Determination of Bacterial and viral pathogen spectra and identification of multi drugs resistance gene *CTX-M1* in *Klebsiella pneumoniae* isolated from acute respiratory infections children aged under-5 from two hospital settings of Dhaka city

A Dissertation Submitted to the Department of Mathematics and Natural Sciences, BRAC University in Partial Fulfillment of the Requirement for the Degree of Masters of Sciences in Biotechnology

Biotechnology Program
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DECLARATION OF AUTHENTICITY

This is to declare that the research work aggregating the results reported in this thesis entitled '**Determination of Bacterial and viral pathogen spectra and identification of multi drugs resistance gene *CTX-M1* in *Klebsiella pneumoniae* isolated from acute respiratory infections children aged under-5 from two hospital settings of Dhaka city'** has been carried out by the undersigned under the joint supervision of Dr.Kaiissar Mannoor Sr. Scientist and Head of ideSHi(institute of developing Science and Health initiatives) and Prof. Dr. Naiyyum Choudhury, Professor of Microbiology and Biotechnology Programme, Department of Mathematics and Natural Science, BRAC University, Dhaka serving as external and internal supervisors respectively . It is further declared that the research work presented here is original and submitted in the partial fulfillment for the degree of Masters of Science in Biotechnology, BRAC University, Dhaka, Bangladesh.

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TO

MY BELOVED PARENTS

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Abstract

This study aimed to determine bacterial and viral pathogen spectra and identify multidrug resistance genes in bacterial isolated from under-5 children with acute respiratory infections from two hospital settings of Dhaka city. Nasal swabs were collected from 150 under-five children hospitalized with clinical signs of ARIs. Screening of viral pathogens targeted ten respiratory viruses using RT-qPCR. Bacterial pathogens were identified by bacteriological culture methods and antimicrobial susceptibility of the isolates was determined following Clinical and Laboratory Standard Institute (CLSI) guidelines. About 78% (n=117) of specimens were positive for pathogens. Of 117 infected cases, 4.27% (n=5) had only single bacterial pathogens, whereas 44% (n=61) cases had only single viral pathogens. The remaining 58% (n=88) cases had coinfections. In viral suspected cases, human rhinovirus was detected as the predominant virus (32%), followed by RSV (17.33%), HMPV (16%), HBoV (13%), HPIV-3 (12%), and adenovirus (9%). *Streptococcus pneumoniae* was the most frequently isolated bacterial pathogen (8%), where *Klebsiella pneumaniae, Streptococcus* spp., *Enterobacter agglomerans*, and *Haemophilus influenzae* were 6%, 3.3%, 2%, and 2%, respectively. Of 15 multidrug-resistant bacteria, a *Klebsiella pneumoniae* (KPn) isolate exhibited resistance against more than 10 different antibiotics and extended spectrum beta-lactamase (ESBL) *CTX- M1* gene was found in multidrug-resistant KPn using conventional PCR. Sanger sequencing had been used for confirmation of amplified *CTX- M1*. Both ARI incidence and pathogen detection rates were higher during post-monsoon and winter, peaking in September. Pathogen detection rates and coinfection incidence in less than 1-year group were significantly higher (P=0.04123) than in 1-5 years age group. Human Boca Virus (HBoV) had a significant involvement between single and confections with P value of 0.0152.

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List of Abbreviation:

ARIs	Acute Respiratory Infections
RSV	Respiratory syncytial virus
RT qPCR	Single plex Reverse Transcriptase Real time PCR
ADV	Adeno Virus
HRV	Human Rhino Virus
HMPV	Human Metapneumo Virus
SPn	*Streptococcus pnumoniae*
Kpn	*Klebsiella pneumoniae*
Ssp	*Streptococcus spp*
AST	Antibiotic Sensitivity Testing
ESBL	Extended Spectrum beta-lactamase
MDR	Multi Drug Resistance
CTX-M	Cefotaximase - Munich
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
µL	Microliter
PCR	Polymerase chain reaction
UV light	Ultraviolet light
MgCl2	Magnesium Chloride
TBE	Tris/Borate/EDTA
RNA	Ribonucleic Acid
BMRC	Bangladesh Medical Research Council
ideSHi	Institute for developing Science and Health initiatives

Chapter 1: Introduction

1.1 Background

Acute Respiratory Infections (ARIs) are the most important and significant infections worldwide, especially in developing countries- like Bangladesh. Among ARIs mostly pneumonia is persistent and pervasive deterrent to public health. The ARI related death rate is more in developing region like Africa and Southeast Asia. Pneumonia is one of the major public health problems in children under five years of age. It is an infection of respiratory system and most commonly caused by virus or bacteria and spreads by direct contact with infected people. According to the World Health Organization (WHO), 1.9 to 2.2 million children die annually due to ARI infections which occurs 70% in Africa and Asia [1, 2]**.** The leading risk factors of ARI are poverty, lack of measles immunization, indoor air pollution, overcrowding, malnutrition, lack of exclusive breastfeeding and low birth weight [3]. The definition of ARI covers all infections of the respiratory tract. However a majority of respiratory death are imposed to acute lower respiratory infections (ALRIs). The incidence of ALRIs in < 5 years is estimated to be 0.22 episodes per child year which most occurring in India, China, Pakistan, Bangladesh, Indonesia and Nigeria **[**4, 5**].**

The causative agents for ARIs include bacteria, virus and fungi. The most commonly ARIs causing bacterial organism are *Streptococcus pneumonia, Haemophilus influenza* and *Staphylococcus aureus* etc. On the other hand, atypical bacteria such as *Mycoplasma pneumoniae*, *Chlalamydophila pneumonie* and *Klebsiella pneumoniae* are less frequently reported [6, 7]. Respiratory syncytial virus (RSV), human influenzae virus type A and B, human parainfluenzae type 1, 2, and 3, Adeno Virus and human metapneumo viruses are more frequently detected in under five children with ARIs. Respiratory viruses are indicates as a major contributors to ARI and they are associated with almost 60% cases of ALRIs [8, 9]

Although numerous pathogens are associated with ARIs, the clinical manifestations of ARIs are almost similar in all cases, irrespective of causative agents. So, detection of the potential causative agents is the prerequisite for proper treatment and viral detection can reduce the irrational and overuse of antibiotics. ARIs due to bacterial infections have become a global concern especially because of the emergence of an increasing number of multi-drug resistant bacteria. Although some studies have examined the viral etiology of ARIs in the under-five children in Bangladesh, information is lacking on bacterial pathogens and their antibiotic

resistance/sensitivity pattern [10, 11]. Lack of information regarding etiological agents influences the use of inappropriate and board spectrum antibiotics which are turn foster antimicrobial resistance.

1.2 Epidemiology

Acute Respiratory Infections (ARIs) are the leading cause of acute illnesses worldwide and remain the leading cause of infant and under five children mortality, accounting for around two million deaths each year in developing countries [12]. Early diagnosis and prompt treatment of ARIs- especially pneumonia, is lifesaving [13]. The percentages of Global Distribution of cause-specific mortality among under five children, pneumonia has the highest percentage (19%) among the other cases (Fig.1.1).

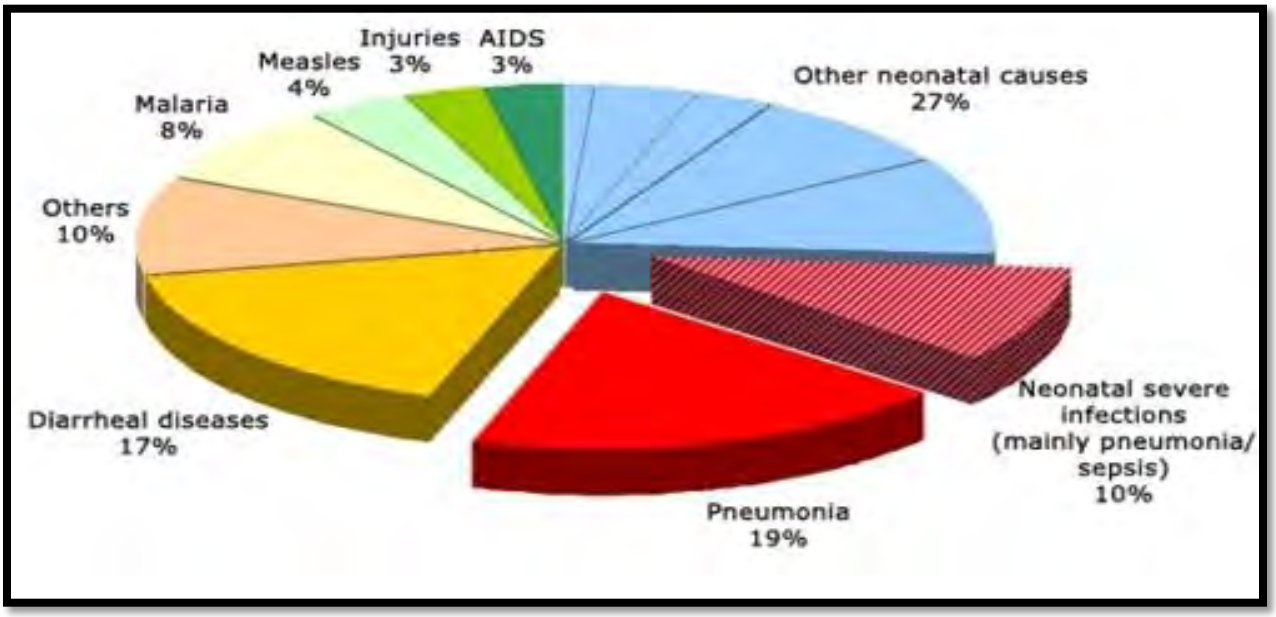


Fig1.1: Global distribution of cause-specific mortality among children under-five, 2000-2003. (Source: Child Health Epidemiology Resources Group (CHERG), with additional data from UNICEF)

It is estimated that 10% of all under-five children deaths are caused by severe infections during this time (2000-2003). A significant proportion of these infections are caused by pneumonia/sepsis (a blood-borne bacterial infection). If these numbers were taken into

consideration in the overall account of under-5 deaths, pneumonia would make up to 3 million deaths, or 29%, of the under-five children deaths each year [14]

1.3 ARIs Mortality of under 5 age of children

The incidence of ARIs in under-5 children is estimated to be 0.29 and 0.05 episodes per child-year in developing and develop countries respectively, which account into 151 million and 5 million new episodes in every year. In developing countries, under five children are the most susceptible to infection and death from ARIs, with the high risk of mortality occurring during the neonatal period [15]. Around19% of childhood deaths in under-5 are due to Acute Lower Respiratory Infections (ALRIs - pneumonia, bronchiolitis and bronchitis), among them 90% occurring by pneumonia. Over two-thirds of pneumonia mortality was held in Africa, South Asia and Southeast Asia (Figure 1.2). Overall, in developing countries, infants and young children have 7-10 episodes of ARI each year, one tenth of which was severe enough to require hospitalization. Although most episodes are self-limiting infections of the upper acute respiratory tract infections (UARIs) such as bronchitis and otitis media, resulted a huge economic burden to the public health system and increase the risk of progression to more severe disease [16].

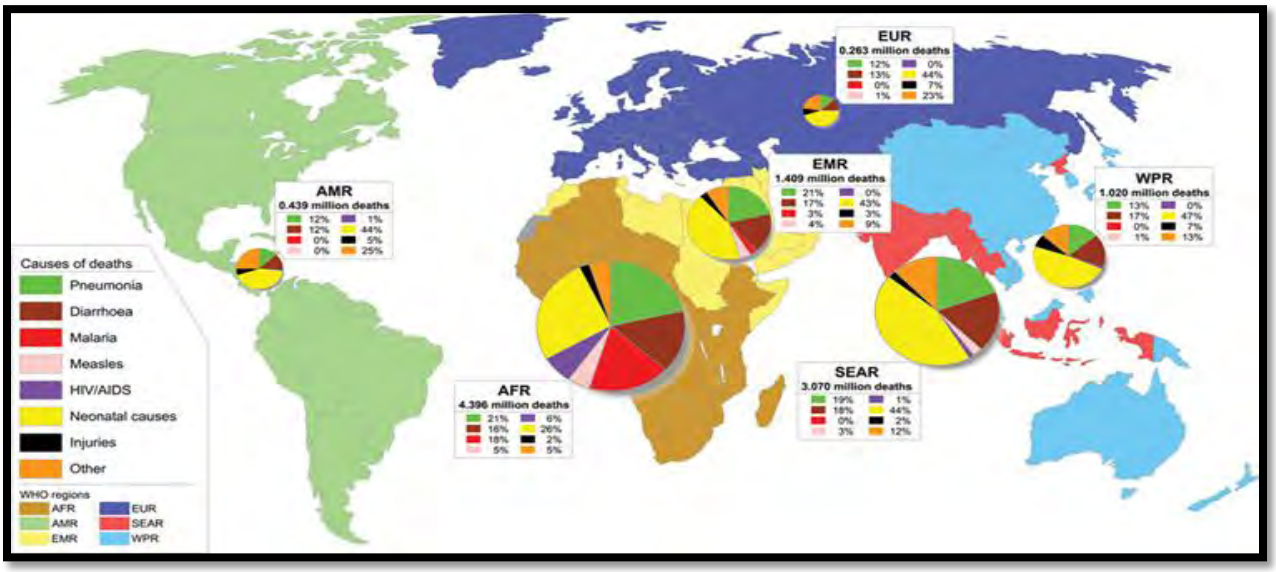


Fig1.2: Distribution of deaths from pneumonia and other causes in children aged less than 5 years, by WHO region, In the Figure AFR, African Region; AMR, Americas Region; EMR, Eastern Mediterranean Region; EUR, European Region; SEAR, South-East Asia Region; WPR, Western Pacific Region. (Source: https://www.who.int/bulletin/volumes/86/5/07-048769/en/)

1.4 Causative Agent

Respiratory infections are caused by Bacteria or virus that manifest in any part of the respiratory tract, including the nose, middle ear, throat, larynx, air passages, and lungs. The main etiological agents responsible for ARIs in children are given below:

- *Streptococcus pneumoniae* (SPn)
- *Haemophilus influenzae* (Hi)
- Respiratory syncytial virus (RSV)
- Human parainfluenzae virus type 3 (HPIV-3)
- Group B streptococcus in newborns

Although the pathogens vary with the child's age, their immune condition, and the environment, most ARIs episodes are caused by viruses. However, the etiology of Acute Respiratory Infections (ARIs) depends on certain factors. For example, *pneumocystis jiroveci*, the main pathogen of pneumonia in areas where HIV prevalence is high, has a higher proportion of gram-negative bacteria than other areas with lower HIV prevalence [17].
The subversive impact of Acute Respiratory Infections (ARIs) is apparent not only due to its high mortality, but its significant rate of morbidity and co-morbidity as well; low birth weight, malnourished and non-breastfed children and those living in overcrowded conditions put children at even higher risk of getting, and dying from pneumonia.

In developing countries bacterial infection plays a impotant role in causing pneumonia than it does in developed countries. Nasopharyngeal infections rates are twice as high in developing countries in comparison to developed countries due to higher exposure to risk factors which increase bacterial colonization of the upper airways. This explains the higher frequency of bacterial ARIs in poor society. In case, the incidence of pneumonia in a developing country is 10 times higher than that in the United States of America [18]. Acute Respiratory Infections related mortality is higher during infancy and particularly in neonatal period where more than half of the deaths occur [19].

1.5 Prevention

In general, the most effective prevention of ARIs is vaccination. The vaccines for diphtheria, pertussis, and tetanus, regularly included in immunization programs, are very effective in preventing some infections which can lead to ARIs. Vaccines are available for *H. influenzae* serotype b (Hib) and *S. pneumoniae*(pnuemococcus) infection, and are frequently used in the developed and some developing countries [20]. However, the high cost of procuring and storing of vaccines in resource poor settings has kept many developing countries from utilizing them in the past.

The effectiveness of the Hib vaccine has been found through different studies based in developing countries, specifically in South America, South Asia, Southeast Asia, and parts of Africa. In fact, the Hib vaccine was shown to reduce the infections caused by Hib despite irregular supply of the vaccine (Child Health Research Project). Studies in the Gambia have been found the efficacy of a pneumococcal conjugate vaccine, substantially improving child survival (Child Health Research Project). Its ability to decrese the number of childhood deaths was evident in these studies. Not only was the vaccine very effective in reducing the number of childhood pneumonia, but of those who were given the vaccine, there were 16% fewer deaths from any cause, not just pneumonia. It is believed that vaccination can prevent 1 in every 7 deaths in the rural developing world setting.The Hib vaccineand and pneumococcal conjugate vaccine was recommended by WHO's Strategic Advisory Group of Experts (SAGE) in 2006, 2007 respectively for all countries, especially those with high child mortality rates [21].

There are many global initiatives to accelerate the introduction of these two vaccines to all the developing countries. One such a initiative funding by the GAVI Alliance, which targets the lowest income, "GAVI eligible" countries. The Hib vaccine initiative, through this funding has been reached 62 of the 72 GAVI eligible countries [22]. Another , the Accelerating Vaccine Introduction (AVI), aims to widely distribution up the uptake of the pneumococcal and rotavirus vaccines to developing countries. 47 countries could be using the pneumococcal vaccine by 2015 thought this initiative [21].

Although the above efforts, primary prevention in the form of vaccination remains difficult in developing countries. There are many areas focus their prevention efforts on reducing the environmental and behavioral factors that can lead to ARIs. The most commonly targeted areas are given below:

- Malnutrition
- Vitamin A and zinc deficiency
- Low-birth weight
- Absence of breastfeeding
- Incomplete immunizations
- Poor hygiene
- Low socioeconomic status and
- Indoor air pollution (including tobacco smoke and smoke from biomass-burning stoves)

1.6 Treatment

Early recognition of sign, symptoms and the use of affordable antibiotics can prevent 30 to 60% of ARIs related under-5 child deaths [23]. The most effective intervention for ARIs is treatment with an appropriate, low-cost antibiotic are cotrimoxazole, amoxicillin, ampicillin, and procaine penicillin. In case both *S. pneumoniae* and *H. influenzae* have shown significant resistance to these standard antimicrobial drugs in the past decade. This burden a serious problem in both develop and developing countries [24]. Antibiotics, such as azithromycin, levofloxacin, or cefuroxime are effective against drug resistant strains but are costly, making them impractical for routine treatment in the developing countries [25].

While treatment with antibiotics is the foundation of ARIs intervention, it is also important that health workers encourage right health-seeking behavior for caretakers of children. UNICEF estimate only 53% of caretakers seeks care for their children with ARI symptoms. This lack of access has many contributing factors, including:

- Local health facilities are not well stocked with necessary supplies [26]
- Distance and travel time to a health facility [27]

- Cost, cultural reasons, and lack of child care for other children at home [28]

Above problems with the referral systems for pneumonia have led to several recent studies trying to make treatment available at the home for even severe pneumonia.

1.7 Acute Respiratory Infections Bug develops to Broad-spectrum antibiotics

Lower respiratory tract infections (LTRIs) such as pneumoniae are the most common infectious diseases with Potential life-threatening complications. Streptococcus pneumoniae is the most predominant pathogen (51.7%) followed by *Haemophilus influenzae* and *Klebsiella pneumoniae* (33.83%), (17.19%) and the emergence of fluoroquinolone resistance among this LRTIs bacterial pathogens has now been documented in many countries. At present, fluoroqoinolone are substituted by the 3rd generation Cephalosporins, which are frequently used by Clinicians. Recent studied have focused on rapidly acquiring resistance to these antibiotics used in LRTI's. Resistance to Cephalosporins is plasmid-born and emerging rapidly, which is the factor responsible for decrease the choice of drugs of LRTI treatment. Horizontal transfer of drug-resistant gene is not immediate concern for treating clinician but in future, drug resistance will pose a potential problem. Their presence plus the potential for plasmid and mediated quinolone resistance will be serious therapeutic problem in future. [28-31].

