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Article

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. Misdiagnosis can hamper such success while leading to improper advising of antibiotics. In Bangladesh, the majority of the diagnostic centers detect and identify pathogens through culture and biochemical test-based methods and suggest antibiotics based solely on the results of disk-diffusion methods. This pilot study tried to validate the identity of the isolates characterized by diagnostic facilities near Dhaka. One hundred and twenty pre-characterized clinical isolates were analyzed biochemically and genotypically. Random Amplification of Polymorphic DNA-PCR, *rcsA*, and *phoA* genes-based PCR, and Loop-Mediated Isothermal Amplification (LAMP)-based identification of *Klebsiella pneumoniae* and *Escherichia coli*, respectively, followed by 16S rRNA sequencing confirmed misidentification of some clinical pathogens of other genera as *Klebsiella* spp. and *E. coli*. According to the antibiotic susceptibility testing guidelines, antibiotic choice, sensitivity pattern, and breakpoint measurement are different for each group of organisms. The lack of adherence to proper standards results in misdiagnosis and may facilitate the development of antibiotic resistance. The pilot study observers misidentification of clinical pathogens identified by the diagnostic centers. Well-characterized rapid molecular techniques like LAMP are suggested in clinical diagnosis to avoid misdiagnosis and subsequently circumvent antibiotic resistance development.

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Keywords: Misdiagnosis; Random Amplification of Polymorphic DNA; 16S rRNA Sequencing, Loop-Mediated Isothermal Amplification, Antibiotic Resistance.

1. Introduction

Bacterial identification is generally accomplished through labor-intensive biochemical tests. Such applications may be ineffective if the findings are needed for medical diagnosis on a quick emergency basis. However, biochemical approaches may not always identify bacteria accurately and frequently provide unacceptable numbers of incorrect conclusions [1-2]. Culture-based techniques are considered the gold standard for pathogen detection, but they are limited by being laborious, time-consuming, requiring trained human resources, the use of standard reagents, and specificity [3].

Nowadays, pathogen identification is carried out by utilizing API, Vitek-2/MS, NMR spectroscopy, or MALDI-TOF in developed countries or advanced hospital diagnostic settings as an alternative to culture-based approaches [4-6]. In addition to these procedures, diagnostic facilities regularly utilize nucleic acid amplification techniques such as PCR, qPCR, 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 of reference laboratories due to their high-cost association and a high technical necessity [11]. Unlike conventional amplification methods, isothermal amplification methods such as Loop-mediated isothermal amplification (LAMP) is a molecular approach known for being simple, fast, cost-effective, and highly reliable if properly designed and suggested for usage in resource-constrained areas [12-15].

Escherichia coli and *Klebsiella 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]. *K. 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]. Additionally, a high risk of mortality is associated with patients infected with antibiotic-resistant strains of these microbes [19].

Antibiotic resistance is a major threat to healthcare systems worldwide. Annually, 700,000 people lose their lives worldwide due to infection with antibiotic-resistant bacteria. It is estimated that resistance will be more fatal than cancer by 2050 [20]. In line with such observations, World Health Organization (WHO) has designated antimicrobial resistance (AMR) as one among the top ten worldwide public health problems confronting humanity [21]. Antibiotic resistance develops naturally. However, the misuse of antibiotics in humans and animals accelerates the process in one direction. The factors that are contributing to the development of antibiotic resistance are inappropriate prescription practices, lack of adequate patient education, unauthorized antibiotic sales, insufficient

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diagnostic facilities, missed diagnosis, and non-human antimicrobial usage in animals [22-23].

This pilot study collected the previously characterized clinical bacterial isolates from two reputed hospital-based diagnostic facilities near Dhaka, Bangladesh. We attempted to determine their proper identity by applying different molecular techniques. Interestingly, we confirmed apparent misdiagnosis in those diagnostic center-identified (DCI) clinical isolates, suggesting one of the possible causes of developing antibiotic resistance in low-to-middle income countries (LMICs) like Bangladesh.

2. Materials and Methods

2.1 Study Samples

One hundred twenty isolates previously characterized by diagnostic facilities were collected from 2017 to 2018. The isolates were stored in -80 ˚C and sub-cultured annually. Before working with the isolates, the cultures were thawed at room temperature and grown in minimal media for recovery, according to the protocol described by Paliy et al. [24]. To ensure the purity of the isolated, they were cultured on MacConkey agar (Scharlau, Spain) and Eosin Methylene Blue (EMB) Agar (Himedia, India) media.

2.2 Phenotypic Characterization of the Isolates

Immediately upon receiving the isolates, biochemical tests were carried out by the laboratory for characterization and identification [3, 25]. Before starting this work, biochemical tests such as Indole production, Methyl Red, Voges Proskauer, Citrate utilization, Catalase, motility, Triple sugar iron (TSI), and sugar utilization were performed to cross-check the previous characterization performed by our laboratory. Further phenotypic validation was carried out using API 20E kit (BioMérieux, France).

2.3 Molecular Detection

2.3.1 Boiling Method for Total DNA Extraction

Chromosomal DNA of the isolates was extracted using the boiling DNA method. In short, isolated colonies from the nutrient agar plate were grown in a test tube overnight in a 5 ml nutrient broth at 37°C. 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 200µl of PCR-grade water (Gibco). After that, each microcentrifuge tube was kept at 90-95°C for 10 minutes in a heat block (Thermo Fisher Scientific), which was then immediately 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 [26].

2.3.2 Randomly Amplified Polymorphic DNA (RAPD) Analysis

Extracted bacterial DNA was subjected to RAPD genotyping. Random primer (10 bp) 5'- AAGAGCCCGT-3' (Primer 1247) was selected for observing their band pattern to classify them into different groups. The PCR reactions were performed in 25 µl volumes containing 12.5 µl master mix (GoTaq® G2 Green Master Mix(2X)), 2µl of primer, 4µl

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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 finally 72°C for 30 seconds for a total of 45 cycles in Takara Thermocycler [27]. Amplicons were characterized in 1% agarose gel.

