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Original article

Phylogenetic diversity and mutational analysis of New Delhi Metallo-β-lactamase (NDM) producing *E. coli* strains from pediatric patients in Pakistan

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ABSTRACT

The evolution of NDM genes (bla_{NDM}) in *E. coli* is accounted for expansive multidrug resistance (MDR), causing severe infections and morbidities in the pediatric population. This study aimed to analyze the phylogeny and mutations in NDM variants of *E. coli* recovered from the pediatric population. Carbapenem-resistant clinical strains of *E. coli* were identified using microbiological phenotypic techniques. PCR technique used to amplify the bla_{NDM} genes, identified on agarose gel, and analyzed by DNA sequencing. The amino acid substitutions were examined for mutations after aligning with wild types. Mutational and phylogenetic analysis was performed using Lasergene, NCBI blastn, Clustal Omega, and MEGA software, whereas PHYRE2 software was used for the protein structure predictions. PCR amplification of the bla_{NDM} genes detected 113 clinical strains of *E. coli* with the contribution of bla_{NDM-1} (46%), bla_{NDM-4} (3.5%), and bla_{NDM-5} (50%) variants. DNA sequencing of bla_{NDM} variants showed homology to the previously described bla_{NDM-1}, bla_{NDM-4}, and bla_{NDM-5} genes available at GenBank and NCBI database. In addition, the mutational analysis revealed in frame substitutions of *Pro60Ala* and *Pro59Ala* in bla_{NDM-4} and bla_{NDM-5}, respectively. The bla_{NDM-1} was ortholog with related sequences of *E. coli* available at GenBank. The phylogenetic analysis indicated that the NDM gene variants resemble other microbes reported globally with some new mutational sites.
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1. Introduction

Multidrug-resistant (MDR) *Escherichia coli* is a substantial threat to the healthcare system and is associated with adverse outcomes. *E. coli* contain several virulence factors leading to various diseases, including urinary tract infection, diarrhea, and neonatal meningitis. Infections with MDR *E. coli* account for much higher mortality rates in the pediatric population (WHO, 2010). Genetic mutations are pivotal to MDR development (Cherry et al., 2017). Carbapenems were the mainstay for a long time to treat the infections with extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases producing *E. coli.* However, new carbapenemases like New Delhi metallo-β-lactamase (NDM) have created a potential threat for their utility (Paterson and Doi, 2007).

The NDM was first identified in New Delhi from an Indian-returned Swedish patient, and subsequently, several clones have been reported from diversified bacterial strains (Rolain et al., 2010). Nearly 24 variants of bla_{NDM} genes were reported among bacteria based on different substitutions of amino acids in their protein structure. Out of these, the NDM-1 to 7 variants, being widely prevailed globally, has become a dominant concern (Wu et al., 2019). The information obtained from the latest bioinformatics analysis

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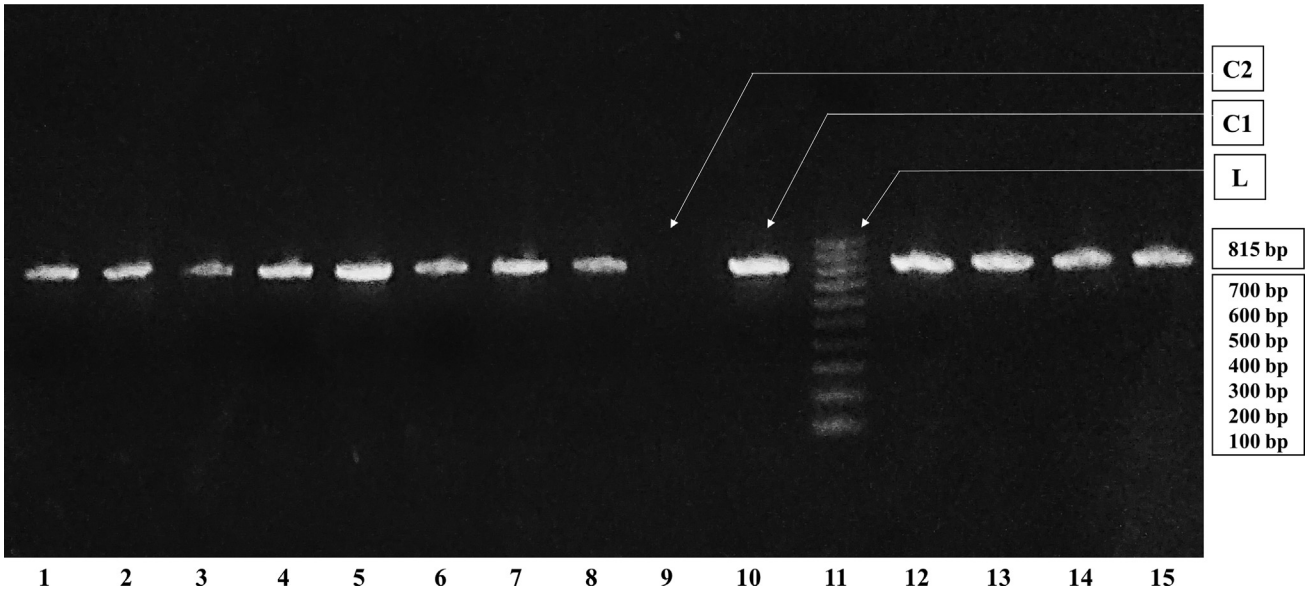