1.8 Objectives of the study

- The purpose of this study was to examine both viral and bacterial pathogens of ARIs among under-five children of two hospital settings (Dhaka Medical Collage Hospital and Shaheed Suhrawardy Medical Collage and Hospital) in Dhaka city
- To identification in vitro antibiotic resistance/ sensitivity assay of ARIs bacterial pathogens
- Confirmation of the multi-drugs resistance gene
- Determination of seasonality of isolated ARI pathogens and
- Co-infection pattern of isolated ARIs pathogens

Chapter 2: Methods and Materials

2.1 Ethics Statement

The study was approved by the Ethical Review Committee of Bangladesh Medical Research Council (BMRC), Ref: BMRC/NREC/2013-2016/29. Written consent was taken from the patients' parents and/ or legal guardians since the eligible participants of the study were under five years of age. The risk and benefits had been clearly stated in the consent form.

2.2 Place of the Study

The study was conducted in the laboratory facility of Institute for Developing Science and Health initiatives (ideSHi), Dhaka Bangladesh. The studies subjected were enrolled into the study from two hospitals in Dhaka, namely Shaheed Suhrawardy Medical Collage and Hospital (SSMCH) and Dhaka Medical Collage Hospital (DMCH).

2.3 Hospital Authority Permission

Before starting the sample collection permission was taken from the Director of the Shaheed Suhrawardy Medical Collage and Hospital (SSMCH) and the Dhaka Medical Collage Hospital (DMCH).

2.4 Study Participants

All the study participants enrolled in the study who admitted to pediatrics ward of the both hospitals. Enrolled all children age were < 5 years. Pediatric ward doctor was suspected ARI case clinically. Written informed consent was taken from all participants. All Nasal swabs were taken from the suspected patients by the study technologist.

2.5 Inclusion Criteria

Acute Respiratory Infection:

- Subjective Fever
- Rapid, labored or noisy breathing
- Cyanosis
- Respiratory Distress Symptoms
- Cough and running noise.

2.6 Period of Study

The study was carried out from September 2014 to July 2015.

2.7 Scheme of Nasal Swab Collection

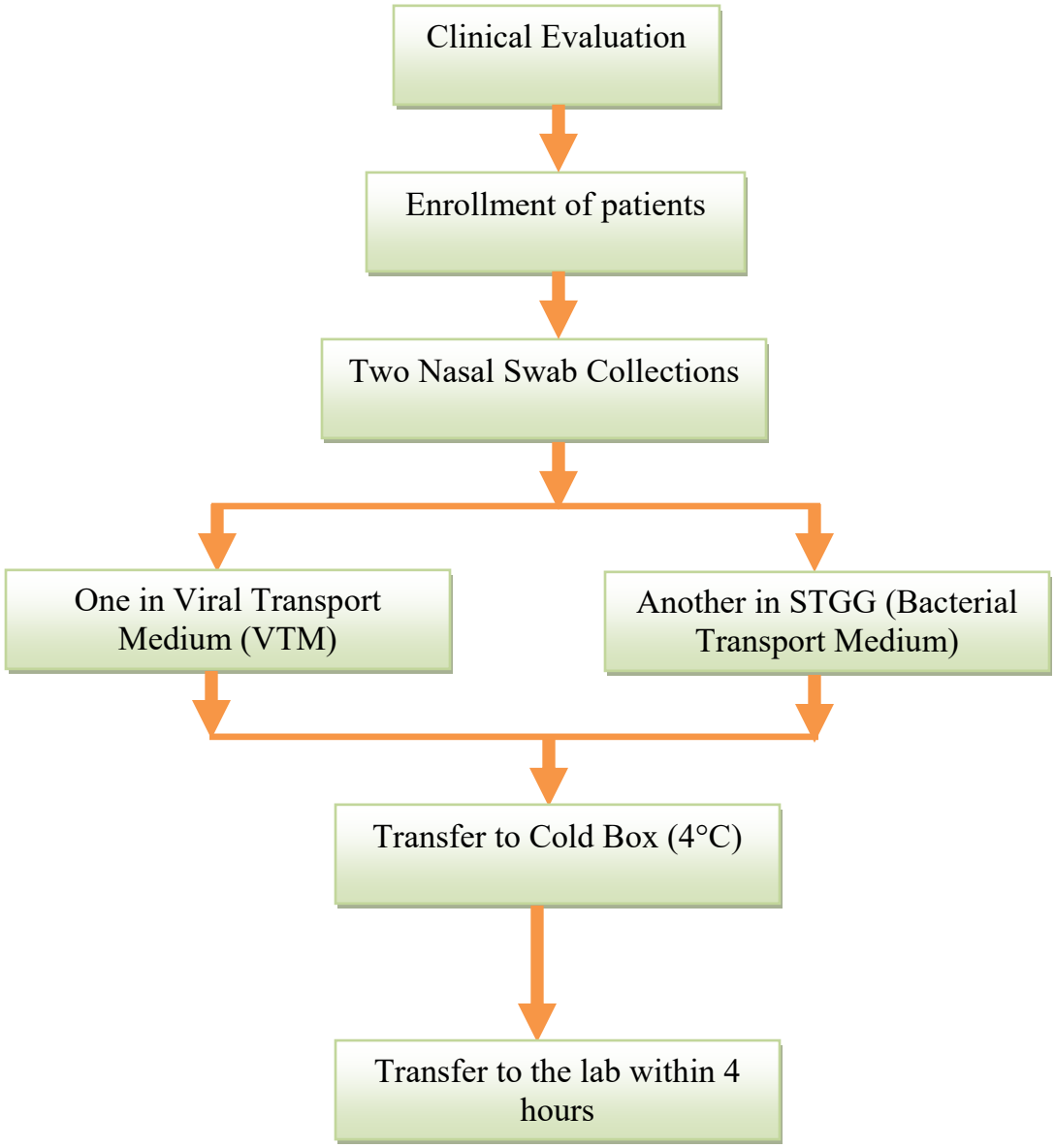


Fig. 2.1 Schedule of Nasal Swab Collection

2.8 Overview of the Study Plan

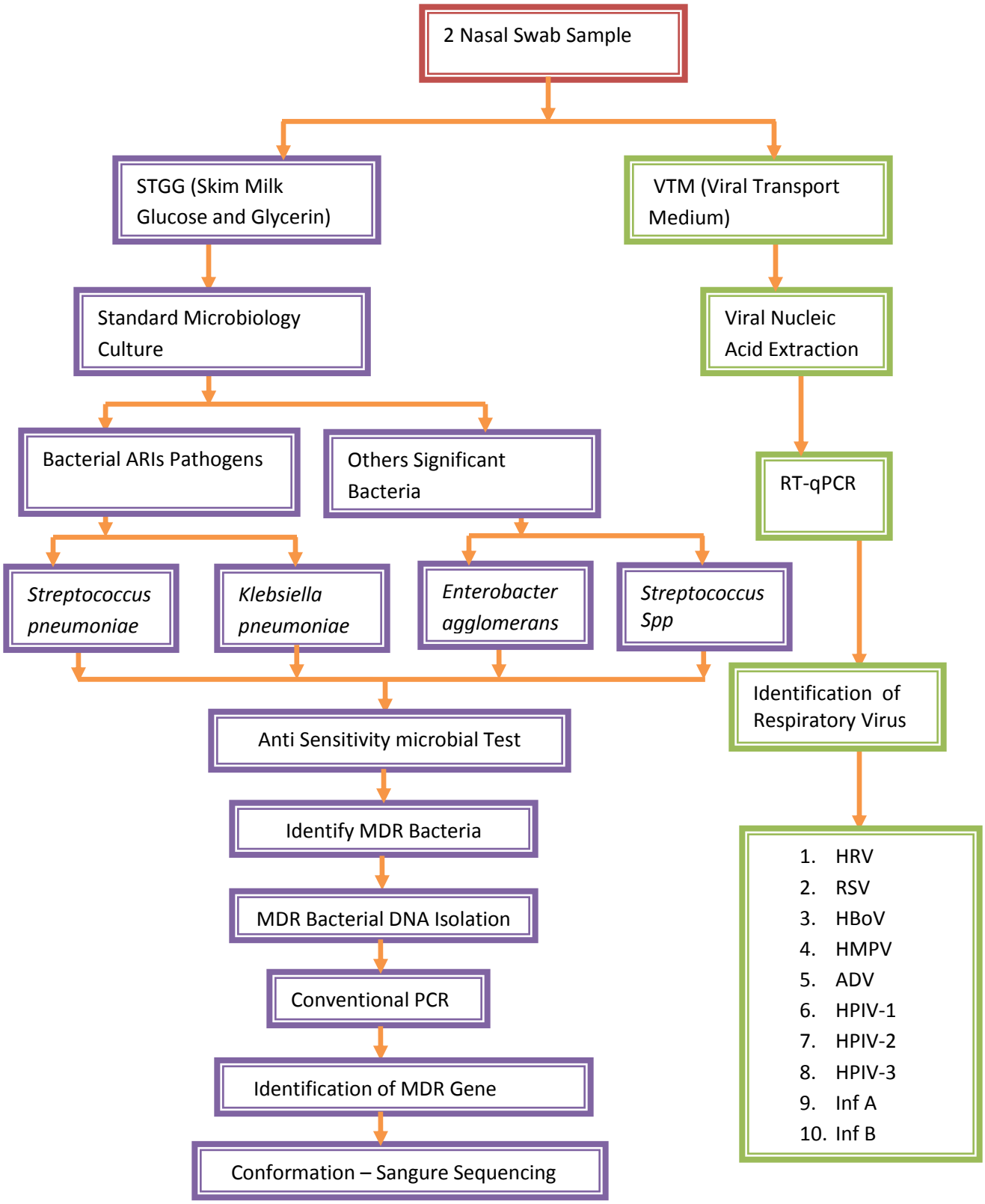


Fig. 2.2 Flowchart of the study plan

2.8.1 Identification and Characterization of *Streptococcus Pneumoniae*

The following specialized tests such as Gram stain, Catalase and Optochin were used to identify colonies on a Blood Agar Plate (BAP) that match pneumoncocci. *Streptococcus Pneumoniae* was identified by the test simultaneously. Bile solubililty test was used as a confirmatory test.

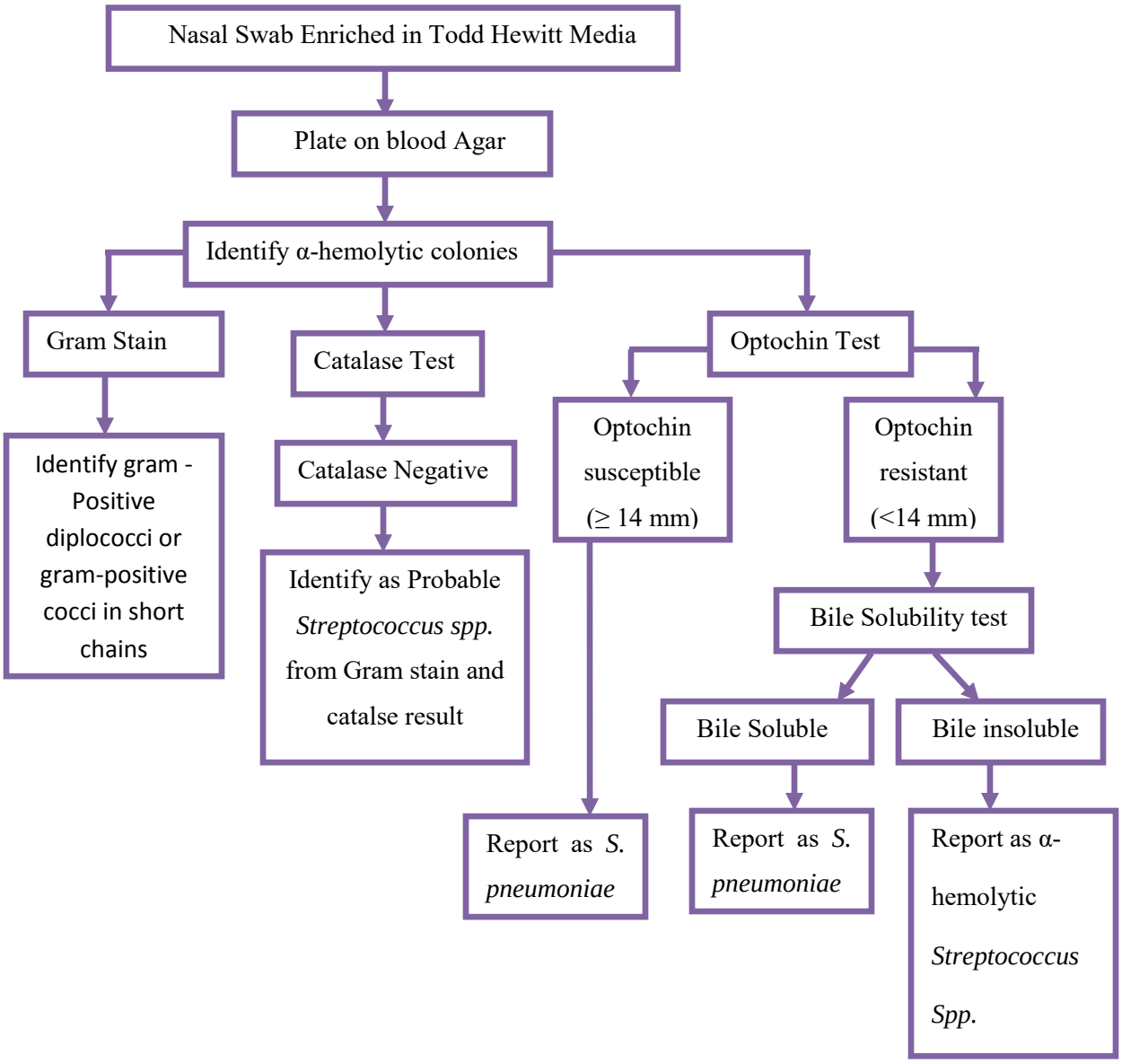


Fig. 2.3 Flowchart for identification and characterization of *S. pneumonia* and *Streptococcus spp*

2.8.1.1 Materials

1) STGG Media

2) Todd Hewitt Media

3) 0.5% sheep blood (BAP) with 5µg Gentamicin per ml

4) Optochin Disc

5) 0.85% Saline

6) Rabbit Serum

7) Sterile distilled water

8) Inoculating platinum loop

9) Screw capped 1.5 ml Vials

10) *Streptococcus pneumoniae* specific antibiotics (Source-oxoid, UK)

2.8.1.2 Methods

2.8.1.2.1 Nasal swab specimen Enrichment in Todd Hewitt Media

1) Within 4-6 hours after collection, 200 µl of NS-STGG transferred into 5 ml enrichment broth that had been combined with 1 ml rabbit serum. The Enrichment broth was made by preparing 100 ml Tood Hewitt broth with 0.5 g dissolved yeast extract (THY).

2) The inoculated enrichment broth was vortexed and incubated for 4 hours at 35-37°C in a CO_2 incubator or candle jar.

3) After the incubation of THY enrichment media, a loop of 10 µl culture was inoculated on Blood Agar Media consistence of 5 µl gentamicin per ml. Then it was incubated at 35-37°C for 24-48 hours in inaerobic condition.

2.8.1.2.2 Pneumococcal Isolates Detection and Identification

1) After overnight incubation, the BAP growth was carefully examined for typical pneumococcal colonies surrounded by a greenish zone of α-hemolysis.

2) Suspected pneumococcal colonies were in parallel subjected to gram stain, catalase test and optochin test.

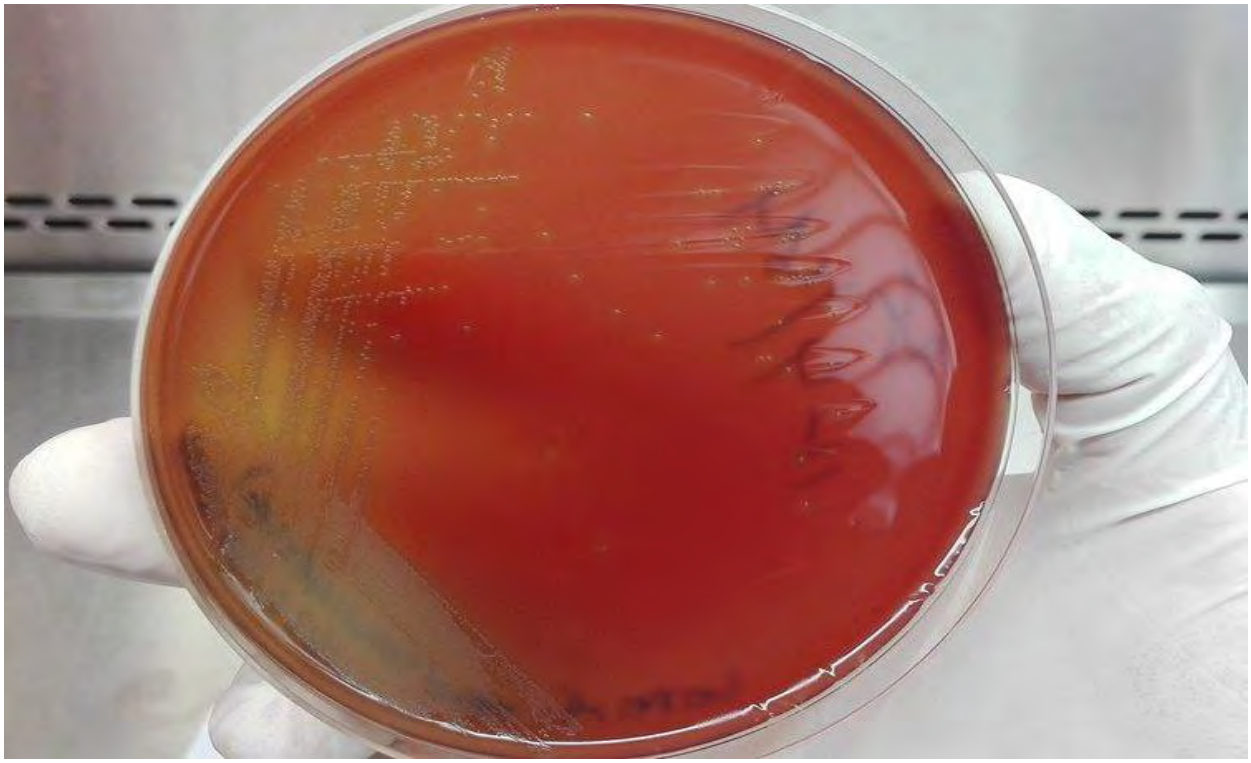


Fig. 2.4 *Streptococcus pneumoniae* on BAP.

2.8.1.2.3 Gram Staining

1) Using a sterile loop, suspected pneumococcal colonies were picked and a thin smear prepared on a glass slide.

2) The smear was then air dried and heat fixed by passing the slide through flame several times.

3) The smear was stained with crystal violet and allowed to rest for 30 seconds.

4) The crystal violet was then decanted and the slide gently washed with water.

5) The smear was stained with Gram's iodine solution and allowed to rest for 30 seconds.

6) The iodine solution was then decanted and the slide gently washed with water.

7) The smear was immediately decolorized with 20% acetone ethanol.

8) Without waiting for too long, the smear was gently rinsed with tap water.

9) The smear was then stained with safranin and allowed to rest for 30 seconds.

10) Finally, the slide was gently rinsed with tap water, blotted dry with clean tissue paper, air dried and examined under oil immersion using 100X objective lense.

2.8.1.2.4 Catalase Test

Catalase is the enzyme that breaks down hydrogen peroxide (H_2O_2) into H_2O and O_2. The oxygen is given off as bubbles in the liquid. The catalase test is primarily used to differentiate between gram-positive cocci. Members of the genus *Staphylococcus* are catalase-positive and members of the *Streptococcus* and *Enterococcus* are catalase-negative.

1) From overnitght growth on the BAP, a platinum loop was used to carefully remove a colony and place it on a glass slide. Special care was taken not to transfer any of the blood agar to the slide as erythrocytes in the blood agar will case a false postive reaction.

2) 1.0 ml of 3% H_2O_2 was added to the slide and mixed with the bacteria.

