Reassessment of Clinical Pathogens Diagnosed by Sub-urban Facilities of Dhaka: Necessity of Comprehensive Techniques to Manage Antibiotic Resistance

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Abstract

Background. The determinative bacteriology currently available in the suburbs of Dhaka city mainly involves culture-based identification techniques. Incorporation of extensive biochemical characterization could enhance the efficiency of the existing methods and reduce the risk of wrong medication. The study was aimed, in this connection, at reassessment of clinical pathogens in the suburbs of Dhaka city through biochemical and molecular analysis. **Methods.** To assess the accuracy of identification of clinical pathogens by the diagnostic facilities of suburbs of Dhaka city, we were provided with previously identified clinical strains from different diagnostic facilities along with their clinical data. The etiological agents, were analyzed based on the cultural characteristics on different selective agar media and biochemical properties. The API20E profiles of the pathogens were analyzed to identify the organisms. Furthermore, to verify the results of API20E, 16S rRNA genes were sequenced and their phylogenetic relationship was checked through NCBI database. **Result.** The gram-negative clinical strains that the diagnostic facilities provided were *Escherichia coli*, *Klebsiella*, and *Pseudomonas*. We further reassessed their identities among which two-third of those clinical strains were correctly identified as ***E. coli*** while half of those were correctly reported as *Klebsiella*. In addition to it, some of the DFI strains were also identified as *Enterobacter*, *Yersinia*, *Acinetobacter* as well as some unknown bacterial genera. These results were confirmed initially by biochemical tests followed by API20E and 16S rRNA sequencing. Finally, through antibiogram we also observed that the reconfirmed *E. coli* and *Klebsiella* strains were resistant to various antibiotics, such as ampicillin, cefotaxime, ciprofloxacin, azithromycin etc. **Conclusions.** Our findings allude to the fact that diagnostics facilities though are able to identify gram-negative bacteria within clinical strains, they are unable to identify the causative agents properly. We also hypothesize that misidentification of bacterial pathogens may promote the dissemination of antibiotic resistance.

Keywords: Misidentification, antibiotic resistance, suburb diagnostic facilities, reassessment.

Abbreviations: DFI- diagnostic facility identified; AR- Antibiotic resistance; MDR- multi-drug resistance; NMR- nuclear magnetic resonance; MALDI-TOF- matrix-assisted laser desorption/ionization-time of flight; rRNA- ribosomal RNA; PCR- polymerase chain reaction.

Significance | Importance of the quality assurance of the service of the suburb diagnostic facilities to reduce the misuse of antibiotics.

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Introduction

Each year, approximately 56.4 million deaths occur worldwide and bacterial infection is one of the key player, especially in the low and lower-middle income countries including Bangladesh (WHO, 2018). One of the significant causes of death due to bacterial infection is antibiotic resistance. Although, antibiotic

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resistance (AR) is a natural phenomenon, the condition is accelerating due to misuse and overuse of antibiotics, misidentification of pathogens, misdiagnosis, poor sanitary conditions, and drug quality etc. (Ventola, 2015; Ayukekbong, et al. 2017).

Proper bacterial identification is very important to decrease the spread of the infectious diseases and facilitate appropriate patient management (Jesumirhewe, et al. 2016). Correct identification also reduces side effects and slows the generation of antibiotic resistance (Abayasekara, et al. 2017). Conventional bacterial identification relies on phenotypic characteristics, genotypic traits and immunological (serological) analysis. In developing countries, diagnosis of bacterial infections is carried out mostly on culture-based techniques, where pathogens are identified based on their morphological and biochemical characteristics (Cheesbrough, 2006). Although these culture-based methods are low-priced and provide both qualitative and quantitative results on the bacterial populations present in the clinical samples (Rhoads, et al. 2012), they are not always reliable, slow, and require proper skills, knowledge, and standard reagents.

Alternate to the culture-based methods, in developed countries or in sophisticated hospitals and diagnostic settings, pathogen identification is carried out using API system (Smith, et al. 1972), Vitek-2 system (Jossart, 1999), NMR spectroscopy (Yatmaz, et al. 2016), MALDI-TOF (Singhal, et al. 2015), or Next-generation sequencing (Sanschagrin, et al. 2014). Along with these techniques, nucleic acid amplification techniques like PCR, qPCR, and isothermal amplification are also used regularly in those diagnostic facilities for this purpose (Yoshii, et al. 2017; Gadkar, et al. 2018). Although Bangladesh has recently been promoted to middle-income country, still many of its people live under poverty level and cannot afford expensive diagnosis. As a result, rural, sub-urban, and even most of the urban diagnostic facilities in Bangladesh use culture-based techniques. But due to complex nature of microorganisms, lack of proper microbiological skills, and use of old or sub-standard reagents, improper identification could occur (Laupland, et al. 2013). Consequently, misidentification may lead to improper treatment and antibiotic resistance in the population.

