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Misdiagnosis of Bacterial Pathogens by the Diagnostic Centers: A Potential Route for Antibiotic Resistance

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ABSTRACT

Successful treatment against infectious agents depends on rapid and accurate detection of the causative organisms. Lack of proper identification may facilitate improper antibiotic recommendations. Apart from a few advanced diagnostic facilities in developing countries, most facilities identify pathogens through culture-based methods and suggest antibiotics based solely on the results of disk-diffusion tests. In this pilot study, we tried to validate the identity of the clinical isolates precharacterized by diagnostic facilities. One hundred precharacterized clinical isolates were collected and analyzed phenotypically, biochemically, and genotypically. We employed random amplified polymorphic DNA-polymerase chain reaction (PCR), *rcsA*, and *phoA* genes-based PCR and loop-mediated isothermal amplification (LAMP) methods to validate the identification of *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*), respectively. Further validation through phylogenetic analysis based on 16S ribosomal deoxyribonucleic acid (rDNA) sequencing was also performed. Phenotypical, biochemical, and phylogenetic analyses found that 30% and 46% misidentification among the diagnostic center identified *E. coli and Klebsiella* spp., respectively. Moreover, 16S rDNA sequencing confirmed that the representatives of the misidentified organisms belonged to *Enterobacter*, *Acinetobacter*, and *Pseudomonas* genus. Furthermore, LAMP successfully detected the clinical *E. coli* within 60 minutes. In this study, we recommend proper monitoring and validation of different tests performed in clinical facilities to avoid misidentification, thus facilitating the avoidance of possible routes responsible for developing antimicrobial resistance.

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INTRODUCTION

Background

Bacterial identification is generally accomplished through labor-intensive and time-consuming culture-based methods involving various biochemical tests. Such applications may be ineffective if the findings are needed for prompt emergency medical diagnosis. Furthermore, biochemical approaches may not always identify bacteria accurately and frequently provide incorrect conclusions [1,2]. Even though culture-based techniques are considered the gold standard for pathogen detection, these drawbacks include the need for trained personnel, standard reagents, labor intensiveness, time constraints, and limited specificity [3].

Developed countries or advanced hospital diagnostic facilities utilize analytical profile index assays, Vitek-2/mass spectroscopy (MS), nuclear magnetic resonance spectroscopy, or matrix-assisted laser desorption/ionization-time of flight MS as an alternative to culture-based approaches for pathogen detection [4–6]. In addition to these procedures, diagnostic facilities regularly utilize nucleic acid amplification techniques such as polymerase chain reaction (PCR), real-time PCR, isothermal amplification, and next-generation sequencing [7–10]. Though the diagnostic application has been implemented to identify signature sequences within species, these tests are not implemented outside reference laboratories due to their high cost and technical necessity [11]. Unlike conventional amplification methods, isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) is a molecular approach known for being straightforward, fast, cost-effective, and highly reliable if adequately designed and suggested for usage in resource-constrained areas [12–15]. *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are components of the commensal gut flora, and they are also the common opportunistic pathogens often associated with urinary tract and bloodstream infections [16]. *Klebsiella pneumoniae* is reported to be responsible for nearly one-third of all Gram-negative bacterium-related infections, such as urinary tract infections, cystitis, pneumonia, surgical wound infections, endocarditis, and septicemia [17]. On the other hand, although most *E. coli* species are harmless, few strains have been associated with various human infections categorized as diarrheagenic and extraintestinal pathogens, with various pathotypes and natural hybrid strains [18]. In addition, a high mortality risk is associated with patients infected with antibiotic-resistant strains of these microbes [19].

Importance of the study

Antibiotic resistance is a significant threat to healthcare systems. Annually, 700,000 people lose their lives worldwide due to infection with antibiotic-resistant bacteria. The fatality rate due to antibiotic resistance is estimated to be more than cancer by 2050 [20]. In line with such observations, the World Health Organization has designated antimicrobial resistance (AMR) as one of the top 10 worldwide public health problems confronting humanity and 12 families of bacteria posing a great threat, as treating infections against these is becoming quite tricky due to multidrug resistance [21]. Among these are the Enterobacteriaceae family members, such as *E. coli* and *Klebsiella* etiological agents, especially for urinary tract infections. Though AMR develops naturally, the misuse of antibiotics by humans accelerates the process. The factors contributing to AMR development are inappropriate prescription practices, inadequate patient literacy levels, unauthorized antibiotic sales, insufficient diagnostic facilities, and uncontrolled antimicrobial usage in animals [22,23].

In low- and middle-income countries (LMICs), there is often a lack of standardized national diagnostic protocols or monitoring policies. Diagnostic facilities may use inconsistent characterization methods or fail to adhere to protocols, leading to variable and inaccurate outcomes. This inconsistency in pathogen identification can result in improper treatment interventions, potentially contributing to AMR development. More studies are needed to monitor the accuracy of diagnoses in LMICs, as misidentification of clinical pathogens can promote incorrect antibiotic use, fueling AMR.

Aim of the study

This pilot study collected the previously characterized clinical *E. coli* and *Klebsiella* spp. isolates from two reputed hospital-based diagnostic facilities near Dhaka, Bangladesh. We attempted to determine their proper identity by applying different phenotypic, biochemical, and molecular techniques. Interestingly, we confirmed apparent misidentification in those diagnostic center-identified (DCI) clinical isolates, suggesting one of the possible routes of developing AMR in LMICs such as Bangladesh.

MATERIALS AND METHODS

Study samples

One hundred DCIs previously characterized by diagnostic facilities were collected from 2017 to 2018 [24]. The isolates were stored at −80°C and sub-cultured annually. Before working with the isolates, the cultures were thawed and grown in minimal media for recovery. To ensure the purity of the isolates, they were cultured on MacConkey agar (Scharlau, Spain, Catalog number: 01-118-500) and eosin methylene blue agar (Himedia, India, Catalog number: SKU M317) media.