2.3.4 Polymerase Chain Reaction (PCR) of E. coli phoA Gene

The alkaline phosphatase (*phoA*) gene was used for the identification of *Escherichia coli.* **5'-** AAGTTGAAGGTGCGTCAAT-3′-F3 and 5′-CTTGTGAATCCTCTTCGGAG-3′-B3 primers were used to identify *E. coli* isolates more accurately [28]. The PCR reactions were performed in 25 µl volumes containing 12.5µl master mix, 2µl each of the forward and reverse primers, 2µl 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 finally 72°C for 30 seconds for a total of 45 cycles. Amplicon's length of approximately 277 bp was initially confirmed using a molecular ladder as amplification of *phoA*. Later, PCR bands were extracted from agarose gel and sequenced to confirm the specific amplification.

2.3.5 Polymerase Chain Reaction (PCR) of K. pneumoniae rcsA Gene

The *rcsA* gene in *K. pneumoniae* produces capsular polysaccharides, which is an important factor in its virulence. 5'- GGATATCTGACCAGTCGG-3′-KP27F3 and 5'-GGGTTTTGCGTAATGATCTG-3′-KP27B3 primer were used for the detection of *K. pneumoniae* [29]. The PCR reactions were performed in 25 µl volumes containing 12.5µl master mix, 1µl each of primer F and primer R, 4µl 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 as amplification of *rcsA* and later confirmed by sanger sequencing.

2.3.6 Polymerase Chain Reaction (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 25µl PCR reactions, the PCR mixture contained 12.5µl master mix (Promega, USA), 2µl each of the forward and reverse primers, 2µl template DNA, and 8.5 µl nuclease-free water [30].

2.3.7 Phylogenetic Analysis

The PCR products (Takara, Japan) were analyzed on 1% agarose gel using an agarose gel electrophoresis unit (Mupid, Japan). The amplicons were purified using the FavorPrep GEL/PCR Purification Kit, as directed by the manufacturer. After that, the purified PCR products were used for Sanger dideoxy sequencing (3500 Series Genetic Analyzer, Applied Biosystems). The basic 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.

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2.3.8 LAMP (Loop-mediated Isothermal Amplification)

LAMP reactions were conducted in 25-µL volumes containing 15µL Bst Polymerase Isothermal master mix (NEB, UK), 5µL (1X) Primers, 5µL Template DNA [32]. The list of primer sequences required to detect *E.coli* and *Klebsiella 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.

Table 1. Primer sequence of LAMP process for the detection of *E. coli* and *Klebsiella pneumoniae*

Organism	Target Gene	Primer	Sequence
Escherichia coli	Alkaline phosphat-ase *phoA*	F3	AAGTTGAAGGTGCGTCAAT
		B3	CTTGTGAATCCTCTTCGGAG
		FIP	GTGATCAGCGGTGACTATGACCTCTCGATGAAGCC GTACA
		BIP	ATTGTCGCGCCGGATACCCTCATCACCATCACTGC G
		Loop F	AGCGTGTTGCCATCCTTT
		Loop B	CAGGCGCTAAATACCAAAGATG
Klebsiella pneumoniae	Capsular polysacc-harides *rcsA*	KP-27F3	GGATATCTGACCAGTCGG
		KP-27B3	GGGTTTTGCGTAATGATCTG
		KP-27FIP	CGACGTACAGTGTTTCTGCAG TTTTAAAAAACAGGAAATCGTTGAGG
		KP-27BIP	CGGCGGTGGTGTTTCTGAATT TTGCGAATAATGCCATTACTTTCG
		KP-27LB	GAAGACTGTTTCGTGCATGATGA

3. Results

3.1 Isolation and Characterization of Escherichia coli and Klebsiella Isolates

A total of 120 bacterial isolates (samples) were collected from hospital-based diagnostic facilities in Savar, Dhaka, Bangladesh, with diagnostic records termed DCI-(Diagnostic Center identified) isolates. According to diagnostic center records, clinical isolates contained 27.5% *E.coli* and 41.67% *Klebsiella* spp, 11.67% *Pseudomonas* spp, 3.33% *Streptococcus* spp, 13.33% *Staphylococcus* spp, and 2.5% *Proteus* spp. DCI-*E. coli* (33/120) isolates and *Klebsiella* spp. (50/120) isolates were selected for further work because of their high prevalence and clinical significance. Previously, we presumptively re-assessed the identities of these isolates culturing on selective media (MacConkey and EMB) and biochemical tests

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(IMVIC, KIA, Catalase, sugar fermentation such as glucose sucrose, mannitol, xylose), and surprisingly found that 30% of the DCI- *E. coli* and 46% the DCI- *Klebsiella* spp. were misdiagnosed and were other than *E. coli* and *Klebsiella* spp, respectively [25]. We found presumptive misdiagnosis of DCI- *Klebsiella* isolates as *E. coli*, *Enterobacter* spp., *Pseudomonas* spp., and *Acinetobacter* spp. Similarly, DCI- *E. coli* isolates were *Enterobacter* spp., *Klebsiella* spp., *Pseudomonas* spp., and *Yersinia* spp.

Representative isolates from each biochemical profile were further tested on the API 20E system, and identity was confirmed by apiweb™ software (Figure 1).