In this study, we have collected previously characterized clinical bacterial strains from suburb diagnostic facilities and re-confirmed their identity by culture-based techniques, aiming to find out whether the hospitals and diagnostic facilities are correctly identifying the pathogens to help the physicians for prescribing appropriate antibiotics.

Materials and Methods

Sample collection

A total of 100 gram-negative bacterial strains previously isolated and identified by two hospital-based diagnostic facilities in Savar, Dhaka from March to November 2017 were provided to us along with their characterization reports. According to their records, among the 100 clinical strains, 88 were collected from urine samples, 8 from pus, and 4 were isolated from sputum. The bacterial strains were aseptically transported to the laboratory through agar slant culture for further characterization and were preserved at -80ºC.

Phenotypic characterization

After transportation of the bacterial strains to the laboratory, Gram staining was done to distinguish between gram-positive and gram-negative bacteria, and to confirm the purity of the strains. All gram-negative strains were then cultured on MacConkey agar (Scharlau, Spain) and Eosin Methylene Blue (EMB) Agar (Himedia, India) media to analyze the colony morphologies. For presumptive identification, biochemical tests like- Indole production, Methyl Red, Voges Proskauer, Citrate utilization, Catalase, motility, Triple sugar iron (TSI), and sugar utilization tests were performed. The biochemical test results were analyzed according to the "Bergey's Manual of Determinative Bacteriology (1994)".

API 20E profiling for identification

We selected four representative strains based on their biochemical profiles and further confirmed their identity using API 20E kit (BioMérieux, France) that consists of microtubes (cupules) containing dehydrated substrates to detect the enzymatic activity or the assimilation / fermentation of sugars by the inoculated organisms (Holmes, et al. 1978). The tests were performed according to manufacturer's (BioMerieux Inc.) instruction. The numerical profile was used for on-line identification of the strains using APIweb (https://apiweb.biomerieux.com).

Phylogenetic analysis

Chromosomal DNA of each strains were extracted by boiling method (Dashti, et al. 2009). Briefly, bacterial cultures were inoculated in 5 ml nutrient broth and after 18 hours incubation at 37 °C, 1 ml broth from each culture was centrifuged (Tomy, Japan) at 12,000 rpm for 10 min. The supernatant was discarded and 200 µl of sterile nuclease-free water (Thermo Fisher, UK) were added to the pellet. The solution was then boiled in a water bath (Titec, Japan) at 100°C for 10 min, and immediately kept on ice for 10 min. The solution was again centrifuged at 12,000 rpm for 10 min, and the supernatant was collected containing the template DNA.

For 16S rRNA gene sequencing, the template DNA were amplified using primers 5'-AGAGTTTGATCCTGGCTCAG-3'-forward and 5'-CGGTTACCTTGTTACGACTT-3' -reverse (Arriba, et al. 2018). PCR mixture contained 12.5 µl master mix (Promega, USA), 2 µl each of the 10 µM forward and reverse primers, 2 µl template DNA, 8.5 µl nuclease-free water for 25-µl PCR reactions. After performing the PCR (Takara, Japan) the products were analyzed on a 1% agarose gel using agarose gel electrophoresis unit (Mupid, Japan). PCR products were then purified using FavorPrep GEL/ PCR Purification Kit, according to the manufacturer's instruction. The purified PCR products were then used for Sanger dideoxy sequencing (3500 Series Genetic Analyzer,

Figure 1 | **Types and percentages of gram-negative bacterial strains collected from diagnostic facilities.**

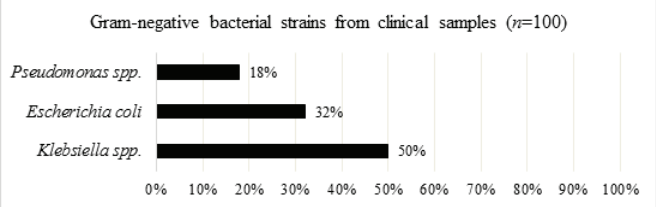


Figure 2A | **Presumptive identification of DFI *Escherichia coli* strains according to the biochemical test.**