Phenotypic characterization of the isolates

Immediately upon receiving the isolates, biochemical tests were carried out in our laboratory for characterization and identification. To cross-check this earlier characterization, we performed biochemical tests such as indole production, methyl red, Voges-Proskauer, citrate utilization, catalase, motility, triple sugar iron (TSI), and sugar utilization before starting this work.

Molecular detection

Boiling method for total DNA extraction

The chromosomal DNA of the bacterial isolates was extracted using the modified boiling DNA method [25]. Colonies isolated from the nutrient agar (Himedia, India, Catalog number: SKU M001) plate were grown overnight at 37°C in a test tube containing 5 ml of nutrient broth (Himedia, India, Catalog number: SKU M002). 1 ml culture was placed in a 1.5 ml microcentrifuge

tube, and cells were separated by centrifugation at 12,000 rpm for 10 minutes. The pellets were resuspended in 200 µl of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, USA, Catalog number: 10977015). Following this, each microcentrifuge tube was kept at 90°C–95°C for 10 minutes in a block heater (Thermo Fisher Scientific, USA, Catalog number: 88870003), then placed on ice for 10 minutes. The tubes were then centrifuged for 10 minutes at 10,000 rpm. The supernatant was collected into a new microcentrifuge tube and kept at −80°C for long-term storage.

Randomly amplified polymorphic DNA (RAPD) analysis

Extracted bacterial DNA was subjected to RAPD genotyping. Random primer (10 bp) 5'-AAGAGCCCGT-3' was selected to observe their band pattern to classify them into different groups. The PCR reactions were performed in 25 µl volumes containing 12.5 µl GoTaq® G2 Green Master Mix (2×) master mix (Promega, USA, Catalog number: M7822), 2 µl of primer, 4 µl template DNA, and 6.5 µl nuclease-free water. PCR condition was set at 94°C for 30 seconds, followed by 36°C for 15 seconds, and 72°C for 30 seconds for 45 cycles in TaKaRa PCR Thermal Cycler Dice® Touch (Takara, Japan, Catalog number: TP350) [26]. Amplicons were characterized in 1% agarose gel. Binary data charts were produced based on the presence or absence of specific bands at specific locations for constructing dendrograms. The DendroUPGMA program (Universitat Rovira i Virgili (URV), Tarragona, Spain) was used to create dendrograms from binary data charts. The program calculates a similarity matrix, transforms similarity coefficients into distances, and makes a clustering using the unweighted pair group method with arithmetic mean (UPGMA) [27].

PCR of E. coli phoA gene

The alkaline phosphatase (*phoA*) gene was used to identify *E. coli.* 5'-AAGTTGAAGGTGCGTCAAT-3'-F3 and 5'-CTTGTGAATCCTCTTCGGAG-3'-B3 primers were used to identify *E. coli* isolate more accurately [28]. The PCR reactions were performed in 25 µl volumes containing 12.5 µl master mix, 2 µl each of the forward and reverse primers, 2 µl template DNA, and 8.5 µl nuclease-free water. PCR condition was set at 94°C for 30 seconds, followed by 50°C for 15 seconds and 72°C for 30 seconds for 45 cycles. Amplicon's length of approximately 277 bp was initially confirmed using a molecular ladder to amplify *phoA*. Later, PCR bands were extracted from agarose gel and sequenced to confirm the specific amplification.

PCR of K. pneumoniae rcsA gene

The *rcsA* gene in *K. pneumoniae* produces capsular polysaccharides, an essential factor in its virulence. 5'-GGATATCTGACCAGTCGG-3'-KP27F3 and 5'-GGGTTTTGCGTAATGATCTG-3'-KP27B3 primer was used for the detection of *K. pneumoniae* [29]. The PCR reactions were performed in 25 µl volumes containing 12.5 µl master mix, 1 µl each of primer F and primer R, 4 µl template DNA, and 6.5 µl nuclease-free water. PCR condition was set at 94°C for 30 seconds, followed by 55°C for 15 seconds, and 72°C for 30 seconds for 45 cycles. Amplicon's length of approximately 170 bp was initially confirmed using a molecular ladder to amplify *rcsA* and later confirmed by Sanger sequencing.

PCR of 16S rRNA gene

5'-AGAGTTTGATCCTGGCTCAG-3' forward and 5'-CGGTTACCTTGTTACGACTT-3' reverse primers were used to amplify the template DNA for 16S rRNA gene sequencing. For 25 µl PCR reactions, the PCR mixture contained 12.5 µl master mix, 2 µl each of the forward and reverse primers, 2 µl template DNA, and 8.5 µl nuclease-free water [30].

Phylogenetic analysis

The PCR products were analyzed on 1% agarose gel using an agarose gel electrophoresis Mupid-2plus system (Takara Bio, Japan, Catalog number: AD110). The amplicons were purified using the FavorPrep GEL/PCR Purification Kit (Favorgen Taiwan, Catalog number: FAGCK 001), as directed by the manufacturer. After purification, PCR products were used for Sanger dideoxy sequencing (3500 Series Genetic Analyzer, Applied Biosystems). The primary local alignment search tool (BLAST) was used to identify close phylogenetic relatives by comparing partial sequences to the GenBank database of the National Center for Biotechnology Information (NCBI) [31]. The phylogenetic tree was generated using BioEdit, ApE plasmid editor, and MEGA 11 software.

Loop-mediated isothermal amplification

LAMP reactions were conducted in 25 µl volumes containing 15 µl *Bst* 2.0 Polymerase Isothermal master mix (NEB, UK, Catalog number: M0537S), 5 µl (1×) Primers, and 5 µl Template DNA [32]. The list of primer sequences required to detect *E. coli* and *K. pneumoniae* is shown in Table 1. For detection by LAMP, the template DNA was boiled in a water bath at 95°C for 5 minutes before being immediately transferred to ice. Master mix, primer, and template DNA were mixed to prepare the reaction solution and incubated at 63°C temperature in a water bath for 30 minutes. The enzymatic reaction was stopped by incubating the tubes at 80°C temperature for 2 minutes. The LAMP products were visualized by subjecting them to gel electrophoresis in 1% agarose gel.