3) The bacterial suspension on the slide was observed immediately for vigorous bubbling.

2.8.1.2.5 Optochin Test

Streptococcus pneumoniae strain are sensitive to the chemical optochin (ethylehydrocupreine hydrochloride). Optochin sensitivity allows for the presumptive identification of alpha-hemolytic streptococci as *S. pneumoniae.*

1) To perform the optochin susceptibility test, the suspect alpha-hemolytic colony was streaked into blood agar plates (BAP) in confluent lines.

2) A 5µg optochin disc with 6 mm diameter was placed in the streaked area.

3) The plate was then incubated in a CO_2 incubator or candle jar at 35-37° for 18-24 hours.

4) After incubation the growth on the BAP near the optochin disc was observed and the zone of inhibiton measured.

5) A zone of inhibiton of diameter 14 mm or greater indicated sensitivity and allowed for presumptive identification of pneumococci. A smaller zone of inhibiton (<14mm) or no zone of inhibiton indicated that the bile solubility test was required.

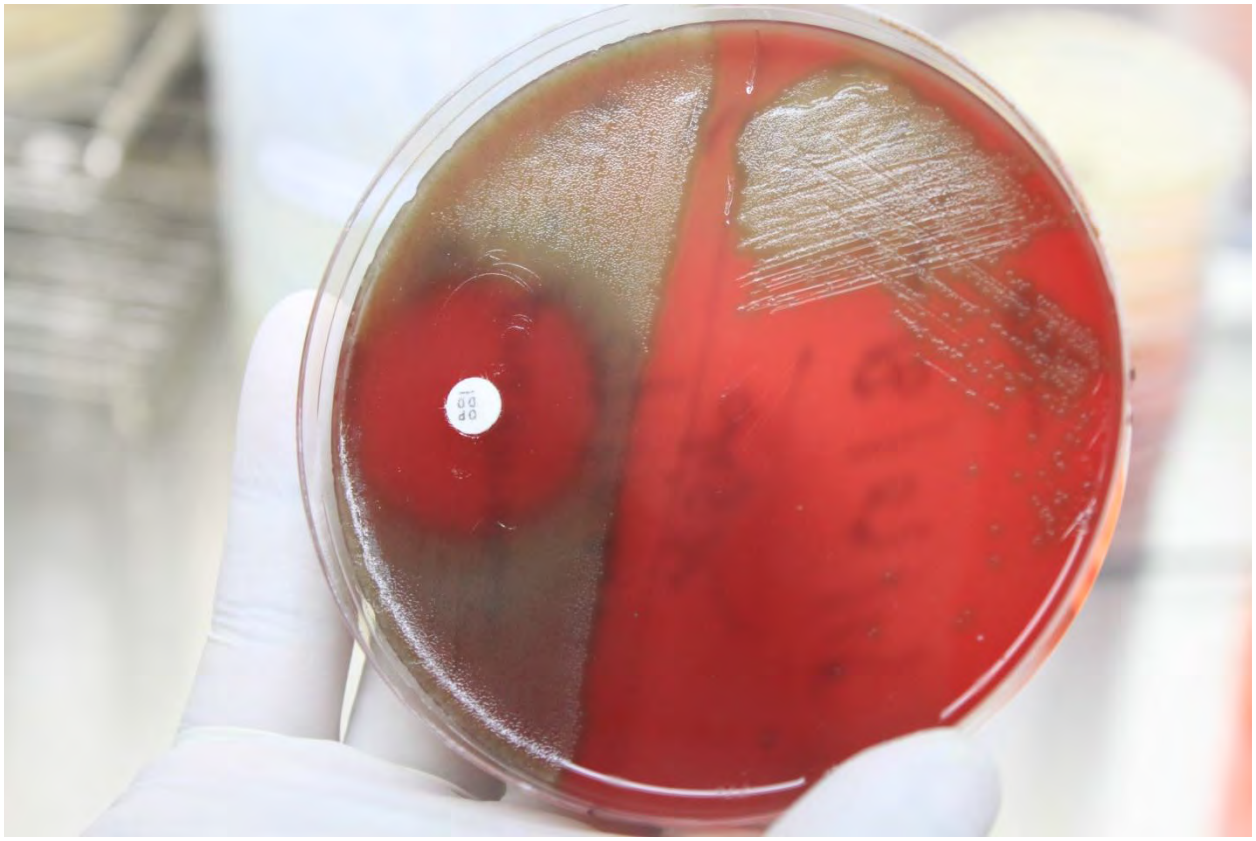


Fig. 2.5 Optochin test of suspected *streptococcus pneumoniae* on BAP.

2.8.1.2.6 Bile Solubility Test

The bile (sodium deoxycholate) solubility distinguised *S. pneumoniae* form all other alpha-hemolytic sterptococci. *S. pneumoniae* in bile soluble, whereas all other alphai-hemolytic streptococci are bile resistant. Sodium deoxycholate (2% in water will lyse the pneumococcal cell wall.

1) A suspension (McFarland No. 1) was prepared from an overnight culture in 1 ml of 0.5% saline.

2) The suspension was divided in two tubes (test and control) of 0.5 ml.

3) 0.5 ml of 2% sodium deoxycholate (bile salts) was added to the test tube and 0.5 ml normal saline was added to the control tube.

4) The suspension were then vortexed and incubated in CO_2 incubator or candle jar at 35-37°C for upto 2 hours.

5) *S. pneumoniae* test tube turned completely transparent without any turbidity, while any other alpha-hemolytic streptococci test tube remained turbid after 2 hours of incubation.

2.8.1.2.7 Anti-Microbial Susceptibility Test

The antimicrobial sensitivity of the isolated pathogen was determined by the disc diffusion technique of the modified Kirby Bauer (1966) method using Mueller-Hinton agar and commercially available antimicrobial discs (Oxoid, Hampshire United Kingdom). Following antibiotics and their concentration per disc were used for the sensitivity test:

Table 2.1: List of antibiotics for the AST of *Streptococcus pneumoniae* according to the Clinical and Laboratory Standards Institute (CLSI) Guidelines; Supplement M100-S24.

Catagory	**Antibiotics**	**Concentration per disc**
First Line Antibiotics	Penicillin-G (P)	10 iu
	Ampicillin (Amp)	10 mcg
	Erythromycin (E)	15 mcg
	Azithromycin (Azm)	15 mcg
	Trimethosporine + Sulphamethoxazole (Sxt)	1.25/ 23.75 mcg
	Ceftriaxone (Cro)	30 mcg
	Cefixime (Cfm)	5 mcg
	Levofloxacin (Lev)	5 mcg
2nd line	Vancomycin	30 mcg

2.8.1.2.8 Method

1) Fresh Mueller + 5% Blood agar plates were dried in an incubator at 37°C for 30 minutes before use.

2) With a sterile wire loop, half of one well isolated colony from a pure culture was suspended in 2 ml of Mueller-Hinton broth in a sterile screw capped tube. The inoculated media was then incubated at 37°C in an aerophilic incubator for 2 hours.

3) The turbidity of the inoculum was adjusted to 0.5 McFarland by adding more organism or more broth, thus achieving a log phase containing approximately $1.5x10^8$ organism/ml.

4) A sterile cotton swab was immersed into the bacterial suspension and the excess suspension was removed by rotating the swab with a firm pressure against the inner side of the tube above the fluid levels. The inoculum was then lawned evenly on the entire surface of a Mueller-Hinton+ 5% Blood Agar plate in three different planes (by rotating the plates approximately 60º angle each time) to get a uniform distribution of the organism.

5) The inoculum was then allowed to dry for 15 minutes at room temperature with lids closed.

6) The disc were then placed 15 mm away from the edge of petri dish on the lawned surface on the Mueller-Hinton+ 5% Blood Agar using a sterile needle while keeping at least 25 mm gap in the between the discs. The discs were gently pressed down to ensure contact.

7) The plate was then incubated at 37°C for 18-24 hours in an anaerobic condition.

2.8.1.2.9 Measurement of Inhibition Zone

After overnight incubation, each plate was examined and the diameter of complete inhibition zone was measured with the help of a scale placed beneath the surface of the petri dish without opening the lids. Zone of inhibition was measured in mm in two directions at right angle to each other through the center of each disc and the average of the two reading was taken.

2.8.1.2.10 Interpretation of Zone

The zone of inhibition of growth produced by each antimicrobial agent on the test organisms was compared with the produced on control organisms. Depending on the diameter of the clear zone

of inhibition around the discs the test organism were categorized into Sensitive (S), Intermediate (I) and Resistant (R) to the respective anti microbial agent.

2.8.2 Identification and Characterization of *Klebsiella pneumonia*

The following tests were performed to confirm the identity of cultures that morphologically appeared to be *Klebsiella pneumonia*. *Klebsiella pneumonia* can be identified using biochemical tests including Triple Sugar Iron agar (TSI), Motility Indole Urea (MIU) and Citrate. When the biochemical tests confirmed *Klebsiella spp.*, further confirmation was achieved using analytical profiling index (API 20 E).

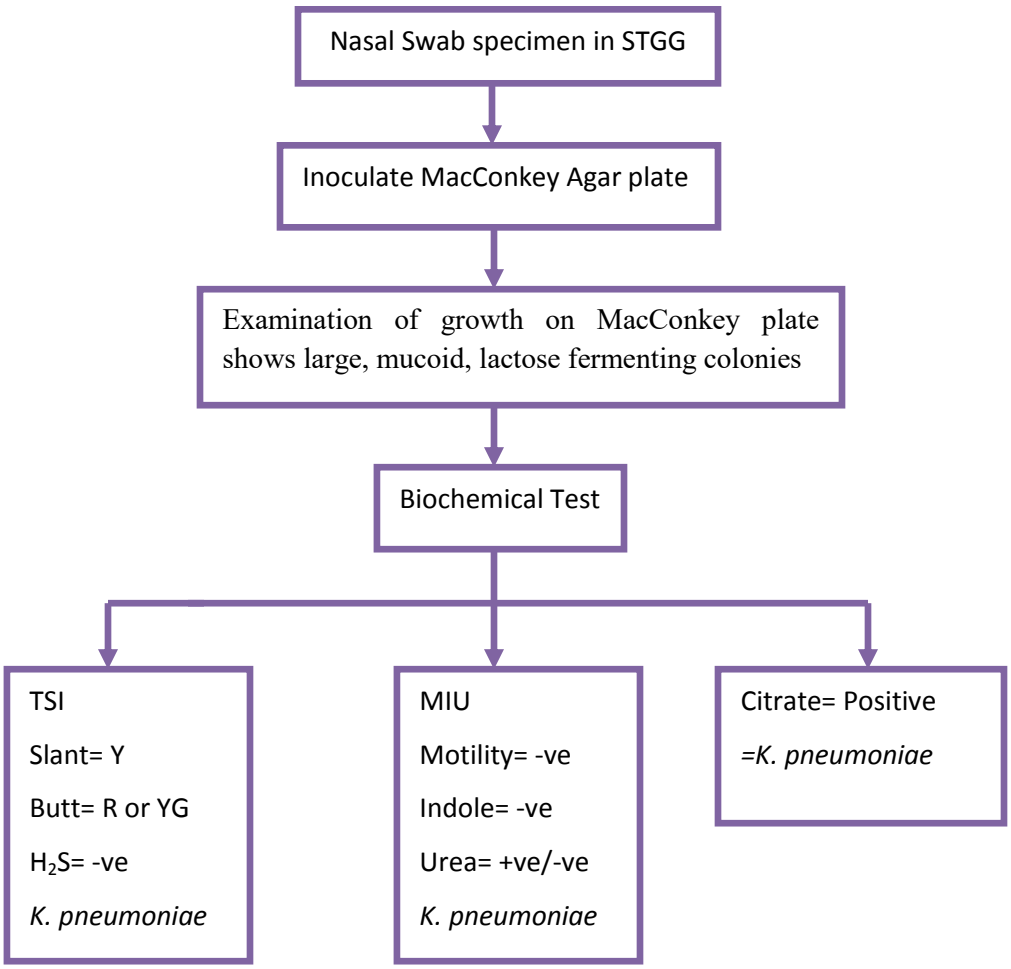


Fig. 2.6 Flow chart for identification and characterization of *Klebsiella pneumonia* isolate.

2.8.2.1 Materials

1) NS-STGG

2) MacConkey Agar Media

3) Inoculating platinum loop

4) 0.85% Normal Saline

5) Sterile distilled water

6) Screw capped 1.5 ml Vials

7) *Klebsiella pneumoniae* specific antibiotics (Source-oxoid, UK)

2.8.2.2 Methods

2.8.2.2 .1 Nasal swab culture in MacConkey Agar Media

1) Within 4-6 hours after collection, one loop (~10 µl) NS-STGG was used to inoculate on MacConkey Agar media, streaked for isolated colonies and incubated for 24-48 hours at 35-37ºC in an aerophilic incubator.

2) 0.5 ml of the NS-STGG was transferred into screw-cap 1.5 ml vials (cryotube) and stored at -70ºC.

2.8.2.2 .2 *Klebsiella pneumonia* Isolates Detection and Identification

1) After overnight incubation, the MacConkey Agar plate growth was carefully examined for *Klebsiella pneumoniac* colonies.

2) *K. pneumoniae* appear as large, usually mucoidal lactose fermenting colonies on a MacConkey Agar plate.

2.8.2.2 .3 Gram staining

Gram staining was done in the same process as described in section 2.8.1.2.3.

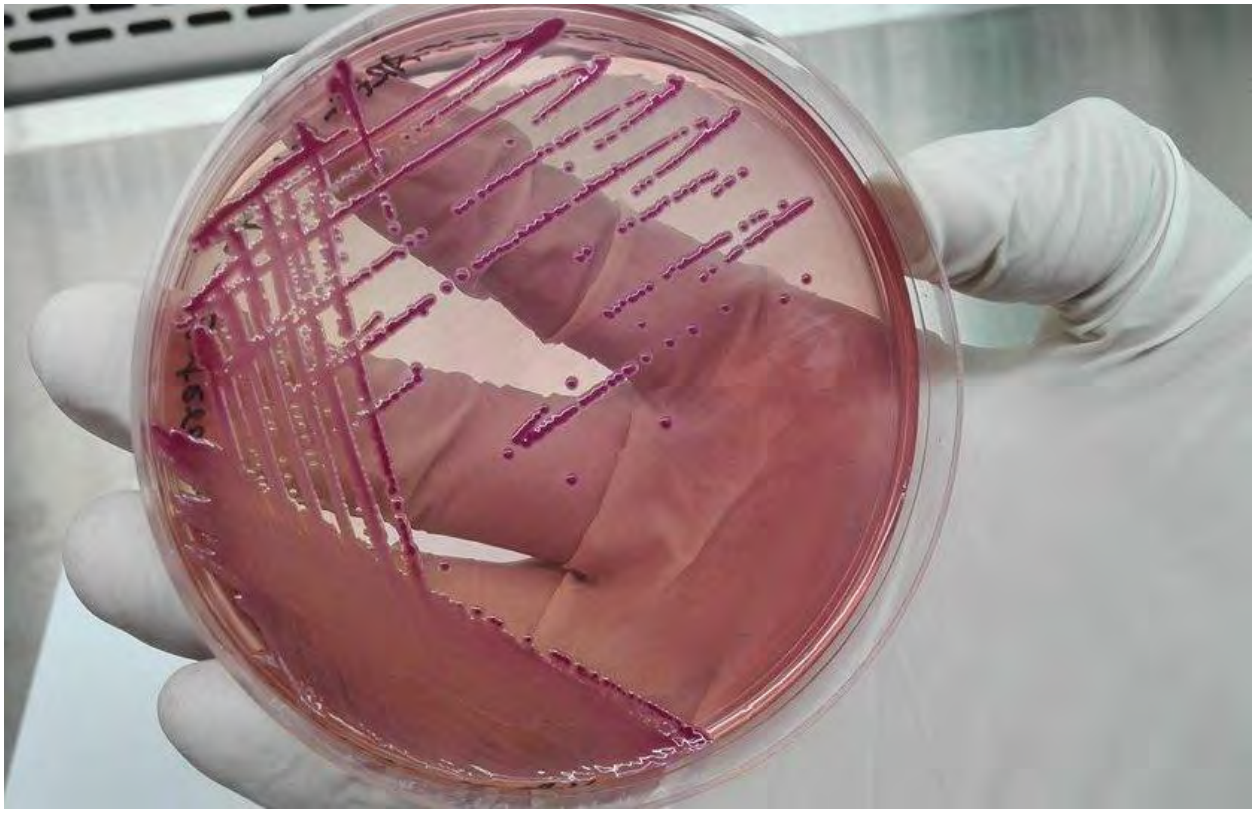


Fig. 2.7 *Klebsiella pneumoniae* colonies on MacConkeys agar plate.

2.8.2.2 .4 Biochemical Tests

2.8.2.2 .4.1 Triple Sugar Iron Agar

Triple Sugar Iron (TSI) Agar was used for the differentiation of gram negative enteric bacilli based on carbohydrate fermentation and the production of hydrogen sulfide. TSI Agar contains three sugars (dextrose, lactose and sucrose). Carbohydrate fermentation is indicated by the production of gas and a change in the color of the p^H indicator from red to yellow. To facilitate the detection of organisms that only ferment dextrose, the dextrose concentration is one –tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline p^H. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension. After depletion of the limited dextrose, organism able to do so will begin to utilize the lactose or sucrose.

2.8.2.2 .4.1.1 Procedure

1) To prepare the TSI agar, 6.5% of the TSI Agar powder (Difco, BD) was suspended in 100 ml of purified water, mixed thoroughly and then heated with frequent agitation boiled for 1 minute to completely dissolve the powder. The solution was then dispensed into screw cap glass tubes and autoclaved at 120ºC minutes following they were allowed to cool in a slanted position so that deep butts were formed.

2) To inoculate, a cool sterile needle was touched at the center of the isolated colony which was then used to stab the medium in the butt of the tube, and then streak back and forth along the surface of the slant.

3) The inoculated TSI agar was then incubated with loosened cap at 34ºC in an aerophilic incubator for 18-24 hours for carbohydrate fermentation, gas production and hydrogen sulfide production.

4) To enhance the alkaline condition of the slant, free exchange of air was permitted by closing the tube cap loosely. Also, the incubation was not continued any longer than 24 hours at the acid reaction in the slant of lactose and sucrose may revert to an alkaline reaction.

2.8.2.2 .4.1.2 Result Interpretation

Following incubation, the reactions produced by the question was compared with those produced by known control organism.

1) Carbohydrate fermentation was indicated by a yellow coloration of the medium. If the medium in the butt of the tube turned yellow (acidic), but the medium in the slant turned red (alkaline), it meant the organism only ferments dextrose (glucose).

2) A yellow (acidic) color in the slant and butt indicated that the organism ferments dextrose, lactose and/ or sucrose.

3) A red (alkaline) color in the slant and butt indicated that the organism is a non fermenter.

4) Gas production was indicated by splitting and cracking of the Media.

2.8.2.2 .4.2 Motility Indole Urea

Motility Indole Urea Agar (MIU) is a semisolid medium designed for detection in Enterobacteriaceae of urease activity, motility and indole production. Its component include tryptone , sodium chloride, potassium dihydrogen phosphate, phenol red and agar. Tryptone is a pancreatic digest of casein. Casein is the main protein of milk and is a rich source of amino acid and nitrogen. This hydrolysate has high tryptophan content and is therefore used in media for testing the indole reaction. Sodium chloride maintains the osmotic balance. Potassium dihydrogen phosphate buffers the medium, which red is a pH indicator. The small amount of agar makes the medium semisolid. Bacterial motility can be observed directly from examination of the tubes following incubation. Growth spreads out of the line of incubation if the organism is motile. Highly motile organisms provide growth throughout the tube. Growth of non motile organisms only occurs along the stab line. Urease activity can be observed by a change of color to red. When organisms utilize urea, ammonia is formed during incubation which makes the reaction of these media alkaline, producing a red-pink color. Consequently, urease production may be detected by the change in the phenol red indicator. Organism that posses the enzyme " tryptophanase" degrade the amino acid tryptophan to indolepyruvic acid, from which indole can be formed through deamination.