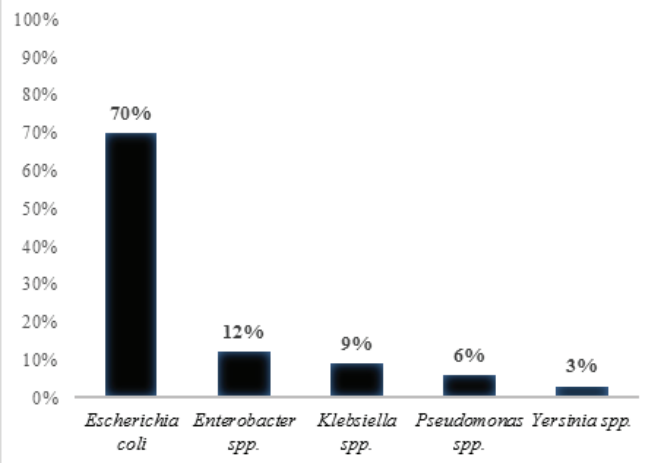


Figure 2B | **Presumptive identification of DFI *Klebsiella* strains according to the biochemical properties.**

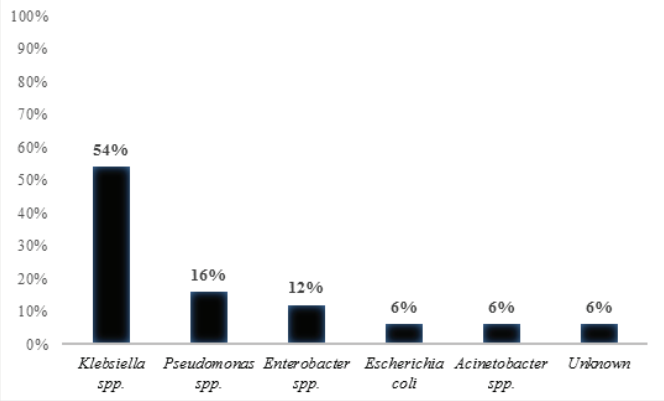
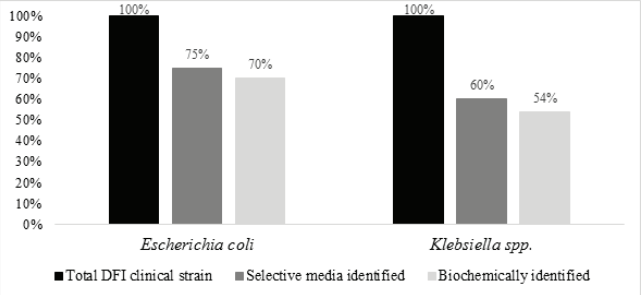


Figure 3 | **Reassessment of clinical pathogens' identity.**



Applied Biosystems).

Partial sequences were compared to GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.gov/GenBank) by the basic local alignment search tool (BLAST) to identify close phylogenetic relatives. Phylogenetic tree was constructed with the help of BioEdit, ApE plasmid editor, and MEGA 7 softwares.

Antimicrobial susceptibility testing

Bacterial susceptibility tests to antimicrobial agents was done by the disk diffusion technique according to the Clinical & Laboratory Standards Institute (CLSI) guidelines. Antibiotic discs used in this study were ampicillin (AML) (10 µg), azithromycin (AZM) (15 µg), ceftriaxone (CRO) (30 µg), cefotaxime (CTX) (30 µg), colistin sulfate (CT) (10 µg), gentamycin (CN) (10 µg), imipenem (IPM) (10 µg), meropenem (MEM) (10 µg), nitrofurantoin (F) (300 µg), tetracycline (TE) (30 µg), ciprofloxacin (CIP) (30 µg), trimethoprim-sulphamethoxazole (SXT) (24 µg), and nalidixic acid (NA) (300 µg) (Oxoid, UK). Fresh inoculum was prepared in 5 ml nutrient broth by growing the culture for 2-6 hours until the optical density (OD) reached similar to that of 0.5 McFarland standard. After that the broth culture was spread on the surface of the Mueller-Hinton agar medium using sterile cotton swab. Sterile antimicrobial disks were then dispensed onto inoculated plates and observed for the zone of inhibition after overnight incubation. The results were interpreted using CLSI guidelines (http://clsi-m100.com/).