RESULTS

Isolation and characterization of *E. coli* and *Klebsiella* spp. isolates

One hundred precharacterized bacterial isolates were collected from diagnostic facilities. Previously, 33 *E. coli* and 50 *Klebsiella* isolates were selected for further study due to their prevalence and clinical significance [24]. Presumptive re-identification through culturing on selective media and biochemical testing revealed 30% of presumed *E. coli* and 46% of suspected *Klebsiella* isolates were misidentified by the diagnostic centers (Tables 2 and 3). Based on the biochemical profile, the misidentified isolates belonged to genera including *Enterobacter*, *Acinetobacter*, and *Pseudomonas*.

Molecular characterization of the bacterial isolates

RAPD results

RAPD profiling revealed eight different band patterns among the DCI-K (Fig. 1A). All isolates belonging

to the first seven groups displayed the same intra-group band pattern, whereas those in group 8 showed different banding patterns from each other. However, although K17 and K45 showed the same RAPD profile biochemically, they differed. On the contrary, based on the banding pattern of 16 of all 33 isolates, DCI-E isolates were represented by three groups. The other 17 isolates exhibited dissimilar band patterns, and they all were represented by group 4 (Fig. 1B). These banding patterns were used to select representative isolates for characterization through PCR and sequencing.

Table 1. The primer sequence of the LAMP process for detecting *E. coli* and *K. pneumoniae*.

Organism	Target Gene	Primer	Sequence
E. coli	Alkaline phosphatase *phoA*	F3	AAGTTGAAGGTGCGTCAAT
		B3	CTTGTGAATCCTCTTCGGAG
		FIP	GTGATCAGCGGTGACTATGACC TCTCGATGAAGCC GTACA
		BIP	ATTGTCGCGCCGGATACCCTCA TCACCATCACTGC G
		Loop F	AGCGTGTTGCCATCCTTT
		Loop B	CAGGCGCTAAATACCAAAGATG
K. pneumoniae	Capsular polysaccharides *rcsA*	KP-27F3	GGATATCTGACCAGTCGG
		KP-27B3	GGGTTTTGCGTAATGATCTG
		KP-27FIP	CGACGTACAGTGTTTCTGCAG
			TTTTAAAAAACAGGAAATCGT TGAGG
		KP-27BIP	CGGCGGTGGTGTTTCTGAATT
			TTGCGAATAATGCCATTACTTTCG
		KP-27LB	GAAGACTGTTTCGTGCATGATGA

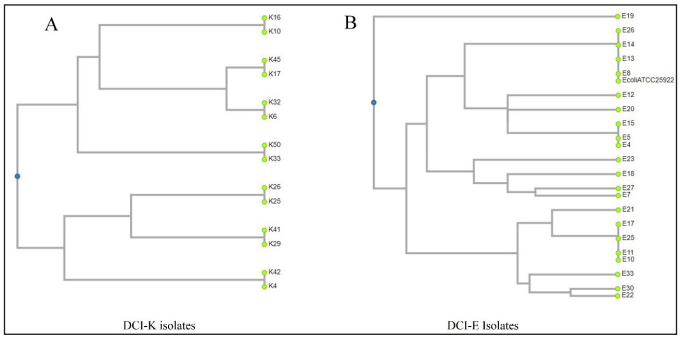

Figure 1. UPGMA dendrograms of the representative DCI-K and DCI-E isolates, showing the formation of clusters based on their RAPD profiles. (A) DCI-K4 and DCI-K42 from group 1, DCI-K10 and DCI-K16 from group 2, DCI-K25 and DCI-K26 from group 3, DCI-K17 and DCI-K45 from group 4, DCI-K6 and DCI-K32 from group 5, DCI-K33 and DCI-K50 from group 6, and DCI-K29 and DCI-K41 from group 7 were selected as the representative isolates of the respective DCI-K groups. They were plotted as distinct clusters based on their similarities in the RAPD band pattern. (B) In the cases of DCI-E isolates, DCI-E8, DCI-E13, DCI-E14, and DCI-E26 from group 1 showed RAPD band patterns such as *E. coli* ATCC 25922 and were clustered together, whereas DCI-E4, DCI-E5, and DCI-E15 from group 2, DCI-E10, DCI-E11, DCI-E17, and DCI-E25 from group 3 were clustered together as seen in the dendrogram. On the contrary, E7DCI-E12, DCI-E18, DCI-E19, DCI-E20, DCI-E21, DCI-E22, DCI-E23 DCI-E27, and DCI-E30 showed different band patterns and were plotted distantly in the dendrogram.

Table 2. Biochemical test results of DCI-K (according to hospital record) pathogens.

Isolates ID	IMViC				Catalase	Motility	TSI agar test			H_2S	Sugar fermentation			Presumptive result
	IN	MR	VP	Ci			Slant	Butt	Gas		Xy	Man	Sor	
Ref. stain *K. pneumoniae*	-	-	+	+	+	−	A	A	+	−	+	+	+	*K. pneumoniae*
DCI-K1, DCI-K2, DCI-K3, DCI-K4, DCI-K5, DCI-K8, DCI-K9, DCI-K10, DCI-K12, DCI-K13, DCI-K15, DCI-K16, DCI-K25, DCI-K26, DCI-K28, DCI-K29, DCI-K31, DCI-K33, DCI-K35, DCI-K39, DCI-K41, DCI-K42, DCI-K45, DCI-K46, DCI-K47, DCI-K49, DCI-K50	−	V	V	+	+	−	A	A	+	−	+	+	+	Probable *K. pneumoniae*
DCI-K6, DCI-K14, DCI-K19, DCI-K17, DCI-K32, DCI-K40	-	-	−	−	+	+	A	A	+	−	+	+	+	Probable *Enterobacter* spp.
DCI-K7, DCI-K11, DCI-K23, DCI-K34	+	+	−	−	+	+	A	A	+	−	+	+	+	Probable *E. coli*
DCI-K20, DCI-K21, DCI-K24, DCI-K27, DCI-K38, DCI-K43, DCI-K48	−	−	−	+	+	+	K	K	−	−	−	−	−	Probable *Pseudomonas* spp.
DCI-K22, DCI-K36, DCI-K30	−	−	−	+	+	−	K	K	−	−	−	−	−	Probable *Acinetobacter* spp.
DCI-K18, DCI-K37, DCI-K44	−	+	−	+	+	+	K	K	−	−	−	−	−	Not identified

IMViC (IN = indole, MR = methyl red, VP = Voges Proskauer, Ci = citrate), Ca = catalase Mo = motility, TSI (triple sugar iron), A = acid, K = alkaline, Sugar (Xy = xylose, Ma = mannitol, So = sorbitol.

