Original Article

Pediatric pneumonia patients in Jashore city, Bangladesh: screening of drug resistance pattern and molecular identification

Nigar Sultana Meghla[1], Md. Mohsin Kobir[2], Mamun Or Rashid[3], Najmuj Sakib[4], Md. Shazid Hasan[5], Md. Tanvir Islam[6], Ovinu Kibria Islam[7]

ABSTRACT

Background

The most common cause of death among children is pediatric pneumonia. The increase of antibiotic abuse to treat pediatric pneumonia patients in Bangladesh is alarming for the rise of multidrug-resistant (MDR) bacteria. However, there is a dearth of research available regarding this. Hence, the current study was conducted on a pilot scale to identify the MDR bacteria from pediatric pneumonia patients by molecular approaches.

Methods

Saliva and nasal swab samples were collected for 3 months (July-September 2019) from 3children with suspected pneumonia at two different hospitals in Jashore city, Bangladesh. Routine biochemical tests were performed to identify the isolates presumptively. All isolates were tested for antimicrobial resistance according to CLSI guidelines using 17 antibiotics. Finally, amplified ribosomal DNA restriction analysis (ARDRA) and 16S rRNA sequencing with phylogeny were performed to confirm the bacteria at the species level.

Results

From the samples collected from the patients, 47 isolates were obtained. The biochemical tests presumptively identified *Acinetobacter* spp., as the most prevalent type, which was followed by Staphylococcus spp., and *Enterobacter* spp. Surprisingly, every isolate was MDR, resistant to at least four antibiotics. Cefepime and Cefotaxime showed the highest (i.e., 100% and 87%, respectively) resistance pattern. In addition, the isolates showed elevated resistance towards Amoxicillin, Vancomycin, Streptomycin, Azithromycin, Erythromycin, Norfloxacin, and Tetracycline. On the contrary, they were susceptible to Imipenem and Meropenem. Subsequently, the isolates were typed into three different groups by ARDRA; the strains *Acinetobacter baumannii*, *Enterobacter hormaechei,* and *Staphylococcus pasteuri* were confirmed.

Conclusion

The rapid revolution of multidrug resistant organisms has an impact on morbidity, mortality and healthcare associated cost. Unrestricted use of antibiotics should be stopped and surveillance is necessary for controlling the emergence of resistant bacteria.

Keywords

pediatric pneumonia; antimicrobial resistance; multidrug resistance; ARDRA; 16S rRNA sequencing.

INTRODUCTION

Antimicrobial resistance (AMR) has been rising sharply over the years and is becoming a subject of continuous challenge for the world. There is clear evidence on the rise of AMR among pneumonia isolates identified from different Asian countries[1,2] although a huge gap exists in Bangladesh regarding the prevalence of multidrug resistant isolates from pneumonia patients. Pneumonia patients are mainly identifiable with the difficulties in their respiratory system while presenting symptoms like rapid breathing, noisy breathing, etc.[3]. People with chronic illnesses, weak immune systems, older adults, infants, and small children are more likely to suffer from pneumonia. Though plethora of organisms can cause pneumonia including bacteria, virus, fungi and sometimes protozoa, most life-threatening infections are caused by bacteria. Various bacteria

1. Nigar Sultana Meghla, Department of Microbiology, Jashore University of Science and Technology
2. Md. Mohsin Kobir, Department of Microbiology, Jashore University of Science and Technology
3. Mamun Or Rashid, Department of Microbiology, Jashore University of Science and Technology
4. Najmuj Sakib, Department of Microbiology, Jashore University of Science and Technology
5. Md. Shazid Hasan, Department of Microbiology, Jashore University of Science and Technology
6. Md. Tanvir Islam, Department of Microbiology, Jashore University of Science and Technology
7. Ovinu Kibria Islam, Department of Microbiology, Jashore University of Science and Technology

Correspondence

Md. Tanvir Islam, Professor, Department of Microbiology, Jashore University of Science and Technology. E-mail: mt.islam@just.edu.bd" as corresponding author along with "Ovinu Kibria Islam, Associate Professor, Department of Microbiology, Jashore University of Science and Technology. E-mail: ovinu@just.edu.bd

