).

The susceptibility towards sulfamethoxazole-trimethoprim is due to the absence of a trimethoprim resistance gene. The *sul1* gene present in BR-MHR107Ecl encodes only sulphonamide resistance, but not trimethoprim resistance.

The correlation of some genes (*qacE*-like, disinfectant resistance; *aph(6)-Id*, streptomycin resistance; *sul1*, sulfonamide resistance) with phenotypic data could not be determined because of the experimental set-up – we did not test the susceptibility of the isolate towards disinfectants, streptomycin and sulfonamide.

It was previously reported that *Enterobacter* spp. is characterized by their capacity to produce extended spectrum β -lactamases (ESBLs) and carbapenemases, which confer resistance to multiple antimicrobial agents (Bush, 2010; Yaikhan et al., 2025), representing a major challenge in managing EEC infections.

The genome of the BR-MHR107Ecl strain co-harbored an additional quaternary ammonium compound-resistance gene (*qacE*-like). The *qacE* gene, a plasmid-mediated gene and its variant *qacEΔ1* were shown to encode an efflux pump protein which mediate resistance to antiseptics and intercalating dyes in Gram-negative bacteria such as *C. freundii*, *E. cloacae* and *Pseudomonas aeruginosa* as previously reported (Kazama et al., 1998, 1999; Kücken et al., 2000; Chang et al., 2007; Gomaa et al., 2017). It was reported that the decrease in antibiotic and antiseptic susceptibility of clinical isolates of *Acinetobacter baumannii* was related to the presence of *qacE* gene (Gomaa et al., 2017). The association of the antibiotic resistance genes with biocide resistance

determinants on the same integron was previously reported in Gram-negative bacteria in China (Wang et al., 2008).

The detection of *qacE*-like resistance gene in *E. cloacae* in the present study is of specific concern since the irrational use of biocides in dairy farm environment may have promoted the selection of antibiotic-resistant Gram-negative bacteria as previously reported and discussed elsewhere (Russell, 2000; Hegstad et al., 2010; Schwaiger et al., 2014; Davies and Wales, 2019; Boyce, 2023; James et al., 2023; Muloi et al., 2023; Cui et al., 2024). To the best of the authors' knowledge, genome sequences of *E. cloacae* isolates have never been reported from milk samples obtained from bovine mastitis.

PlasmidFinder analysis revealed five different plasmid replicons Col(pHAD28), IncFIA(HI1), IncFIB(K), IncR, and repB(R1701) which are frequently detected in *Klebsiella* spp. It was previously reported that IncR plasmid has been frequently associated with *bla*_{DHA-1} (Mata et al., 2011; Yuan et al., 2023). ISfinder analysis detected six different insertion sequences including IS5075, IS30, IS26, *ISEcl10*, IS5 and *ISEhe3* in different contigs in the BR-MHR107Ecl genome. Most hits were more than 99% identity in the different contigs of the genome. Myriads of insertion sequences have been associated with the carriage of ESBL and aminoglycoside resistance genes such as *aph(3'')-Ib-like*, and *aph(6)-Id*, indicating that the insertion sequences might be involved in conferring multidrug resistance in BR-MHR107Ecl strain.

It was reported that misuse of antibiotics in agriculture and animal production sectors provides selection pressure which enhances the evolution of antimicrobial-resistant bacteria in human and animal populations (Chantziaras et al., 2014). Additionally, the environment where dense populations of different animal species are housed could serve as a reservoir for the interspecies and intraspecies spillover and dissemination of antimicrobial-resistant bacteria and resistance genes (Van Balen et al., 2014; Adams et al., 2018). Further investigations utilizing the One Health principles are required to delineate the main source and the origin of BR-MHR107Ecl and to determine possible zoonotic potential of the strain.

It was reported that the ECC bacteria are relatively uncommon in foodborne diseases compared to other bacterial pathogens (Pintor-Cora et al., 2023). Hence, the potential presence of antimicrobial-resistant strains of ECC in the food chain including the strain BR-MHR107Ecl reported in the present study in bovine mastitis, and the potential dissemination of their antimicrobial resistance genes to humans could pose a public health risk.

In the current study, different virulence genes such as *astA* and *nlpI* encoding for the heat-stable enterotoxin EAST-1, and the lipoprotein NlpI precursor, respectively were detected in the genome of BR-MHR107Ecl strain. It was previously reported that *astA* is a structural gene in enteroaggregative *E. coli* encoding a distinct low-molecular-weight putative enterotoxin (Savarino et al., 1993). In the present study, *astA* has been detected in BR-MHR107Ecl strain associated with bovine clinical mastitis.