Detection of E. coli and K. pneumoniae using phoA and rcsA genes specific PCR

The alkaline phosphatase gene was used to characterize 33 DCI-E isolates and 3 isolates biochemically positive for *E. coli* among the DCI-*Klebsiella* spp. samples. *Vibrio cholerae* ATCC (14035), *K. pneumoniae* reference strain, *Micrococcus luteus* ATCC [4698 (150307)], and *Staphylococcus aureus* ATCC (6538) were used as negative controls in the experiment. Twenty-three of the 33 DCI-E isolates and 2 from the DCI-K displayed a banding pattern similar to *E. coli* reference strains (JM109, V517, BL21, and DE3) and *E. coli* ATCC 25922 (Fig. 2A). Similarly, the *K. pneumoniae* strains amongst the DCI-K isolates that were biochemically determined to be *Klebsiella* spp. were identified using the *K. pneumoniae-specific* capsular polysaccharide gene *rcsA*. Reference strain was used as positive controls, while *V. cholerae* ATCC (14035), *M. luteus* ATCC (4698), *S. aureus* ATCC (6538), *Salmonella typhi* ATCC (14028), *Bacillus cereus* ATCC (14574), and *E. coli* ATCC (25922) were used as the negative controls. No bands were observed following PCR amplification and gel electrophoresis in negative control lanes. However, 13 of 27 isolates were biochemically positive for *Klebsiella* spp. gave a banding pattern similar to *K. pneumonia*e reference strains (Fig. 2B).

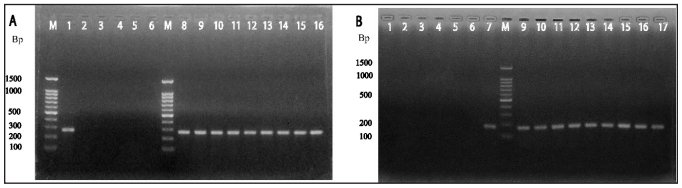

Figure 2. Gel electrophoresis analysis of PCR amplified (A) *phoA* gene and (B) *rcsA* gene. Agarose gel electrophoresis (on 1.5% agarose gel) image showing the amplified products by PCR, which was done by using *phoA* primer (~270 bp) and *rcsA* primer (~176 bp). Lane M represents the molecular marker spanning 100–1,500 base pairs (bp). (A) Lane 1 corresponds to *E. coli* ATCC (25922) as a positive control. Lanes 2–6 represent negative control, with lane 2 designated as general negative control, lane 3 as *K. pneumoniae* reference stain, lane 4 as *V. cholerae* ATCC (14035), lane 5 as *M. luteus* ATCC (4698), and lane 6 as *S. aureus* ATCC (6538). The sample lanes (8–16) contain the amplified *phoA* products (located between 200 and 300 bp sized band of DNA marker) showing positive results in the form of distinct bands. (B) Lanes 1–6 corresponds to negative control with as lane 1 as *V. cholerae* ATCC (14035), lane 2 as *E. coli* ATCC (25922), lane 3 as *M. luteus* ATCC (4698), lane 4 as *S. typhi* ATCC (14028), lane 5 as *S. aureus* ATCC (6538), and lane 6 as *B. cereus* ATCC (14574). Lane 7 represents *K. pneumoniae* reference strain as the positive control, and lanes 9–17 show positive results (bands) for *rcsA* gene-positive clinical isolates. These band sizes are ~176 bp and located just below 200 bp.

Sequencing and homology alignment of amplified phoA and rcsA genes

phoA and *rcsA* amplicons were found in 25 and 17 isolates of presumptively characterized *E. coli* and *K. pneumoniae,* respectively. Three amplicons from each group were subjected to sequencing using the forward primers. *phoA* and *rcsA* gene sequences from *E. coli* (Accession No. MG1655) and *K. pneumoniae* (Accession No. NNP58345) were retrieved for homology alignment. Multiple sequence alignments (MSAs) of these gene sequences were performed using ClustalW multiple alignment tools. MSA found the amplicons specific to their respective genes (Fig. 3).

16S rRNA PCR, sequencing, and phylogenetic analysis

Representative isolates from the RAPD pattern and biochemical tests were subjected to 16S rRNA PCR for further confirmation of the match and mismatches of bacterial identification by diagnostic centers. The PCR products were gel extracted and sequenced. The sequences were submitted to Genebank. Sequencing confirms the misidentification of clinical pathogens by diagnostic centers (Table 4).

Closely related reference 16S rRNA sequences were downloaded from the NCBI database, and sequences were aligned with the ClustalW MSA tool. The alignments were subjected to phylogenetic tree development using the maximum likelihood method and the Tamura-Nei model (Mega 11). A bootstrap value of 1,000 was set to minimize the error of distant measurement. The phylogenetic tree generated with selected DCI-E and DCI-K shows matched and mismatched isolates in different branches (Fig. 4).