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are associated with the disease that makes antibiotic as the first choice of treatment[4]. Because of high mortality rate among children, it is a matter of concern to provide the effective treatment. But it is becoming difficult day by day because of the emergence of resistant strains; resistance and/or multi-drug resistance to Penicillin, Azithromycin, Erythromycin, Ampicillin, Meropenem, Ceftriaxone, Tetracycline, Ciprofloxacin, Ofloxacin, Gentamycin, Chloramphenicol, Levofloxacin, Cefepime, Cefotaxime, Vancomycin and Imipenem has already been reported in many countries[5-10]. Moreover, multi-drug resistant strains are emerging which may result in a post-antibiotic era where people may die in very mild diseases like the pre-antibiotic era[11]. The rate of various organisms being resistant to multiple antibiotics is higher in developing countries because of their lack of knowledge and using antibiotics in nonbacterial infection[12].

Pneumonia has been known as potentially lethal disease for nearly two centuries. The condition was the single most significant cause of child death over 2000-2015 period[12]; developing countries are at the highest risk. However, there is no proper information about the overall burden of pneumonia in Asia[13,14]. In 2008, Bangladesh ranked 10th in terms of post-neonatal pneumonia mortality among the entire world[15]. Herein Bangladesh, around a fifty thousand of children die of pneumonia every year; severe illness of lower respiratory tract is responsible for up to 52% of the mortality rate among children aged less than 5-years[16]. The situation is even worse for the country when there are plenty of evidences available for inappropriate use of next generation antibiotics in the hospitals to treat pediatric pneumonia patients[17-19]. For the identification of isolates involved in pneumonia, biochemical tests are performed in most of the cases. However, the biochemical method doesn't provide accurate identification always[20]. ARDRA (amplified ribosomal DNA restriction analysis) can be used as a fingerprint to identify isolates, while restriction sites on RNA operons are conserved based on phylogenetic patterns[21]. As 16S rRNA is conserved for every organism, and its function has not changed over time, it is the gold standard for the identification of a species as well as their phylogenetic analysis[22,23]. As a result, the present study aimed to determine the bacteria associated with pediatric pneumonia in a selected region of Bangladesh and their antibiotic resistance patterns. The study may help to evaluate the organisms involved with the disease along with the choice of treatment for the disease and to assist the policy makers in taking necessary steps to minimize the chance of emerging multi drug resistant organisms across the country.

MATERIALS AND METHODS

Sample Collection and Survey

Samples were collected between July 2019 and September 2019 from two different hospitals (i.e., Jashore 250-bed General hospital and Jashore Shishu hospital) located in Khulna division, Bangladesh. Doctors initially examined all the children (15 days to 4 years of age) based on their radiological test for pneumonia. For the purpose of the study, children with suspected pneumonia cases were chosen from those hospitals' pediatric ward. A total of 39 patients met the criteria for the samples. Saliva and nasal secretions from every patient were collected with sterile cotton bud and then stored in a transport media (skimmed-milk Tryptone Glucose Glycerol media, Oxoid Limited, England) for transporting them to the laboratory. Cough reflex, and secretion of the bronchus accumulated in the pharynx were collected following swabbing.

Morphological analysis

From the transport media, samples were inoculated into blood agar media (Oxoid Limited, England). Afterwards, isolates were cultured again to determine their morphological and biochemical characteristics. The colony morphology of the isolated bacteria was examined using Bergey's Manual of Determinative Bacteriology[24]. Their hemolysis characteristics have also been tested. Finally, Gram staining was performed based on Gram-staining protocol[25].

Cultural and Biochemical Identification of Isolates

Bacterial isolates found on the blood agar plate were purely cultured and then detected by performing standard biochemical tests and microscopy. Different biochemical tests like oxidase, catalase, citrate utilization, motility, indole, urease, MR-VP test, and triple sugar iron test were conducted to identify the isolated bacteria presumptively. Microscopy was used to identify the morphology of the isolates. The tests were performed according to the standard microbiology laboratory manual[26].