The detection of the strain BR-MHR107Ecl in bovine mastitis therefore carries potential risks due to its plausible introduction into the food-chain and the farm-to-fork continuum, resulting in its possible transmission in raw milk, food, other livestock, and eventually to humans. Future community-wide surveillance involving livestock and environmental samples will provide an updated picture on the population dynamics of *E. cloacae* ST84 and its clinical relevance.

Phylogenetic analysis showed that the closest relative to BR-MHR107Ecl (Figure 1) was isolate NK-BR1453 (GenBank accession number DALQOB000000000) assembly name PDT001543683.1 isolated from a human rectal swab sample in Nigeria in 2016 as previously reported (Portal et al., 2024), albeit the relatively high number of SNPs (n=317, Table 3) which

suggests recent ancestry.

The detection of carbapenem-resistant *Enterobacteriaceae* including *E. cloacae* harboring *bla*_{KPC-2} isolated from livestock, humans and environmental samples was detailed elsewhere (Tuhamize and Bazira, 2024). In a recent study from Spain, antimicrobial-resistant ECC strains were isolated from fresh vegetables and their farm environments including samples from soil, irrigation water, air, and farm worker hands as previously reported (Pintor-Cora et al., 2023). In the present study, analysis revealed that BR-MHR107Ecl genome harbored beta-lactam resistance genes *bla*_{DHA-1} and *bla*_{CMH-3-like} which highlights the strain could be of clinical significance. The detection of carbapenem-resistant *Enterobacteriaceae* in livestock raises significant concerns about the potential dissemination of antibiotic-resistant bacteria to humans posing a grave risk to public health (Murray et al., 2022; Pintor-Cora et al., 2023; Tuhamize and Bazira, 2024). Carbapenem-resistant *E. cloacae* strains carrying *bla*_{NDM-1}, *bla*_{KPC}, and *bla*_{OXA-48-like} genes were isolated from different environmental wastewater samples including river water, soil, vegetables samples in South Africa as previously reported (Ebomah and Okoh, 2021). These reports highlight the increasing importance of One Health approach-based studies to mitigate potential risks associated with antimicrobial resistance to public health (Lammie and Hughes, 2016; Robinson et al., 2016 b; White and Hughes, 2019; Wernli et al., 2020; Aslam et al., 2021; de Mesquita Souza Saraiva et al., 2022; Velazquez-Meza et al., 2022; Jesudason, 2023; World Health Organization, 2023; Huang et al., 2024). Furthermore, the dissemination of antimicrobial resistance among various sources including humans, wild animals, livestock, plants, and their environments, necessitate a One Health comprehensive approach and oriented stewardship to control antimicrobial resistance across various environments.

The detailed genetic characterization of *Enterobacter* species associated with bovine mastitis will provide sufficient data on the complex genetic organization of mobile genetic elements, antimicrobial resistance, and virulence genetic elements. Genomic characterization of mastitis bacteria will help understand their complex etiology, interplay and ability to induce mastitis in udder-healthy dairy animals.

Conclusions

To the authors' knowledge, the present study is the first at the time of writing this manuscript to report the detection and genome characterization of *E. cloacae* isolated from bovine mastitis using whole genome sequencing analysis. Results of the present study highlight the crucial role of whole genome sequencing tools in pathogen detection and the importance of continued surveillance for mastitis-associated bacteria, which continue to pose serious global impacts on animals, human health, and food security. Moreover, BR-MHR107Ecl ST84 is the first reported *E. cloacae* associated with bovine mastitis as of 17 May 2025. The draft genome of BR-MHR107Ecl from bovine clinical mastitis revealed different virulence genes, several antimicrobial resistance genes, and five plasmid replicons, indicating significant pathogenic and antimicrobial resistance potential. According to *Enterobacter cloacae* PubMLST database, *E. cloacae* ST84 strain B13 was isolated from a human urine sample in Bangladesh. Therefore, the genome sequence of the reported strain will serve as a valuable reference point for molecular epidemiological studies to delineate the dissemination of multidrug resistant, foodborne and mastitis-associated pathogens between farm animals and humans. Additionally, the findings of the present study will help understand the antimicrobial resistance in livestock production systems-associated *E. cloacae*. The findings of the present study provide substantial new insights into the field and are sufficiently novel to contribute significantly to the existing literature, thus broadening

the overall impact of the research.

