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Isolation and Molecular Detection of *Klebsiella pneumoniae* from Children Affected by Pneumonia in Dinajpur District, Bangladesh

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The study was conducted to isolate and identify *Klebsiella pneumoniae* from children affected by pneumonia in Dinajpur district. This research includes sample collection from pneumonia affected children, isolation and identification of *Klebsiella pneumoniae* from those samples, molecular detection and antibiotic sensitivity of the identified bacteria. In this research, the samples were collected from nasal secretion of children between 6 months to 10 years old. Then isolation, identification and molecular characterization of *Klebsiella pneumoniae* from those samples were done. The collection of samples and research work was carried out from May, 2020 to April, 2021. All research work was performed in the Bacteriology laboratory of Microbiology Department, HSTU,

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Dinajpur. To conduct the study, a total 60 samples were collected from 4 different hospitals in Dinajpur district. Then the samples were brought to the Bacteriology laboratory, Department of Microbiology, HSTU and divided into 5 age category. These samples were then processed and cultural tests were performed in various differential and selective media. Then selected samples were chosen for biochemical tests. After analysing biochemical test results, further selected samples were passed for molecular test to identify the presence of *Klebsiella pneumoniae*. At last antibiotic sensitivity tests were performed.

In this test, specific primer for *Klebsiella pneumonia*e was used to detect the presence of bacteria in the samples. After the cultural, biochemical and molecular tests, total 7 (11.66%) samples were positive among 60 isolates. Among those samples, 2 samples were positive between 6 months to 1 year age for the detection of *Klebsiella pneumoniae* which is around 14.28%, 2 samples were positive between 1 year to 3 years age which is around 15.38%, 1 sample was positive between 3 years to 5 years age which is around 10%, 1 sample was positive between 5 years to 7 years age which is around 8.33% and 1 sample was positive between 7 years to 10 years age which is around 9.09%. Then molecular detection was done by performing PCR test using specific primer for *Klebsiella pneumoniae*. At last antibiotic sensitivity test was performed which shows that *Klebsiella pneumoniae* is resistant to Amoxicillin, Ampicillin, Doxycycline, Erythromycin, Penicillin G and sensitive to Gentamicin, Streptomycin, Azithromycin, Levofloxacin, Tetracycline, Neomycin. All the results resemble recent studies as it is a normal inhabitant of human nosocomial pathway but it holds potential threats for children because in immunocompromised condition, infections can be occurred by *Klebsiella pneumoniae* which can lead to serious illness becoming more and more resistant to antibiotics.

Keywords: Pneumonia; Klebsiella pneumonia; isolation and identification; molecular detection of pneumonia; children, Dinajpur.

1. INTRODUCTION

Pneumonia is a form of acute respiratory infection that affects the lungs. The lungs are made up of small sacs called alveoli, which fill with air when a healthy person breathes. When an individual has pneumonia, the alveoli are filled with pus and fluid, which makes breathing painful and limits oxygen intake. Pneumonia is the single largest infectious cause of death in children worldwide. Pneumonia killed 740180 children under the age of 5 in 2019, accounting for 14% of all deaths of children under five years old but 22% of all deaths in children aged 1 to 5. Pneumonia affects children and families everywhere, but deaths are highest in South Asia and sub-Saharan Africa. Children can be protected from pneumonia, it can be prevented with simple interventions, and treated with low-cost, low-tech medication and care. (WHO report 2021)

Klebsiella pneumoniae is a gram-negative, encapsulated, non-motile bacterium that is found in the environment and has been associated with pneumonia in patient populations with alcohol use disorder or diabetes mellitus. The bacterium typically colonizes human mucosal surfaces of the oropharynx and gastrointestinal (GI) tract. Once the bacterium enters the body, it can display high degrees of virulence and antibiotic resistance. Today, *K. pneumoniae* is considered the most common cause of hospital-acquired pneumonia in the United States, and the organism accounts for 3% to 8% of all nosocomial bacterial infections [1].

Typical *K. pneumoniae* is an opportunistic pathogen that is widely found in the mouth, skin and intestines, as well as in hospital settings and medical devices. Opportunistic *K. pneumoniae* mostly affects those with compromised immune systems or who are weakened by other infections. Colonization of the GI tract by opportunistic *K. pneumoniae* generally occurs prior to the development of nosocomial infections, and *K. pneumoniae* colonization can be further found in the urinary tract, respiratory tract and blood. *K. pneumoniae* biofilms that form on medical devices (e.g., catheters and endotracheal tubes) provide a significant source of infection in catheterized patients. Nosocomial infections caused by *K. pneumoniae* tend to be chronic due to the two following major reasons: *K. pneumoniae* biofilms formed in vivo protect the pathogen from attacks of the host immune responses and antibiotics and nosocomial isolates of *K. pneumoniae* often display multidrug-resistance phenotypes that are commonly caused by the presence of extended-

spectrum β-lactamases or carbapenemases, making it difficult to choose appropriate antibiotics for treatment. (Zhenhong Chen et al. 2014).