2.8.2.2 .4.2.1 Procedure

1) To prepare the MIU agar, 4.1 g gel (Nacl 0.5g, Agar 0.4g, KH_2PO_4 0.2g, Peptone 3.0g) was suspended in 90 ml dH_2O, followed by 200µl of phenol red (0.25%) was added and autoclaved at 121ºC for 15 minutes. The preparation was then allowed to cool to 50ºC following which 10 ml of 20% filter sterilized urea supplement was aseptically added to it.

2) To inoculate, the center of an isolated colony growing on an agar plate was touched using a cool sterile needle, which was then used to stab through the center to more than half the depth of a media column tube for effective transfer.

3) Prepared indole reagent was added to filter paper with dimensions of 3 cm length and 0.5 cm width and subsequently allowed to dry.

4) Prepared one third of indole paper was entered into MIU test tube with sterile cotton swab.

5) The inoculated MIU agar tube was then incubated with loosened cap at 35 to 37ºC in aerophilic incubation for 24-48 hours.

2.8.2.2 .4.2.2 Result Interpretation

1) Motility was observed as growth extending from the line of inoculation or by the presence of diffused turbidity of the medium. Non-motile organisms grew only along the line of inoculation.

2) Urease activity was observed as a color transformation of the media to a pink color.

3) Indole production was also indicated by a pink color change of lndole paper. No color was produced in the case of a negative indole test.

2.8.2.2 .4.3 Citrate Agar Test

Simmons citrate Agar Test is used for the differentiation of different gram-negative bacteria on the basis of citrate utilization. Organisms able to utilize ammonium dihydrogen phosphate and Sodium citrate as the sole source of nitrogen and carbon, respectively, grow on this medium and produce an alkaline reaction as evidence by a change in the color of the bromthymol blue indicator from green (neutral) to blue (alkaline).

2.8.2.2 .4.3.1 Procedure

1) For preparing the citrate agar media, 2.3 g of Simmons Citrate Agar Powder (Oxoid, UK) was suspension in 100 ml of deionized water, mixed thoroughly for completely dissolve the powder and autoclaved at 121ºC for 15 minutes. Then was added 5ml of the autoclaved media in test tubes and allowed settled in a slanted position.

2) Using a cold sterile inoculation loop a pure culture was picked up and used for inoculating the slant by streaking.

3) The inoculating citrate agar was then incubated at 35-37ºC in an aerophilic condition for 24-48 hours.

2.8.2.2.4.3.2 Result Interpretation

1) A positive reaction was indicated by growth with an intense blue color in the slant.

2) A negative reaction was indicated by no change of the of the citrate agar color.

2.8.2.2.4.3.3 Typical Biochemical Reaction of *Klebsiella pneumonia*

Table 2.2: Biochemical Test Reference Shit for *Klebsiella Pneumoniae*

Organism Name	KIA				MIU			Citrate
	Slant	Butt	Gas	H_2S	Motility	Indole	Urea	
Klebsiella pneumoniae	A/K	A	+	-	-	-	± (63%)	± (95%)

A= Acid, K= Alkaline

2.8.2.3 Analytical Profile Index (API 20E)

2.8.2.3.1 Principle

API 20 E is a standardized identification system for *Enterobacteriaceae* and other non-fastidious, Gram-negative rods which use 20 miniaturized biochemical test and a database. This plastic strip holds 20 mini-test chambers containing dehydrated media. In this device inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism produces color change by the addition of reagents. The reactions are taken according to the reading table and identification is obtained by referring to the API guideline. Following tests are included:

1. ONPG: Test for β- galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside.

2. ADH: Decarboxylations of the amino acid arginine by arginine dihydrolase.

3. LDC: Decarboxylations of the amino acid by lysine decarboxylase.

4. ODC: Decarboxylations of the amino acid ornithine by ornithine by ornithine decarboxylase.

5. CIT: Utilization of citrate as only carbon source.

6. H_2S: Production of hydrogen sulfide.

7. URE: Test of the enzyme urease.

8. TDA (Tryptophan deaminase) : Detection of the enzyme tryptophan: Reagent to put - ferric chloride.

9. IND: Indole test-production of indole from tryptophan by the enzyme tryptophanase.

10. VP: The Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway.

11. GEL: Test for the production of the enzyme gelatinase which liquefies gelatin.

12. GLU: Fermentation of glucose (hexose sugar).

13. MAN: Fermentation of the mannose (hexose sugar).

14. INO: Fermentation of inositol (cyclic polyalcohol).

15. SOR: Fermentation of sorbitol (alcohol sugar).

16. RHA: Fermentation of rhamnose (methyl pentose sugar).

17. SAC: Fermentation of sucrose (disaccharide).

18. MEL: Fermentation of melibiose (disaccharide).

19. AMY: Fermentation of amygdalin (glycoside).

20. ARA: Fermentation of arabinose (Pentose sugar).

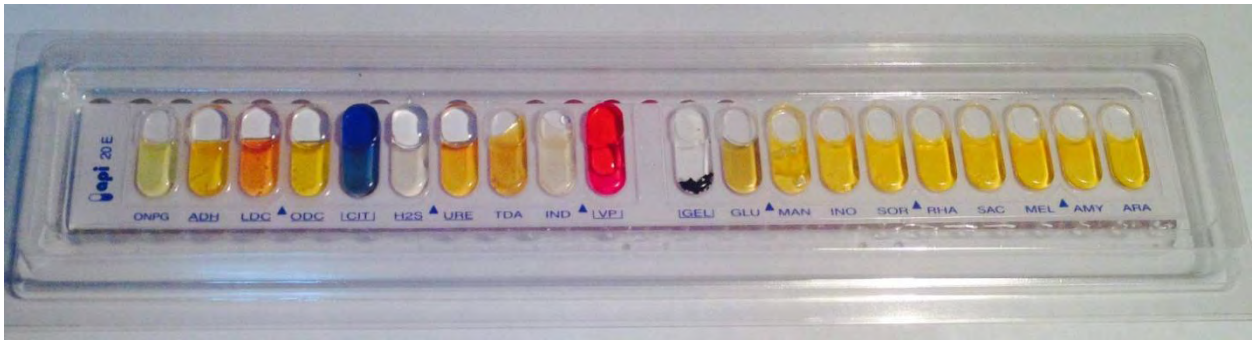


Fig. 2.8 Schematic representation of an API 20 E strip.

2.8.2.3.2 Materials

1) Pipettes

2) Ampule protector

3) Ampule rack

4) BSL-2 Laboratory

5) Reagent

a. API NaCl 0.85 % Medium

b. API Suspension Medium

c. API 20 E reagent kit

d. TDA

e. JAMES

f. VP 1 + VP 2

g. NIT 1 + NIT 2

h. Zn reagent

i. Oxidase

j. Mineral oil

k. API 20 E Analytical Profile Index Reference Guideline

2.8.2.3.3 Procedure

2.8.2.3.3.1 Preparation of the strip

1) Prepare an incubation box with tray and lid. Distribute about approximately 5 ml of distilled water into the honey-combed wells of the try.

2) Firstly remove the strip from its packet then place the strip in the incubation box.

2.8.2.3.3.2 Preparation of the inoculum

1) Open an ampule then 5ml API NaCl 0.85% was taken.

2) Using a sterile loop, a single well-isolated colony was removed from an isolation plate. More than 24 hours cultures were avoided.

3) Efficient emulsification was done to achieve a homogeneous suspension which was used immediately after preparation.

2.8.2.3.3.3 Inoculation of the Strip

1) Using the pipette both tube and cupule of the tests CIT, VP, and GEL were filled with the bacterial suspension.

2) In the case of other tests, only the tube and not the cupule were filled.

3) For the tests ADH, LDC, ODC, H_2S and URE anaerobic condition was created by adding overlaying mineral oil.

4) Then incubation box was closed and incubated at 36º C ± 2 for 18-24 hours,

2.8.2.3.3.4 Inoculation of Results

1) Identification was obtained with the numerical profile.

2) On the result sheet the test, reside into groups of 3 and a value 1, 2 or 4 is indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests of the API 20 E strip. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

3) The identifications were done by looking up the numerical profile in the list of the database.

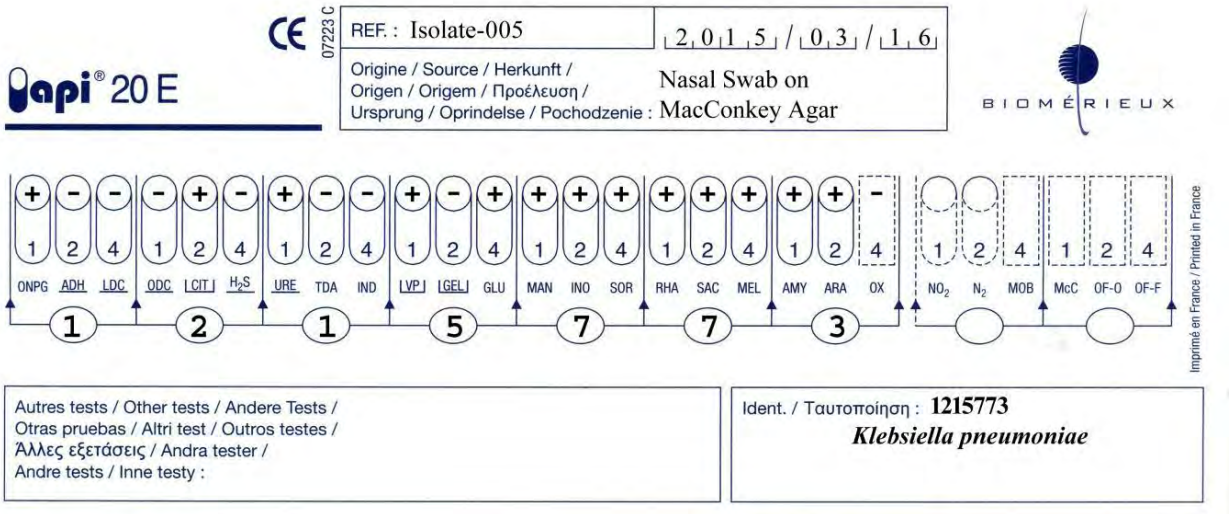


Fig. 2.9 Representation of the numbers designated to tubes on API strip used for generating a numerical profile.

Table 2.3: API 20 E test reference

Tests	Reaction	Result	
		Negative	Positive
ONPG	ß-galactosidase	colorless	yellow
ADH	Arginine dihydrolase	yellow	red / orange

LDC	Lysine decarboxylase	yellow	red / orange
ODC	Ornithine decarboxylase	yellow	red / orange
CIT	Citrate utilization	pale green / yellow	blue-green / blue
H2S	H2S production	colorless / greyish	black deposit / thin line
URE	Urease	yellow	red / orange
TDA	Tryptophane deaminase	yellow	reddish brown
IND	Indole production	pale green / yellow	pink
VP	Acetoin production	colorless	pink / red
GEL	Gelatinase	no diffusion	diffusion of black pigment
GLU	Fermentation / oxidation	blue / blue	green yellow / greyish yellow
MAN	Fermentation / oxidation	blue / blue	green yellow
INO	Fermentation / oxidation	blue / blue	green yellow
SOR	Fermentation / oxidation	blue / blue	green yellow
RHA	Fermentation / oxidation	blue / blue	green yellow
SAC	Fermentation / oxidation	blue / blue	green yellow
MEL	Fermentation / oxidation	blue / blue	green yellow
AMY	Fermentation / oxidation	blue / blue	green yellow
ARA	Fermentation / oxidation	blue / blue	green yellow
OX	Cytochrome-oxidase	colorless	Purple

2.8.2.3.3.5 Typical Reactions of *Klebsiella pneumoniae* in API

Table 2.4: Typical Reactions of *Klebsiella pneumoniae* in API

ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL
+	-	+	-	+	-	V	-	-	V	-
GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2
+	+	+	+	+	+	+	+	+	+	-

2.8.2.3.3.6 Anti-Microbial Susceptibility Test

The anti-microbial sensitivity testing was done by the same method except using Muller-Hinton Agar plate as described in the section **2.8.1.2.7.** The following antibiotics were used for the anti-microbial test given below-

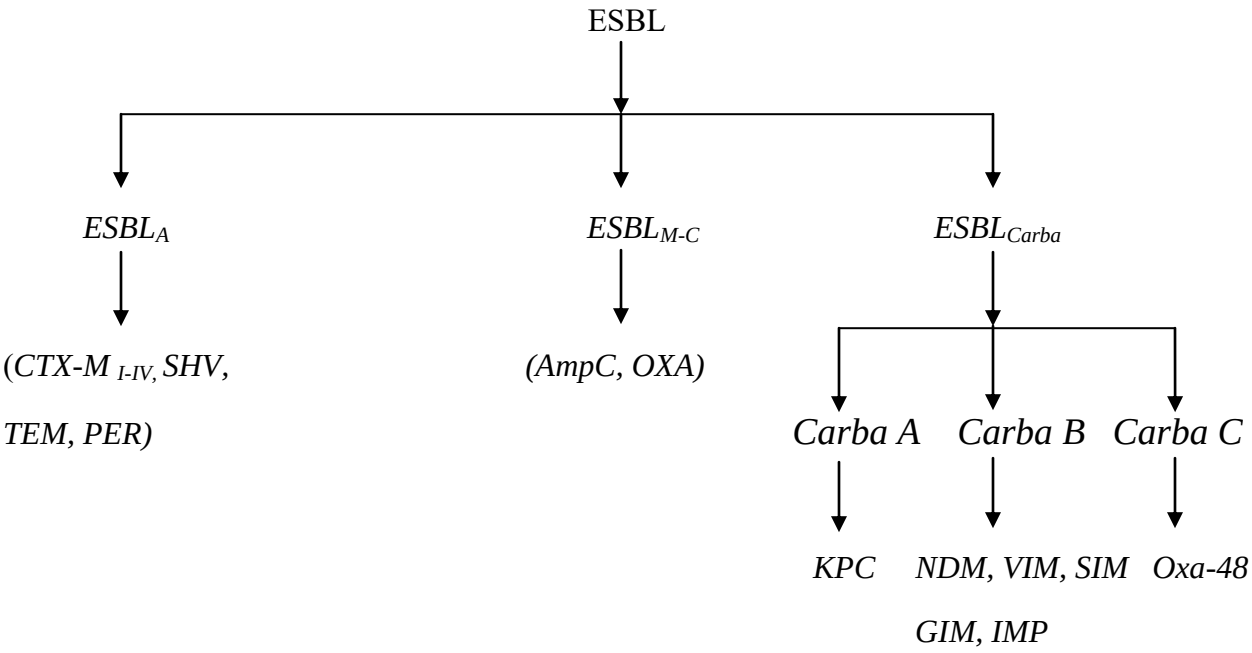
Table 2.5: Antibiotics Panel for the AST of the Klebsiella *pneumonia*. Antibiotics source: Oxoid

Catagory	**Antibiotics**	**Concentration per disc**
First Line Antibiotics	Gentamycin -10 (CN)	10 mcg
	Tobramycin (TOB)	10 mcg
	Ceftriaxone (CRO)	30 mcg
	Cefixime (CFM)	5 mcg
	Ciprofloxacin (CIP)	5 mcg
	Ceftriaxone (CRO)	10mcg
	Imepenem (IPM)	10mcg
	Meropenem (MEM)	5 mcg
	Azithromycin (AZM)	15 mcg
2nd line	Amikacin (AK)	30 mcg
	Netilmicin (NET)	30 mcg
	Tazobactam/ Piperacillin (TZP)	120 mcg
	Carbenicillin	100 mcg
	Polymyxin B (PB)	300 U

2.8.3 Detection of Extended –Spectrum β- Lactamase (ESBL) gene from MDR *Klebsiella pneumonia* strain

ESBL produced by bacteria in order to hydrolyzed beta-lactams antibiotics, the group includes Penicillin,Cephalosporins, Monobactams, and Carbapenems. ESBLs are often located on bacterial plasmids that are transferable from one strain to another strain of bacteria. The rates of ESBL-expression among nosocomial Enterobacteriaceae isolates, particularly *Klebsiella pneumoniae*, have been raised substantially in many countries.

ESBL has been classified into 3 groups and subgroups:



2.8.3.1 Bacterial DNA *(*MDR *Kelbsiella pneumonia)* Extraction

1. Taken 4 to 6 separate colony from MacConkey Agar Media by the using of the sterile platinum loop.

2. Suspended in a 1.5 ml eppendrof tube containing 300 µl Deionized water then wait for 1-2 minutes.

3. The suspension was vortex for 15 seconds.

4. The tube was heated at 95-99º C in a water bath for 9 minutes.

5. The suspension was centrifuged at 13000 rpm for 10 minutes.

6. The supernatant was transferred into another RNase and DNase free eppendrof tube.

7. Then the separated supernatant was used as the PCR template, If delay immediate store at -20ºC.

2.8.3.2 CTX-M group I gene specific Primers

The following CTX-M1 gene specific primers were used for detection of this from MDR *Klebsiella pneumonia* isolates by conventional PCR.

Table 2.6: CTX-M group I gene specific primer

Primer	Sequence	Size
CTX-M1-3F (Forward)	AATCACTGCGCCAGTTCACGCT	22 nt
CTX-M1-R2 (Reverse)	AGCCGCCGACGCTAATACA	19 nt

* nt- Neucleotide.

2.8.3.3 Preparation of the PCR master mix

Master mixer was prepared using all the components of PCR. The different PCR component was added into a 1.5 ml DNAse and RNAse free eppendrof and the total volume with template was 10 µl. Taq DNA polymerase was added just before strating the PCR reaction.

Table 2.7: PCR master mix use for CTX-M Group I conventional PCR

PCR Reagent	Optimization Quantity (µl)
2.5 mm dNTPs	1.0
10X Buffer	1.0
25 mm $MgCl_2$	0.25
Nuclease Free water	3.75
CTX-M1-3F (Forward)- Primers	0.50
CTX-M1-R2 (Reverse)- primers	0.50
Template	3.0
Total Volume	10

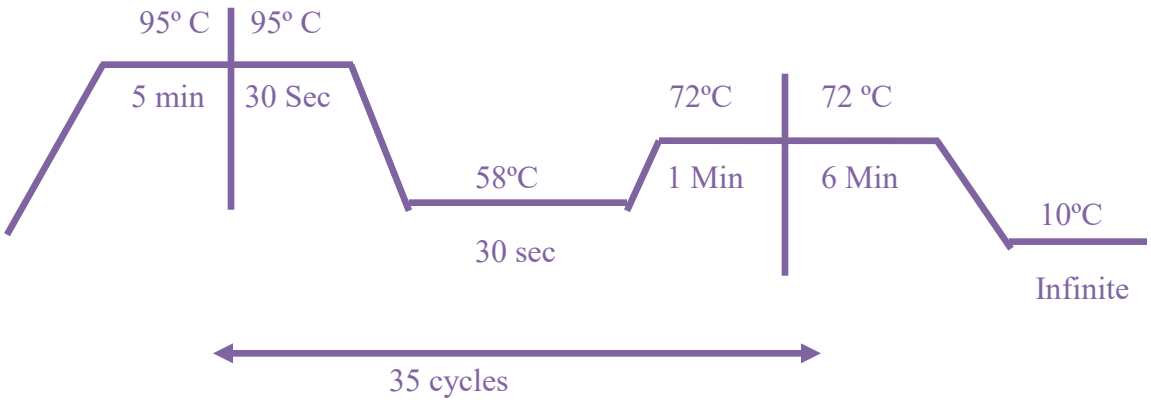
* At the end 0.1 µl Taq polymerase was added in the master mixer tube.