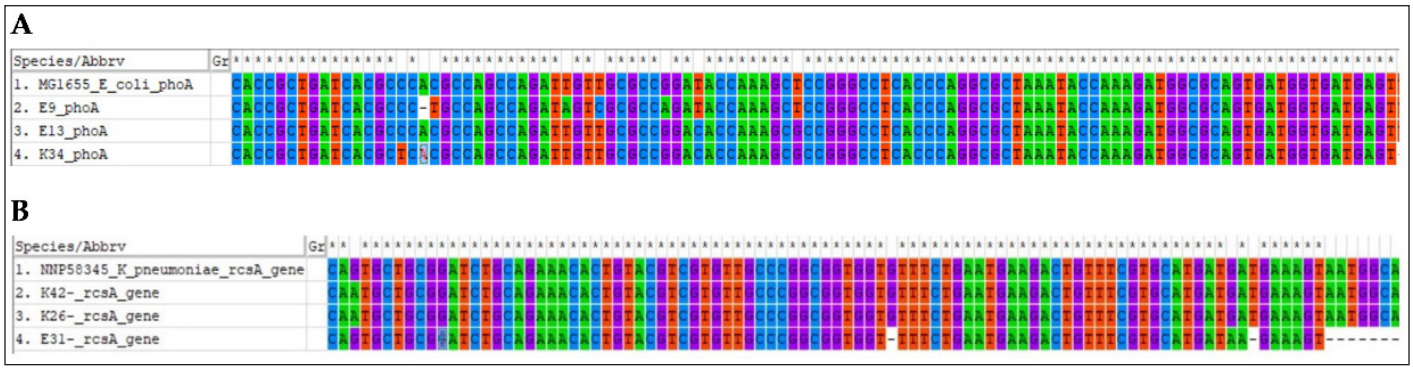

Figure 3. Homology alignment of *phoA* and *rcsA* gene. (A) *phoA* amplicon sequences from three isolates, two from DCI-E (DCI-E9 and DCI-E13) and one DCI-K (DCI-K34), were aligned with the *phoA* gene sequence of reference *E. coli* (MG1655), and the amplicons matched the reference sequence. (B) *rcsA* amplicon sequences from three isolates, two from DCI-K (DCI-K26 and DCI-K42) and one DCI-E (DCI-E31), were aligned with the *rcsA* gene sequence of reference *K. pneumoniae* (NNP58345), and the amplicons matched the reference sequence.

Table 3. Biochemical test results of selected DCI-E (according to hospital record) pathogens.

Isolates ID	IMViC				Catalase	Motility	TSI agar test			H2S	Sugar fermentation			Presumptive result
	IN	MR	VP	Ci			Slant	Butt	Gas		Xy	Man	Sor	
ATCC *E. coli* 25922	+	+	−	−	+	+	A	A	+	−	+	+	+	Confirmed *E. coli*
DCI-E1, DCI-E2, DCI-E3, DCI-E4, DCI-E5, DCI-E7, DCI-E8, DCI-E9, DCI-E13 DCI-E14, DCI-E15, DCI-E16, DCI-E18, DCI-E20, DCI-E26, DCI-E27, DCI-E28, DCI-E29, DCI-E32, DCI-E33	+	+	−	−	+	+	A	A	+	−	+	+	+	Probably *E. coli*
DCI-E24	+	+	-	-	+	+	A	A	+	−	+	-	-	Probably *E. coli*
DCI-E6, DCI-E10, DCI-E11, DCI-E17, DCI-E19, DCI-E25	-	-	+	+	+	+	A	A	+	−	+	+	+	Probably *Enterobacter* spp.
DCI-E31	−	+	+	+	+	−	A	A	+	−	+	+	+	Probably *Klebsiella* spp.
DCI-E12, DCI-E23	−	−	−	+	+	+	K	K	−	−	−	−	−	Probably *Pseudomonas* spp.
DCI-E21, DCI-E22, DCI-E30	−	+	+	−	+	−	A	A	+	−	+	+	+	Probably *Klebsiella* spp.

IMViC (IN = indole, MR = methyl red, VP = Voges Proskauer, Ci = citrate), TSI (triple sugar iron) test (A = acid, k = alkaline), Sugar (Xy = xylose, Man = mannitol, Sor = sorbitol).

Table 4. Comparative identification of bacterial pathogens by the diagnostic centers, biochemical profiling, *phoA*, and *rcsA* gene-specific PCR, and 16S rRNA sequencing.

Sample code	DCI identification	Biochemical profile	Gene	16S rRNA sequencing	Accession no.	Comment
DCI-E6	*E. coli*	*Enterobacter* sp.	-	*E. cloacae*	OM066748	Mismatch
DCI-E9	*E. coli*	*E. coli*	*phoA*	*E. coli*	OM066744	Match
DCI-E10	*E. coli*	*Enterobacter* sp.	*phoA*	*E. coli*	OM066745	Match
DCI-E12	*E. coli*	*Pseudomonas* sp.	-	*Pseudomonas putida*	OM066749	Mismatch
DCI-E13	*E. coli*	*E. coli*	*phoA*	*E. coli*	OM066746	Match
DCI-E18	*E. coli*	*E. coli*	*phoA*	*E. coli*	OM066747	Match
DCI-E31	*E. coli*	*Klebsiella* sp.	*rcsA*	*K. pneumoniae*	OM066742	Mismatch
DCI-K2	*Klebsiella* sp.	*Klebsiella* sp.	-	*Acinetobacter* sp.	OM066757	Mismatch
DCI-K14	*Klebsiella* sp.	*Enterobacter* sp.	-	*Enterobacter hormaechei*	OM066755	Mismatch
DCI-K22	*Klebsiella* sp.	*Acinetobacter* sp.	-	*Acinetobacter baumannii*	OM066751	Mismatch
DCI-K23	*Klebsiella* sp.	*E. coli*	*phoA*	*E. coli*	OM066750	Mismatch
DCI-K26	*Klebsiella* sp.	*Klebsiella* sp.	*rcsA*	*K. pneumoniae*	OM066753	Match
DCI-K27	*Klebsiella* sp.	*Pseudomonas* sp.	-	*P. aeruginosa*	OM066759	Mismatch
DCI-K30	*Klebsiella* sp.	*Acinetobacter* sp.	-	*A. baumannii*	OM066752	Mismatch
DCI-K34	*Klebsiella* sp.	*E. coli*	*phoA*	*E. coli*	OM066758	Mismatch
DCI-K40	*Klebsiella* sp.	*Enterobacter* sp.	-	*E. hormaechei*	OM066756	Mismatch
DCI-K42	*Klebsiella* sp.	*Klebsiella* sp.	*rcsA*	*K. pneumoniae*	OM066754	Match

Rapid molecular identification of clinical isolates by LAMP method

Biochemical, *phoA,* and *rcsA* gene PCR-positive *E. coli* (DCI-E9, DCI-E10, DCI-E13, DCI-K23, and DCI-K34) (Fig. 5A) and *K. pneumoniae* (DCI-E31, DCI-K26, and DCI-K42) were subjected to LAMP analysis (Fig. 5B). All *phoA* and *rcsA* gene-positive isolates showed multiple band patterns in 1% agarose gel.