Data availability statement

The strain reported in this study was submitted to public databases for molecular typing and microbial genome diversity for curation and is publicly available in *Enterobacter cloacae* PubMLST database, under ID number 532. This whole genome sequencing project has been deposited at DDBJ/ENA/GenBank under the BioProject number PRJNA716986, BioSample accession number SAMN26025963 and GenBank accession number JALBGO000000000. The version described in this paper is the first version JALBGO000000000.1. The sequence was submitted to the Sequence Read Archive (SRA) under the accession number SRR18182114.

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Conflict of interests

None to declare.

CRedit authorship contribution statement

M.H.R.: conceptualization, methodology, investigation, formal analysis, validation. M.E.Z.: conceptualization, methodology, data curation, formal analysis, investigation, visualization, validation, writing – original draft, writing – review & editing, project administration, supervision; E.C.E: formal analysis, methodology, visualization, data curation, software, validation, writing – original draft, writing – review & editing; L.F.: writing – original draft, writing – review & editing, formal analysis, methodology, software, visualization, data curation, software, validation; M.F.R.K.: methodology, investigation; J.A.: methodology,

investigation; M.R.: conceptualization, validation, supervision, project administration; A.S.: visualization, data curation, validation; writing – review & editing; H.M.S.: visualization, data curation, validation; writing – review & editing; F.A.M.G.: visualization, data curation, validation; writing – review & editing; A.N.: visualization, data curation, validation; writing – review & editing; S.F.: visualization, data curation, software, validation, writing – original draft, writing – review & editing; M.B.R.: conceptualization, validation, funding acquisition, supervision, project administration, writing – review & editing.

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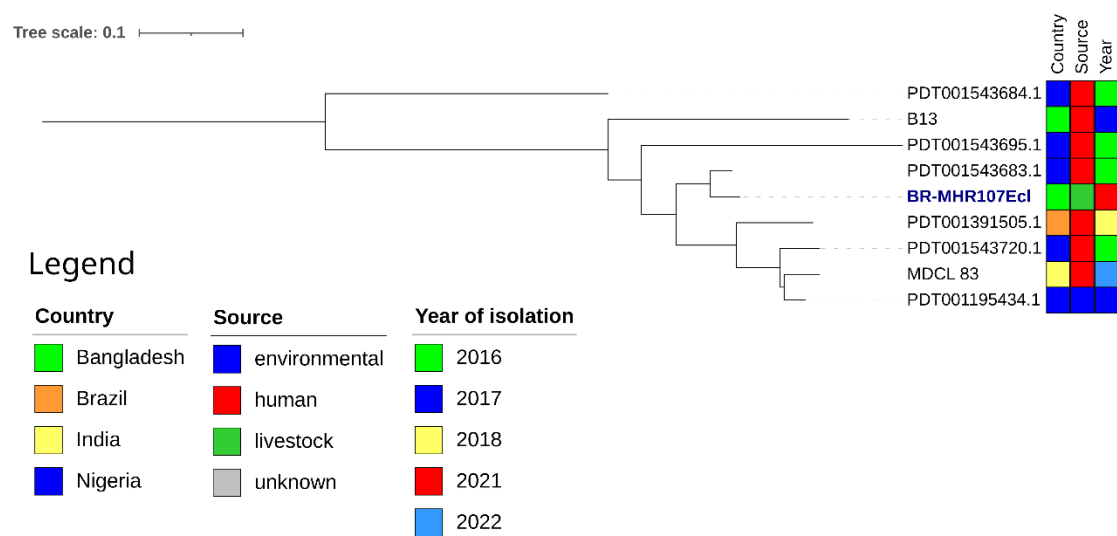


Figure 1. Mid-point rooted core genome-based phylogenetic tree of ST84 *Enterobacter cloacae* isolates from PubMLST database and BR-MHR107Ecl. The tree was produced using ParSNP, annotated using iTOL v. 1.8 and amended using Inkscape v. 0.9.1

Table 1. *Enterobacter cloacae* isolates available at the PubMLST database used in the phylogenetic comparison of BR-MHR107Ecl with other ST84 isolates