2.8.3.4 Thermal cycling profile used in PCR

The thermal cycling profile were programmed in Polymerase Chain Reaction for 35 cycles are givennn below:

Table 2.8: Thermal cycling profile used to amplify CTX-M group I gene

Steps of Thermal Cycling	**Temperatuer**	**Time**	**No. of Cycles**
Initial denaturation	95º C	5 Minutes	1 (First)
Denaturation	95º C	30 Seconds	35
Annealing	58ºC	30 Seconds	35
Elongation	72ºC	1 Minutes	35
Final Elongation	72ºC	6 Minutes	1 (Last)
Final hold up temp.	10ºC	Infinite Period	-



* Expected Band size of the CTX-M-I gene- 841 bp.

Fig. 2.10 Thermal cycling profile for CTX-M group I primers.

2.8.3.5 Agarose Gel electrophoresis and Visualization of PCR products

The amplified PCR products were analyzed by agarose gel electrophoresis on 1% agarose gel. Gel was prepared using 0.5g Ultra pure agarose in 50 ml 1X TBE buffer (Invitrogen Ultra Pure) and was melted in a microwave for 2 minutes 10 seconds at medium-high temperature. 1.5 µl gel red was added to agarose gel and poured on the gel casting plate and then allowed 15 to 20 minutes at room temperature for solidifying the gel. 2 µl loading dye (6X bromophenol blue)

was mixed with 5 µl of PCR products and loaded into the wells. To estimate the size of PCR products 1 µL of 1 kb Plus DNA ladder (Cat. No. 10787018, InvitrogenTM) was mixed with 3 µl loading dye into a well at one corner of the gel along with experimental sample DNA. Then the PCR products were separated at 150 V for 45 minutes. The band of the PCR products were observed using the Gel documentation system XR+ (BioRad, USA).

2.8.4 Sequencing

2.8.4.1 Sanger Sequencing Procedure (Source: Qiagen)

1) Calculation of the number of cycles of sequencing was determined according to the measured template concentration. Templates used for the reaction were the purified PCR products of each sample to be confirmed.

2) Tubes containing this template were spun and 10-20 ng/µl (depending on the template concentration) was added to the 8-tube PCR strip.

3) Nuclease free water was then added to each mixture to make up a total volume of 10 µl per reaction.

4) Following this, the PCR tubes were centrifuged at 4000 RPM for 3 minutes before the PCR strip was placed in the Mastercycler gradient (Cat. No. 4095-0015, USA Scientific) Thermal Cycler and subjected to the following thermal cycling profile: initial denaturation at 95°C for 10 minutes, then 25 cycles of denaturation at 95°C for 10seconds; annealing at 55°C for 5 seconds; and extension at 72°C for 4 minutes followed by a final extension step at 72°C lasting for 6 minutes.

5) Upon completion of the cycle sequencing, the reaction plate was centrifuged at 41,000 RPM for 1 minute.

6) Next, for each 10 µl reaction, 45 µl of SAM solution and 10 µl of X-terminator (Applied Biosystems, USA) were added.

7) Homogenization of each solution was ensured by vortexing for a minimum of 30 seconds, and wide bore micropipette tips being used to aliquot the viscous X-terminator solution to maintain required volumes. Both of these reagents aid the removal of impurities, in particular salts which interfere with the electrokinetic injection as well as excess ddNTPs leftover which may generate dye blobs which contribute to significant background noise in the final read.

8) The reaction plate was then sealed and vortexed for 30 minutes.

9) Finally, the mixture was centrifuged for 2 minutes at 4,100 RCF before being collected for capillary electrophoresis. For this process, 10 µl of supernatant were transferred to fresh sequencing tubes, covered with Septa mat., and then placed into the ABI PRISM 310 capillary electrophoresis machine**.**

10) Remaining supernatant was stored at 4°C for future use.

2.8.4.1 Analysis of Sequence Data

Data obtained through sequencing were assessed and analyzed using Chromas Lite 2.4 software which enabled evaluation of sequencing reads. This program was used to export each .ab1 file to FASTA format for further processing.

2.8.5. Respiratory Viral Pathogen Detection: Nasal swab specimen collection in viral Transport Medium (VTM)

The viral transport medium is used as a combined collection and transport medium for identification of viral infection from nasal swab sample. It is a standard media for stabilization of this sample. Its components include DMEM powder with high glucose, Na-pyruvate, $NaHCO_3$ powder, HEPES Buffer, Pen-Strep, L-glutamate , Bovine serum albumin and Amphotericin B. The nasal sample were collected into viral transport medium and stored at 4ºC sample box then sent to the ideSHi laboratory within 4-6 hours. After came to the lab it was stored at -70 freeze.

2.8.6 Purification of Respiratory Virus RNA

2.8.6. 1 Principle

The pureLink® (invitrogen™) Viral RNA / DNA Kits can be used for efficient purification of nucleic acid from fresh or frozen cell-free biological fluids such as VTM-NS. The purified RNA is devoid of protein and nuclease which is suitable for use in downstream application that allows viral detection.This kit complies efficient lysis of viral particles using at elevated temperature. The viral particles in the VTM-NS specimen are lysed using Proteinase K , Lysis Buffer with Carrier RNA , 96-100 % Ethanol and silica spin column.The Viral RNA molecules bind to the silica-based column and impurities such as proteins, nuclease, and cell debris are removed by thorough washing with Wash buffer. Then the RNA is eluted in nuclease-free water. In this process, takes total time is ~ 45 minutes. Figure 2.9 shows the steps for purification of RNA

from VTM-NS sample (Figure reprinted from https://tools.thermofisher.com/purelink viral RNA/DNA manual pdf.)

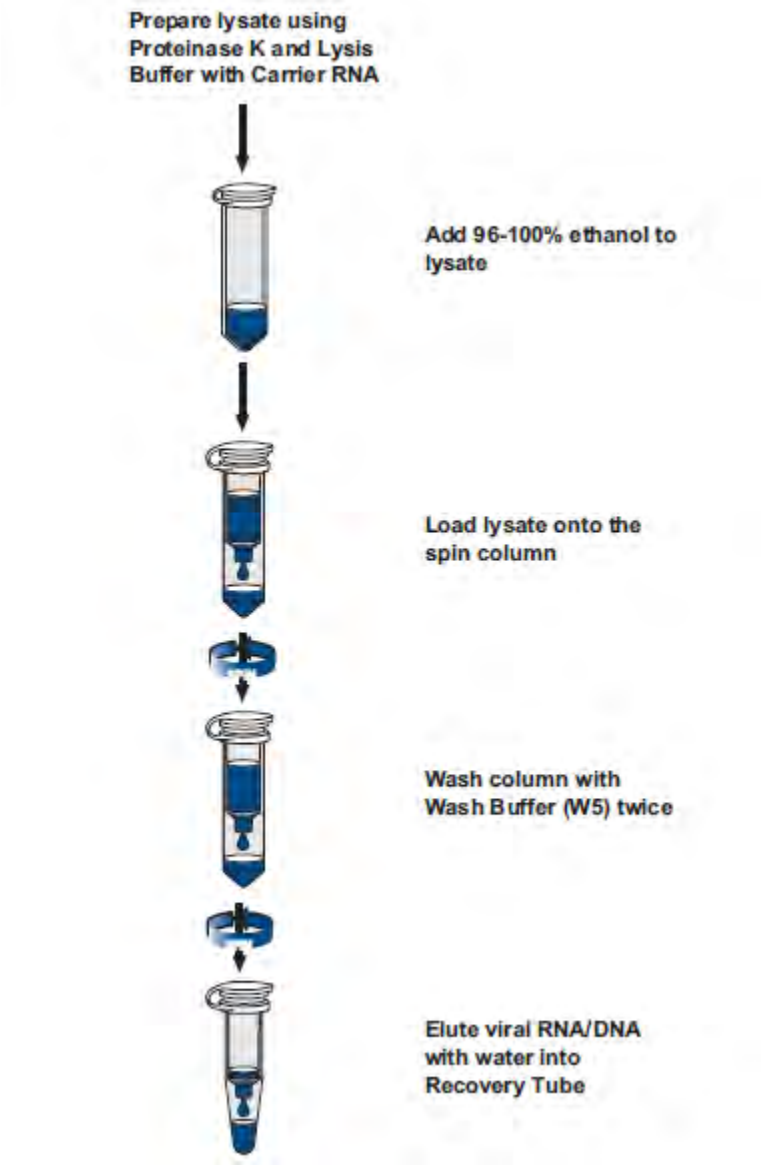


Fig. 2.11 Purification of Viral RNA from VTM-NS specimen.

2.8.6.2 Equipment and reagents for purification of viral RNA

Equipment

- Biosafety cabinet (Level II)
- Micro- centrifuge
- Water bath

- Refrigerator (-70oC)

Materials

- VTM-NS sample
- Sterile Nuclease-free 1.5 ml microcentrifuge tube
- Sterile, RNase-free 0.9% NaCl
- 200-1000 µL Pipettes
- 1-20 µL Pipettes

Reagents

- Wash Buffer
- Sterile, RNase -free Water
- Viral Spin Column in Collection Tubes
- Wash Tubes and Recovery Tube
- Lysis Buffer containing Carrier RNA
- 96–100% ethanol (Molecular Biology Grade)

2.8.6.3 Procedure

Twenty-five microliter proteinase K were taken into the 1.5 ml eppendrof microcentrifuge tube. Then 200 µl VTM-NS sample were added in this eppendrof tube. It ensured that for <200 µl sample was adjusted by the sterile RNase -free 0.9% NaCl up to final volume. Two ml lysis buffer was taken into a 2ml eppendrof tube with 58.8 µl carrier RNA and were mixing by the pipette. The prepared 200 µl lysis buffer was into the sample tube and mixed by vortexing for 15 seconds. The lysate tube was incubated at 56°C for 15 minutes. After the incubation 250 µl 96% molecular ethanol was added to the lysate tube and vortexed for 15 seconds. All lysate tube were incubated at room temperature for 5 minutes. Then was transferred above lysate (~675 µl) into the viral spin column. The spin column was centrifuged at ~6800 x g for 1 minutes. Then the spin column was placed into the clean wash tube (2 ml). The centrifuged was did at ~6800 x g for 1 minutes. Flow-through was discarded and placed the spin column back into the wash tube. Then was added 500 µl wash buffer into the spin column. The spin column with wash buffer was centrifuged at ~6800 x g for 1 minutes (two times was washed the spin column). Then the

spin column was placed into another clean wash tube. The spin column with wash tube was centrifuged at 14000 rpm for 1 minutes to dry the membrane completely. Then the washed tube was discarded and the spin column was placed into the 1.5ml recovery tube. Then 50 µl sterile RNase-free water was added to the center of the column. The column with recovery tube was incubated at room temperature for 1 minutes. Then was centrifuged at 14000 rpm for 1 minutes. The spin was removed and recovery tube contained viral RNA. The purified viral RNA was stored at -70°C before downstream analysis.

2.8.7 Detection of Respiratory Virus using q-RT PCR

2.8.7.1 Principle

Real-time polymerase chain reaction, also called quantitative Real time polymerase chain reaction (qPCR) or kinetic polymerase chain reaction, is a laboratory technique based on the polymerase chain reaction. It is used to amplify and simultaneously quantify a targeted NA molecule. It enables both detection and quantification of a specific sequence in a NA sample. All samples are tested to detect the presence of respiratory viruses [respiratory syncytial virus (RSV), human parainfluenza viruses type 1 to 3 (HPIVl, HPIV2, HPIV3), human metapneumo virus (hMPV) and Adeno virus]. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified NA is quantified as it accumulates in the reaction in real time after each amplification cycle. Two common methods of quantification are (1) the use of fluorescent dyes that intercalate with double-stranded DNA, and (2) modified DNA-oligonucleotide probes that fluoresce when hybridized with a complementary DNA. Frequently, to measure gene expression, real-time polymerase chain reaction is combined with reverse transcription to quantify messenger RNA (mRNA) in viruses, cells or tissues. The abbreviations used for real-time PCR is qRT-PCR and for real-time reverse transcription PCR is qRT-PCR.

2.8.7.2 Equipments and Reagents

Equipments

- Real time qPCR machine

- Biosafety Cabinet class II (BSC II)
- PCR work station
- Refrigerator (4^0C, 20^0C and -80^0C)
- Microcentrifuge
- Table top centrifuge
- Vortex-mixer
- 1-10 µl, 2-20 µl, 100 µl and 1 ml Micropipettes.

Materials

- 1.5 mL Microcetrifuge tubes
- Aerosol barrier pipette tips
- 96 well PCR plates recommended for the respective RT-PCR machine
- Ultra clear optical flat cap stripes
- PCR color tube rack

Reagents

- PCR grade RNase free dH_2O .
- SuperScript® III Platinum® One-step Quantitive RT-PCR system (invitrogen)
- Primers and probes of respiratory syncytial virus (RSY), human parainfluenza viruse type 1 to 3 (HPIV 1, HPIV 2, HPIV 3), human Metapneumo virus (hMPV) and Adeno virus and Influenzae A , Influenzae B.
- RNP (Ribonucleoprotein).

2.8.7.3 Pimers and Probes for Influenzae Virus Panel q-RT PCR

Primers Name	Nucleotide Sequence 5'-3´
RSV Forward	GGC AAA TAT GGA AAC ATA CGT GAA
RSV Reverse	TCT TIT TCT AGG ACA TTG TAY TGA ACA G
RSV Probe1	6 FAM-CTG TGT ATG TGG AGC CTT CGT GAA GCT- BHQl
Inf A Forward	GAC CRA TCC TGT CAC CTC TGA C
Inf A Reverse	AGG GCA TTY TGG ACA AAK CGT CTA
Inf A probe	FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1
Inf B Forward	TCCTCAACTCACTCTTCGAGCG
Inf B Reverse	CGGTGCTCTTGACCAAATTGG
Inf B probe	JOE-CCAATTCGAGCAGCTGAAACTGCGGTG-BHQ1
HMPV Forward	CAA GTG TGA CAT TGC TGA YCT RAA
HMPV Reverse	ACT GCC GCA CAA CAT TTA GRA A
HMPVprobe 1	6 FAM-TGG CYG TYA GCT TCA GTC AAT TCA ACA GA-BHQl
HPIVl Forward	ACA AGT TGT CAA YGT CTT AAT TCR TAT
HPIVl Reverse	TCG GCA CCT AAG TAR TTY TGA GTT
HPIVl Probe	6 FAM-ATA GGC CAA AGA **"T"**TG TTG TCG AGA CTA TTC CAA
HPIV2 Forward	GCA TTT CCA ATC TAC AGG ACT ATG A
HPIV2 Reverse	ACC TCC TGG TAT AGC AGT GAC TGA AC
HPIV2 Probe	6 FAM-CCA TTT ACC **"T"**AA GTG ATG GAA TCA ATC GCA AA
HPIV3 Forward	TGG YTC AAT CTC AAC AAC AAG ATT TAA G
HPIV3 Reverse	TAC CCG AGA AAT ATT ATT TTG CC
HPIV3 Probel	6 FAM-CCC RTC TG"T" TGG ACC AGG GAT ATA CTA CAA A
Adeno Forward	GCC CCA GTG GTC TTA CAT GCA CAT C
Adeno Reverse	GCC ACG GTG GGG TTT CTA AAC TT
Adeno Probe	6 FAM-TG CAC CAG ACC CGG GCT CAG GTA CTC CGA-BHQl

2.8.7.4 Principle

Real-time polymerase chain reaction, also called quantitative Real time polymerase chain reaction (qPCR) or kinetic polymerase chain reaction, is a laboratory technique based on the polymerase chain reaction. It is used to amplify and simultaneously quantify a targeted NA molecule. It enables both detection and quantification of a specific sequence in a NA sample. All samples are tested to detect the presence of respiratory viruses [respiratory syncytial virus (RSV), human parainfluenza viruses type 1 to 3 (HPIVl, HPIV2, HPIV3), human metapneumo virus (hMPV) and Adeno virus]. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified NA is quantified as it accumulates in the reaction in real time after each amplification cycle. Two common methods of quantification are (1) the use of fluorescent dyes that intercalate with double-stranded DNA, and (2) modified DNA-oligonucleotide probes that fluoresce when hybridized with a complementary DNA. Frequently, to measure gene expression, real-time polymerase chain reaction is combined with reverse transcription to quantify messenger RNA (mRNA) in viruses, cells or tissues. The abbreviations used for real-time PCR is qRT-PCR and for real-time reverse transcription PCR is qRT-PCR.

Procedure:

Steps	Description
1	Basic Rules for working with BSL-3 practices
2	All activities regarding NA extraction from isolates which might contain Highly Pathogenic Influenza Viruses are located in the BSL-2 laboratory but with BSL-3 practices.
3	Before entering to the laboratory, wear personal protective Equipments: long sleeve lab coat, respirator, toe covered shoes, shoe covers, head cover and goggles. Wear powder free gloves during the NA extraction procedure.
4	All procedures should be done inside the BSC II. Switch on the BSC II and run for 10 minutes before starting the work. Before starting NA extraction the work surface in the BSC II should be cleaned with 0.5% Bleach and wiped with RNaseZap/0.1 MHCL solution to remove any traces of RNase enzyme.

<table>
<tr><td>5</td><td>Be sure all the necessary items for the procedure are inside the BSC II before you start, including two unbreakable containers containing 0.5% Bleach: One for disposal of tips and vials and another for pipettes.</td></tr>
<tr><td>6</td><td>All the items should be wiped with 70% alcohol before putting it inside the BSC II. And wiped with 0.5% bleach before taking it out.</td></tr>
<tr><td>7</td><td>q-RT PCR for Respiratory viruses</td></tr>
<tr><td>8</td><td>Mix the components of the one step RT-PCR kit (dH_20, 2X buffer, dNTPs and enzyme) in the PCR work station with the respective primers (type specific for respiratory viruses) to make up a volume of 15 µLfor each reaction tube as bellow:
<table>
<tr><th>Component</th><th>Concentration</th><th>ul/rxn</th></tr>
<tr><td>Buffer</td><td>2X</td><td>10.0</td></tr>
<tr><td>Enzyme</td><td>25X</td><td>0.7</td></tr>
<tr><td>Forward primer</td><td>100 µM</td><td>0.2</td></tr>
<tr><td>Reverse primer</td><td>100 µM</td><td>0.2</td></tr>
<tr><td>Probe</td><td>25 µM</td><td>0.2</td></tr>
<tr><td>Reaction enhancer</td><td></td><td>0.7</td></tr>
<tr><td>Water</td><td></td><td>3.0</td></tr>
</table></td></tr>
<tr><td>9</td><td>Prepare an excel sheet designating particular PCR tube location in 96 well PCR tube-plate for respiratory viruses respectively</td></tr>
<tr><td>10</td><td>According to the excel sheet point order, 15 µL of the corresponding PCR master mixture is aliquoted for respiratory viruses</td></tr>
<tr><td>11</td><td>Add 5 µL of RNA template to each PCR tube</td></tr>
<tr><td>12</td><td>Set the following thermal cycling protocol in the qRT-PCR machine:</td></tr>
</table>

<table>
<tr><td>13</td><td>
<table>
<tr><td></td><td>Temperature (^{0}C)</td><td>Duration</td><td>Cycles</td></tr>
<tr><td>Cycle 1</td><td>50</td><td>30 min</td><td>1</td></tr>
<tr><td>Cycle 2</td><td>95</td><td>3 min</td><td>1</td></tr>
<tr><td rowspan="2">Cycle 3</td><td>95</td><td>15 sec</td><td rowspan="2">45</td></tr>
<tr><td>55</td><td>45 sec (collect fluorescence)</td></tr>
</table>
</td></tr>
<tr><td>14</td><td>Result should be taken considering the relative Cycle threshold (CT)values with
the positive control. Normally avoid high CT values (>40)</td></tr>
</table>

Chapter 3: Results

3.1 Demographic characteristics of study population

In this study a total 150 under-five children were enrolled from September 2014 to July 2015. All the children were examined for clinical signs and symptoms by the physicians to determine Acute Respiratory Infections (ARIs). 62.7% (n=94) of hospitalized children were male, while the rest 37.3% (n=56) were female. The mean age of the patients was 9.7 months. The children age group was divided into two categories including ≤ 1 years 80% (n=120) and < 1-5 years 20% (n=30).