Fig. 1. Agarose gel electrophoresis of amplified PCR products. Each batch of the gel contained a 100 bp leader (L) to estimate the gene size, a positive control (C1) showing the detection of bla_{NDM}, a negative control (C2) showing the absence of bla_{NDM}.

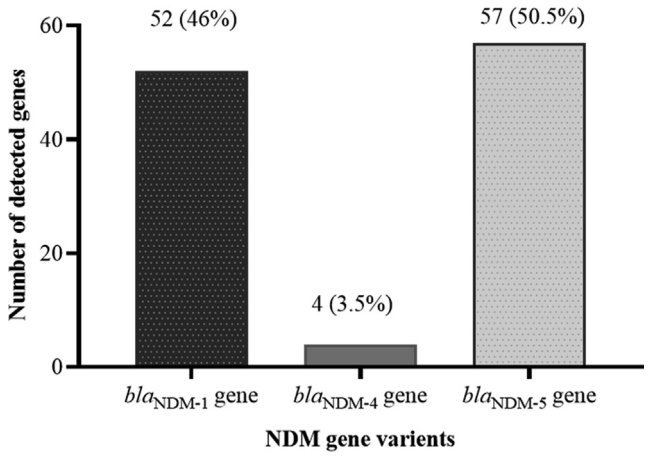

Fig. 2. Distribution bla_{NDM} variants in *E. coli* (n = 113).

of the sequenced DNA of NDM producing *E. coli*, their protein 3D format, and phylogenetics has become imperative for evolving breakthrough antibacterial agents because such structural aspects and genetic features influence the mechanisms of antibiotic-hydrolysis (Gromiha et al., 2019). Understanding structural and genetic features of NDM variants that affect the hydrolysis of antibiotics can assist in designing the new and distinct NDM inhibiting agents (Rolain et al., 2010). A few local studies have been carried out in Pakistan in this context. However, they bear a different focus point, divergence in their scopes such as involving adult patients only (Fakhuruddin et al., 2012; Nahid et al., 2013), specific bacteria (Amin et al., 2013), limited coverage from wards (Javed et al., 2016), or specimen types (Anis-ur-Rehman et al., 2008).

The majority of such in-depth genetic studies have been conducted on the local adult population and with various limitations in few local studies, including children. This study aimed to report the currently prevalent NDM variants of *E. coli*, infecting children, identifying their mutation sites, and studying their phylogenetics. This will aid in targeted antibiotic therapy through precise and timely identification of culprit MDR mutated strain of bacteria to reduce morbidity and mortality among children.

2. Materials and methods

2.1. Study design and specimen collection

From April 2017 to March 2019, all in- and out-patient specimens were obtained from The Children's Hospital and The Institute of Child Health, Lahore, Pakistan, after taking ethical approval by the Institutional Review Board (Ref No. 09/Ch/ICH) and from the University of Punjab, Lahore (Ref No. D/6001-ACAD).

2.2. Isolation and phenotypic characterization of MBL producing E. coli

The specimens analyzed during the study include blood, urine, cerebrospinal fluid (CSF), pus swabs, peritoneal dialysis (PD) catheter, central venous pressure (CVP) line, endotracheal tube (ETT), wound swab, ear tip, and tracheal secretions. The specimens were cultured on standardized blood, chocolate, CLED, and MacConkey agar plates and incubated at 37 °C overnight (Qamar et al., 2017). *E. coli* were screened out through Gram's staining, colony morphology, biochemical and API 20 E (bioMerieux, France) strip tests. Carbapenem resistance was detected by Kirby Baur disc diffusion test. Modified Hodge test was employed to identify carbapenemase-producing (CP) *E. coli*. The combined disc (CDT) and double-disc synergy test (DDST) were performed for detection of MBL production (Wayne, 2018). Their antibiogram was also established as detailed in our previous study (Nosheen et al., 2020), which presented the MDR in the bacteria under study.