In Bangladesh, pneumonia is responsible for around 28% of the deaths of children under five years of age. Around 50,000 children die of pneumonia every year. An estimated 80,000 children under five years of age are admitted to hospital with virus-associated acute respiratory illness each year; the total number of infections is likely to be much higher. Again, 45 per cent of the pneumonia-related deaths are occurring at health facilities, which strongly indicate the lack of readiness of the health facilities to provide appropriate treatment for childhood pneumonia (icddr,b).

Out of the 4,007 pneumonia patients 5 years or younger meeting the criteria for clinical and radiographic pneumonia admitted to a Bangladeshi hospital (median age, 7.6 to 7.95 months), 45% (1,814) had blood cultures, of which 108 (6%) were positive. Children were more likely to have a positive blood culture if they were severely underweight (up to 3 standard deviations by weight-by-age), and children with bacteremia had lower mean hemoglobin levels (9.8 vs 10.3 grams per deciliter) and were more prone to severe sepsis and respiratory failure than those without. Most positive cultures (83 [77%]) showed gram-negative pathogens, including Pseudomonas (22) and Enterobacteriaceae (46, including *Escherichia coli* (17), *Salmonella enterica* (14), and *Klebsiella pneumoniae* (11).Gram-positive pathogens were most commonly Pneumococcus (7) and *Staphylococcus aureus* (6). With the exception of *K pneumoniae*, these pathogens are typically not associated with a primary respiratory infection, which suggests that young children with both clinical and radiographic evidence of pneumonia may have other or additional underlying source(s) of illness, particularly in a population where concomitant malnourishment and diarrheal illness are common [2].

The optimal treatment of infections caused by *Klebsiella pneumoniae* isolates is unknown. Their evolving resistance mechanism(s) and the lack of agents with Gram-negative activity in the development pipeline represent a major treatment dilemma for clinicians. Currently, very limited data are available from in vitro infection models or animals, and research into these avenues is necessary. Observational studies and clinical outcome data are urgently needed in order to determine the optimal treatment for KPC infections. Lastly, infections caused by *Klebsiella pneumoniae* organisms further emphasize the need to study combination therapy and rational treatment strategies [3].

Objectives of the present study:

- To isolate and identify *Klebsiella pneumoniae* from the children affected by pneumonia
- Molecular detection of the bacteria
- Perform antibiotic sensitivity test of bacteria

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Selection of study area and patient

The laboratory work of this study was done in the bacteriology laboratory of Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200. This study was completed within the area of Dinajpur District. Pneumonia affected children between 6 months to 10 years of age were selected as patients for conducting the study.

2.1.2 Study period

This Research work was carried out from May, 2020 to April, 2021.

2.1.3 Collection of samples

The collection of sample were done in Dinajpur district. Samples were collected from four reputed hospitals. Necessary information's were noted and special measures were taken while collecting samples due to COVID-19 pandemic situation. Samples were collected using sterile cotton bar from nasal secretion of the children affected by pneumonia. Total 60 samples were collected and 7 of them were suspected to have *Klebsiella pneumoniae*.

2.1.4 Media for culture

Nutrient Broth media, Nutrient Agar media, MacConkey Agar media, EMB Agar media, Muller-Hinton Agar, Semisolid Agar Media, Tryptophan Broth Media, Broth Media, Simmons' citrate agar media.

Table 1. Sample collection

Age of Patients	Dinajpur Sadar Hospital, Dinajpur	M Abdur Rahim Medical College Hospital, Dinajpur	Arbindh Child Hospital, Dinajpur	Green Life Diagnostic and Hospital, Dinajpur	Sample Type	Number of collected samples
6 months to 1 year	4	3	4	3	Nasal secretion	14
1 year to 3 years	2	4	3	4	Nasal secretion	13
3 years to 5 years	2	3	2	3	Nasal secretion	10
5 years to 7 years	3	4	3	2	Nasal secretion	12
7 years to 10 years	3	3	2	3	Nasal secretion	11

Total Number of collected samples = 60

2.1.5 Reagents

Phosphate Buffer Saline (PBS) solution, Urea 40% solution, Methyl- red solution, Kovac's Reagent, Glycerine, Crystal violate dye, Grams iodine, Alcohol, Safranin.