3.2 Spectrum of bacterial pathogens in <5 ARIs suspected patients

Among the 150 nasal swab specimens 19% (n=29) were culture positive (Fig 3.1A). Taking into account both single and co-infection cases of bacterial infections, 32 bacterial pathogens were isolated from 29 positive specimens; and the most commonly isolated bacteria were *S. pneumoniae* (12/32; 38%), followed by *K. pneumaniae* (9/32; 28%), *Streptococcus spp.* (5/32; 16%), *E. agglomerans* (3/32; 9%), and *H. influenzae* (3/32; 9%) (Fig 3.1B). Two bacterial co-infections were found in 3 positive specimens.

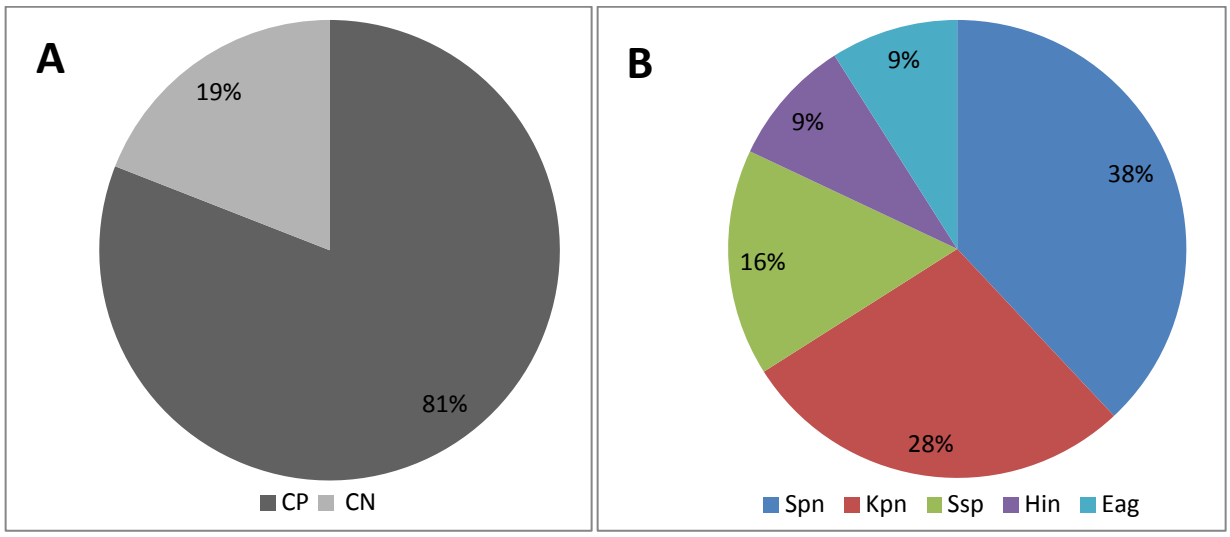


Fig. 3.1: Detection and identification of bacterial pathogens isolated from 150 under-five children hospitalized with ARIs. A shows the rates of culture positive and culture negative specimens. **B** shows the proportion of different bacterial strains among culture positive specimens. Here, CP= Culture Positive, CN= Culture Negative, Spn=*Streptococcus pneumoniae,* Kpn=*Klebsiella pneumaniae,* Hin= *Haemophilus influenzae,* Eag= *Enterobacter agglomerans,*

Ssp=*Streptococcus spp.*

3.3 Age Dependent Distribution of Bacterial load of <5 ARIs suspected children

The Respiratory Bacterial pathogens prevalence rate was high in <1 year age group and detection rate were *Streptococcus pneumoniae* 8.33% (10/12), *Klebsiella pneumaniae* 5.83% (7/9), *Streptococcus spp.* 4.17% (5/5), *Enterobacter agglomerans* 2.5% (3/3), and *Haemophilus influenzae* 0.13% (1/3). On the other hand the bacterial detection rate in 1-5 years of ARI suspected children was found to be *Streptococcus pneumoniae* 6.67% (2/12), *Klebsiella pneumaniae* 6.67% (2/9) and *Haemophilus influenzae* 6.67% (2/3). *S. spp* and *E. agglomerans* was not found in this age group (Fig 3.2). In this two age group a significant P value was generated in case of *Haemophilus influenza* (HI) (P= 0.04123, RR= 0.4118 [0.08296- 2.044], OR= 0.1176 [0.0103- 1.344]).

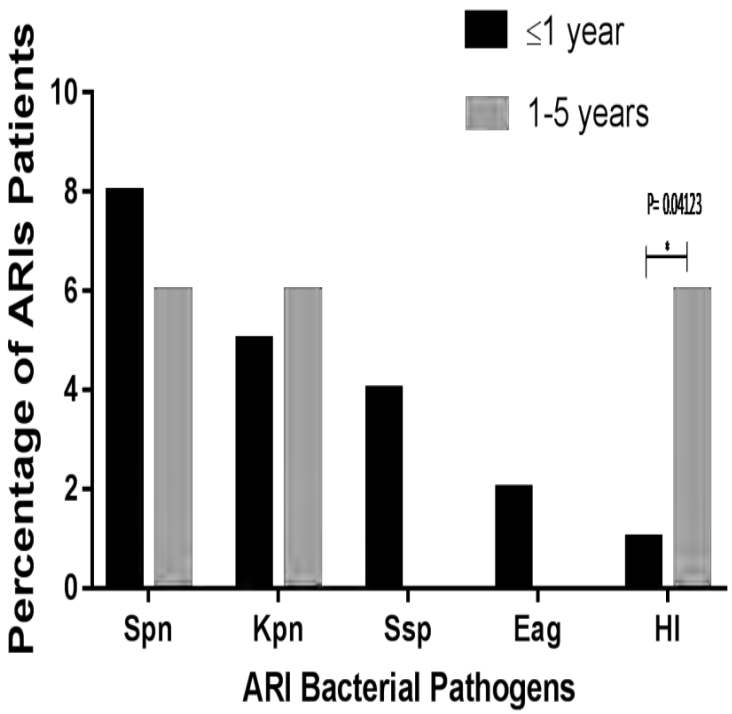


Fig. 3.2: Age dependent distribution of major bacterial pathogen of ARIs suspected under 5 hospitalized patients. Fisher's exact test was performed for each group p<0.05 was considered significant (significant p value was found; HI, P= 0.04123*). In the figure, Spn=*Streptococcus*

pneumoniae, Kpn=*Klebsiella pneumaniae*, Hin= *Haemophilus influenzae*, Eag= *Enterobacter agglomerans*, Ssp=*Streptococcus spp.*

3.4 In Vitro Antibiotics sensitivity/resistance pattern of isolated bacterial strain

3.4.1 *Streptococcus pneumoniae*

Table 1 show that *Streptococcus pneumoniae* was the most prevalent respiratory bacterial pathogen with a resistance pattern of 92% to Cotrimoxazole, 67% to both Erythromycin and Azythromycin, 17% to cefixime, 8% to ceftriaxone and a susceptibility of 100% to Penicillin, Ampicillin and levofloxacillin.

3.4.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae was found to be 100% resistance to Azithromycin, 56% to Ceftriaxone, Cefixime and Ciprofloxacin respectively, 44% to Tobramycin, and 33% to Gentamicin. In addition Meropenem and Imipenem showed 100% sensitivity.

3.4.3 *Streptococcus spp*

The resistance pattern of the streptococcus species showed 80% to Cefexime, 40% to penicillin and 60% to Ampicillin, Erythromycin and Gentamicin respectively. Ceftriaxone was found to be 100% susceptible.

3.4.5 *Enterobacter agglomerans*

Antibiotic resistance of Enterobacter agglomerans were 100% for Azithromycin, Ceftriaxone, Cefixime, Gentamycin, Tobramycin respectively and 33% sensitive to Imipenem and Meropenem.

3.4.6 *Haemophilus Influenzae*

The resistance pattern of *Haemophilus influenzae* was found to be as 67% for Cotrimoxazole , 33% for Ampicillin, Ceftriaxone, Amoxyclave and Chloramphenicol respectively. Azithromycin was 100% susceptible.

Table3. 1: Resistance pattern of the isolated bacteria against first line of antibiotics

Antibiotics	**Bacterial isolates**				
	Streptococcus pneumoniae n=12	*Klebsiella pneumaniae* n=9	*Streptococcus spp.* n=5	*Enterobacter agglomerans* n=3	*Haemophilus influenzae* n=3
Penicillin	0	-	2 (40%)	-	-
Ampicillin	0	-	3 (60%)	-	1 (33%)
Erythromycin	8 (67%)	-	3 (60%)	-	-
Azithromycin	8 (67%)	9 (100%)	-	3 (100%)	0
Cotrimoxazole	11 (92%)	-	-	-	2 (67%)
Ceftriaxone	1 (8%)	5 (56%)	0	3 (100%)	1 (33%)
Cefixime	2 (17%)	5 (56%)	4 (80%)	3 (100%)	-
Levofloxacin	0	-	-	-	-
Gentamicin	-	3 (33%)	3 (60%)	3 (100%)	-
Tobramycin	-	4 (44%)	-	3 (100%)	-
Ciprofloxacin	-	5 (56%)	-	3 (100%)	-
Imipenem	-	0	-	1 (33%)	-
Meropenem	-	0	-	1 (33%)	-
Amoxyclave	-	-	-	-	1 (33%)
Chloramphenicol	-	-	-	-	1 (33%)

*Modified disc diffusion method was used for profiling of antimicrobial susceptibility/resistance patterns. Bacterial strains were identified as either sensitive or resistant to an antibiotic based on the diameter of inhibition zone interpretative, as manifested by Clinical and Laboratory Standards Institute (CLSI) 2014, M100-S24 Supplement Information.

3.5 Multidrug Resistance Strains

Two multidrug resistant bacterial pathogens - *Klebsiella pneumaniae* and *Enterobacter agglomerans* isolates were resistant for first line of antibiotics as well as for second line of antibiotics (Table 2).

Table3. 2: Resistance pattern of two multi-drug resistant bacteria (*Enterobacter agglomerans* and *Klebsiella pneumoniae*) to second line of antibiotics.

Bacteria	**Name of the Antibiotics**				
	Amikacin	Pipercilin/ Tazobactam	Carbenicillin	Polymyxin B	Netilmicin
E. agglomerans	Resistant	Resistant	Resistant	Resistant	Resistant
K. pneumoniae	Resistant	Resistant	Resistant	Resistant	Resistant

*Modified disc diffusion method was used for profiling of antimicrobial susceptibility/resistance. Bacterial strains were identified as either sensitive or resistant to an antibiotic based on the diameter of the inhibition zone interpretative, as manifested by Clinical and Laboratory Standards Institute (CLSI) 2014, M100-S24 Supplement Information.

3.6 Extended-Spectrum Beta-lactamase *CTX-M1* resistance gene detection

One of the *klebsiella pneumoniae* strains showed resistance to 6 out of 8 first line antibiotics and to all five second line antibiotics used for antibiogram (Table 1 & 2). As such, we had an interest in this strain showing multidrug resistance. Some studies suggested that the presence of extended-spectrum beta-lactamase (ESBL) genes in *K. pneumoniae* give them the ability to develop resistance towards multiple drugs. *CTX-M1* is one such antibiotic resistant gene, which falls under the classification of $ESBL_{A.}$ Therefore, for the detection of *CTX-M1* gene, PCR was performed with specific primers, and the PCR product was analyzed using a 1% agarose gel electrophoresis (Figure 3.2).

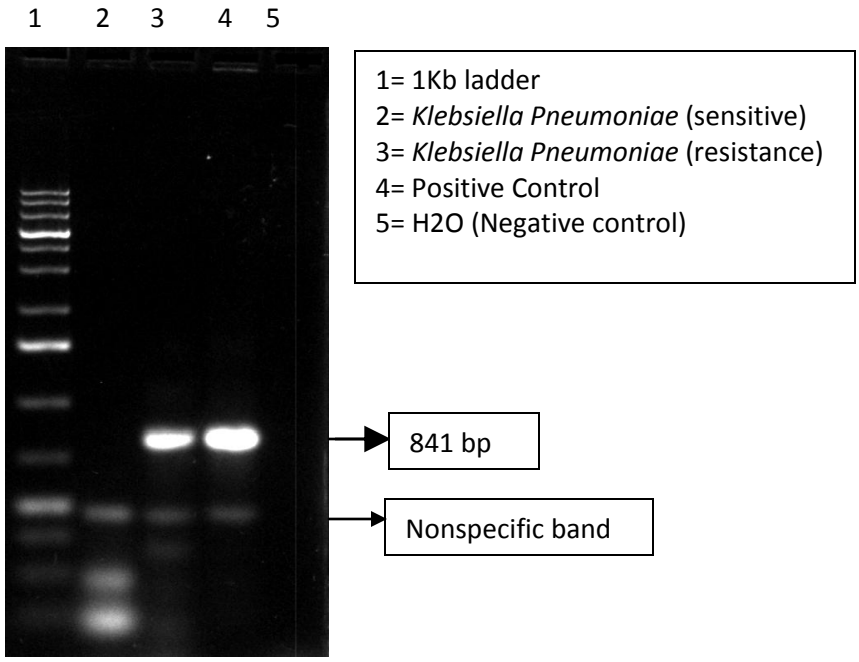


Fig. 3.3: PCR result for the presence of CTX-M1 gene in *Klebsiella pneumoniae.* After extraction of the total bacterial genome and amplification using gene specific primers, the band was resolved by the 1% agarose gel electrophoresis. The expected CTX-M1 band size was 841 bp. In the figure, Lane 1: 1kb plus ladder, Lane 2 & 3 *klebsiella pneumoniae* sensitive and resistant strains, respectively; Lane 4 & 5 Positive and negative controls, respectively.

3.7 Purity and amount of purified PCR product

After purification of PCR products by spin column-based method, the purity and concentration of the DNA were measured using the NanoDrop 2000/200c (Thermo Scientific). The ratio of OD at 260/280 nm of the extracted PCR product was found to be 1.82 and the concentration was 42.22 ng/µl.

3.8 Sequencing results

Sequencing was done using column purified PCR product and sequencing result had been presented in figure 3.3 to 3.7. The chromatogram depicts the two-dimensional plot with the ordinate axis presenting base calling intensity according to detector response (Figure-3.3). Sequencing data were analyzed using Chromas Lite 2.6.2 tool, which generated a four coloured chromatogram. Different bases were represented in different colors in chromatogram which are as below:

1. Adenosine (A) = Green

2. Guanine (G) = Black
3. Cytosine (C) = Blue
4. Thymine (T) = Red

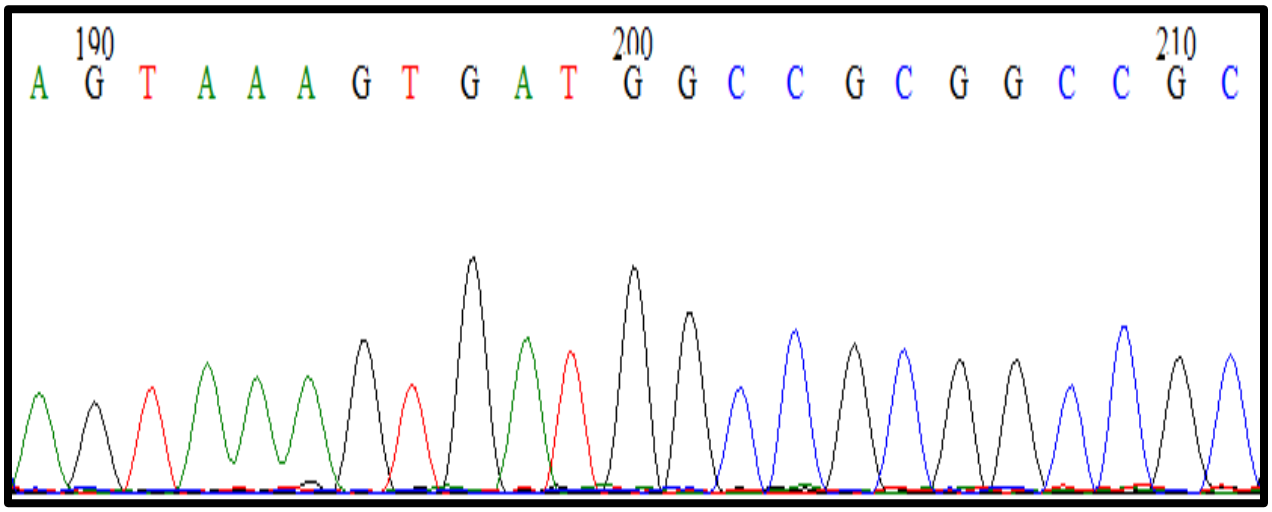


Figure 3.4: Illustration of a part of the chromatogram of sequence. Well-formed, distinct single colored peaks and absence of noise was observed, that indicate refined sequencing with proper concentration of template and primer. In the sequence chromatogram, the area under the pick depicted the component concentration.

The format in which the Nucleotides are shown here by the single letter text-based code is called the FASTA format. This specific format was documented using the Bioedit software. Sequencing data for the bacterial isolate with CTX-M1 antibiotics resistance gene is given below (Figure

>*Klebsiella Pneumoniae* _CTXM_1
CGWGGCGMGGCACCGTCACGCTGTTKTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGG
ACGTACAGCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCAT
TGATTAACACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGT
GCAGCACCAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGA
ATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTG
CGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGT
ACAGCGATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCA
CCGCGTTCGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGT
TTAAACACCRCCATTCCGGGCGATCCGCGTGATACCACTTTCACCTCGGGCAATWGGYGC
AAACTCTTGYGGAATCTGAC

Fig. 3.5: Sequencing data revealed 560 nucleotide bases by using CTXM_1 specific forward primer.

The gene sequencing was analyzed using NCBI Nucleotide Basic local alignment search tool (BLAST) and a list of sequences that were 'most similar' to the query sequence were obtained (figure-3.5).