2.3. Molecular analysis of the bla_{NDM} in E. coli

Freshly grown *E. coli* colonies suspended in 250 μl TE buffer, vortexed well, and kept for 10 min in a boiling water bath. Later, the suspension was centrifuged for five minutes at 14,000 rpm to obtain the DNA template from the supernatant stored at −70 °C until subsequent use (Ejaz et al., 2020). The target gene (815 bp) was amplified by PCR using a maximum of 0.5 μM each of the forward (ATGGAATTGCCCAATATTATG) and the reverse (TCAGCGCAGCTTGTCGGCC) primers (Nordmann et al., 2011). The thermal cycle was optimized and programmed for an initial denaturation step of 10 min at 94 °C, followed by 35 cycles of amplification, each

Fig. 3. Multiple sequence alignment compared the amino acids of proteins in NCBI database. a) NDM-1 gene encoding protein by bla_{NDM-1} gene. b) NDM-4 gene encoding protein by bla_{NDM-4} gene. c) NDM-5 gene encoding protein by bla_{NDM-5} gene.

of which comprised 30 sec at 94 °C, 40 sec at 57 °C, and 45 sec at 72 °C with a final extension at 72 °C for 5 min. Gel stain was used to analyze PCR products after electrophoresis using 1% agarose gels in TAE buffer at 95 V for 45 min.

2.4. DNA sequencing and bioinformatics analyses

Amplified bla_{NDM} products were sequenced in Singapore on 96-capillary 3730xl Genetic Analyzer. The amino acid substitutions were examined for mutations after aligning with wild types. DNA Star, Lasergene molecular biology software version 16.2.0.130 was used for the pairwise sequence alignments. The nucleotide studies were performed using the NCBI blastn suite program, DNA translation, sequence manipulation suite, and multiple nucleotide alignment and Clustal Omega (Sievers et al., 2011).

2.5. Phylogenetic studies and prediction of the 3D protein structure of bla_{NDM} genes of E. coli

The phylogenetic tree of 113 nucleotide sequences of *E. coli* was established with sequenced genetic data of different NDM producing *E. coli*, available at GenBank, through MEGA version 10.0.5. The initial tree for heuristic search was obtained automatically by applying Neighbor-Join (NJ) and Bio-NJ algorithms to a matrix of pairwise distances estimated by the maximum composite likelihood approach. The topology with a superior log likelihood value was selected, and the codon positions comprised were first till third and non-coding (Kumar et al., 2018). The complete dataset included 826 positions. Following the node in the phylogenetic tree, each descendant represented a taxonomic unit, expressing the most recent ancestor, while the length of the edges indicated time estimation. The PHYRE2 software was used to predict the three-dimensional structure of proteins.