2.1.6 Materials used for bacterial genomic DNA isolation

1. Distilled water
2. Ice and ice bags
3. Boiling water

2.1.7 Material used for Polymerase Chain Reaction

Table 2. Materials needed in PCR

Components	Amount
DNA Extract	4µl
Master Mix	13µl
Reverse Primer	1µl
Forward Primer	1µl
Distilled water	6µl

1. Primer used for PCR
   Forward primer (F3) SEQ:

   5' -CCGATAGAGAACTCGAACTG- 3' (20mer)

   Reverse primer (B3) SEQ:

   5' - TCTGATGCATTTTACCCTGAT- 3' (21mer)

2. Agarose Gel 1.5%
3. TAE Buffer
4. 100bp DNA ladder
5. Ethidium Bromide (2.5µl)
6. Distilled water
7. GoTaq® Green Master Mix

2.1.8 Antimicrobial sensitivity discs

Different types of commercially available antimicrobial discs (Oxoid Ltd, UK) were used to determine the drug sensitivity pattern of identified isolate. The following are the antibiotics that were tested against, the selected organism with their disc concentration and zone diameter (EUCAST, 2015).

Table 3. Antimicrobial agents with their disc's concentration

Antimicrobial agent (Disc code)	Potency	Zone diameter nearest whole mm		
		Resistant≤	Intermediate	≥Susceptible
Gentamicin (GEN)	10µg/disc	12mm	12-15mm	15mm
Amoxicillin (AMX)	30µg/disc	13mm	13-18mm	18mm
Azithromycin (AZM)	15µg/disc	13mm	13-18mm	18mm
Erythromycin (E)	15µg/disc	13mm	13-23mm	23mm
Streptomycin (S)	10µg/disc	11mm	11-15mm	15mm
Ampicillin (AMP)	25µg/disc	17mm	17-22mm	22mm
Doxycycline (DO)	30µg/disc	10mm	10-14mm	14mm
Neomycin (N)	30µg/disc	12mm	12-17mm	17mm
Tetracycline (TE)	30µg/disc	11mm	11-15mm	15mm
Penicillin G (P)	10µg/disc	26mm	-	26mm
Levofloxacin (LE)	10µg/disc	12mm	12-16mm	16mm