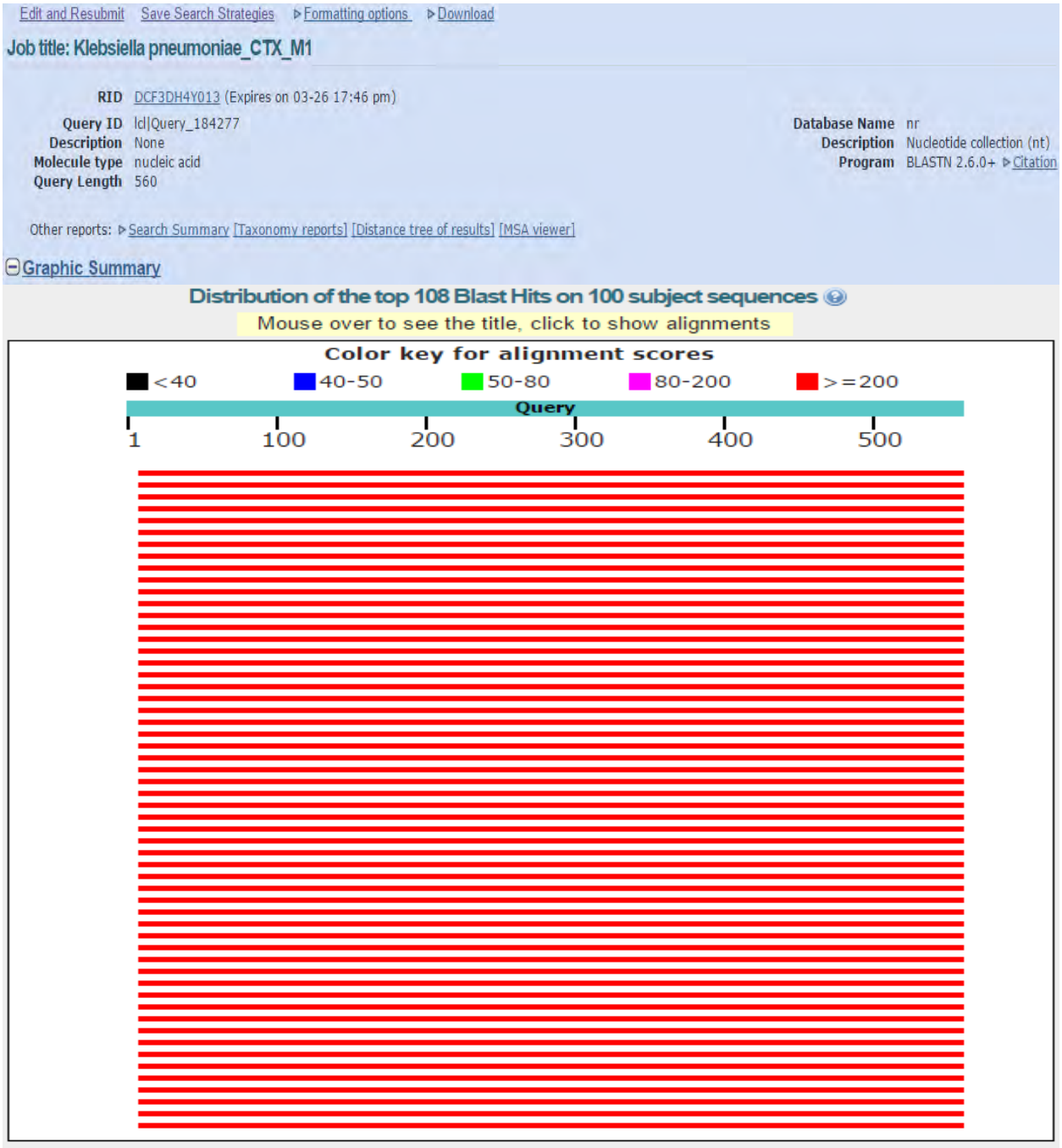


Figure 3.6: BLASTn result shown used the sequence of PCR product amplified by the CTX-M1 specific forward primer of the resistance *klebsiella pneumoniae*.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Escherichia coli strain 358B extended-spectrum beta-lactamase CTX-M-15 (blaCTX-M-15) gene, partial cds	976	976	98%	0.0	99%	KU163311.1
Klebsiella pneumoniae 1098378 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-176, complete	974	974	98%	0.0	98%	NG_048961.1
Escherichia coli 1126619 unnamed blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-170, comple	974	974	98%	0.0	98%	NG_048956.1
Proteus mirabilis 803693 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-167, complete CDS	974	974	98%	0.0	98%	NG_048952.1
Escherichia coli 1125509 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-163, complete CDS	974	974	98%	0.0	98%	NG_048948.1
Klebsiella oxytoca 1125476 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-162, complete CDS	974	974	98%	0.0	98%	NG_048947.1
Klebsiella pneumoniae KK08 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-156, complete CD	974	974	98%	0.0	98%	NG_048940.1
Escherichia coli CNR312 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-150, complete CDS	974	974	98%	0.0	98%	NG_048936.1
Escherichia coli 45.01 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-103, complete CDS	974	974	98%	0.0	98%	NG_048902.1
Escherichia coli TN03 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-28, complete CDS	974	974	98%	0.0	98%	NG_048977.1
Klebsiella pneumoniae blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-22, complete CDS	974	974	98%	0.0	98%	NG_048971.1
Citrobacter freundii CLSiS 2526/96 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-3, complete	974	974	98%	0.0	98%	NG_048979.1
Escherichia coli CTX-M-15 resistance cassette, isolate RS371	974	974	98%	0.0	98%	LN868277.1
Escherichia coli CTX-M-15 resistance cassette, isolate RS288	974	974	98%	0.0	98%	LN868276.1
Escherichia coli CTX-M-15 resistance cassette, isolate RS254	974	974	98%	0.0	98%	LN868275.1
Escherichia coli CTX-M-15 resistance cassette, isolate RS56	974	974	98%	0.0	98%	LN868274.1

Fig. 3.6: Top 16 similar sequences results obtained after alignment by the sequence of isolated resistant *Klebsiella pneumoniae*

After BLASTn analysis, the resistance bacterial sequence was matched with the CTX-M1 gene (Figure- 3.6).

Klebsiella pneumoniae 1098378 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-176, complete CDS