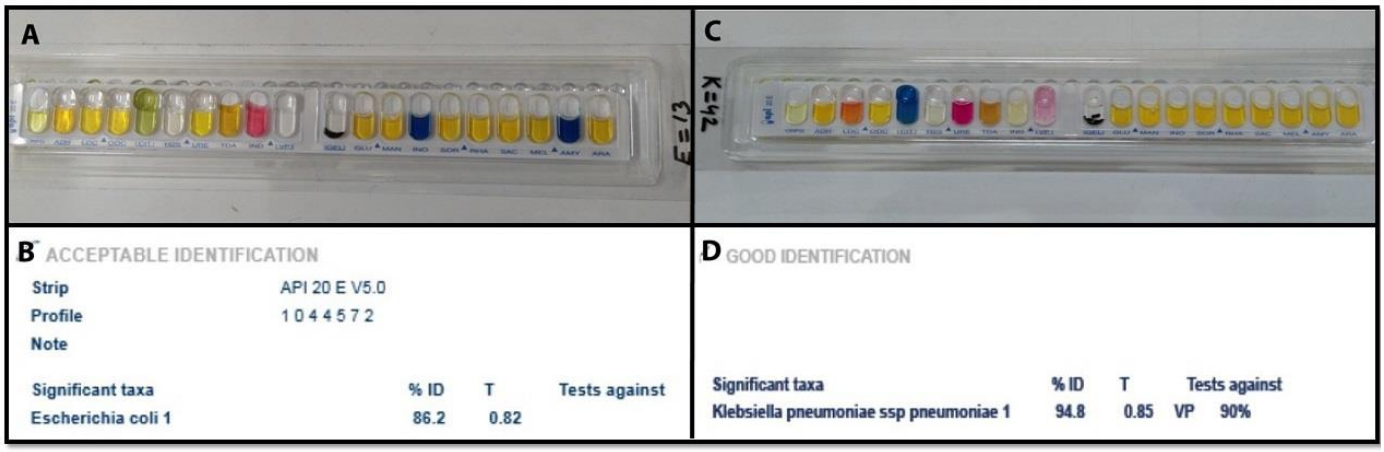

Figure 1. API 20E profiling and identification of clinical pathogens, (A) API 20E profile of DCI-E13, (B) apiweb™ identify DCI-E13 as *E. coli*, (C) API 20E profile of DCI-K42, and (D) apiweb™ identify DCI-K42 as *Klebsiella pneumoniae.*

3.2 MolecularCcharacterization of the Bacterial Isolates

3.2.1 RAPD

Among the PCR-based methods, RAPD is a fast method for generating DNA profiles comparable to pulsed-field gel electrophoresis, which is considered the gold standard in genetic fingerprinting [33]. In this study, we employed our optimized RAPD protocol as an attempt to discriminate among various clinical isolates of *E. coli* and *Klebsiella* spp.

In this pilot study, RAPD for all the DCI-*Klebsiella* spp isolates was performed with primer 1247, where it showed nine different band patterns. Isolates belonging to the first eight groups showed the same intra-group band pattern (Figure 2A), whereas isolates in Group 9 showed different banding patterns from each other (data not shown).

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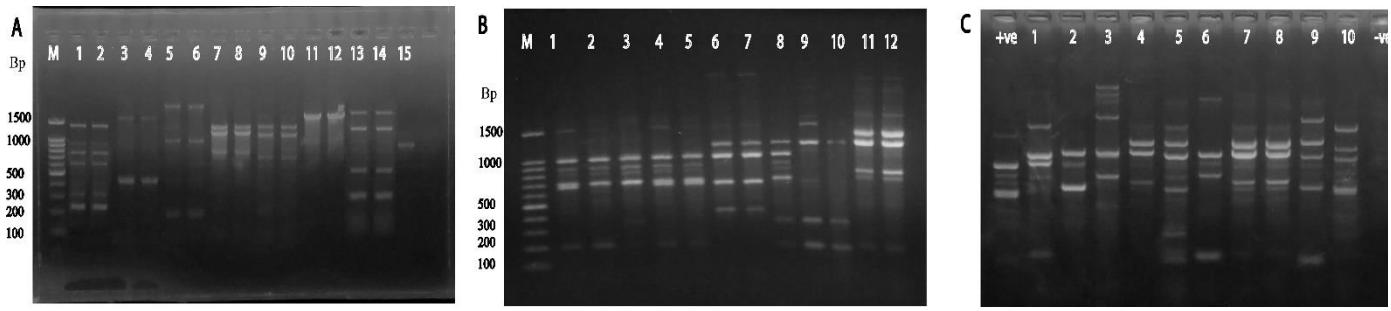

Figure 2. Gel electrophoresis image of RAPD for DCI-*Klebsiella* spp (A), and DCI-*E. coli* (B-C) using primer 1247. M indicates a molecular marker (ladder) that was 100-1500 bp. -Ve indicates the template less control. (A) Lane 1,2=group 2; lane 3,4= group 3; lane 5,6= group 1; lane 7,8= group 4; lane 9,10= group 5; lane 11,12= group 6; lane 13,14= group 7 ; lane 15= group 8. (B) lane 1-4= group; lane 5, 6, 7= group 2; lane 8,9= group 3; and lane 10, 11= group 4. (C) +Ve indicate *E.coli* ATCC 25922 as control Lane 1, 2, 3, 5, 6, 9, and 10 gave different band patterns from each other.

On the contrary, according to the banding pattern 16 (of all 33 isolates), *E. coli* isolates were represented by four groups (Figure 2B). The other 17 isolates did not give similar band patterns, and they all were represented by group 5 (Figure 2C). These banding patterns were used to select representative isolates for characterization through PCR and sequencing.