ID	Isolate	Country	Continent	Year	Source	Detailed source	Disease
73	B13	Bangladesh	Asia	2017	human		
532	BR-MHR107Ecl	Bangladesh	Asia	2021	animal	Lactating cow	other
915	PDT001195434.1	Nigeria	Africa	2017	environmental		
1055	PDT001391505.1	Brazil	South America	2018	human		
1201	PDT001543720.1	Nigeria	Africa	2016	human		
1216	PDT001543695.1	Nigeria	Africa	2016	human		
1219	PDT001543684.1	Nigeria	Africa	2016	human		
1224	PDT001543683.1	Nigeria	Africa	2016	human		
1347	MDCL 83	India	Asia	2022	human	Central line blood	bacteraemia

Table 2. Correlation of phenotypic resistance patterns and the detected antibiotic resistance genes in BR-MHR107Ecl in the present study

Resistance to	Phenotypic	Genotypic	Correlation phenotype vs. genotype	Possible explanation of difference
Amikacin	Sensitive	Not present	Yes	Neither <i>aph(6)-Id</i> nor <i>aph(3'')-Ib</i> -like confer resistance to this particular aminoglycoside
Amoxicillin-clavulanate	Resistant	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMH-3-like}	Yes	Beta-lactamase present
Ampicillin	Resistant	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMH-3-like}	Yes	Beta-lactamase present
Azithromycin	Sensitive	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMH-3-like}	Yes	Beta-lactamase present
Cefoxitin	Resistant	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMH-3-like}	Yes	Beta-lactamase present
Ceftazidime	Resistant	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMH-3-like}	Yes	Beta-lactamase present
Chloramphenicol	Sensitive	Not present	Yes	Antibiotic resistance gene not encoded
Ciprofloxacin	Intermediate	<i>qnrB4</i>	Yes	<i>qnrB</i> -like enzymes express only low-level fluoroquinolone resistance
Disinfectant	Not tested	<i>qacE</i> -like	Correlation not possible due to experimental set-up (not tested phenotypically)	N.A.
Fosfomycin	Sensitive	Not present	Yes	Antibiotic resistance gene not encoded
Gentamicin	Sensitive	Not present	Yes	Neither <i>aph(6)-Id</i> nor <i>aph(3'')-Ib</i> -like confer resistance to this particular aminoglycoside
Meropenem	Intermediate	Not present	Yes	Overexpression of <i>bla</i> _{DHA-1} and/or <i>bla</i> _{CMH-3-like} in combination with porin deficiency mutations
Penicillin G	Resistant	<i>bla</i> _{DHA-1} , <i>bla</i> _{CMH-3-like}	Yes	Beta-lactamase present
Streptomycin	Not tested	<i>aph(6)-Id</i>	Correlation not possible due to experimental set-up (not tested phenotypically)	N.A.
Sulfamethoxazole-trimethoprim	Sensitive	Not present	Yes	Only gene for resistance towards sulfamethoxazole present (<i>sul1</i>) but not towards trimethoprim
Sulfonamide	Not tested	<i>sul1</i>	Correlation not possible due to experimental set-up (sulfonamide tested only in combination with trimethoprim)	N.A.
Tetracycline	Sensitive	Not present	Yes	Antibiotic resistance gene not encoded
Doxycycline	Intermediate	Not present	No	Unknown mechanisms of antibiotic resistance encoded or expressed

N. A. not applicable (not tested phenotypically).

Table 3. Matrix showing the number of single nucleotide polymorphisms (SNPs) of strain BR-MHR107Ecl compared to other *Enterobacter cloacae* ST84 isolates

	532_BR_MHR107Ecl	73_B13	915_PDT001195434_1	1055_PDT001391505_1	1201_PDT001543720_1	1216_PDT001543695_1	1219_PDT001543684_1	1224_PDT001543683_1	1347_MDCL_83
532_BR_MHR107Ecl	0	1979	1142	1169	1218	1891	3144	317	1223
73_B13	1979	0	2368	2399	2444	2523	3108	1934	2447
915_PDT001195434_1	1142	2368	0	853	390	2293	2826	1097	342
1055_PDT001391505_1	1169	2399	853	0	929	2322	2839	1123	934
1201_PDT001543720_1	1218	2444	390	929	0	2369	2902	1173	474
1216_PDT001543695_1	1891	2523	2293	2322	2369	0	3504	1847	2374
1219_PDT001543684_1	3144	3108	2826	2839	2902	3504	0	3099	2907
1224_PDT001543683_1	317	1934	1097	1123	1173	1847	3099	0	1178
1347_MDCL_83	1223	2447	342	934	474	2374	2907	1178	0