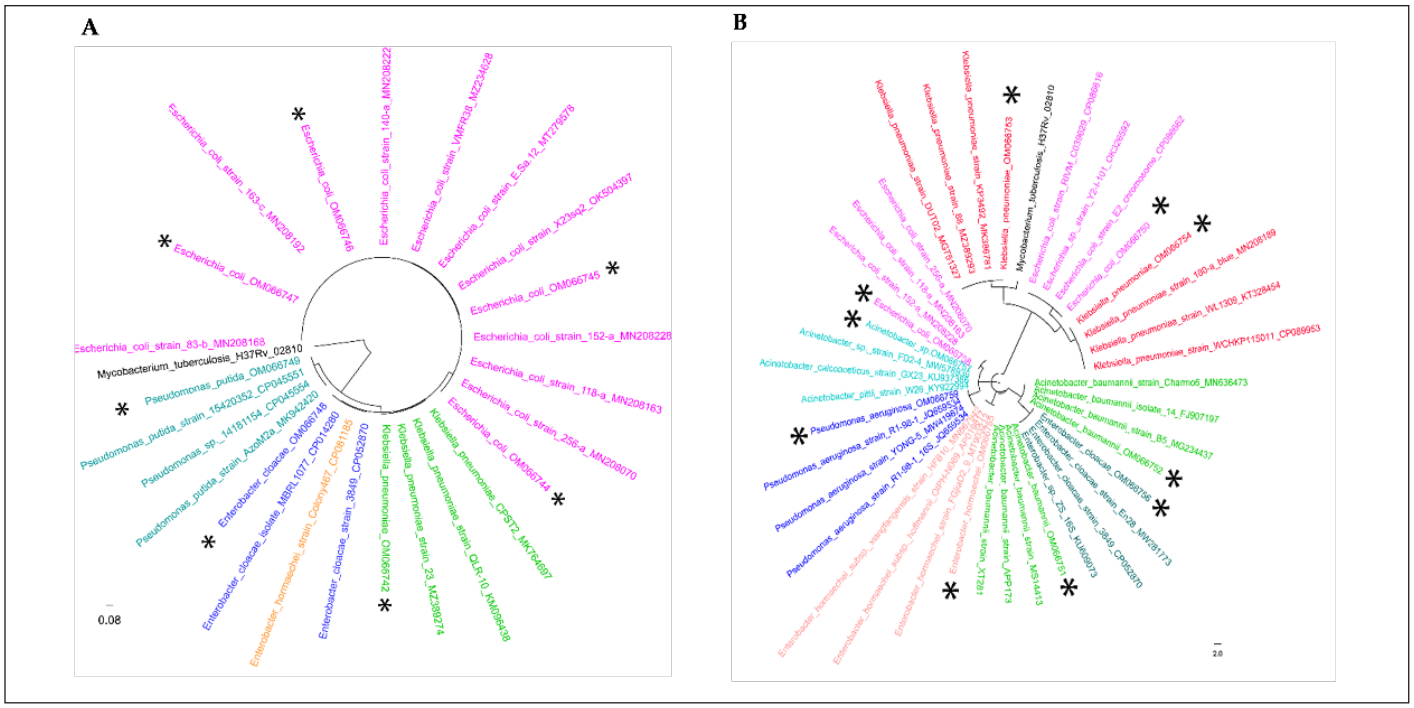

Figure 4. Evolutionary analyses were conducted in MEGA11 using the maximum likelihood method and the Tamura-Nei model. The study isolates are marked with the * sign. (A) Tree with the highest log likelihood (−4,590.91) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1,558 positions in the final dataset. Four of the *phoA* gene-positive isolates, DCI-E9, DCI-E10, DCI-E13, and DCI-E18 (OM066744, OM066745, OM066746, and OM066747), closely clustered with reference *E. coli* isolates. *phoA* PCR negative isolate DCI-E6 (OM066748), DCI-E31 (OM066742), and DCI-E12 (OM066749), closely clustered with *Enterobacter cloacae, K. pneumoniae,* and *Pseudomonas* spp. (B) The tree with the highest log likelihood (−6,340.37) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 41 nucleotide sequences. Codon positions included were first + second + third + noncoding. There were a total of 1,288 positions in the final dataset. Two of the *rcsA* gene-positive isolates, DCI-K26 and DCI-K42 (OM066753 and OM066754), are closely clustered with reference *K. pneumoniae* isolates. *rcsA* PCR negative isolate DCI-K23 (OM066750) and DCI-K34 (OM066758) closely clustered with *E. coli* reference strains; isolates DCI-K2 (OM066757), DCI-K22(OM066751), and DCI-K30 (OM066752) closely clustered with *Acinetobacter* spp. reference strains; isolate DCI-K14 (OM066755) and DCI-K40 (OM066756) closely clustered with *Enterobacter* reference strains, and isolate DCI-K27 (OM066759) closely clustered *Pseudomonas aeruginosa* reference strains.