3.2.2 Detection of E. coli and Klebsiella pneumoniae Using phoA and rcsA Genes Specific PCR

Alkaline phosphatase (*phoA*) gene was used to characterize 33 DCI- *E. coli* isolates and three biochemically *E. coli* positive isolates among DCI-*Klebsiella* spp. Twenty-three isolates from thirty-three DCI- *E. coli* isolates and two isolates from DCI-*Klebsiella* spp. gave banding patterns similar to *E. coli* reference strains as (JM109, V517, BL21 (DE3), and *E. coli* ATCC 25922 (Figure 3A). *Klebsiella pneumoniae* reference strain, *Vibrio cholerae* ATCC (14035), *Micrococcus luteus* ATCC (4698 (150307)), and *Staphylococcus aureus* ATCC (6538) were used as negative controls.

Similarly, *Klebsiella pneumoniae* specific capsular polysaccharide gene *rcsA* was used to identify *Klebsiella pneumoniae* isolates amongst the DCI-*Klebsiella*, which were identified biochemically as *Klebsiella* spp. Reference strains were used as positive controls, while *Vibrio cholerae* ATCC (14035), *Micrococcus luteus* ATCC (4698), *Staphylococcus 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 after PCR amplification and gel electrophoresis in negative control lanes. However, 13 isolates from 27 biochemically positive *Klebsiella spp.* isolates gave the band similar to *Klebsiella pneumoniae* reference strains (Figure 3B).

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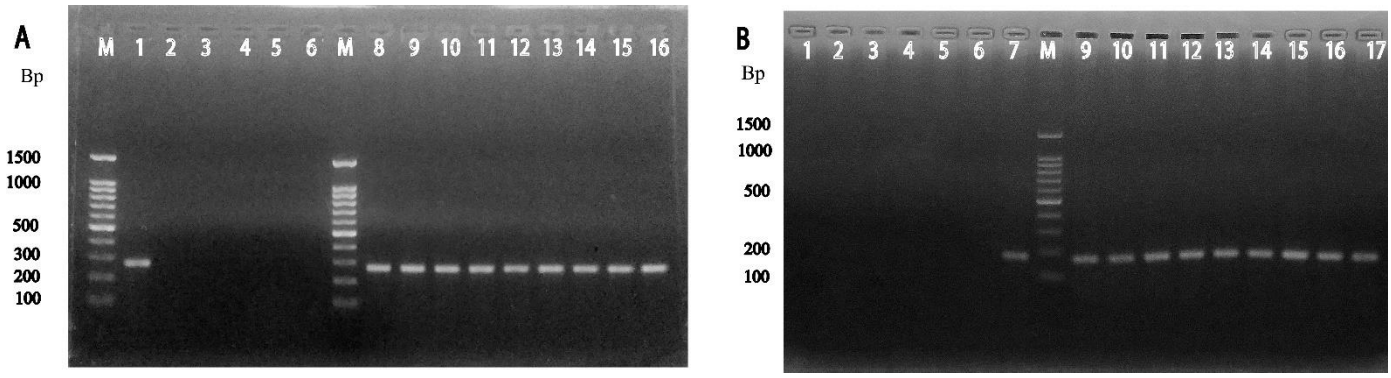

Figure 3. Gel electrophoresis image of PCR amplified *phoA* gene (A) and *rcsA* gene (B). (A) Agarose gel electrophoresis (on 1.5% agarose gel) image showing the amplified products by PCR which was done by using *phoA* primer (~270 bp). This figure indicates the molecular marker, which was 100-1500 base pairs (bp). Lane-1 indicates *E. coli* ATCC (25922) as a positive control. Lanes-2-6 represents negative control, as Lane-3= *Klebsiella pneumoniae* reference stain, Lane-4= *Vibrio cholerae* ATCC (14035), Lane 5= *Micrococcus luteus* ATCC (4698), Lane 6= *Staphylococcus aureus* ATCC (6538). Lanes-8-16 were representative isolates, as lanes 8-16 show positive results (band) (amplified products of *phoA* are located between 200 bp to 300 bp sized band of DNA marker). (B) Lanes-1-6 represents negative control, as Lane-1= *Vibrio cholerae* ATCC (14035), Lane 2= *Escherichia coli* ATCC (25922), Lane 3= *Micrococcus luteus* ATCC (4698), Lane 4=*Salmonella typhi* ATCC (14028), Lane 5= *Staphylococcus aureus* ATCC (6538), and Lane 6= *Bacillus cereus* ATCC (14574). Lane-7 represents *Klebsiella pneumoniae* reference strain as the positive control, lane-8 contained molecular ladder, and lanes-9-17 were *rcsA* gene-positive clinical isolates samples. These band sizes are ~176bp and located just below the 200bp.

3.2.3 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 *Klebsiella* spp., respectively. Three amplicons from each group were subjected to sequencing using the forward primers. For homology alignment, *phoA* and *rcsA* gene sequences from *E. coli* (Accession No. MG1655) and *Klebsiella pneumoniae* (Accession No NNP58345) were retrieved, respectively. Multiple sequence alignments (MSA) of these gene sequences were performed using ClustalW Multiple Alignment tools. MSA found the amplicons specific to their respective genes (Figure 4).