Antimicrobial susceptibility test

The antibiotics used in the antibiotic susceptibility test

were selected based on the criteria of being regularly used in the hospital for controlling the infection of pneumonia. A Kirby-Bauer Disk Diffusion susceptibility test was performed using an antimicrobial agent-impregnated paper disc[27]. 17 different types of antibiotics from 9 classes were used for the study: β-Lactamases (Amoxicillin-10µg), Cephalosporins (Cefepime-30µg, Cefotaxime-30µg), Carbapenems (Imipenem-10µg, Meropenem-10µg), Tetracycline (Tetracycline-10µg), Glycopeptide (Vancomycin-30µg), Aminoglycoside (Streptomycin-10µg, Gentamycin-30µg, Kanamycin-30µg), Macrolides (Erythromycin-15µg, Azithromycin-15µg), Phenicol (Chloramphenicol-30µg) and Quinolones (Ciprofloxacin-5µg, Norfloxacin-10µg, Levofloxacillin-5µg, Nalidixic Acid-30µg). The diameter of the inhibition zone was measured in millimeter unit and used to interpret the result according to the latest CLSI Guideline and denoted as susceptible, intermediate and resistant[28].

Genomic DNA Extraction

The boiled DNA extraction method was used to extract the chromosomal DNA of the selected multi drug resistant isolates. Colonies isolated from the nutrient agar plate were grown overnight in 5 ml nutrient broth at 37°C with aeration using a shaker machine set at 120 rpm. Cells were collected by centrifuging 1.0 mL of the culture for 10 minutes at 13,000 rpm in a 1.5 mL microcentrifuge tube (Eppendorff, Germany). The cell pellets were washed with distilled water by re-centrifugation. In the next step, 200 liters of PCR water were mixed to dissolve and each tube was heated for 10 minutes to 100°C. It was then centrifuged at 10,000 rpm then boied at water bath for ten minutes and finally immediately transferred on ice. The supernatant was collected into a fresh 1.5 mL tube (100-150 µl). A nano photometer (Implen NanoPhotometer®, Germany) was used to quantify extracted DNA, and it was stored at -20°C for five days.

Amplification of 16S rRNA of Bacterial Isolates

As a template for the amplification reaction, purified bacterial DNA was used. In this experiment, two universal primers were used: 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Applied Biosystems SimpliAmp (ThermoFisher Scientific, USA) thermal cyclers were used to perform the reaction in 0.2 mL PCR tubes. PCR reactions were carried out in 15 µL volumes containing: 7.5 µL of commercial master mix (Taq DNA polymerase, dNTPs, MgCl2 and reaction buffer), 0.75 µL of forward primer, 0.75 µL of reverse primer, 1.5 µL of template DNA and nuclease free water up to the volume. During thermocycling, the following conditions were used: initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 1 minute, annealing at a range of 55°C for 1 minute and 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. PCR products were electrophoresed on a 1% agarose gel in TAE buffer containing 0.5g/mL of ethidium bromide. The gel was then visualized through a gel dock system using UV illuminato. The amplified product in all cases measured approximately 1500 bases.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The amplified PCR products were digested using *AluI* (Thermo Fisher Scientific, USA) restriction enzyme. We prepared a 20-liter reaction mixture containing 2 liters of cut smart buffer, 0.2 liters of bovine serum albumin, 0.5 liters of restriction enzyme, 7 liters of PCR product, and 12.8 liters of nuclease-free water. Restriction digestion lasted for 4 hours at 37°C according to the recommendation of the enzyme manufacturer and terminated by heating the mixture at 65°C for 20 minutes to destroy the activity of the enzyme. In 1% TAE buffer at 100 volts and 100 amps, 3% agarose gel was used to separate restriction fragment patterns. The gel was then visualized under a UV-transilluminator, and a photomicrograph was taken in the gel documentation system. The ARDRA profile was then analyzed, and the isolates were grouped based on their band position.