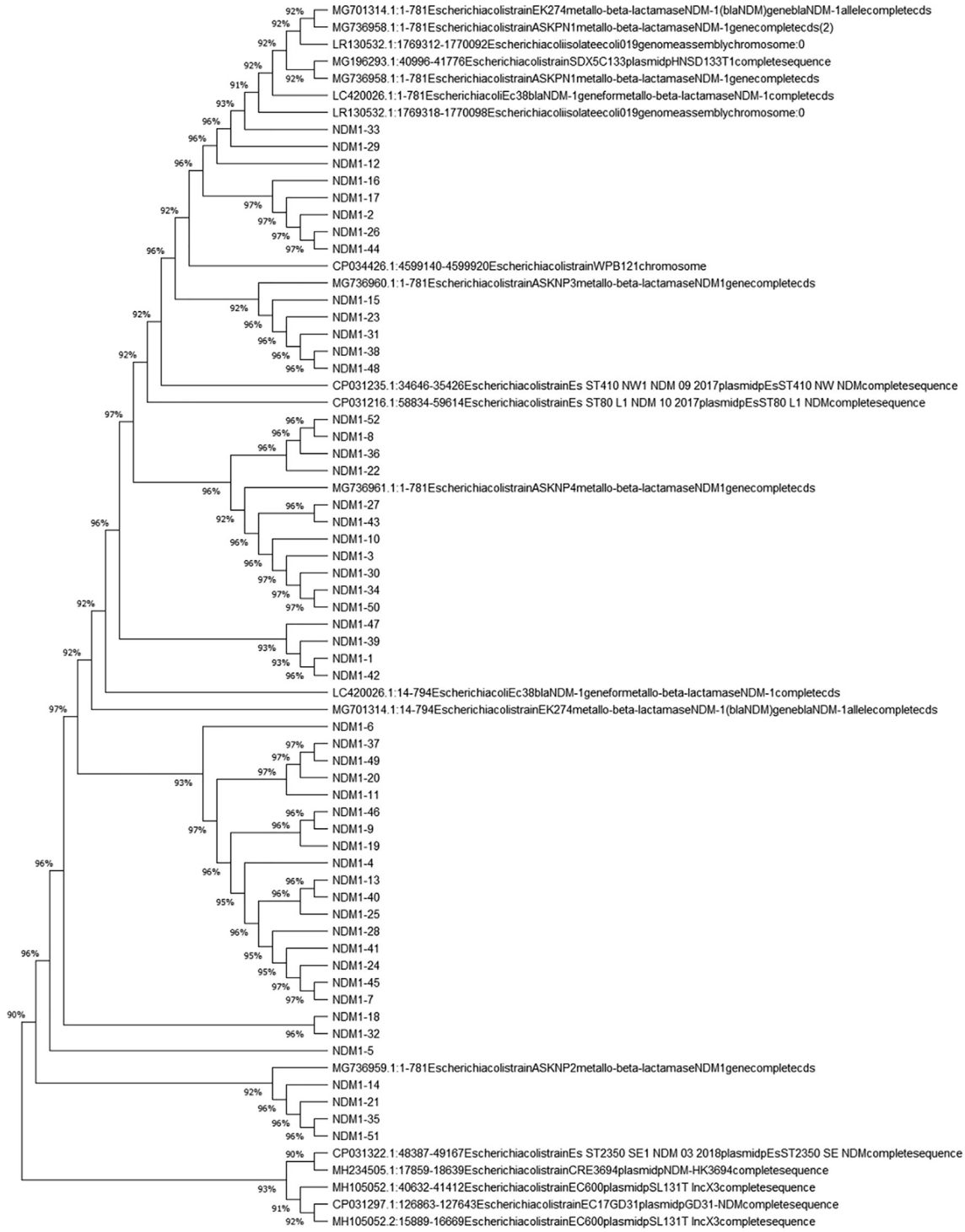

Fig. 4a. Phylogenetic tree of $bla_{\text{NDM-1}}$ genes isolated from clinical strains of *E. coli*.

3. Results

Of 6468 isolated bacterial strains, 1522 (24%) were *E. coli*, out of which 113 (7.4%) were carbapenamase producers.

3.1. Molecular characteristics of bla_{NDM} genes in E. coli

Agarose gel electrophoresis of the amplified PCR gene products confirmed the presence of bla_{NDM} genes among all of the 113 *E. coli* strains (Fig. 1). The detected bla_{NDM} genes variants include 52 (46%) $bla_{\text{NDM-1}}$, 4 (3.5%) to be $bla_{\text{NDM-4}}$ and 57 (50.5%) to be $bla_{\text{NDM-5}}$ (Fig. 2).

3.2. DNA sequence analysis

The chromatograms of $bla_{\text{NDM-1, -4,}}$ and $_{-5}$ genes did not show undesirable ambiguities in contig and no overlapping dirty peaks. Multiple nucleotide sequence alignments of DNA sequencing data of $bla_{\text{NDM-1, -4,}}$ and $_{-5}$ genes of *E. coli* were observed using several bioinformatics software. The amino acids in each column were analyzed to identify their homology with various associated