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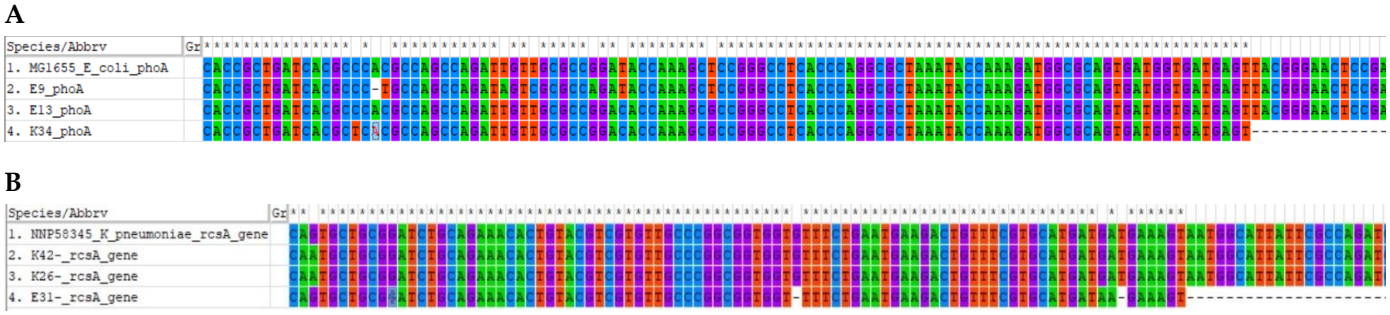

Figure 4. Homology alignment of *phoA* and *rcsA* gene. (A) *phoA* amplicon sequences from three isolates, two from DCI- *E. coli* (E9 and E13), and one DCI-*Klebsiella* (K34), were aligned with *phoA* gene sequence of reference *E. coli* (MG1655), and the amplicons matched the reference sequence. (B) *rcsA* amplicon sequences from three isolates, two from DCI-*Klebsiella* (K26 and K42), and one DCI-*E. coli* (E31) were aligned with *rcsA* gene sequence of reference *Klebsiella pneumoniae* (NNP58345), and the amplicons matched the reference sequence.

3.2.4 16S rRNA PCR, sequencing, and phylogenetic analysis

For further confirmation of the match and mismatches of bacterial identification by diagnostic centers, representative isolates from the RAPD pattern and biochemical tests were subjected to 16S rRNA PCR. The PCR products were gel extracted and sequenced. The sequences were submitted to Genebank. Sequencing confirms misdiagnosis of clinical pathogens by diagnostic centers (Table 2).

Table 2. Comparative identification of bacterial pathogens by the diagnostic centers, biochemical profiling, *pho*A, and *rcsA* gene-specific PCR, and 16S rRNA sequencing.

Sample Code	DCI Identification	Biochemical profile	Gene	16S rRNA Sequencing	Accession No	Comment
E6	*E. coli*	*Enterobacter sp.*	-	*Enterobacter cloacae*	OM066748	**Mis-match**
E9	*E. coli*	*E. coli*	*phoA*	*E. coli*	OM066744	Match
E10	*E. coli*	*E. coli*	*phoA*	*E. coli*	OM066745	Match
E12	*E. coli*	*Pseudomonas sp.*	-	*Pseudomonas putida*	OM066749	**Mis-match**
E13	*E. coli*	*E. coli*	*phoA*	*E. coli*	OM066746	Match
E18	*E. coli*	*E. coli*	*phoA*	*E. coli*	OM066747	Match
E31	*E. coli*	*Klebsiella sp.*	*rcsA*	*Klesbsiella pneumoniae*	OM066742	**Mis-match**
K2	*Klebsiella sp.*	*Klebsiella sp.*	-	*Acinetobacter sp.*	OM066757	**Mis-match**

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K14	*Klebsiella sp.*	*Enterobacter sp.*	-	*Enterobacter hormaechei*	OM066755	**Mis-match**
K22	*Klebsiella sp.*	*Klebsiella sp.*		*Acinetobacter baumannii*	OM066751	**Mis-match**
K23	*Klebsiella sp.*	*Klebsiella sp.*	*phoA*	*Escherichia coli*	OM066750	**Mis-match**
K26	*Klebsiella sp.*	*Klebsiella sp.*	*rcsA*	*Klesbsiella pneumoniae*	OM066753	Match
K27	*Klebsiella sp.*	*Pseudomonas sp.*	-	*Pseudomonas aeruginosa*	OM066759	**Mis-Match**
K30	*Klebsiella sp.*	*Acinetobacter sp.*	-	*Acinetobacter baumannii*	OM066752	**Mis-Match**
K34	*Klebsiella sp.*	*Klebsiella*	*phoA*	*Escherichia coli*	OM066758	**Mis-match**
K40	*Klebsiella sp.*	*Enterobacter sp.*	-	*Enterobacter hormaechei*	OM066756	**Mis-match**
K42	*Klebsiella sp.*	*Klebsiella sp.*	*rcsA*	*Klesbsiella pneumoniae*	OM066754	Match

Closely related reference 16S rRNA sequences were downloaded from the NCBI database, and sequences were aligned with ClustalW multiple sequence alignment tool. The alignments were subjected to phylogenetic tree development using the maximum likelihood method and Tamura-Nei model (Mega 11). The bootstrap value of 1000 was set to minimize the error of distant measurement. The phylogenetic tree was generated with selected DCI-*E. coli* and DCI- *Klebsiella* spp. shows matched and mismatched isolates in different branches (Figure 5).