16S rRNA gene sequencing. Phylogenetic tree preparation and homology analysis

Purification of the amplified PCR product was performed using ExoSAP-ITTM PCR product cleanup reagent (ThermoFisher Scientific, USA). The purified products were then sequenced by using a BigDyeTM Terminator v3.1 Cycle Sequencing Kit from ThermoFisher Scientific. For sequencing, 27F and 1492R primers were used at a concentration of 10 picomoles. A single contig was then assembled from both the forward and reverse sequences. BLAST (Basic Local Alignment Search Tool) was used to find which reference sequences are aligned with the 16S rRNA sequence in the NCBI database (http://www.ncbi.nlm.nih.gov/). Using the Neighbor-Joining method[29], the evolutionary history was inferred. The evolutionary

distances were computed using the Tamura 3-parameter method[29] and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X[30]. For phylogenetic analysis, we used NR 119358.1, NR 042154.1, NR 024669.1, and NR 102933.1 (outgroup).

Results:

Biochemical Poperties of the Isolates

A total of 47 isolates were isolated from the 39 samples and stocked for further analysis. Almost equal number of isolates was found from the Jahsore Shishu Hospital (n=25) and the Jahsore 250 bed General hospital (n=22). Gram staining identified 16 organisms as Gram-positives and 31 as Gram-negatives. From the presumptive test, the most common pathogens found were *Acinetobacter* spp. (40.42 %), followed by *Enterobacter* spp. (25.58 %) and *Staphylococcus* spp. (34 %) **(Table 1)**.

Antimicrobial Resistance Pattern

This study tried to unveil the efficacy of 17 most used antibiotics and the resistant pattern of the bacteria. Meropenem, Imipenem, Gentamycin, Chloramphenicol, Ciprofloxacin, and Levofloxacin showed higher activity (i.e., 100%, 100%, 79%, 75%, 66% and 60%, respectively) against bacterial isolates (**Table 2**). Third generation Cephalosporin resistance was widespread among all the isolates (i.e., 100% and 87% resistance for Cefepime and Cefotaxime, respectively). *Acinetobacter* spp. had the highest prevalence rate (40.42%) which was resistant to Amoxicillin (79%), Chloramphenicol (10%), Ciprofloxacin (42%), Gentamycin (31%), Nalidixic Acid (42%), Vancomycin (84%), Streptomycin (74%), Cefotaxime (89%), Azithromycin (57%), Cefepime (100%), Tetracycline (42%), Norfloxacin (26%), Kanamycin (47%), Erythromycin (68%) and Levofloxacin (26%) (**Table 2).**

The second most prevalent type of bacteria was *Staphylococcus* spp. with 34% prevalence rate, which showed resistance against Amoxicillin (56%), Chloramphenicol (12%), Gentamycin (6%), Vancomycin (44%), Streptomycin (31%), Cefotaxime (81%), Azithromycin (50%), Cefepime (100%), Tetracycline (37%), Norfloxacin (50%), Kanamycin (31%), Erythromycin (50%) and Levofloxacin (12%). The *Enterobacter* spp. had the lowest prevalence with 25.53% and was resistant against Amoxicillin (50%), Chloramphenicol (17%), Ciprofloxacin (33%), Gentamycin (17%), Nalidixic Acid (17%), Vancomycin (42%), Streptomycin (50%), Cefotaxime (91%), Azithromycin (58%), Cefepime (100%), Tetracycline (50%), Norfloxacin (17%), Kanamycin (41%), Erythromycin (33%) and Levofloxacin (8%) **(Table 2)**. It is surprising that all of our presumptively identified bacterial isolates showed resistance pattern towards more than one drug. Therefore, we prepared a table, which illustrated the multidrug resistance patterns **(Table 3)**. All the presumptive isolates resisted at least 4 of the drugs used (out of 17). 27.65% of the total isolates were resistant to five antibiotics, followed by 27.65% for six antibiotics and 14.89% for seven antibiotics. One strain of *Acinetobacter* spp. was found to be resistant against a maximum number of 13-drugs used in this study and 30% of the isolates were resistant to more than 10 types of antibiotics in total. Nearly half of the *Staphylococcus* spp. isolates showed resistance to at least 5 drugs, while about one-third (33.3%) of the *Enterobacter* spp. isolates were resistant to six types of drugs.