2.2 Methods

2.2.1 Experimental layout

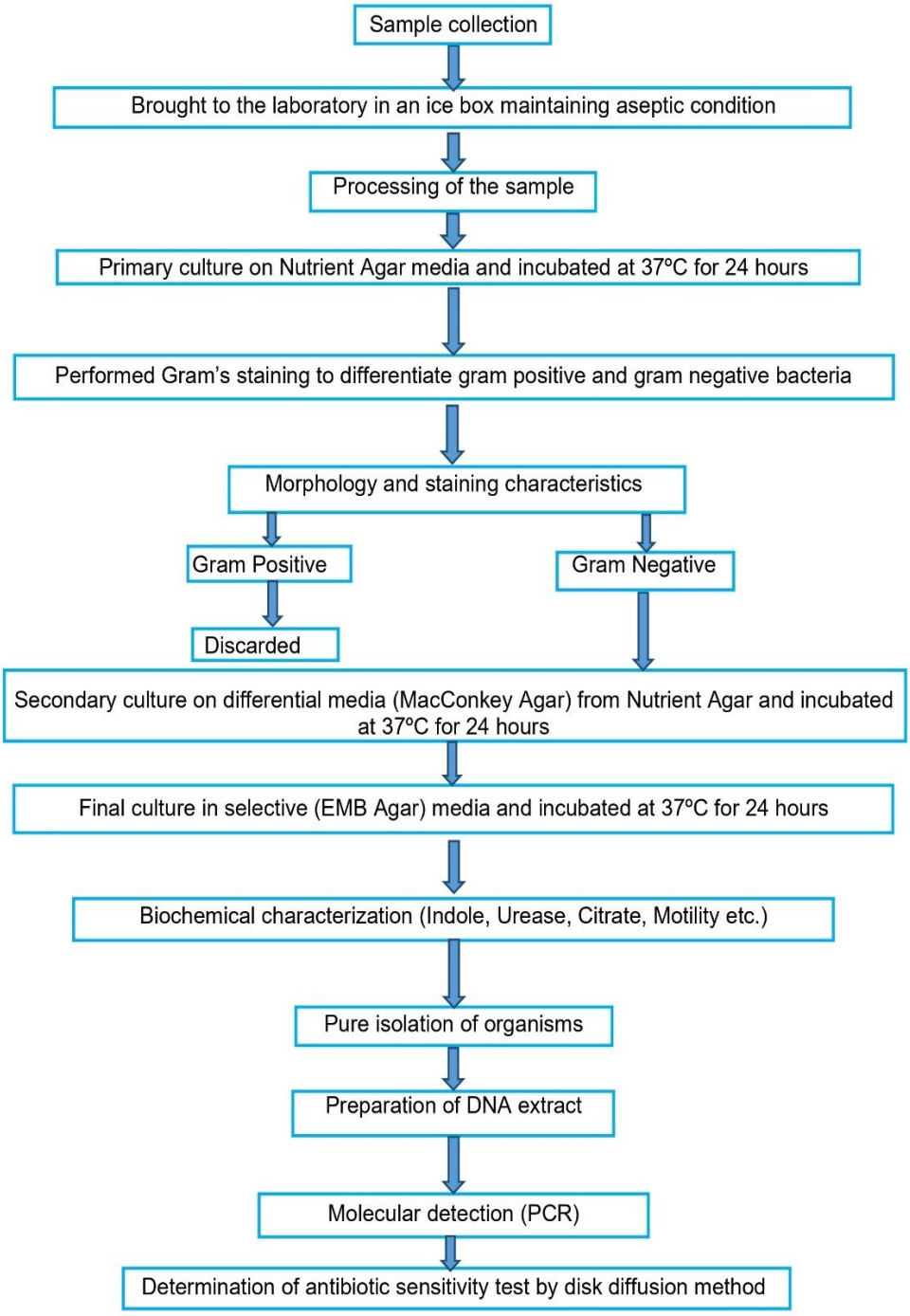

Fig. 1. Experimental layout

2.2.2 Plan of the experiment work at a glance

At first total 60 samples were collected from four different hospitals in Dinajpur district. Then all of the samples were transferred to the bacteriology laboratory of the department of microbiology, HSTU, Dinajpur, Bangladesh. After that samples were processed and primarily cultured on

nutrient agar media. Then morphology of the bacteria is observed by grams staining. After that bacteria was cultured on MacConkey agar media from nutrient agar media. Colony in MacConkey agar media was observed and used to culture in EMB agar media. EMB is a selective media for *Klebsiella Pneumoniae*. Colonies in EMB Agar are observed and suspected colonies are selected for further biochemical test. After biochemical tests, selected samples were finally made into stock culture and DNA extraction was done from the colonies in selective media. At last PCR was done for the confirmation of *Klebsiella pneumoniae* and antibiotic sensitivity tests were performed.

2.2.3 DNA extraction

At first a colony and 200µl distilled water were taken in a tip. Then we boiled it for 10 minutes. After that we kept it in ice for 10 minutes (Ice shock). Then we centrifuged it at 10,000 rpm for 10 minutes and at last the supernatant was collected in another tip and preserved in -4º Celsius temperature.

2.2.4 Working process for PCR

At first 4µl extracted DNA sample, 1µl Forward primer, 1µl Reverse primer, 13µl master mix and 6µl distilled water were taken into a PCR tip and closed properly. Then the tip was placed in PCR machine and appropriate data for every steps of PCR was set in the machine.

Then PCR process was run in the machine and waited for the time when it would be completed. At last PCR tip was taken from the machine and DNA band was analysed after PCR was completed.

2.2.5 Gel Electrophoresis

At first 50ml buffer peptone water and 0.75g agar was taken into a beaker. Then we heated the solution for 2 minutes in a micro oven and mixed it at 30 seconds interval. After that we took out the beaker and added 2.5µl ethylene bromide and mixed the ethylene bromide by shaking the beaker. Then the gel plate was prepared and gel was poured into the plate. Then we let the get solidify. After solidifying, gel was placed in electrophoresis machine. Then 6µl ladder and 25µl PCR sample were placed in two different gel pores. Then top cover was put on the machine and electrophoresis was done for 45 minutes at 82 volt and 4500mAh. After electrophoresis, gel was distained and placed on imaging system in the dark chamber of the gel documentation system. The UV light of the machine was turned on and the image was viewed on the monitor, focused, acquired and saved in a flash drive.

2.2.6 Antibiotic sensitivity test

Antibiotic sensitivity assay of isolated bacteria, bacterial susceptibility to anti-microbial agent was determined in vitro by using the standardized agar disc-diffusion method known as the Kirby-Bauer (K-B) method.

3. RESULTS

3.1 Results of Cultural Examinations

The cultural characteristics of *Klebsiella pneumoniae* on various culture media are presented in the Table 5.