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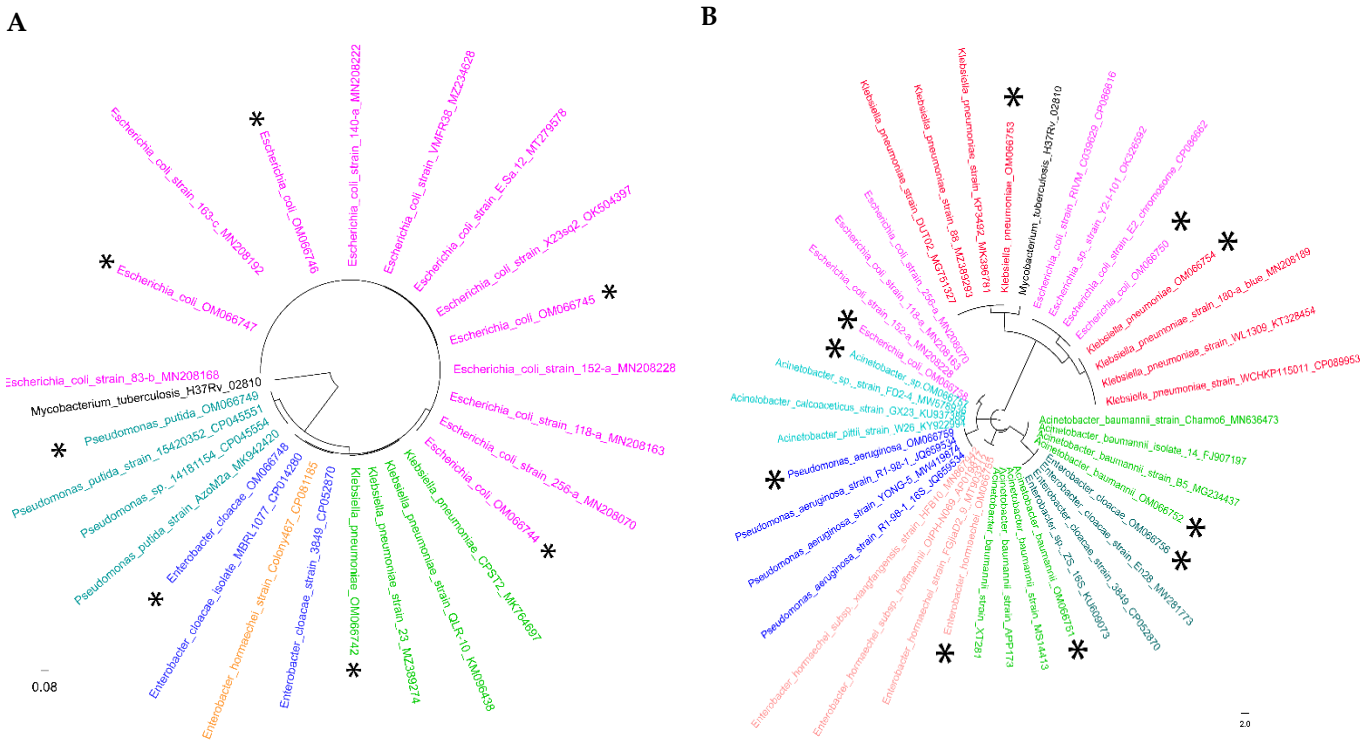

Figure 5. Evolutionary analyses were conducted in MEGA11 by using the Maximum Likelihood method and Tamura-Nei model. The study isolates are marked with the * sign. (A) The tree with the highest log likelihood (-4590.91) is shown. Initial tree(s) for the heuristic search were obtained automatically by 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 1558 positions in the final dataset. Four of the *phoA* gene-positive isolates, E9, E10, E13, and E18 (OM066744, OM066745, OM066746, and OM066747), closely clustered with reference *E. coli* isolates. *phoA* PCR negative isolate E6 (OM066748), E31 (OM066742), and E12 (OM066749), closely clustered with *Enterobacter cloacae, Klebsiella pneumoniae,* and *Pseudomonas* spp. (B) The tree with the highest log likelihood (-6340.37) 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 1st+2nd+3rd+Noncoding. There were a total of 1288 positions in the final dataset. Two of the *rcsA* gene-positive isolates, K26 and K42 (OM066753 and OM066754), are closely clustered with reference *Klebsiella pneumoniae* isolates. *rcsA* PCR negative isolate K23 (OM066750) and K34 (OM066758) closely clustered with *E. coli* reference strains; isolates K2 (OM066757), K22(OM066751), and K30 (OM066752) closely clustered with *Acinetobacter* spp

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reference strains; isolate K14 (OM066755) and K40 (OM066756) closely clustered with *Enterobacter* reference strains, and isolate K27 (OM066759) closely clustered *Pseudomonas aeruginosa* reference strains.

3.2.5 Rapid Molecular Identification of Clinical Isolates by Loop-Mediated Isothermal Amplification Method

Biochemical, *phoA*, and *rcsA* gene PCR positive *E. coli* (E9, E10, E13, and K34) and *K. pneumoniae* (E31, K26, and K42) were subjected to LAMP analysis. All *phoA* and *rcsA* gene-positive isolates showed multiple band patterns in 1% agarose gel (Figure 6).