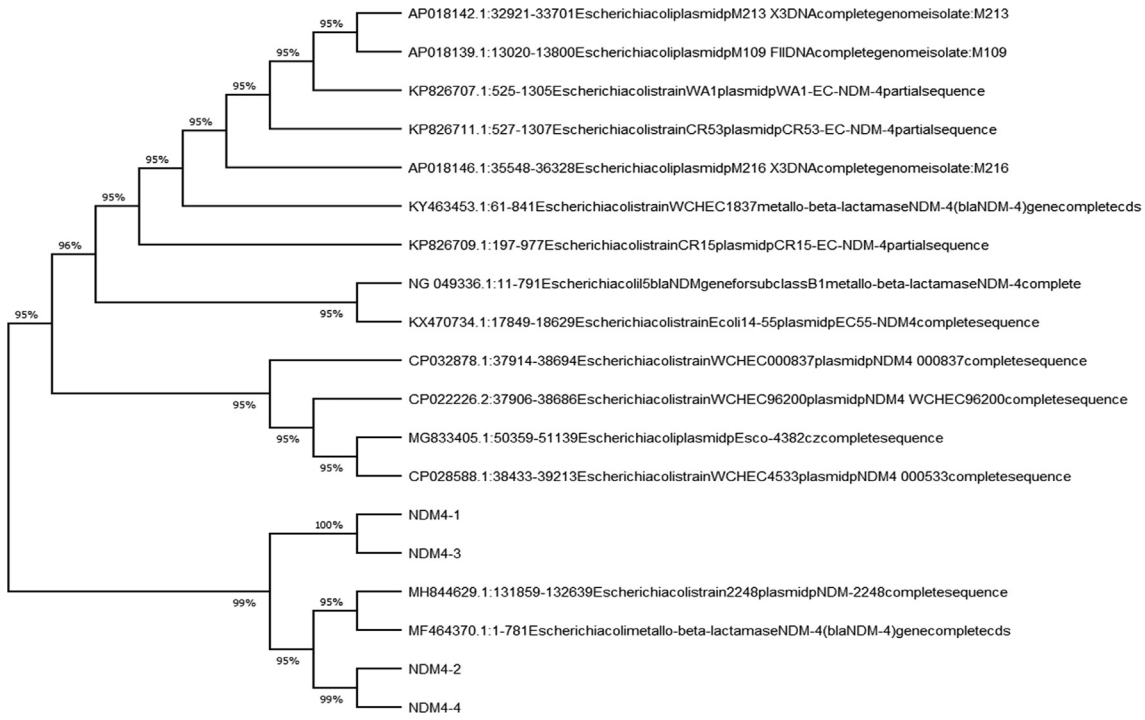

Fig. 4b. Phylogenetic tree of bla_{NDM-4} genes isolated from clinical strains of *E. coli*.

sequences which share a common evolutionary history, available at the GenBank, NCBI database. The isolated bla_{NDM} genes were identical to previously described bla_{NDM-1}, $bla_{NDM-4,}$ and bla_{NDM-5} *E. coli* plasmids present in the GenBank database.

3.3. *E. coli* bla_{NDM} gene mutations

The sequence of the bla_{NDM-1} gene was found ortholog with several related *E. coli* sequences at GenBank (Fig. 3a). However, as shown in Fig. 3b, the bla_{NDM-4} sequence substituted amino acid proline (P) from the GenBank sequence for alanine (A) in the sequence under study, in frame 60. In contrast, bla_{NDM-5} presented substitution in frame 59, A from P of sequence from GenBank (Fig. 3c). These mutations found in *E. coli* genes responsible for producing specific variants of NDM in bacteria affect their ability to recognize substrates and catalyze the antibiotics.

3.4. Phylogenetic studies of bla_{NDM} genes

Figs. 4a, 4b, and 4c depict the phylogenetic tree, which represented the evolutionary history of *E. coli* carrying the bla_{NDM-1}, $_{-4}$, and $_{-5}$ genes individually. All the variants of bla_{NDM-1}, $_{-4}$, and $_{-5}$, along with the same genes carried by other *E. coli* isolated from different areas worldwide and available at GenBank (Fig. 5). The phylogenetic analysis showed that the bla_{NDM-1}, $_{-4}$, and $_{-5}$ genes exhibited minimal variation compared to other *E. coli* strains isolated from different countries that possessed the same genes.

3.5. Prediction of the three-dimensional structure of the bla_{NDM} genes

The three-dimensional crystal structure model of the *E. coli* bla_{NDM-1}, $_{-4}$, and $_{-5}$ genes is shown in Fig. 6, where α-helices are colored blue, β-strands green, loop regions cyan. In contrast, active sites were represented in red color, which presents the amino acid substitution or mutation sites in comparison to other *E. coli* NDM variants previously mentioned.

4. Discussion

Antibiotics misuse has exerted extensive selection pressure on the health care system, as there are limited therapeutic options for MDR infections. This threat is being foreseen to worsen since the first reported case of NDM-1, an incessant upsurge in MDR of bacteria carrying the NDM gene. This study has expanded the *E. coli* characterization from phenotypic to molecular level for identifying NDM-1, −4, and −5 variants, from all in- and out-patients and including all specimen types among children, along with its bioinformatics and phylogenetic analysis. This information is vital as it exerts a direct impact on the selection of targeted antibiotic regimens.

E. coli burden in this study (24%) was comparable with the US (Dahle et al., 2012), higher than Russian (Edelstein et al., 2003), while lesser than reported from Bangladesh (Islam et al., 2016). The variation in incidence could be due to different levels of public hygiene, climatic variation, and health education. The carbapenem resistance of *E. coli* in the current study was in line with Georgian research (Gupta et al., 2011). The present study determined that 7.4% of *E. coli* were carbapenemase producers based on MHT. Three local studies, conducted at Rawalpindi and Lahore, showed 38%, 33.1%, and 10.6% *E. coli* to be CP, respectively (Abbas et al., 2019; Amjad et al., 2011; Javed et al., 2016), while a greater frequency was identified in Greece (Falagas et al., 2010). The greater frequency of carbapenem-resistant *E. coli* infection in uprising countries might be due to poor socio-economic conditions and insufficient compliance towards antibiotic protocols.