Genotyping of the Isolates using Amplified Ribosomal DNA Restriction Analysis

Amplified product for 16S rRNA PCR was found at approximately 1500 base pair position **(Figure 1A)**. ARDRA grouping of the isolates was made based on the molecular size pattern. The isolates showed three different size patterns and thus differentiated into three groups **(Figure 1B)**.

Phylogenetic Analysis of the Strains

16S rRNA sequencing was carried out where one of the isolates was used as a representative of the ARDRA groups. The phylogenetic tree showed the position of the isolates on the basis of evolutionary and represented similar strains to the isolates **(Figure 1C)**. *Acinetobacter baumannii, Enterobacter hormaechei* and *Staphylococcus pasteuri* were found to be related to the ARDRA groups 1, 2 and 3, respectively **(Figure 1)**.

DISCUSSION

The problem of child mortality due to Pneumonia can be aggravated by the emergence of antibiotic-resistant bacteria that contributes to the ineffectiveness of the popular next-generation antibiotics to the disease. Several studies have showed the rise of drug-resistant isolates in Bangladesh as well and thus an increase in child death[31,32]. To tackle the situation, early detection of these isolates is beneficial. Therefore, the present study

Table 1: Biochemical characteristics of presumptive bacterial isolates from pediatric pneumonia patients

In-house Isolates identifiers	Gram staining	Hemolysis	Catalase	Oxidase	Methyl red	Voges Proskauer	Indole	Citrate	Urease	Motility	Glucose	Lactose	Sucrose	Gas	H2S	Presumptive bacteria
B1Sa, B1Sb, B2Nsa, B15Nsa, B6Sb, B9Sb, B11Nsc, B13Sb, B14Nsb, C5Sa, C5Sb, C5Nsc, C6Sd, C7Sa, C8Sa, C9Sb	+	β	+	-	+	+	-	+	+	-	+	+	+	-	-	*Staphylococcus spp.*
B3Sa, B5Nsa, B6Sc, B7Sb, B7Nsb, B8Sa, B9Sa, B9Nsb, B10Sa, B10Nsa, B10Nsb, B11Sc, B11Nsa, B11Nsb, C1Sc, C3Nsa, C4Sc, C6Sa, C8Nsb	-	α	+	-	-	-	-	+	-	-	+	+/-	-	-	-	*Acinetobacter spp.*
B2Sa, B4Sb, B12sa, B12Nsc, B14Nsa, C1Nsc, C2Nsb, C4Sd, C4Nsc, C5Nsa, C6Nsb, C10Sa	-	β	+	-	+	+	+/-	-	+/-	+	+	+	-	+	-	*Enterobacter spp.*

Table 2: Antimicrobial susceptibility test of bacterial isolates

Bacterial isolate	Total No	Antimicrobial susceptibility																	
			AMX	CHL	CIP	GEN	NA	VAN	STR	CTX	AZM	CFP	TET	MEP	NOF	KAN	ERY	IMP	LEV
		S	7	12	13	14	-	8	10	-	8	-	3	16	8	3	5	16	8
Staphylococcus spp.	16	I	-	2	3	1	-	1	1	3	-	-	7	-	-	8	3	-	6
		R	9	2	-	1	-	7	5	13	8	16	6	-	8	5	8	-	2
		S	6	8	8	10	10	4	6	0	5	0	5	12	7	3	7	12	10
Enterobacter spp.	12	I	0	2	0	0	0	3	0	1	0	0	1	0	3	4	1	0	1
		R	6	2	4	2	2	5	6	11	7	12	6	0	2	5	4	0	1
		S	4	15	10	13	11	3	5	-	7	-	9	19	7	10	3	19	10
Acinetobacter spp.	19	I	-	2	1	-	-	-	-	2	1	-	2	-	7	-	3	-	4
		R	15	2	8	6	8	16	14	17	11	19	8	-	5	9	13	-	5
		S	17	35	31	37	21	15	21	-	20	-	17	47	22	16	15	47	28
Total (N)	**47**	I	-	6	4	1	-	4	1	6	1	-	10	-	10	12	7	-	11
		R	30	6	12	9	10	28	25	41	26	47	20	-	15	19	25	-	8