Table 4. Conditions of PCR

Steps	Temperature	Duration	Cycles
1. Initial denaturation	95°C	5 min	01
2. Denaturation	95°C	30 sec	33
3. Annealing	57°C	40 sec	33
4. Extension	72°C	1 min	33
5. Final Extension	72°C	8 min	01
6. Holding	4°C	Hold	-

Table 5. *Klebsiella pneumoniae* cultural examination results

Name of culture media	Colony Morphology
Nutrient Agar	Dome-shaped circular colony measuring around 2-3mm. The colony is mucoid and translucent-opaque having greyish white colour.
MacConkey Agar	Convex circular colony measuring around 2-3mm. The colony is mucoid and opaque having pink-red colour.
EMB Agar	Convex circular colony measuring around 2-3mm. The colony is mucoid and translucent-opaque having pink-purple colour.

3.1.1 Culture on nutrient agar media

Plate 1. Colonies on nutrient agar media

3.1.2 Culture on MacConkey agar media

Plate 2. Colonies on MacConkey Agar Media

3.1.3 Culture on EMB Agar media

Plate 3. Colonies on EMB Agar Media

3.2 Microscopic Examination Result (Gram's Staining)

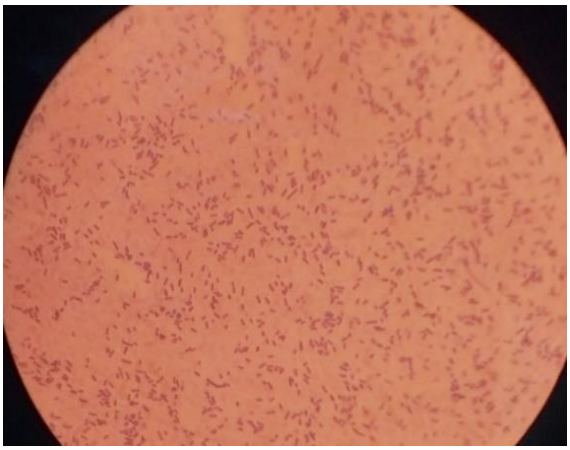

Plate 4. Gram Negative *Klebsiella pneumoniae*

Observation: Rod shaped pink coloured bacteria.

3.3 Results of Biochemical Examinations

The isolated organisms were confirmed by different biochemical tests. Following table represents the results obtained from different biochemical tests for *Klebsiella pneumoniae*.

Table 6. Biochemical test examination results

Biochemical tests	Change of the media	Results
Motility test	Sharp growth confined to the stab line	Negative
Urease test	Colour change of media from orange to red	Positive
Indole test	Reagent colour is not changed.	Negative
Citrate test	Colour change of media from green to blue	Positive