In this study, the PCR identified *E. coli* gene variant NDM-1 (46%), NDM-4 (3.5%), and NDM-5 (50%). However, the above findings varied from others in Egypt (Soliman et al., 2020) and India (Ranjan et al., 2016), presenting different variants of NDM in multiple geographical areas. The current prevalence of the NDM-5 variant among pediatrics was excessive than in Algeria (Sassi et al., 2014). The prevalence of NDM-4 in this study was comparable to that in a Chinese study. (Bi et al., 2018). Furthermore, no other local research has expressed the frequency of

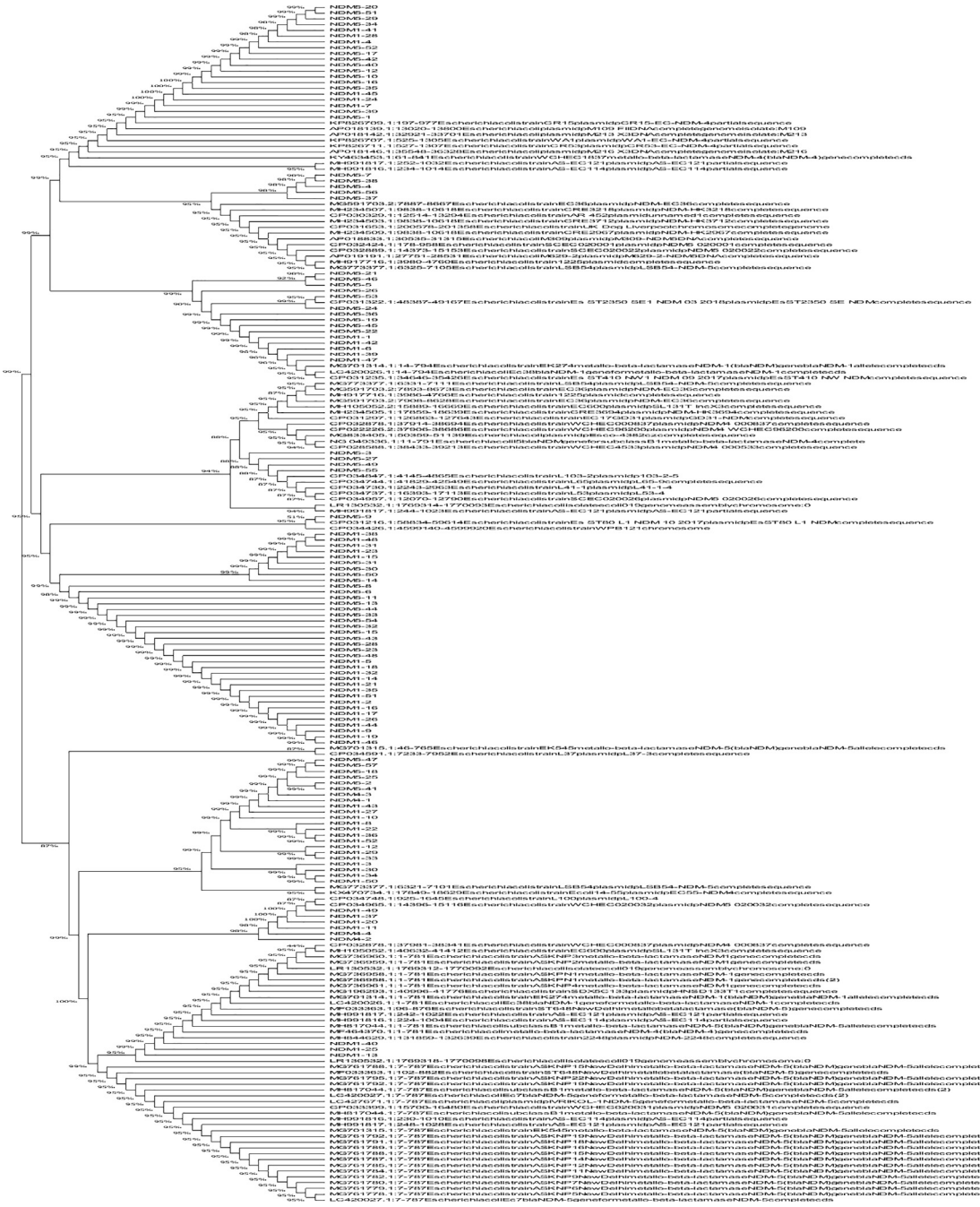

Fig. 4c. Phylogenetic tree of *bla*$_{NDM-5}$ genes isolated from clinical strains of *E. coli*.

specified NDM variants in *E. coli* among Pakistani children. However, it has been reported in a pediatric study on *Klebsiella* (Heinz et al., 2019).

The multiple sequence alignments of *bla*$_{NDM-1, -4,}$ and $_{-5}$ genes of *E. coli* in the present study exhibited no amino acid substitution in *bla*$_{NDM-1}$ gene structure, while in *bla*$_{NDM-4}$, proline60alanine, and *bla*$_{NDM-5}$, alanine59proline substitution from the sequence of GenBank was noted. An Algerian research reported amino acid substitutions in genes of three NDM-5 producing *E. coli* that were similar to those found in India (Sassi et al., 2014). A research study from the UK revealed that their *E. coli* gene differed from NDM-1 by two amino-acid substitutions (Hornsey et al., 2011). Variability in these substitutions could be because of perpetual mutations in the genetic material of *E. coli*, leading to magnified MDR.