AMX= Amoxicillin, CHL= Chloramphenicol, CIP= Ciprofloxacin, GEN= Gentamycin NA= Nalidixic Acid, VAN= Vancomycin, STR= Streptomycin, CTX= Cefotaxime, AZM= Azithromycin, CFP= Cefepime, TET= Tetracycline, MEP= Meropenem, NOF= Norfloxacin, KAN= Kanamycin, ERY= Erythromycin, IMP= Imipenem, LEV= Levofloxacin; S: Sensitive, I: Intermediate, R: Resistant

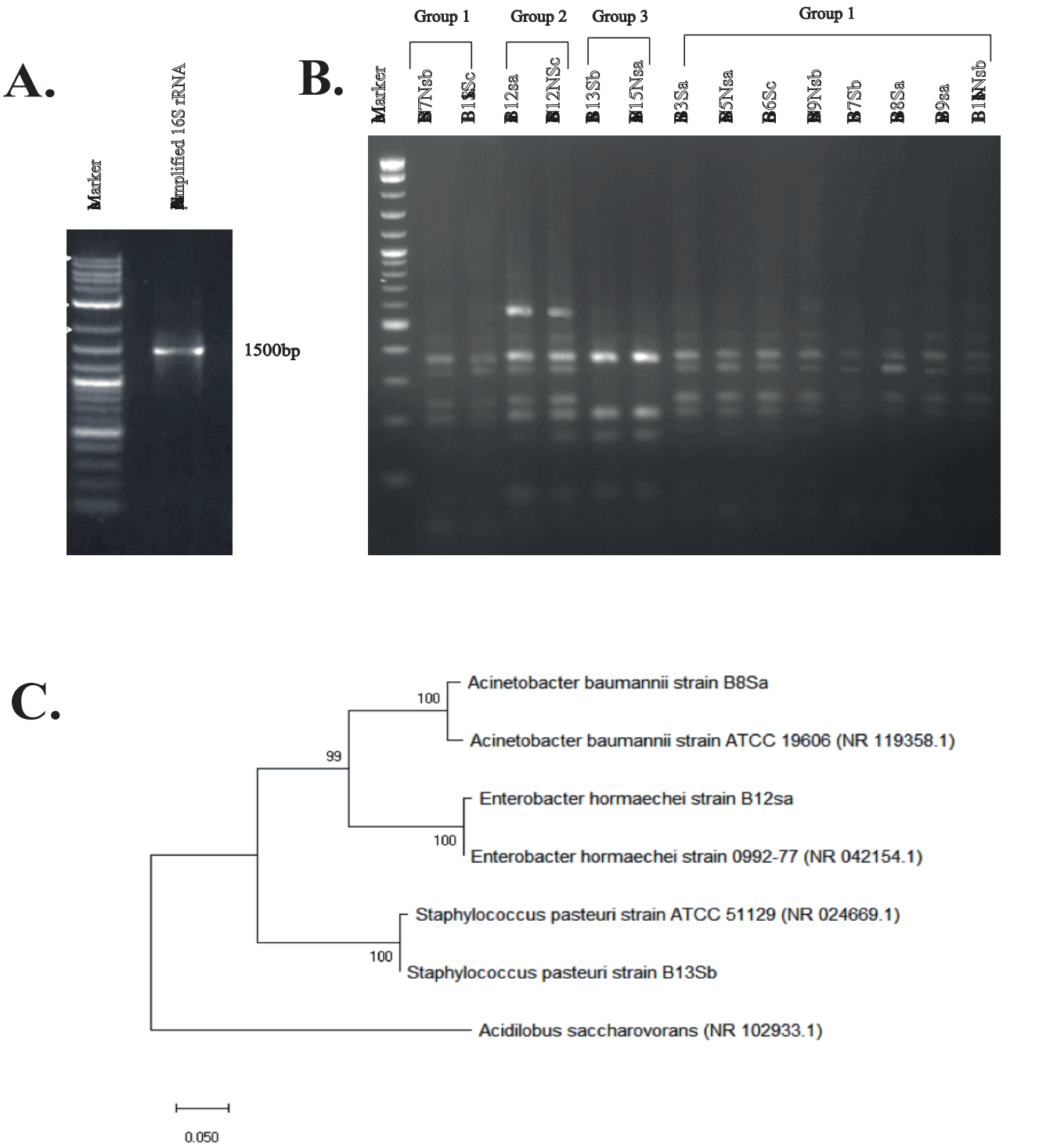

Figure 1. ARDRA genotyping of the isolates and molecular phylogeny analysis. (A) Amplification of 16S rRNA. The visible band was found at approximately 1500 bp for all the samples. 1Kb plus DNA ladder was used as a marker. **(B)** ARDRA pattern of the isolated strains. The isolated strains were divided into three groups based on their pattern. Group 1 consists the isolates: B7Nsb, B11Sc B3Sa, B5Nsa, B6Sc, B9Nsb, B7Sb, B8Sa, B9sa, B11Nsb, Group 2 consists the isolates: B12sa, B12NSc and Group 3 consists the isolates: B13Sb, B15Nsa. **(C)** Molecular Phylogenetic analysis by using Maximum Likelihood method. The optimal tree with the sum of branch length is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Lab isolates B8Sa, B12sa and B13Sb were used as a representative for the groups 1, 2 and 3, respectively.

Table 3: Multidrug resistance pattern of presumptive bacterial isolates

	Antibiotic resistance									