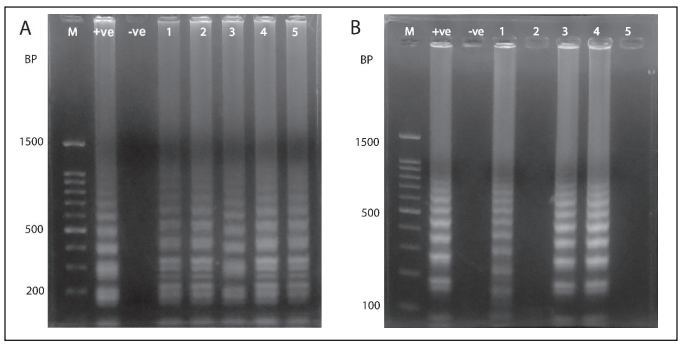

Figure 5. Agarose gel electrophoresis (1% agarose gel) image of LAMP method. A. From left, M indicates molecular marker (100–1,500) bp, *E. coli* ATCC 25922, and *K. pneumoniae* reference strain as the positive and negative control, respectively. Lanes 1–5 represent positive bands of clinical isolates (DCI-E9, DCI-E10, DCI-E13, DCI-K23, and DCI-K34) positive for the *phoA* gene. B. Lane 1 (+ve) was positive control of *K. pneumoniae* reference strain, lane 2 (−ve) was negative control [*E. coli* ATCC (25922)], lanes 1, 3, and 4 were rcsA positive isolates (DCI-E31, DCI-K26, and DCI-K42), and lanes 2 and 5 were blanks.

DISCUSSION

This study suggests that diagnostic tests' accuracy, precision, analytical sensitivity, analytical specificity, reportable range, and internal reference should be validated regularly against gold standards and monitored by proper regulatory authorities [33]. Like other departments of a diagnostic facility, the microbiology department should also strictly follow standards set by the Clinical and Laboratory Standards Institute (CLSI), European Committee on Antimicrobial Susceptibility Testing, and so on, while performing culture and sensitivity assays [34–36]. The false-negative results generated by these centers can increase mortality and morbidity, whereas false-positive results influence wrong interventions, misuse of drugs, and economic burden [2,37].

Previously, there were some reports published on misidentification by diagnostic centers. However, those reports implemented the identification of organisms using biochemical tests [1,24,38]. In this pilot study, we have used molecular techniques in conjunction with biochemical tests to

identify the extent of misidentification, suggesting that rapid and cost-effective assay for clinical diagnostics is suitable for LMICs such as Bangladesh. After the biochemical test, among the DCI-E isolates, 70%, 12%, 9%, 6%, and 3% were found to be *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas* spp., and unknown, respectively, whereas, among all DCI-K isolates, 54%, 12%, 6%, 16%, 6%, and 6% were identified as *Klebsiella* spp., *Enterobacter* spp., *E. coli*, *Pseudomonas* spp., *Acinetobacter* spp., and unknown, respectively (Tables 2 and 3). This high rate of discrepancies in pathogen identification prompted us to use molecular tests to justify our claims.

Molecular techniques are the more sensitive and fast diagnostic tools for detecting pathogens in clinical samples. RAPD is a PCR method requiring no prior knowledge of the genome since the amplified fragments are random. This makes the technique well-regarded for comparing biological systems' DNA and diversity analysis of pathogens in environmental and clinical samples [39–43]. We tried to utilize RAPD to cluster the isolates based on band patterns to reduce the number of tests. UPGMA-based dendrograms showed the representative *Klebsiella* spp., and *E. coli* isolates in distinct clusters based on the similarities among their RAPD band patterns. When DCI-E and DCI-K were analyzed, the former isolates were clustered into four groups, whereas the latter were into eight groups (Fig. 1). The last groups of each DCI isolate contained isolates with unique band patterns that could not be clustered into a specific group. Thus, RAPD helped us to shorten the number of samples that needed further

Table 5. Variation of antibiotic susceptibility pattern in different groups of Gram-negative bacterial pathogens according to CLSI.

Test/ report group	Antimicrobial agent	Disk content	Enterobacterales		*P. aeruginosa*		*Acinetobacter* spp.		Non-Enterobacterales	
			S	R	S	R	S	R	S	R
Penicillins										
	Piperacillin	100	≥21	≤17	≥21	≤14	≥21	≤17	NC	NC
β-Lactam combination agents										
	Ampicillin-sulbactam	20/10[a]	≥18	≤13	NC	NC	≥15	≤11	NC	NC
	Piperacillin-tazobactam	100/10	NC	NC	NC	NC	≥21	≤17	NC	NC
	Ticarcillin-clavulanate	75/10	NC	NC	NC	NC	≥20	≤14	NC	NC
Cephems (parenteral) (including cephalosporins I, II, III, and IV)										
	Ceftazidime	30	≥21	≤17	≥20	≤14	≥18	≤14	NC	NC
	Cefepime	30	≥25	≤18	≥18	≤14	≥18	≤14	NC	NC
	Cefotaxime	30	≥26	≤22	NC	NC	≥23	≤14	NC	NC
	Ceftriaxone	30	≥23	≤19	NC	NC	≥21	≤13	NC	NC
	Cefiderocol	30	≥16	≤11	≥18	≤12	≥15	≤10	NC	NC
Monobactams										
	Aztreonam	30	≥21	≤17	≥22	≤15	NC	NC	NC	NC
Carbapenems										
	Doripenem	10	≥23	≤19	≥19	≤15	≥18	≤14	NC	NC
	Imipenem		≥23	≤19	≥19	≤15	≥22	≤18	NC	NC
	Meropenem		≥23	≤19	≥19	≤15	≥18	≤14	NC	NC
Aminoglycosides										
	Netilmicin	30	≥15	≤12	≥15	≤12	NC	NC	NC	NC
Tetracyclines										
	Doxycycline	30	≥15	≤11	NC	NC	≥13	≤9	NC	NC
	Minocycline	30	≥16	≤12	NC	NC	≥16	≤12	NC	NC
	Tetracycline	30	≥15	≤11	NC	NC	≥15	≤11	NC	NC
Fluoroquinolones										
	Ciprofloxacin	5	≥26	≤21	≥25	≤18	≥21	≤15	NC	NC
	Levofloxacin	5	≥21	≤16	≥22	≤14	≥17	≤13	NC	NC
	Lomefloxacin	10	≥22	≤18	≥22	≤18	NC	NC	NC	NC
	Norfloxacin	10	≥17	≤12	≥17	≤12	NC	NC	NC	NC
	Ofloxacin	10	≥16	≤12	≥16	≤12	NC	NC	NC	NC
Folate pathway antagonists										
	Trimethoprim-sulfamethoxazole	1.25/23.75	NC	NC	≥16	≤10	NC	NC	NC	NC

R = resistant; S = sensitive; NC = not characterized.