In this research, the phylogenetic tree showed that *bla*$_{NDM-1, -4,}$ and $_{-5}$ genes of *E. coli* carry resemblance with various bacteria, reported worldwide, submitted online on GenBank exhibiting fewer gene variations. Phylogenetic trees of *E. coli* have been established at various parts of the world, including Rome (Garcia-Fernandez et al., 2020), China (Zheng et al., 2016), and locally as well (Shahzad et al., 2016), whose focus remained converged on uropathogenic strains of adult males only. In this study, the phylogenetic tree highlights the potential of *E. coli* to disseminate NDM among various bacteria worldwide (Mushtaq et al., 2011), although

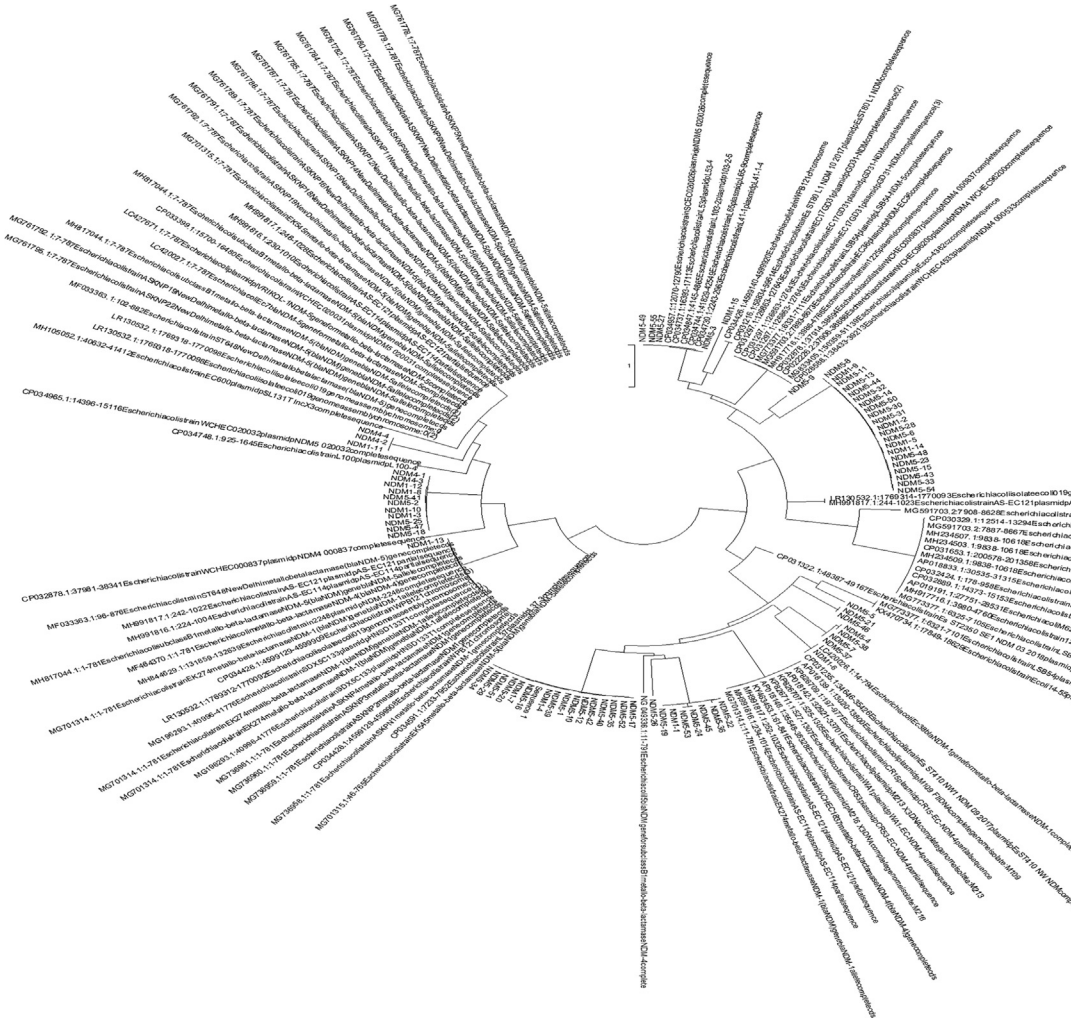

Fig. 5. Phylogenetic tree (circular) of *bla*$_{NDM-1}$, *bla*$_{NDM-4}$, and *bla*$_{NDM-5}$ genes isolated from clinical strains of *E. coli*.