Bacterial isolates	**For 4 drugs No. (%)**	**For 5 drugs No. (%)**	**For 6 drugs No. (%)**	**For 7 drugs No. (%)**	**For 8 drugs No. (%)**	**For 9 drugs No. (%)**	**For 10 drugs No. (%)**	**For 11 drugs No. (%)**	**For 12 drugs No. (%)**	**For 13 drugs No. (%)**
Acinetobacter spp. (N=19)	-	3 (15.7)	3 (15.7)	3 (15.7)	2 (10.5)	2 (10.5)	2 (10.5)	2 (10.5)	1 (5.2)	1 (5.2)
Staphylococcus spp. (N=16)	1 (6.2)	7 (43.7)	6 (37.5)	1 (6.2)	1 (6.2)	-	-	-	-	-
Enterobacter spp. (N=12)	1 (8.3)	3 (25.0)	4 (33.3)	3 (25.0)	-	-	-	1 (8.3)	-	-
Total N= 47	2 (4.2)	13 (27.65)	13 (27.65)	7 (14.89)	3 (6.38)	2 (4.2)	2 (4.2)	3 (6.38)	1 (2.12)	1 (2.12)

was performed in the southwest region of Bangladesh to determine the causative agent of pediatric pneumonia and sensitivity to commonly used antibiotics against the disease.

Streptococcus pneumoniae and *Haemophilus influenzae* is the principal causative agent of pneumonia in many countries[33]. Our study found that *Acinetobacter* spp., *Enterobacter* spp*., and Staphylococcus* spp. are also highly associated with the disease. A similar result was found in a recent study in our neighboring country India[34] and studies conducted among ten countries of Asia region [35,36]. Besides, we found *Enterobacter* spp*., Acinetobacter* spp., and *Staphylococcus* spp. that indicate community-acquired pneumonia[34,37,38]. We didn't find *Streptococcus* spp. as the predominant one; it is possible that clinical pneumonia due to other pathogens masks the presence of *Streptococcus* spp. in a routine culture.