Results

Types of bacterial strains in clinical samples collected from diagnostic facilities

Total 100 gram-negative bacterial strains were collected from the diagnostic facilities along with their characterization reports. According to the facilities' reports, 50%, 32%, and 18% of the provided strains were *Klebsiella*, *E. coli*, and *Pseudomonas*, respectively (Figure-1). For this study, we selected only two groups, i.e. *E. coli* (denoted as DFI *E. coli*) and *Klebsiella* spp. (denoted as DFI *Klebsiella*), because of their abundance for re-characterization.

Determination of morphological and cultural characteristics

At first, we carried out Gram staining of the selected strains for re-confirmation and found that all the strains were gram-negative, indicating that the facilities had correctly selected the gram-negative ones. We initially found few mixed cultures (~5%), but to avoid the complicacy of the analysis, we excluded those from this study and continued the work with 32 diagnostic facility identified (DFI) *E. coli*, and 50 DFI *Klebsiella* strains.

To check out the cultural characteristics of the selected strains on selective and differential media, DFI *Klebsiella* and *E. coli strains* were at first grown on MacConkey agar and later the DFI *E. coli* on Eosin-Methylene Blue (EMB) agar. Based on the colony morphology we presumed that 40% of the provided DFI *Klebsiella* strains were not *Klebsiella* and 25% of the DFI *E. coli* strains were not *E. coli*. For further confirmation, we carried out extensive biochemical characterization of those strains.

Table 1 | **Presumptive identification of DFI *Escherichia coli* strains according to the biochemical test.**

Strain no.	Identification by diagnostic facility	Identification by biochemical properties	Identification by API 20E profiling	Identification by Phylogenetic analysis of 16S rRNA gene sequencing
DFI-E6	*Escherichia coli*	*Enterobacter* sp.	*Enterobacter cloacae*	*Enterobacter cloacae*
DFI-E13	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*
DFI-K27	*Klebsiella* sp.	*Pseudomonas* sp.	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
DFI-K42	*Klebsiella* sp.	*Klebsiella* sp.	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*

Biochemical characterization

Presumptive identification of the selected DFI *E. coli* and *Klebsiella* strains were then done by the biochemical tests, e.g. Indole production, Methyl Red, Voges Proskauer, Citrate utilization, Catalase, motility, Triple sugar iron (TSI), and sugar utilization tests. Through analyzing these biochemical test results, we found that, all the DFI *E. coli* strains did not show the biochemical profiles like *E. coli*. Although 70% of the *E. coli*-claimed strains gave *E. coli*-like properties, 12%, 9%, 6%, and 3% gave *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Yersinia* like features, respectively, (Figure-2A, Supplementary table-1A) that we had presumed.

Similarly, DFI *Klebsiella* strains also showed variable results. It was observed that 54% of the diagnostic facility claimed *Klebsiella* strains gave *Klebsiella*-like properties, whereas, rest of the 16%, 12%, 6%, and 6% strains gave *Pseudomonas*, *Enterobacter*, *E. coli*, and *Acinetobacter*- like properties, respectively. Among the DFI *Klebsiella* strains, 6% could not be identified (Figure-2B, Supplementary table-1B), although those were in pure culture.

Identity of the DFI clinical strains were first reassessed on selective media and then by biochemical tests. It was observed that among the DFI *E. coli*, selective media could presumptively identify 75%, whereas based on biochemical tests it was found that diagnostic facilities incorrectly identified 30% of the strains as *E. coli*. Similarly, based on the biochemical results, about 46% of the DFI *Klebsiella* were found to be incorrectly identified (Figure-3).

API 20E profiling for identification of DFI *Escherichia coli* and DFI *Klebsiella* strains

Four bacterial strains- DFI-E6, DFI-E13 (from the DFI *E. coli* strains) and DFI-K27, DFI-K42 (from the DFI *Klebsiella* spp. strains) were selected for extensive biochemical characterization and identification by API 20E kit. Among these four strains, DFI-E6 and DFI-E13 although provided us as DFI *E. coli*, DFI-E6 showed biochemical properties like *Enterobacter* sp. in our study; whereas, DFI-E13 matched with *E. coli*. The API 20E profiles confirmed the above result as this technique identified DFI-E13 as *E. coli* but DFI-E6 as *Enterobacter cloacae* (Table-1). Similarly, DFI *Klebsiella* strains DFI-K27 and DFI-K42, biochemically showed the features of *Pseudomonas* sp. and *Klebsiella* sp., respectively and API-20E profile analysis identified strain DFI-K27 and DFI-K42 as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, respectively (Table-1) which also validate our claim of misidentification of clinical pathogens by diagnostic facilities.