Table 6. List of antibiotics for which no standard is set for *P. aeruginosa*, *Acinetobacte*r spp., and non-Enterobacterales according to CLSI.

Organism	Antibiotics	Zone of inhibition	Minimum inhibitory concentration standard
Enterobacterales	Ampicillin, mecillinam, amoxicillin-clavulanate, ceftolozane-tazobactam, ceftazidime-avibactam, imipenem-relebactam, meropenem-vaborbactam, cefazolin, cefazolin, ceftaroline, cefotetan, cefoxitin, cefamandole, cefmetazole, cefonicid, cefoperazone, moxalactam, cefuroxime, cefazolin, loracarbef, cefaclor, cefdinir, cefpodoxime, cefprozil, cefetamet, ceftibuten, ertapenem, amikacin, kanamycin, streptomycin, azithromycin, cinoxacin, enoxacin, gatifloxacin, gemifloxacin, grepafloxacin, nalidixic acid, fleroxacin, sulfonamides, trimethoprim, fosfomycin, and nitrofurantoin	CLSI standard	CLSI standard
*P. aeruginos*a		No CLSI standard	No CLSI standard
Acinetobacter spp.		No CLSI standard	No CLSI standard
Non-Enterobacterales		No CLSI standard	No CLSI standard

analysis. However, multiple clustering of the same genus makes RAPD unsuitable for diagnostic use in pathogen identification.

phoA gene encodes bacterial alkaline phosphatase. Under low phosphate conditions, *E. coli* alkaline phosphatase is synthesized and secreted across the inner membrane to the periplasmic space, which plays a critical role in the breakdown of organic phosphate esters [44,45]. Among 33 DCI-E, 23 (70%) isolates and 2 isolates from DCI-K were *phoA* positive (Fig. 2A). Sequencing the *phoA* amplicons confirmed their proper amplification and identification (Fig. 3A). On the other hand, *rcsA* is involved in expressing the K antigen capsule [46] of *K. pneumoniae*. Among *Klebsiella* spp., *K. pneumoniae* is mainly responsible for infectious diseases. *rcsA* gene was selected for molecular detection of *K. pneumoniae* because of its clinical significance [2]. Thirteen (48%) isolates from 27 biochemically positive *Klebsiella* spp. among the 50 DCI-K were *rcsA* gene positive (Fig. 2B). Identities were confirmed by sequencing the *rcsA* amplicons (Fig. 3B).

The absence of *phoA* and *rcsA* genes in 30% DCI-E and 46% DCI-K were further characterized by 16S rRNA PCR followed by sequencing. Misidentification was confirmed in both groups, where *Enterobacter* sp., *Klebsiella* sp., and *Pseudomonas* sp. were misidentified as *E. coli* (Table 4, Fig. 4A). On the other hand, *Enterobacter* sp., *E. coli*, *Pseudomonas* sp., and *Acinetobacter* spp. were misidentified as *Klebsiella*, not only by the diagnostic centers (Table 4, Fig. 4B).

Later, we employed LAMP, a rapid, simple, specific, and cost-effective nucleic acid amplification method developed by Eiken Chemical Co., Ltd., LAMP amplifies DNA with high specificity, efficiency, and rapidly under the isothermal condition where two to three sets of primers (outer, loop, and inner) are used. It is a cost-effective detection method that can be performed with simple instruments, such as a heater or water bath [47]. The LAMP method can detect *E. coli* and *K. pneumoniae* [27,28]. We used the *rcsA* gene and *phoA* gene primer set (Table 1) to detect *K. pneumoniae* and *E. coli* from clinical samples within 60 minutes (Fig. 5). Due to the high-sensitivity nature of LAMP in the case of *K. pneumoniae,* we observed some cross-reacting nonspecific bands with negative controls. Such discrepancies were reported previously, emphasizing the importance of validating these methods before clinical use and overcoming the misidentification of clinical pathogens [48,49].

The impact of the misidentification of clinical pathogens on AMR development is yet to be understood. However, Table 5 shows that, according to CLSI, antibiotic suggestion and susceptibility patterns in the disk-diffusion method vary among bacterial groups. Although the pattern or suggested antibiotics are the same for the genera belonging to Enterobacterales, the largest group of pathogenic bacteria [50], the situation varies outside this order. For non-Enterobacterales, no standards are set for the disk-diffusion method. Moreover, sensitivity patterns of many antibiotics (Table 6) are only known for Enterobacterales. Therefore, we hypothesized that misidentification followed by improper antibiotic suggestions may lead to AMR development.

LIMITATION OF THE STUDY

This pilot study works with a limited number of clinical pathogens; however, the scenario of misidentification was observed. Further experimental proof is required to identify the prospective route of antibiotic resistance development, which is beyond the scope of this study.

CONCLUSION

Here, we found that traditional culture and biochemical processes are not always accurate, whereas molecular techniques are more precise and ensure proper identification. Since antibiotic susceptibility varies among genera, misidentification by the diagnostic facilities/laboratories may lead to the wrong antibiotic prescribing and, consequently, may result in antibiotic resistance development. Therefore, healthcare authorities of a country, especially in the LMICs, should develop policies for the healthcare and diagnostic institutes to provide standard services and regularly monitor the practices. Policies should be included in a country's antibiotic stewardship program to mitigate diagnostic centers' misidentification of clinical pathogens. Diagnostic centers and healthcare facilities should follow international and national guidelines for correctly identifying clinical pathogens and their susceptibility to antibiotics. Moreover, healthcare workers should be adequately trained to perform and interpret the test results.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the

International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The study protocol was approved by the Biosafety, Biosecurity, and Ethic Committee of Jahangirnagar University Bangladesh [Ref No: BBEC, JU/M-2022(5), dated 02-02-2022].

DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

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