With the highest prevalence of 40.42%, one-third of the presumptively identified *Acinetobacter* spp. isolates were resistant to more than ten types of antibiotics in the present study. Similarly, previous work reported multi-drug resistant (MDR) Acinetobacter spp. for pneumonia patients as well [39]. Resistance to Chloramphenicol (10.5%), Norfloxacin (26.3%), and Levofloxacin (26.3%) were the lowest, along with imipenem and meropenem, making them useful for treatment. Prevalence of *Enterobacter* spp. was 25.53%, and one of the isolates showed resistance to 11 drugs. Though MDR *Enterobacter spp.* was reported in previous studies[40], resistance to this high number of antibiotics is a matter of concern as the isolate can be found regularly in the environment. *Enterobacter spp*. showed sensitivity to Imipenem, Meropenem, Gentamycin, Levofloxacin, and Nalidixic acid, thus making them the preferred way of controlling the disease. 43% of the *Staphylococcus* spp. showed resistance against five of the drugs used in this study. However, the situation is worse than the situations previously identified[41]. It may be because of the continuous rise of antibiotic resistance due to unrestricted antibiotic use that is putting pressure on the pathogens to become more resistant and virulent, thereby [42]. However, Chloramphenicol, Ciprofloxacin, Gentamycin, Imipenem, and Meropenem were sensitive for most *Staphylococci* spp. isolates.

Third-generation cephalosporin was the choice of treatment for childhood pneumonia even a few years ago[36,43]. However, resistant to third-generation Cephalosporin is currently increasing worldwide[44]. Similarly, we also observed resistance to cephalosporin for almost all the isolates, narrowing the scope of treatment. Interestingly, sensitivity to Imipenem and Meropenem was found for all the isolates. Intermediate sensitivity to Kanamycin and Levofloxacin was observed in 26% and 23% of the cases, respectively, suggesting that organisms are becoming resistant to these antibiotics over time. Besides, resistance to Chloramphenicol and Ciprofloxacin is also emerging[17].

It is to be noticed that the prevalence of antibiotic resistance was high in our study. Gram-negative strains

were found to be dominant over gram-positive strains. The resistance of gram-negative isolates is of high concern, as they cannot be treated with inexpensive antibiotics[45]. Resistance to β-lactam, Cephalosporin, and other antimicrobial agents among clinical isolates of gram-negative bacteria is rising worldwide[9,46,47]. High resistance to Amoxicillin, Streptomycin, Vancomycin, Kanamycin, Erythromycin, and Tetracycline was also found in the study. As the isolates were from pediatric patients, the newborns might be in great danger of acquiring multi-drug resistant organisms, which can result in a considerable threat for humankind. The return of pre-antibiotic era has become the reality in many countries of the world. Our study suggests that we are also walking through the same way.

Following the ARDRA grouping of the isolates, the phylogenetic analysis by 16srRNA sequencing identified *Acinetobacter baumannii, Enterobacter hormaechei* and *Staphylococcus pasteuri* as the potential candidates associated with the present study. A previous study suggests that *Acinetobacter baumannii* can be an opportunistic pathogen and a potential nosocomial-pneumonia agent[48]. Also, *Enterobacter* spp. was found to be involved in community-acquired pneumonia in a very recent study[49]. Furthermore, 16srRNA sequencing was defined as a successful non-culture method in detecting organisms associated with pneumonia in a study conducted a few years ago[50].

However, we are aware that there were some limitations in our study; for instance, our study may not reflect the true etiologic pathogen, as the more stringent procedures (e.g., the culture of Bronchoalveolar lavage) were not performed. Furthermore, the current data do not reflect the resistance pattern of the whole country as it was performed in a small region.

CONCLUSION

Discordant initial empirical antimicrobial therapy by healthcare personals has significantly increased the likelihood of pneumonia-related mortality. Injudicious usage of antibiotics has augmented the risk of worst clinical outcomes and death. Further study is suggested to find out the genes responsible for resistance by molecular techniques, which allows faster detection of antibiotic resistance. Continuous monitoring and surveillance of antimicrobial resistance for pneumonia-associated organisms is necessary for curbing the emergence of resistant bacteria and better therapeutic outcomes.

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Authors Contribution: The study's data collectors and concept owners are OKI and MTI. The total study was carried out by NSM, MMK, and MOR, who drafted the articles. The study design was completed by NS, MSH, MTI, and OKI. They also approved the final text and made intellectual contributions to the interpretation and presentation of the results.

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