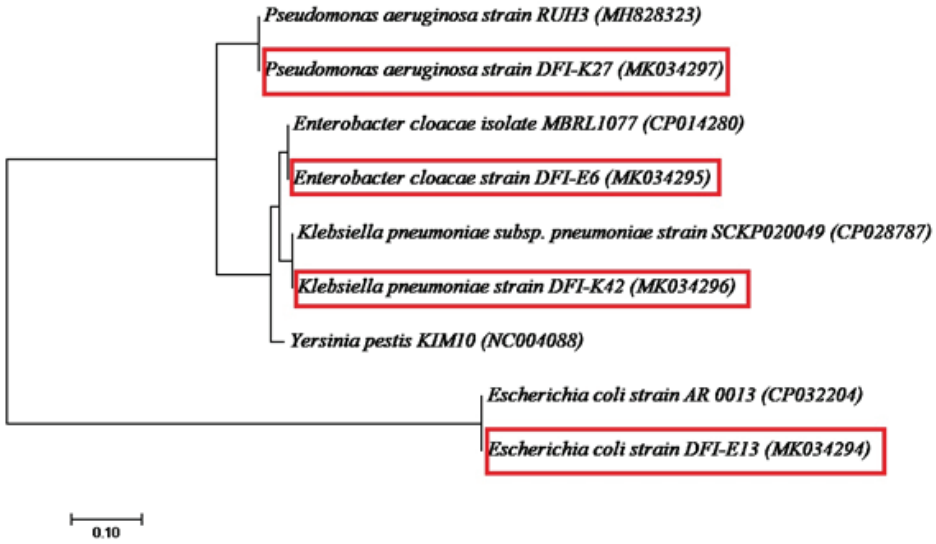
Phylogenetic analysis

Final identification of the selected four stains- DFI-E6, DFI-E13, DFI-K27, and DFI-K42 were analyzed by phylogenetic analysis through 16S rRNA gene sequence analysis. The accession number for strains DFI-E6, DFI-E13, DFI-K27, and DFI-K42 are MK034295, MK034294, MK034297, and MK034296, respectively. After analyzing the 16S rRNA gene sequences, strain DFI-E13 and DFI-K42 were identified as *E. coli* and *K. pneumoniae*, respectively (Table-1, Figure-4). Whereas, strain DFI-E6 and DFI-K27 were identified as *Enterobacter cloacae* and *Pseudomonas aeruginosa*, respectively which were provided as *E. coli* and *Klebsiella* sp. by the diagnostic facilities.

Antibiotic resistance analysis

We carried out antibiogram with 16 DFI *E. coli* and 29 DFI *Klebsiella* strains. According to antibiogram profiling it was found that among the DFI *E. coli* strains, more than 70% strains were resistant against Ceftriaxone, Tetracycline, Azithromycin, Ampicillin and Ciprofloxacin, whereas >90% strains were resistant against Trimethoprim-sulphamethoxazole and Nalidixic acid. Interestingly, all of the analyzed strains were Cefotaxime resistant. In case of Colistin sulfate, Gentamycin, Imipenem, Meropenem, Nitrofurantoin, a low resistant profile (≤30%) was observed but Imipenem was found highly effective (100% sensitive) (Figure-5). In case of DFI *Klebsiella* strains, 100% of the strains were found to be resistant against Ampicillin, whereas >90% strains were Cefotaxime resistant and >70% strains Ceftriaxone resistant, whereas 50-60% of the strains were resistant against Colistin sulfate, Gentamycin, Azithromycin, Meropenem, Nitrofurantoin, Trimethoprim-sulphamethoxazole, and Nalidixic acid. Less than 30% of the strains showed resistance to Imipenem (Figure-5).