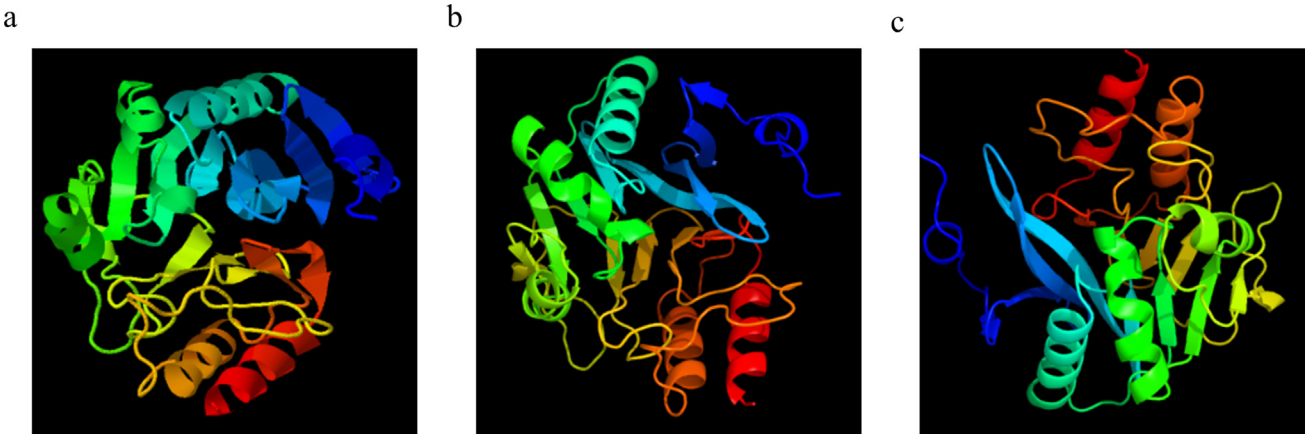

Fig. 6. 3D protein structure of NDM isolated from clinical strains of *E. coli*. a) *bla*$_{NDM-1}$, (b) *bla*$_{NDM-4}$, and (c) *bla*$_{NDM-5}$ genes.

the protein crystal structure models of *bla*$_{NDM-1, -4,}$ and $_{-5}$ genes, support the knowledge of genomics and functionality of proteins, adding to the formerly present data which, in turn would be advantageous in formation of better MBL inhibitors.

The precise identification of bacteria and its resistance mechanism has become imperative because it directly influences choosing a targeted regimen. There is a grave necessity for comprehensive studies to provide statistics of the diversity of *bla*$_{NDM}$ gene variants in children and efficient monitoring in hospitals to control MDR and support the antibiotic policy optimization. The present study is limited with NDM variants; however, NDM lineages suggest working on the phylogenetic analysis of other car-

bapenamase genes. Some new mutated sites found in NDM-1, −4, and −5 proteins suggest further need to design antibacterial drugs targeting these active sites to combat NDM harboring bacterial strain. The dissemination of MDR *E. coli* can be alleviated by the proficient screening of such bacteria, targeted therapy, and stringent strategies for rational use of antibiotics and infection control.

5. Conclusion

The study of substitution of amino acid showed orthology of $bla_{\text{NDM-1}}$ with related sequences in GenBank. The $bla_{\text{NDM-4}}$ and $bla_{\text{NDM-5}}$ exhibited substitutions, presenting mutation sites. *E. coli* strains harbored more NDM-5 than NDM-1 and NDM-4 variants. The phylogenetic analysis revealed that the $bla_{\text{NDM-1}}$, $bla_{\text{NDM-4}}$, and $bla_{\text{NDM-5}}$ genes from strains circulating among the pediatric population exhibited minimal variation to the NDM gene structure of *E. coli* strains, isolated from various countries around the globe. The variations in NDM genes may have occurred as a result of horizontal gene transfer from diverse geographical extents and environmental reservoirs, providing deep insight into phylogenetics and biological bioinformatics domains. The new protein mutations presented in this study have not been explored from clinical strains isolated from pediatric patients in this region of the world. The 3D crystal structure of bla_{NDM} presents a structural comparison with other MBLs subclasses, which might help to design mechanism-based inhibitors, bridge research, and develop new chemicals and modified medicines.

6. Consent for publication

All authors consent to publish this manuscript in Saudi journal of Biological Science.

7. Availability of data and material

The data used and analyzed during the current study available from the corresponding author.

Author contributions

Sumbal Nosheen, Nadeem Irfan Bukhari, and Hasan Ejaz data curation conceived and designed the study. Sumbal Nosheen, Kashaf Junaid, Naeem Anwar, and Sonia Younas helped with specimen processing, performed formal analysis, optimized methodology, and wrote the original draft. Fahad Ahmad and Sumbal Nosheen performed software analysis. Nadeem Irfan Bukhari and Hasan Ejaz supervised, administered, and provided resources for the project. Hasan Ejaz, Nadeem Irfan Bukhari and Naeem Anwar performed final editing. All authors critically reviewed the manuscript and approved the final version.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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