3.3.1 Motility test

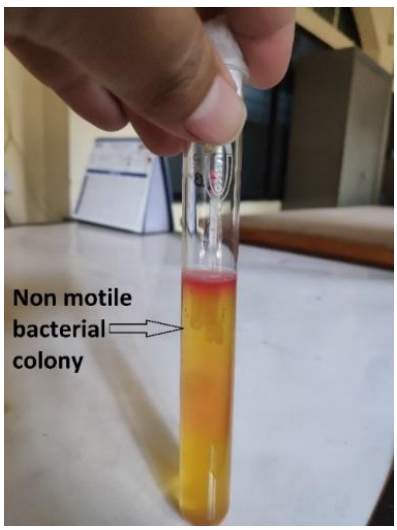

Plate 5. *Klebsiella pneumoniae* showing negative result on Motility test

3.3.2 Urease Test

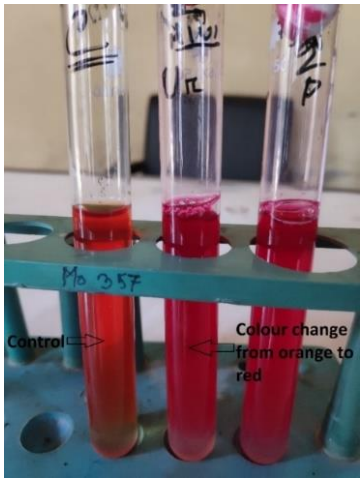

Plate 6. *Klebsiella pneumoniae* showing positive result on Urease test

3.3.3 Indole Test

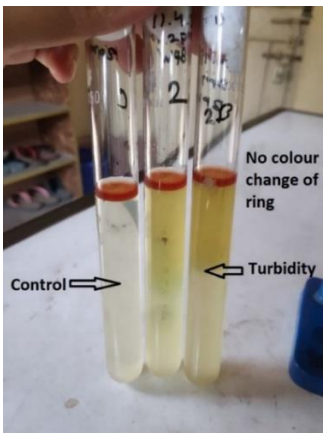

Plate 7. *Klebsiella pneumoniae* showing negative result on Indole Test

3.3.4 Citrate Test

Plate 8. *Klebsiella pneumoniae* showing positive result on Citrate test

3.4 PCR Test Result

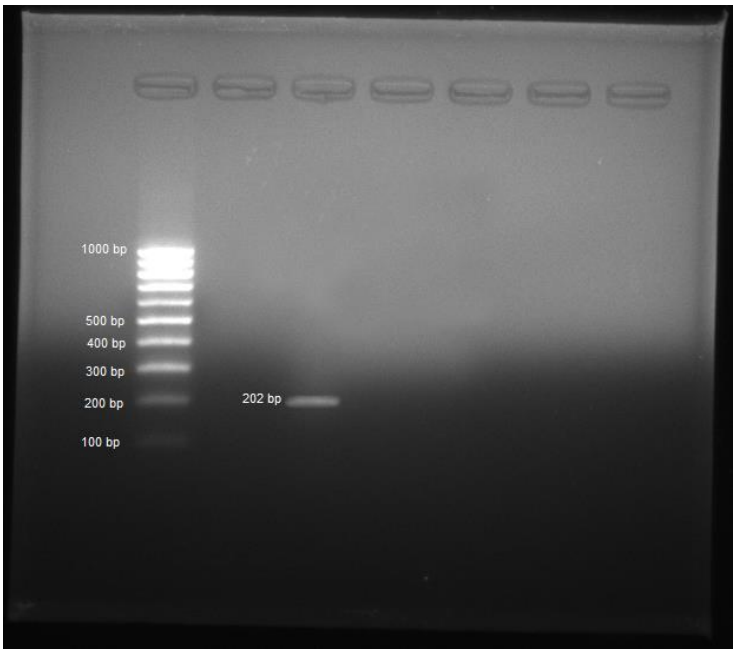

Fig. 2. PCR Test result of *Klebsiella pneumoniae* showing band 202bp

3.5 Age Wise Frequency of the Positive Isolates

Table shows the frequencies of positive isolates according to five age categories. These categories are 6 months to 1 year, 1 year to 3 years, 3 years to 5 years, 5 years to 7 years and 7 years to 10 years age. The frequencies of the presence of *Klebsiella pneumoniae* according to these age categories are 14.28% (6 months to 1 year), 15.38% (1 year to 3 years), 10% (3 years to 5 years), 8.33% (5 years to 7 years) and 9.09% (7 years to 10 years).

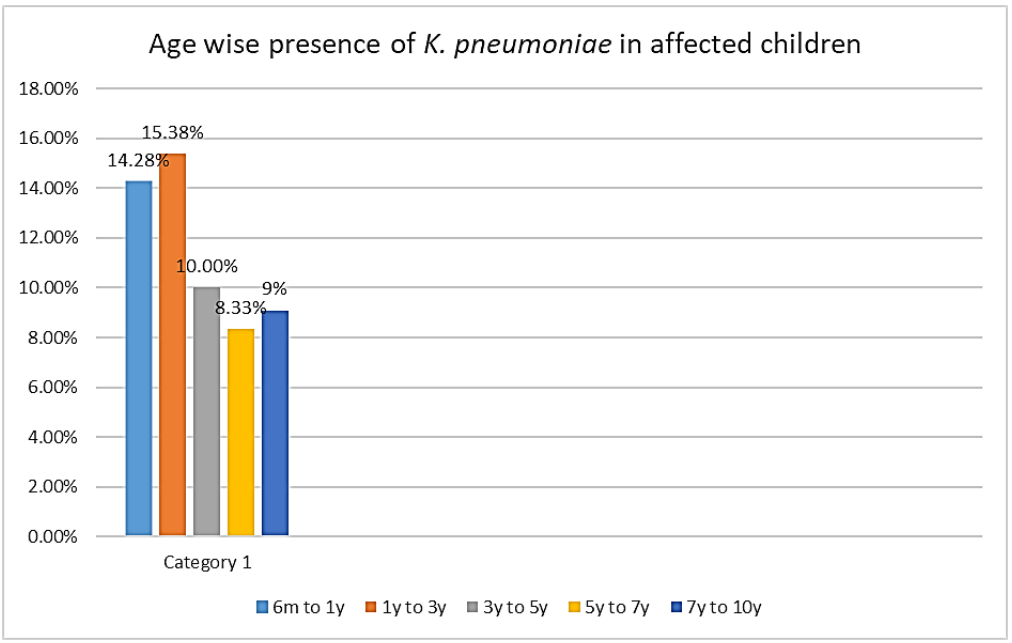

Fig. 3. Age wise percentage of the presence of *Klebsiella pneumoniae* in affected children