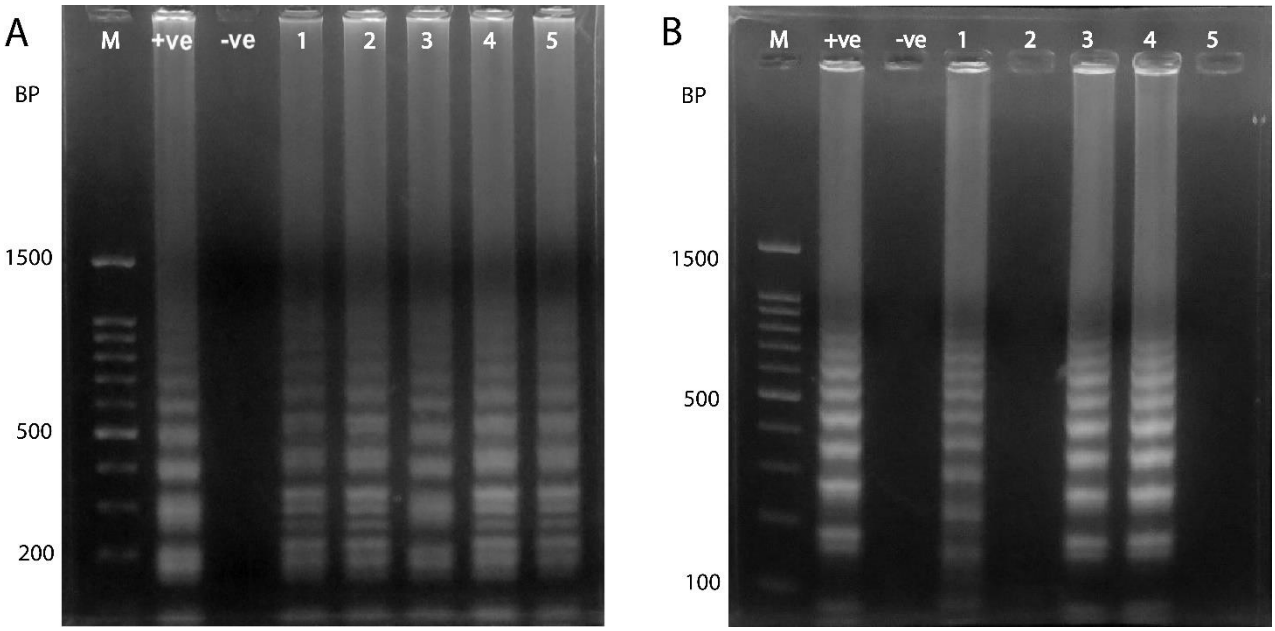

Figure 6. Agarose gel electrophoresis (1% agarose gel) image of LAMP method. M indicates molecular marker (100 -1500) bp. (A) Lane 1 (+ve) was positive control as *E. coli* ATCC 25922, lane 2 was negative control (*Klebsiella pneumoniae* reference strain), and lanes 1-5 were clinical isolates of *phoA* gene PCR positive (E9, E10, E13, K23, and K34). (B) Lane 1 (+Ve) was positive control of *Klebsiella pneumoniae* reference strain, lane 2 (-Ve) was negative control (*Escherichia coli* ATCC (25922)), lanes-1, 3, and 4 were *rcsA* positive isolates (E31, K26, and K42), and lanes 2 and 5 were blanks.

4. Discussion

4.1 Misdiagnosis of Pathogens by Diagnostic Facilities

This study suggests that tests' accuracy, precision, analytical sensitivity, analytical specificity, reportable range, and reference internal should be validated in regular intervals against gold standards and need to be monitored by proper regulatory authorities [34]. Similar to other departments of a diagnostic facility, the microbiology department should also strictly follow standards set by CLSI, EUCAST, etc., while

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performing culture and sensitivity assays [35-37}. The false-negative results generated by these centers can lead to increase mortality and morbidity, whereas false-positive results influence wrong interventions, misuse of drugs, and economic burden [2,38].

Previously, there were some reports published on misdiagnosis by diagnostic centers. However, those reports implemented the identification of organisms using biochemical tests [1,25,39]. In this pilot study, we have used molecular techniques in conjunction with biochemical tests to identify the extent of misdiagnosis, suggesting that rapid and cost-effective assay for clinical diagnostics is suitable for LMIC like Bangladesh. After the biochemical test, among the DCI-*E. coli* 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-*Klebsiella* isolates, 54%, 12%, 6%, 16%, 6%, and 6% were identified as *Klebsiella* spp., *Enterobacter* spp., *E. coli*, *Pseudomonas* spp, *Acinetobacter* spp., and unknown, respectively. 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 polymerase chain reaction method, requiring no prior knowledge of the genome since the amplified fragments are random. This makes the method well-regarded for comparing biological systems' DNA as well as diversity analysis of pathogens present in environmental and clinical samples [40-44]. We tried to utilize RAPD to cluster the isolates on the basis of band patterns to reduce the number of tests. When DCI- *E. coli* and DCI- *Klebsiella* were analyzed, the former were clustered into five groups, whereas the latter into nine groups (Figure 2). The last groups of each DCI-isolates contained isolates with unique band patterns which could not be clustered into a specific group. Thus, RAPD helped us shorten the number of samples needed to be further analyzed. 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, where it plays a critical role in the breakdown of organic phosphate esters [45,46] Among 33 DCI-*E. coli*, 23 (70%) isolates and two isolates from DCI-*Klebsiella* were *phoA* positive (Figure 3A). Sequencing the *phoA* amplicons confirmed their proper amplification and identification (Figure 4A). On the other hand, *rcsA* is involved in expressing the K antigen capsule [47] of *Klebsiella pneumoniae*. In *Klebsiella* spp. *Klebsiella pneumoniae* is mainly responsible for infectious diseases. *rcsA* gene was selected for molecular detection of *Klebsiella pneumoniae* because of its clinical significance [29]. Thirteen (48%) isolates from 27 biochemically positive *Klebsiella* spp among the 50 DCI-*Klebsiella* were *rcsA* gene positive. Identities were confirmed through sequencing of the *rcsA* amplicons (Figure-4B).

The absence of *phoA* and *rcsA* genes in 30% DCI-*E. coli* and 46% DCI-*Klebsiella*, respectively, 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 2, Figure 5A). On the other hand,

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Enterobacter sp., *E. coli*, *Pseudomonas sp.*, and *Acinetobacter* spp were misdiagnosed as *Klebsiella*, not only by the diagnostic centers (Table 2, Figure 5B).

Later, we employed Loop-mediated Isothermal Amplification (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's a cost-effective detection method that can be performed with simple instruments, such as a heater or water bath [48]. The LAMP method can directly detect *E. coli* and *Klebsiella pneumoniae* [29,29]. We used the *rcsA* gene and *phoA* gene primer set (Table 1) to detect *Klebsiella pneumoniae* and *E. coli*, respectively, from clinical samples within 60 minutes (Figure 6A and 6B). Due to the high sensitivity nature of LAMP in the case of *Klebsiella 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 [49,50].