Figure 4 | **Phylogenetic tree constructed based on 16S rRNA gene sequence data by Maximum Likelihood method. DFI *E. coli* strain DFI-E13 was found to be *E. coli*, whereas strain DFI-E6 was identified as *Enterobacter cloacae*. Similarly, in DFI *Klebsiella* strain DFI-K42 was found to be *K. pneumoniae*, on the other hand strain DFI-K27 was identified as *P. aeruginosa*.**



Discussion

Diagnostic accuracy is essential for proper treatment and safe use of antibiotics. To reduce the misuse of antibiotics in health care and prevent the development of antibiotic resistance, the physicians require proper diagnosis of diseases and related pathogens. As most of the people of low and lower-middle income countries cannot afford expensive diagnosis, the diagnostic facilities sometimes provide cheap diagnosis. As a result, due to improper characterization, lack of trained personnel, and out of date reagents, inaccurate diagnosis may occur (Faiz, et al. 2011).

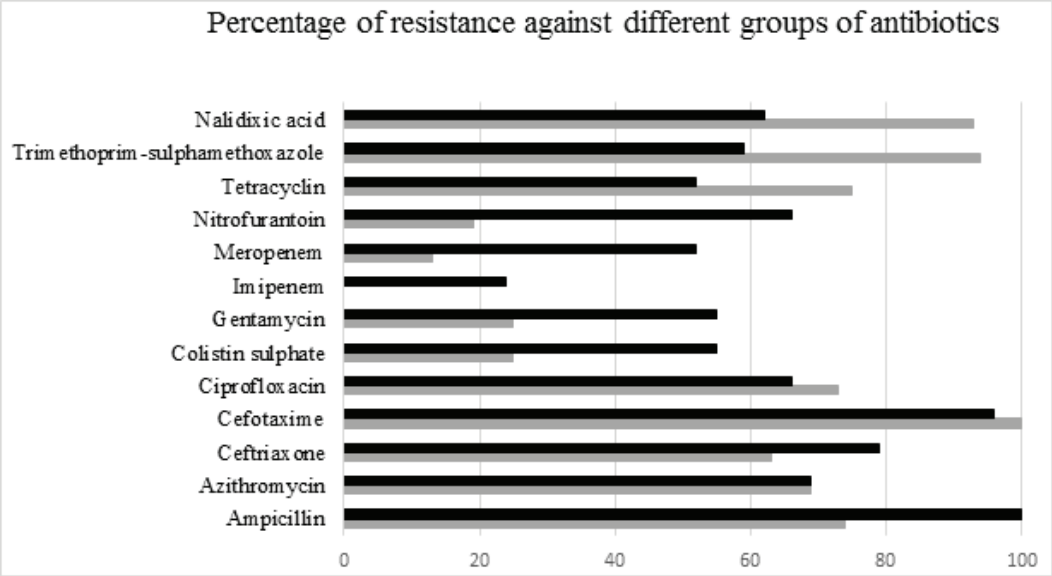
In this work, during the study period, the selected suburb diagnostic facilities had reported only the presence of *E. coli*, *Klebsiella*, and *Pseudomonas* in their gram-negative clinical samples (Figure-1), which was an unusual phenomenon, because presence of other gram-negative bacterial population, such as *Enterobacter*, *Yersinia*, *Proteus,* etc., along with these pathogens is common (Yasmeen, et al. 2015). This result led us to assume that, these facilities might have been unable to correctly identify the pathogens and made us interested in designing the present study. Previously, various research groups had published reports on the inappropriate or misuse of antibiotics without proper diagnosis in Bangladesh and other developing countries (Ronsmans, et al. 1996; Baqui, et al. 2004; Akter, et al. 2004; Tangcharoensathien, et al. 2018). Here, we tried to correlate between misidentification of etiological agents and inappropriate use of antibiotics, which eventually lead to antibiotic resistance.

For re-assessment of the identification results of the clinical pathogens by the diagnostic facilities, we carried out detailed phenotypical (morphological, cultural and biochemical) characterization of 100 DFI bacterial strains provided by the selected diagnostic facilities. After careful analyses, we concluded that about 30% and 46% of the previously identified *E. coli*, and *Klebsiella* strains, respectively, were misidentified (Figure-2A, 2B, 3, 4). We identified those 30% misidentified DFI *E. coli* strains as *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Yersinia* (Figure-2A, Figure-4, Table-1). Similarly, the 46% incorrectly identified DFI *Klebsiella* strains were identified as *Pseudomonas*, *Enterobacter*, *Escherichia coli*, *Acinetobacter,* and as some unknown bacteria (Figure-2B, Figure-4, Table-1). From our results, it can be presumed that, the suburb diagnostic facilities might not identify the pathogens properly.