Table 7. Age wise frequency of positive isolates

Age range	Samples collected	Samples giving positive result	Frequency as percentage
6 months to 1 year	14	2	14.28%
1 year to 3 years	13	2	15.38%
3 years to 5 years	10	1	10%
5 years to 7 years	12	1	8.33%
7 years to 10 years	11	1	9.09%

Table 8. Result of antibiotic sensitivity test for *Klebsiella pneumoniae*

Name of antibiotics	Zone of diameter (mm)	Interpretation
Gentamicin	32	S
Streptomycin	25	S
Penicillin G	20	R
Erythromycin	0	R
Azithromycin	19	S
Levofloxacin	37	S
Tetracycline	28	S
Neomycin	26	S
Doxycycline	8	R
Ampicillin	0	R
Amoxicillin	0	R

S= Susceptible and R= Resistant

3.6 Total Frequency of Positive Isolates

Among 60 samples collected in this study, a total number of 7 isolates gave positive result which represent 11.66% presence of *Klebsiella pneumoniae* in children affected by pneumonia in Dinajpur District.

3.7 Results of Antibiotic Sensitivity Test

Antimicrobial susceptibility testing was performed using Muller-Hinton agar (Himedia, India) plates as recommended by the Clinical and Laboratory Standards Institute. Isolates of *Klebsiella pneumonia*e was subjected to antibiotic sensitivity tests for nasal secretion sample from pneumonia affected children. The results of antibiotic sensitivity tests are given in the Table 8.

4. DISCUSSION

According to the this study, the highest presence of *Klebsiella Pneumoniae* was found among 1 years to 3 years children (15.38%) which resembles another study of R podschun *et al.* [4] who isolated *Klebsiella pneumoniae* from hospitalized patients and the presence rate of *Klebsiella pneumoniae* in his study was 19% in pharynx sample. During the study, it was found that the least presence of *Klebsiella pneumonia* was in 5 years to 7 years children (8.33%) which represents a study done by Farida *et al.* [5] who isolated *Klebsiella pneumoniae* from hospitalized patients and the presence of positive isolates were 7% in children.

Presence of *Klebsiella Pneumoniae* between the 6 months to 1 years is 14.28%, 3 years to 5 years is 10% and 7 years to 10 years is 9.09%. The overall presence of *Klebsiella Pneumonia* among children affected by pneumonia is 11.66% (7 among 60 samples) which resembles the study done by Patwari *et al.* [6] who got the presence of *Klebsiella pneumoniae* in hospitalized children around 13% and recent study by Lianna Matt McLemon *et al.* (2021) in Bangladesh who described that 11 *Klebsiella pneumoniae* isolates are found among 83 cultures.

Isolation of the samples were performed onto Nutrient agar, MacConkey agar & EMB agar media. Identification of the bacteria was performed by morphological, cultural and biochemical tests. In Nutrient agar Dome-shaped circular mucoid colony having greyish white colour (Plate 1), in MacConkey agar convex circular mucoid colony having pink-red colour (Plate 2) and in EMB agar convex circular mucoid colony having pink-purple colour (Plate 3) indicate *Klebsiella Pneumoniae*.

The microscopic examination of Gram's stained smears from EMB agar showed that the isolated bacteria were Gram negative, rod shaped pink