4.2 Misdiagnosis Can Be a Prospective Route of Antibiotic Resistance Development

Antibiotic resistance is a global public health threat and, if not dealt with now, will become a severe issue, rendering surgical procedures risky and life-threatening. The root causes of antibiotic resistance development are poor drug quality, wrong antibiotic suggestions, wrong doses, excessive human and animal usage, environmental pollution from hospital untreated hospital sewage samples [22, 51-53]. Along with these causes, we hypothesize that inaccurate identification of pathogens by diagnostic centers can also lead to antibiotic resistance.

If we check Table-3, we can find that, according to CLSI, antibiotic suggestion and susceptibility patterns in the disk-diffusion method varies among groups of bacteria. Although for genus belonging to the *Enterobacterales*, the largest group of pathogenic bacteria [54], the pattern or suggested antibiotics are the same, the situation changes outside this order. For non-*Enterobacterale*, no standards are set for the disk-diffusion method. Moreover, sensitivity patterns of a large number of antibiotics (Table 4) are only known for *Enterobacterales*.

Table 3. Variation of antibiotic susceptibility pattern in different groups of gram-negative bacterial pathogens according to CLSI.

Test/ Report Group	**Antimicrobial Agent**	**Disk Content**	***Enterobacterales***		***Pseudomonas 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										

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	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
	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										
	Trimethopri-sulfamethoxazole	1.25/ 23.75	NC	NC	≥ 16	≤ 10	NC	NC	NC	NC

Note: NC= Not characterized; a For *Acinetobacter spp.* 10/10

Table 4. List of antibiotics for which no standard is set for *Pseudomonas aeruginosa*, *Acinetobacter* spp., and Non-*Enterobacterales* according to CLSI.

Organism	Antibiotics	Zone of inhibition	Minimum Inhibitory Concentration standard

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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
Pseudomonas aeruginosa		No CLSI Standard	No CLSI Standard
Acinetobacter spp.		No CLSI Standard	No CLSI Standard
Non-Enterobacterales		No CLSI Standard	No CLSI Standard

Therefore, we hypothesize that there are three possibilities for developing antibiotic resistance due to the misdiagnosis of bacterial pathogens. Firstly, it has been observed in our study that the diagnostic center makes the mistake of classifying bacteria. Due to the lack of proper classification of bacteria, *Acinetobacter* and *Pseudomonas* are also classified as *Klebsiella* spp and so on (Table 2). The misclassification of the genus results in the selection of antibiotics that may not be designated or characterized to that particular genus (Table 3 and 4). This can result in the development of antibiotic resistance without resolving the issues of underlying infection.

Secondly, in accordance with the CLSI, the zone of inhibition for antibiotics is not defined for non-*Enterobacterales*, and a few cases for *Pseudomonas aeruginosa*, or *Acinetobacter* spp., such as Ampicillin-sulbactam, Piperacillin-tazobactam, Ticarcillin-clavulanate, Cefotaxime, Ceftriaxone, Aztreonam, Netilmicin, Doxycycline, Minocycline, Tetracycline, Lomefloxacin, Norfloxacin, and Ofloxacin (Table 2). However, it is to be noted that, through minimum Inhibitory Concentration, i.e., breakpoint determination, it has been suggested to identify the antibiotic sensitivity for these antibiotics. Nonetheless, due to misdiagnosis and lack of micro-dilution facilities, patients are discriminately administered antibiotics without proper characterization. Without knowing what happens in such cases, raises the concern of antibiotic resistance development.

Thirdly, it is possible that when the culture and sensitivity testing is done following the CLSI guideline, the zone of inhibition can vary between two different genera of organisms (Table 3). In such a case, the organism's misdiagnosis can prevent the prescription of antibiotics against which the organism was sensitive. This, in turn, may lead to the usage of the last resort of antibiotics, such as colistin, and contribute to the rapid development of the resistance to the new generation of antibiotics.

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5. Limitation of the study

This pilot study aims to discover the diagnostic centers' misidentification of bacterial pathogens in the clinical sample. The limitation of the work is the number of samples and centers that were tested. Moreover, samples were selected on the basis of biochemical profiles and RAPD band patterns for molecular analysis, which may represent an understatement of the misdiagnosis percentage. We also suggested a rapid, cost-effective, and accurate diagnostic alternative to a conventional biochemical test. Although LAMP specifically detected the *E. coli*, cross-reaction was found in a few cases with negative samples with *Klebsiella pneumoniae.* The further experimental proof is required to identify the prospective route of antibiotic resistance development, which is beyond the scope of this study.

6. Recommendations

Healthcare authorities of a country, especially in the LMICs, should develop policies for the healthcare and diagnostic institutes to provide the standard services and regularly monitor the practices. In a country's antibiotic stewardship program, policies should be included to mitigate the misdiagnosis of clinical pathogens by the diagnostic centers. Diagnostic centers and healthcare facilities should follow the international and national guidelines, such as CLSI, EUCAST, etc., for correctly identifying clinical pathogens and their susceptibility to antibiotics. Moreover, healthcare workers should be adequately trained to perform the tests and interpret the test results.

6. Conclusion

In this pilot study, we used culture and molecular-based methods to validate pathogens' identity determined by diagnostic centers. Traditional culture and biochemical methods are time-consuming and are not always accurate. On the other hand, molecular methods are more accurate and require less time. According to the elaborated biochemical test, our pilot study found an error of 30% and 46% among the DCI- *E. coli* and DCI-*Klebsiella* spp. *phoA* and *rcsA* gene-targeted PCR and LAMP methods accurately identified *E. coli* and *Klebsiella pneumoniae*. According to CLSI, antibiotic susceptibility varies among genera. We hypothesize that misidentification by the diagnostic center can lead to the wrong antibiotic prescribing, consequently, may result in antibiotic resistance development.

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