These facilities may have performed minimal biochemical tests to get the results. But again, using only biochemical tests it is difficult to claim that the diagnostic facilities have identified the pathogens incorrectly. Use of more extensive biochemical tests like API profiling or using other commercial biochemical kits, along with phylogenetic characterization of 16S rRNA gene can properly identify any pathogen (Harris, et al. 2003). For that purpose, we selected four representative strains from the two bacterial groups (DFI *E. coli* and DFI *Klebsiella*) and further characterized those using API 20E biochemical profiling and 16S rRNA gene sequence analysis. Here, we showed that DFI *E. coli* (DFI-E6) and DFI *Klebsiella* (DFI-K27) were an *Enterobacter* sp. and a *Pseudomonas* sp., respectively (Figure-4, Table-1).

Moreover, antibiotic resistance is one of the major threats to health and food security. Some of the main causes of antibiotic resistance are: overuse of antibiotic, patients' lacking knowledge about antibiotic misuse, overuse of antibiotic in veterinary and fish farming, poor disposal facilities of hospital and clinical

Figure 5 | **Percentage of resistance against different classes of antibiotics by DFI *Escherichia coli* (Gray) and DFI *Klebsiella* (Black)strains.**



wastes, lack of personal hygiene and sanitation, and lack of newly discovered antibiotics (Davies, et al. 2010). In our previous works with antibiotic resistance spreading, we found that untreated hospital liquid waste carries huge amount of active antibiotics as well as multi-drug resistance (MDR) bacteria (Adnan, et al. 2013). Many other studies also reported that many MDR-bacteria are spreading through the veterinary wastes and poultry litters in the environment (Ahmed, et al. 2013; Nahar, et al. 2014).

Similar to the results of our previous works, when we characterized the DFI strains for antibiotic resistance, we found an alarming situation. All DFI *E. coli* and DFI *Klebsiella* tested were found resistant to more than three antibiotics and some were resistant against all thirteen drugs tested (Data not shown). Although, resistance situation of DFI *E. coli* were found to be less severe than DFI *Klebsiella* strains (Figure-5), the result became inconclusive with DFI data, as disc diffusion method relies on measurement of zone of inhibition which varies between bacterial groups. According to CLSI guideline antibiotic resistance profile of non-*Enterobacteriaceae* should be tested using broth dilution method. Therefore, if the causative organisms are misidentified, wrong antibiotic could be prescribed to the patients, leading to antibiotic resistance (Filce, et al. 2015).

For proper health care and to control the antibiotic resistance dissemination, it is essential that hospitals and diagnostic facilities should increase their accuracy in identifying the etiological agents. Inexpensive novel diagnostic techniques like isothermal amplification can be explored to tackle the situation (Hudson, et al. 2014; Maurer, et al. 2017; Liu, et al. 2017), along with various subsidiary rapid detection kits. Furthermore, it is essential to employ properly trained microbiologists who have the knowledge and experience for proper identification of the etiological agents. Our results moreover stress that, proper quality assurance mechanisms should be placed within the diagnostic setups to ensure the proper and better identification of etiological agents. Thus, creating awareness of the severity of misidentification of proper causative microbes to the misuse of antibiotics, can bring about a control on the spread of antibiotic resistance.

Conclusion

In this work, we tried to check if suburb hospitals and diagnostic facilities in the densely populated regions are properly providing healthcare to its patients by correctly identifying the etiological agents. We found that, the diagnostic facilities could not identify two groups of pathogens – *E. coli* and *Klebsiella* spp. accurately. and the patients might have been prescribed with wrong antibiotics. We are afraid that, if this situation persists in other diagnostic facilities also, then antibiotic resistance could arise. In future, more diagnostic facilities should be brought under scrutiny and not only *E. coli* or *Klebsiella*, rather all diagnostic tests, especially with etiological agent detection and antibiotic susceptibility, should be validated and certified before reaching public healthcare.

Author contributions

SFB performed the study and wrote the manuscript. MRU and MJU performed the experiments. SAM revised the manuscript. MUK and AAT contributed by critical suggestions and resources. MRJ and NA co-supervised the work and revised the manuscript. NA designed the research, supervised the whole work and reviewed the manuscript critically.

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Competing financial interests

Authors have declared that no competing interest exists.

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