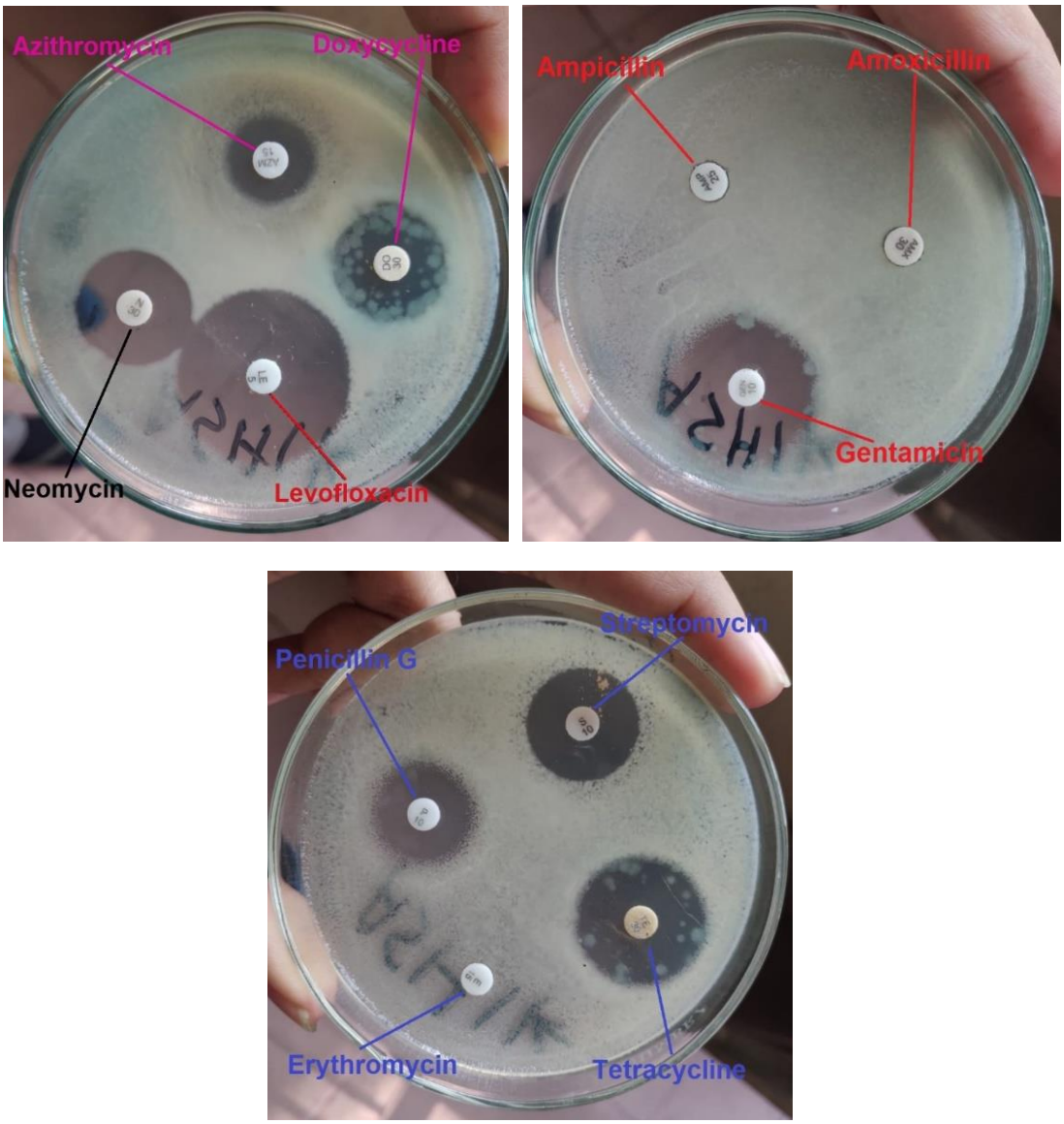

Plate 9. Antibiotic sensitivity test for *Klebsiella pneumoniae*

coloured (Plate 4). *Klebsiella pneumoniae* showed negative result to Motility test (Plate 5), positive result to Urease test (Plate 6), negative result to Indole test (Plate 7) and Positive result to Citrate test (Plate 8). Specific primer for *Klebsiella pneumoniae* was used in PCR and amplified 202bp fragments of DNA confirmed the identity of *Klebsiella pneumoniae* [7].

Again, results of the antibiotic sensitivity test of this study reveals that the isolated *Klebsiella pneumoniae* is sensitive against Gentamicin, Streptomycin, Neomycin, Azithromycin, Levofloxacin, Tetracycline and resistant to Penicillin G, Erythromycin, Doxycycline, Amoxicillin, Ampicillin (Plate 9). Result of this study resembles the study result of Farhan Essa Abdullah et al. [8] and Ujjwal Rimal et al. [9, 10-15].

5. CONCLUSION AND SUMMERY

Although the frequency rate of the presence of *Klebsiella pneumonia* among pneumonia affected children is not so high, it holds potential threat to children. Again followed by recent studies, we can say that day by day *Klebsiella pneumoniae* is becoming resistant to most of the antibiotics. As a result it is becoming more of a threat for humans, especially for children.

In this study, the prevalence rate of the presence of *Klebsiella pneumoniae* among the pneumonia affected children is 11.66 percent which is related to the normal incidence rate of the nosocomial presence of *K. pneumoniae* in human children as a natural inhabitant. Again followed by antibiotic sensitivity test of this study, we can say that the infections caused by *Klebsiella pneumoniae* can be treated by Gentamicin, Streptomycin, Neomycin, Azithromycin, Levofloxacin and Tetracycline.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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