Sequence ID: NG_048961.1 Length: 876 Number of Matches: 1

Range 1: 42 to 590 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
974 bits(527)	0.0	544/553(98%)	5/553(0%)	Plus/Plus

```
Query  9    GGC-ACCGTCACGCTGTTKTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACA  67
            ||| ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  42   GGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACA  101

Query  68   GCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAA  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102  GCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAA  161

Query  128  CACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCAC  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162  CACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCAC  221

Query  188  CAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTT  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222  CAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTT  281

Query  248  AAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAA  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282  AAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAA  341

Query  308  GCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGA  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  GCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGA  401

Query  368  TAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTT  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402  TAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTT  461

Query  428  CGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTTAAACA  487
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  462  CGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACG-TTAAACA  520

Query  488  CCRCCATTCCGGGCGATCCGCGTGATACCACTTTCACCTCGGGCAATWGGYGCAAACTCT  547
            || |||||||||||||||||||||||||| ||||||||||||||| || ||||||||
Sbjct  521  CCGCCATTCCGGGCGATCCGCGTGATACCAC-TTCACCTCGGGCAAT-GGCGCAAACTC-  577

Query  548  TGYGGAATCTGAC  560
            || ||||||||||
Sbjct  578  TGCGGAATCTGAC  590
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Fig. 3.7: One of the matched sequenced within sixteen blaCTX-M gene for class A of the extended-extended-spectrum beta-lactamase (ESBL). Sequence identity matched perfectly except for 6 points in sequence.

Maximum identity and e-value was observed in the BLASTn result which revealed that the amplified sequence belong to the CTXM subclass of $ESBL_{A.}$.BLASTn results of homologous sequence annotated to *Klebsiella pneumoniae,* Escherichia *coli* and *Proteus mirabilis* strains harboring the resistance gene this indicates the primer pair and amplified the gene of interest (Figure- 3.7). In this study, extended β lactamase producing gene had been detected from the resistant strain of *klebsiella pneumoniae.* The BLASTn result indicated that the same ESBL gene was found in three different bacterial species, which suggested that horizontal resistance gene transfer of $ESBL_A$ are often plasmid encoded.

3.9 Spectrum of Viral pathogens under-5 ARIs suspected patients

150 nasal swab specimens were collected and tested by the RT-qPCR for detecting 10 different respiratory viruses. 74% (n=111) was found to be positive by RT-qPCR with both the single and co -viral infection, rest of 26% (n=39) had negative results **(Figure 3.8)**. The maximum repeatedly detected virus was HRV (48/150; 32%) followed by RSV, HMPV,HBoV,HPIV-3 and adenovirus with detection rate of 17.33% (26/150), 16% (24/150), 13% (20/150), 12% (18/150) and 9% (13/150) respectively. On the other hand Influenza A was found in three (2%) specimen, **(Figure 3.9).**

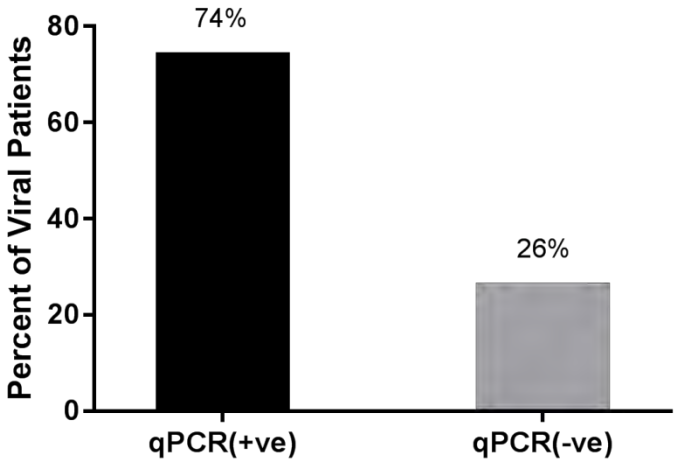

Fig. 3.8: Detection and identification of viral pathogens isolated from 150 hospitalized under-five patients nasal with suspected ARIs. Black Bar Diagram represents percentage of

total viral outcome where white background is negative.

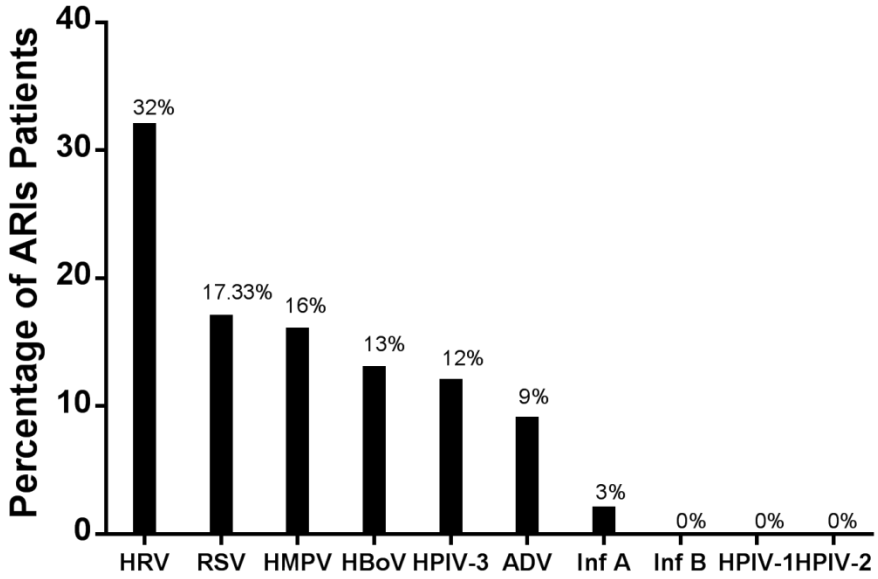

Figure 3.9. Show the prevalence of different respiratory viruses among the positive patients nasal swab specimens. In the Figure, HRV= Human Rhino Virus, RSV= Respiratory Syncytial Virus, HMPV= Human metapneumovirus, HBoV= Human Boca Virus, HPIV- 3= Human Parainfluenzae Virus 3, ADV= Adeno Virus, Inf A= Influenzae Virus A, Inf B= Influenzae Virus B, HPIV- 1= Human Parainfluenzae Virus 1, and HPIV- 2= Human Parainfluenzae Virus 2.

Even though the number of positive viral infection patients had 111, the total number of respiratory viruses detected 152 because of the presence of multiple viral pathogens in same specimen.

3.10 Age-dependent distribution of major viral pathogens in under-5 years ARI patients

The Respiratory viral pathogens identification rate was high in <1-year age group compared with 1-5 years patients. Human Rhino Virus (HRV) was found to be 35% (42/48), Respiratory Syncytial Virus (RSV) 19.17% (23/26), Human metapneumovirus 15% (18/24), Human Boca Virus (HBoV) 12.5% (15/20), Human Parainfluenzae Virus 3 (HPIV-3) 14.17 (17/18), Adeno Virus (ADV) 9.17 (11/13) and Influenzae Virus A (InfA) 2.5% (3/3), moreover the result showed negativity for Influenza B, Human Parainfluenzae Virus 1 (HPIV-1) and Human

Parainfluenzae Virus 2 (HPIV-2). As opposed to the viral detection rate 1-5 years of ARI suspected children was found to be HRV 20% (6/48), RSV 10% (3/26), HMPV 20% (624), HBoV 16.67% (5/20), HPIV-3 3.33% (1/18), and ADV 6.67% (2/13). On the other hand Influenza A (Inf A), Influenza B (Inf B), Human Parainfluenzae Virus 1 (HPIV-1) and Human Parainfluenzae Virus 2 (HPIV-2) showed a negative results (**Fig 3.10).**

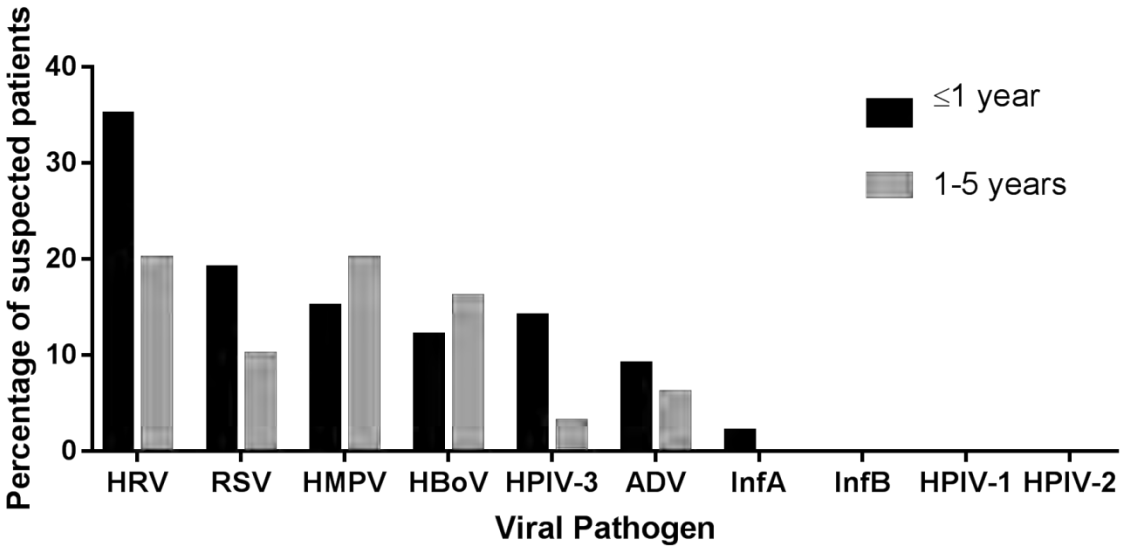

Fig. 3.10: Age dependent distribution of major viral pathogen of ARIs suspected in under 5 years hospitalized patients. In the Figure, HRV= Human Rhino Virus, RSV= Respiratory Syncytial Virus, HMPV= Human metapneumovirus, HBoV= Human Boca Virus, HPIV- 3= Human Parainfluenzae Virus 3, ADV= Adeno Virus, Inf A= Influenzae Virus A, Inf B= Influenzae Virus B, HPIV- 1= Human Parainfluenzae Virus 1, and HPIV- 2= Human Parainfluenzae Virus 2.

3.11 Bacterial and Viral Co infection Frequency

According to the research findings, respiratory viruses such as influenza are predisposing to secondary bacterial pulmonary infection. The co-occurrence of 2 or more pathogens in the host was analyzed based on the viral-viral, viral-bacterial, viral-viral-bacterial and viral-bacterial-bacterial patterns. Out of 32 bacterial isolates, a total 27 (84%) coinfection was detected in this study. The coinfection percentage of *Streptococcus pneumoniae, Klebsiella pneumoniae, Streptococcus species, H. Influenzae* and *Enterobacter agglomerans* were 40.74%, *29.63%,*

11.11%, 11.11% and 7.41% respectively. On the other hand, the respiratory viral panel is HRV, RSV, HMPV, Adenovirus, HBoV, HPIV-3 and Inf A were 36.36%, 13.64%, 12.5%, 13.64%, 12.5%, 9.09% and 2.27 % respectively. Out of 54 coinfection cases, 39 showed pair-wise confection of respiratory pathogens, whereas 14 showed pathogen co-occurrences in a tri-partner manner. In one case, up to four pathogens were detected in one specimen.

Table3.3: The table demonstrates the coinfections spectrum found in ≤5 suspected ARI children.

Coinfection with two pathogens	**No. of Patients**	**Coinfection with three or more than three pathogen**	**No. of Patients**
RSV+ S. pneumoniae	4	HRV+HPIV-3+ *K. pneumoniae*	2
RSV+ HRV	1	HRV+ *S. pneumoniae+ K. pneumoniae*	1
HRV + HMPV	3	RSV+ *S. pneumoniae+ H. influenza*	1
HRV + ADV	4	RSV+ ADV+ *E. Agglomerans*	1
HRV + HPIV-3	4	RSV+ HPIV-3+ *S. pneumoniae*	1
RSV+ ADV	2	HRV+ HBoV+ *K. pneumoniae*	1
HRV + HBoV	3	ADV+ HBoV+ HPIV-3	1
HRV + *K. pneumoniae*	2	HRV+ RSV+ *E. Agglomerans*	1
HMPV+ *S. pneumoniae*	2	HRV+ HBoV+ *Streptococcus spp.*	1
HRV+ *Streptococcus spp.*	1	HRV+ HPIV-3+ *S. pneumoniae*	1
HMPV+ *K. pneumoniae*	1	HRV+ HBoV+ HPIV-3	1
HPIV-3+ ADV	1	HPIV-3+ ADV+ RSV	1
HPIV-3+ HRV	1	HMPV+HRV+K. pneumoniae	1
HMPV + *Streptococcus spp.*	1		**n=14**
S. pneumoniae+ H. influenzae	1	**HRV+ HBoV+ HMPV+ HPIV-3**	1
HBoV+ HMPV	1		**n=1**
RSV+ *H. influenzae*	1		
Inf A+ HRV	1		
Inf A+ ADV	1		
HMPV+ ADV	1		
HRV+HBoV	2		
HPIV-3+Boca	1		
	n=39		

Coinfection in pair wise patterns of *Human Rhino Virus* was 1 RSV, 3 HMPV, 6 HBoV, 5 HPIV-3, 4 ADV, 1 Inf A and 2 KPN and 1 Ssp. On the other hand, co-occurrence in tri-partner virus were 1 RSV, 3 HBoV, 3HPIV-3 and bacteria were 2 Spn, 4 KPN, 1 Ssp and 1 Eag. In case of Respiratory Syncytial Virus, the dual partner coinfection rate was 1 HRV, 2 ADV and tri-partner virus were 1 HRV, 2 HPIV-3, 2 ADV and bacteria were 1 SPN, 2 Eag. *Haemophilus influenza* coinfection with RSV was 1 in pair-wise and a tri-partner manner. HMPV Coinfection sequence were HRV (three), HBoV (One), ADV (One), SPN (Two), KPN (One), Ssp (One) and tri-partner infection was HRV (One) HBoV (One) and HPIV-3 (One). Coinfection partners of adenovirus were confined to viruses including 4 HRV, 2 RSV, 1 HMPV, 1 HPIV-3, 1 Inf A in pair-wise fashion and Tri-partner coinfection were 2 RSV, 1 HBoV, 2 HPIV-3 where there was only one co-detection of bacterial isolate namely *Enterobacter agglomerans*. The pair-wise coinfection of Human parainfluenzae virus 3 were (five) HRV, 1 ADV and tri-partner was (four) HRV, (one) RSV, (one) HMPV, (three) HBoV, (one) ADV and bacterial isolates were (one) SPN and (two) KPN in tri-partner co-occurrences. In case of Influenzae A only two co infection in pair-wise manner was found HRV (1) and ADV (1) **(Table-3.3)**. Interestingly in one cases four viral co-infection patterns was found and the sequence was HRV+ HBoV+ HMPV+ HPIV-3 **(Table-3.3)**.

The bacterial co-infection partner with viruses and bacteria was analyzed. In case of *Streptococcus pneumoniae* the pair-wise coinfection were HRV (2), RSV (4), HMPV (2) and HI (1) and tri-partner had found to be (two) HRV, (two) RSV, (two) HPIV-3, (one) KPN and (one) HI . *Klebsiella pneumoniae* coinfection in dual manner were 2 HRV, 1 HMPV and tri-partner to (five) HRV, (one) HMPV, (one) HBoV, (two) HPIV-3 and (one) SPN. In case of *Streptococcus* species, the pair wise coinfection was HRV (2), HMPV (1) and HBoV (1). In addition, coinfection in tri-partner was not found of Ssp infected case. *Haemophilus influenza* coinfection in dual and tri partner were RSV, SPN in (n=1), (n=1) respectively. On the other hand, *Enterobacter agglomerans* were (one) HRV, (two) RSV and (one) Adenovirus and no dual partner coinfection was detected. **(Table-3.3)**

The distribution pattern of major viral and bacterial pathogens among single and mixed infection was analyzed. In case of viral coinfection, HRV, HBoV, HPIV-3, ADV and Inf A were higher compare with single infection in 32/19, 11/10, 8/4, 12/1 and 2/1 respectively. Moreover RSV (12/13) and HMPV (11/13) single infection were higher than coinfection of ARIs suspected patients. Interestingly in HBoV cases generating significant p value of 0.0152. In case of HRV, RSV, HMPV, HPIV-3 and Inf A there was no significant differences observed in the frequency of each viral single and coinfection (**Figure- 3.11).**

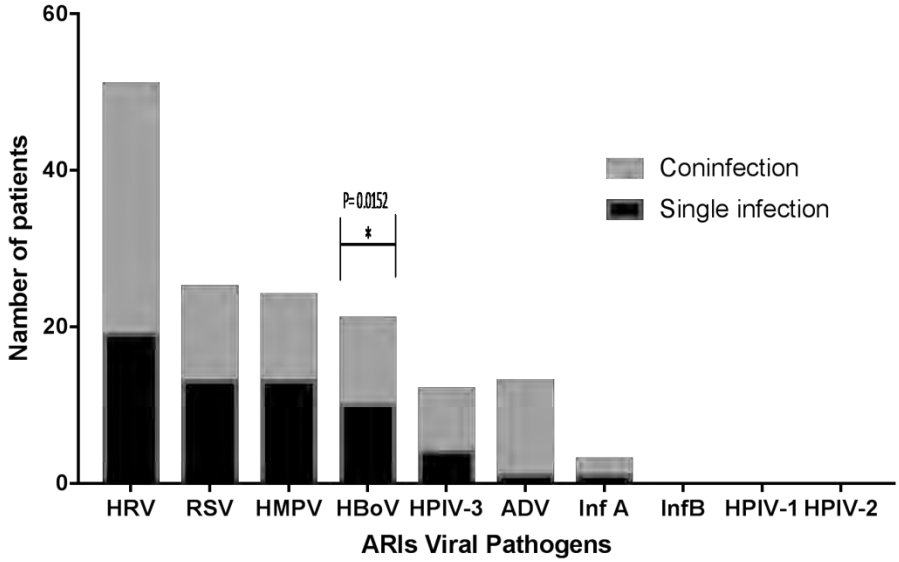

Fig. 3.11: Comparision of frequencies of viral identification in single infections and coinfections Fisher's exact test was performed for each group $p<0.05$ was considered signifact (significant p value was found; HBoV, P= 0.0152*). In the Figure, HRV= Human Rhino Virus, RSV= Respiratory Syncytial Virus, HMPV= Human metapneumovirus, HBoV= Human Boca Virus, HPIV- 3= Human Parainfluenzae Virus 3, ADV= Adeno Virus, Inf A= Influenzae Virus A, Inf B= Influenzae Virus B, HPIV- 1= Human Parainfluenzae Virus 1, and HPIV- 2= Human

Parainfluenzae Virus 2.

On the other hand, the bacterial coinfection partners of *Streptococcus pneumoniae, Klebsiella pneumoniae, Streptococcus species, Haemophilus influenza* and *Enterobacter agglomerans* were (n=11), (n=8), (n= 3), (n=3) and (n=2) respectively. The single bacterial infection showed less number of patients compared with coinfection and the single bacterial infection was found in SPN (1), KPN (1), Ssp (2) and Eag (1). In case of *Haemophilus influenza* no single infection was found. The comparisons between frequencies of single and coinfection of isolated bacteria are SPN, KPN, Ssp, HI and Eag did not generate any significant p value **(Figure-3.12).**

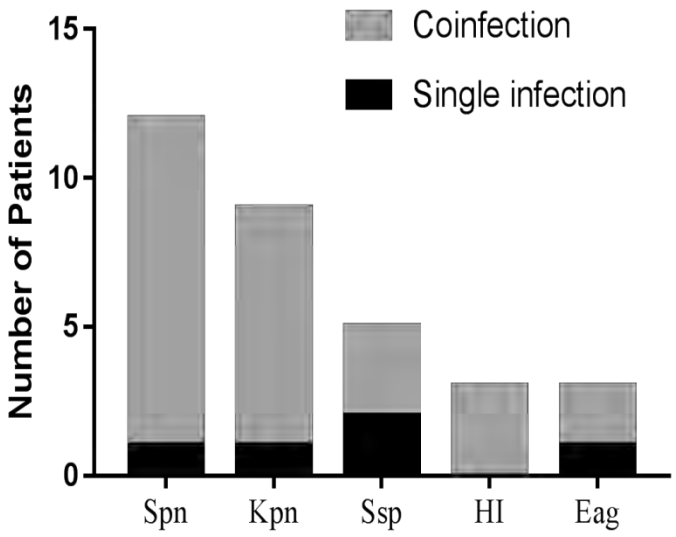

Figure 3.12: Differentiation of frequencies of bacterial identification in single infections and coinfections. Fisher's exact test was performed for each group and no significant p value was found (p= <0.05 was considered to be significant). Spn=*Streptococcus pneumoniae,* Kpn=*Klebsiella pneumaniae,* Hin= *Haemophilus influenzae,* Eag= *Enterobacter agglomerans.*

3.12 Viral Seasonality

The viral seasonal variation was observed in terms of specimen collection throughout the year during study period. HRV highest peak was found in September (n=8) and November (n=8). Second highest peak was found in December (n=7). In January, October and February, April months the same number of HRV was found, (n= 6) and (n=4) respectively. On the other hand,

in March, June and July months the HRV were found to be n= 3, n=1 and n=1 respectively. Similar to HRV the number of RSV infected patients had been found in September, 19 (76%) patients were hospitalized. The RSV hospitalization rate were (n= 3) 12%, (n=2) 8% and (n=1) 4% in October, November and December respectively, whereas no findings of RSV infected patients in other months. These findings suggest that the RSV findings were highly confined in September. HMPV circulation reached its peak (54.17%, n=13) in January, followed by (25%, n=6) and (20.83%, n=5) in February and December and no HMPV- infected patients were found to other months.

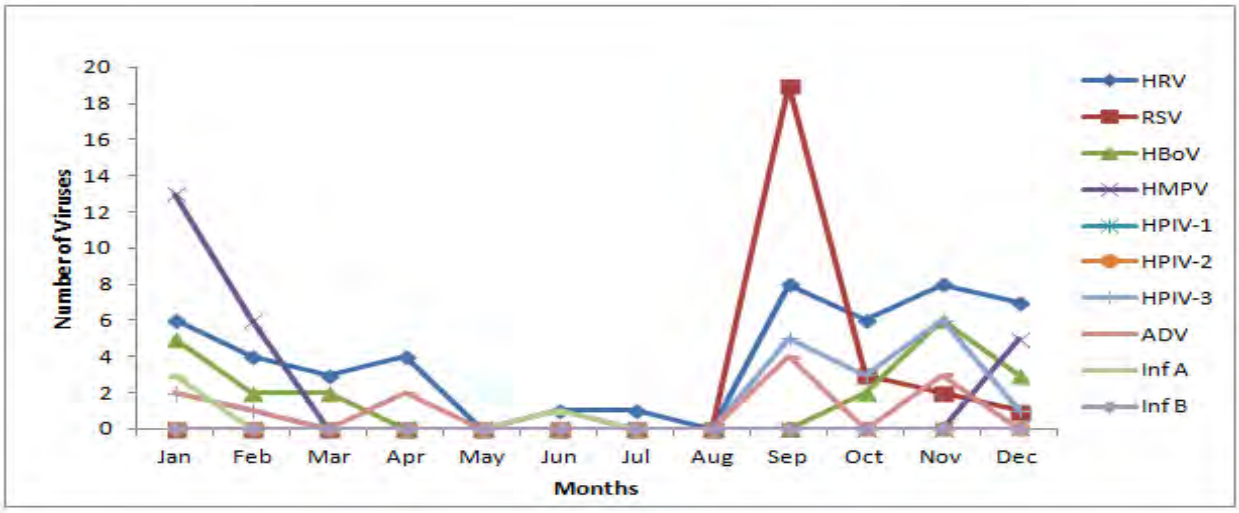

Figure 3.13: **Monthly distributions of respiratory viral pathogens of ARIs patients.**

HBoV had the fourth highest detection virus during the study period. The HBoV Suspected patient in January, February, March, October, November and December were n=5 (25%), n=2 (10%), n=2 (10%), n=2 (10%), n= 6 (30%) and n=3 (15%) respectively. In addition to April, May, June, July, August and September months could not found any HBoV suspected patient. In case of HPIV-3, the hospitalization patients found to be (n= 2) 11.11%, (n=1) 5.56%, (n=5) 27.78%, (n=3) 16.67%, (n=6) 33.33%, and (n=1) 5.55% in January, February, September, October, November and December respectively. Hospitalization rates of Adeno Virus Infected patients were 15.38% (n=2), 7.69% (n=1), 15.38% (n=2), 7.69% (n=1), 30.77(n=4) and 23.08 (n=3) in January, February, April, June, September and November respectively. In the months of March, May, July, August, October and December none of the Adeno Virus suspected patients were found. On the other hand, only 4 Influenza A virus were found out in January (n=3) and

June (n=1) months. Others viruses of the viral panel including HPIV-1, HPIV-2 and Influenza B were not suspected between the study periods in hospitalized patients **(Figure- 3.13).**

3.13 Bacterial Seasonality

The seasonality of Acute Respiratory Infections (ARIs) causing bacterial pathogens were observed in *Streptococcus pneumoniae* (SPN), *KLebsiella pneumoniae* (KPN) and *Haemophilus Influenzae* (HI). In addition, there are two more bacteria *Streptococcus species* (Ssp) and *Enterobacter agglomerans* (Eag) that were found in this study. *S.pneumoniae* was the most prevalent respiratory pathogen and highest peak was found in September showing, hospitalization of 7

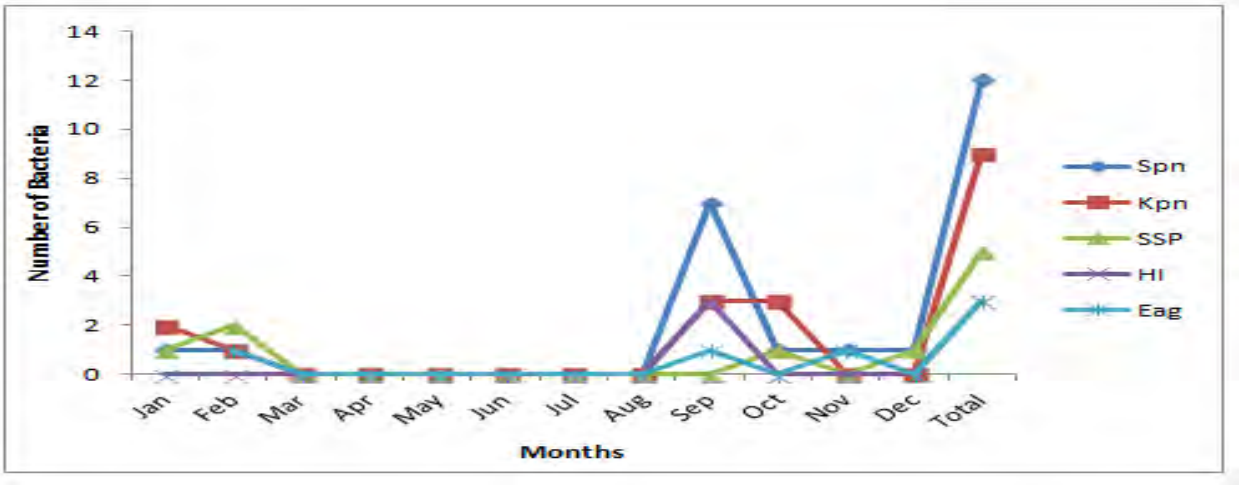

Fig. 3.14: Monthly distributions of Respiratory Bacterial Pathogens in suspected <5 ARIs patients.

(58.33%) out of 12 patients. There was only one patient (8.33%) enrolled in each of the months of January, February, October, November and December for *S. pneumoniae* infected hospitalized patients. On the other hand, from March to August no SPN suspected case were found. The second most prevalent pathogen *k. pneumoniae* showed its highest peak circulation in September and October were (n = 3) 33.33%, (n = 3) 33.33% followed by (n=2) 22.22%, (n=1) 11.11% in January and February. In case of *Streptococcus spp* suspected patient found to be February (n=2) 40% and January n=1 (20%), October n=1 (20%), December n=1 (20%) infected case were same

respectively. Interestingly, all the *Haemophilus Influenzae* (HI) n=3 (100%) Circulation peak were confined to September. On the other hand, one *Enterobacter agglomerans* (Eag) (33.33%) infected patient were found in February, September and November respectively.

Chapter 4: Discussion

Discussion

This study demonstrated bacterial and viral ARIs (Acute Respiratory Infections) pathogens in under five children. Major Findings of our study are- (I) Prevalence of Bacterial and Viral ARIs pathogens. (II) Age dependent distribution pattern of ARIs pathogens. (III) In vitro antibiotic resistance/ sensitivity assay for bacterial pathogens. (IV) Confirmation the multi-drugs resistance gene. (V) Determination of seasonal variation in term of ARI specimen collection rates as well as seasonality of isolated ARI pathogens. (VI) Co-infection pattern of isolated ARIs pathogens.

The standard microbiological culture had been performed of nasal swab sample from clinically ARIs suspected under five children during the study period. Detection rate of the isolated bacteria was 19% along with co viral infection. The most commonly isolated bacteria were *Streptococcus pneumoniae* (38%), *Klebsiella pneumaniae* (28%) and *Haemophilus influenzae* (9%). Along with others two bacteria were also detected, *Streptococcus spp.* (16%) and *E. agglomerans* (9%). *Streptococcus pneumoniae* is a predominant cause of mortality and morbidity to under five children despite the vaccination schedule, it has been responsible for at least 18% of severe respiratory cases and 33% of death worldwide (11). Others study was conducted by Kenya, Zambia, Nepal and Brazil reported that *S. pneumonie* is the most frequently isolated bacteria and the identification rate from 15.8 to 54.8% 9 [33-36].

K. pneumoniae had been described as the most common cause of lower respiratory tract infection in Jordan and India [37, 38]. It is known that bacterial colonization is mostly facilitated by physical damages of respiratory cells caused by viral infection. However, Yu et al. reported that bacterial colonization following viral infection has no effect on clinical manifestation of ARIs [39]. Many of the bacteria found as coinfection partners with virus in this study, are pathogenic. When bacteria enter a body, the host must elicit immune responses against the invading organisms. However, the immune responses are not always beneficial and sometimes these responses can be harmful, such as in case of hyper immune responses. Even the immune responses elicited by one organism may compromise the immune responses elicited by another organism. Although the pathogenic role of bacterial coinfections with viruses is unclear, they might have involvement in disease pathogenicity. One study reported that, colonization of pathogenic bacteria in airway stimulates topical immune responses [40] and this phenomenon

may play important role in compromising host immune responses. Most of the bacteria isolated during the study period including *S. pneumoniae, K. pneumaniae*, *H. influenzae,* and *E. agglomerans* fall in either community-acquired or nosocomical categories, suggesting appropriate preventive measures could limit bacterial involvement in ARIs.

Increasing antibiotic resistance to commonly prescribed antibiotics makes bacterial infections a major threat to public health worldwide. As we have described in result section, our *in vitro* antibiotic resistance patterns were concerning in the sense that most of the isolates except *S. pneumaniae,* such as *K. pneumaniae*, *Streptococcus* species, *H. influenzae,* and *E. agglomerans* exhibited alarming levels of resistance against the majority of available first line of antibiotics. Even two of 15 multidrug-resistant isolates (*K. pneumaniae* and *E. agglomerans*) displayed resistance not only against all the first-line of antibiotics but also against all the second-line of antibiotics. Despite the advances in therapeutic and preventive measures, the emerging resistance to the antibiotics is a growing concern among clinicians and other health professionals worldwide. The indiscriminate and unreasonable use of antibiotics have contributed to the emergence of resistance, which may turn out to be a leading cause of morbidity and mortality in the developing countries. A tailored antibiotic treatment is in demand to tackle this major issue, but the dearth of information regarding the etiological agents and antibiotic sensitivity patterns in countries like Bangladesh have made it difficult. The information regarding antibiotic resistance patterns provided here in this study is expected to raise alertness among physicians, community people, as well as among policy makers of public and private sectors in the country.

Extended spectrum beta-lactamase (ESBL) family enzymes are induced in certain resistant bacteria; hydrolyze extended-spectrum cephalosporins antibiotics with an oxyimino side chain as well as the oxyimino-monobactam drugs [41]. Identification of different ESBL genes by gene detection techniques reveled better understanding of the ESBL production. A study found that out of 246 samples tested, 58.1% were detected as ESBL positive by genotypic methods which indicates its greater sensitivity when compared to phenotypic methods (44.3%) [42]. CTX-M β-lactamases have emerged as the most significant ESBL enzyme in humans. The *blaCTXM* gene encodes a 291 amino acid enzyme. A single amino acid change in blaCTX-M can constitute a new CTX-M type [43]. In our study *CTX-M1* gene was found in 13 antibiotics resistant *Klebsiella pneumoniae*. In addition, CTXM-1 gene in *Klebsiella pneumoniae* rendered it resistant to multiple antibiotics. This is an alarming subject as sensitive opportunistic

microorganisms are turning into multiple drug resistant strains. It is high time to take necessary steps for preventing spread of antibiotic resistant genes from resistant to sensitive bacteria by raising awareness about health and hygiene and also by imposing restriction to unnecessary use of antibiotics

Single-plex reverse transcription real-time PCR (RT-qPCR) method could detect the presence 152 viruses in 111 (74%) children. The findings corroborate the concept of viruses being the most common causative agent of ARIs and the data are consistent with hospital-based findings in Bangladesh reported by Homaira *et al.* [11] and the comparable results were also published from Laos and Egypt [44,45]. However, many studies in other regions including Brazil, Niger, Zambia, Spain, and Mexico reported higher viral detection rates ranging from 65-87% [33,44-49]. The disparity in viral detection rates among these study findings might be due to variation in numbers of viruses that were included in the viral panel of each study group. Our viral panel included ten common respiratory viruses including respiratory syncytial virus (RSV), human rhinovirus (HRV), influenza A and B viruses, human parainfluenza viruses type 1 to 3 (HPIV1, HPIV2, HPIV3), human metapneumo virus (HMPV), human bocavirus (HBoV), and adenovirus. However, the panel did not include coronavirus which may account for a certain percentage of infections. 32% specimens were positive for HRV, which was predominantly detected as a viral pathogen. Adenovirus was was found to 9% in suspected patients. RSV was the second most prevalent virus identified in 17.33% cases. However, unlike HRV, RSV has been reported as a recurrent cause of ARI in both children younger than five years old and adult, with incidence reaching 26% (Laos PDR), 33% (Mexico), 35% (Niger), 37% (Brazil), 51% (Germany), 57% (India), and 76% (Spain) of the investigated cases [44,48-49,50-51]. RSV can cause both upper and lower respiratory tract infections. After RSV, HMPV (16%), HBoV (13%) and HPIV-3 (12%) came out as the third, fourth, and fifth most prevalent viral agents. However, we observed relatively low pathogen detection rates for HPIV-1, HPIV-2, influenza A, and influenza B viruses. Thus after RSV, HMPV, HBoV, and HPIVs came out as the frequent causes of hospitalization of children with respiratory illness and the findings are consistent with other published data[52]. Approximately 70–89% cases of bronchiolitis had been reported to associate with RSV infection and 3–25% with parainfluenza viruses and there werek very few cases of bronchiolitis which could be attributed to human influenza virus and adenovirus [53, 54]. Several studies have described frequencies of 3.3–19% for HMPV infections in hospitalized children

with respiratory tract infections [7, 55,56], although HMPV has been known to cause severe respiratory infections in the elderly [57,58]. The frequency of HBoV identified in this study (13%) was also comparable with HBoV detection rates in Spain (9.9%) and Vietnam (7.2%) [59, 60].

Age-dependent distribution of respiratory virus data of our study shows that viral infection is mostly concentrated in less than one-year age group particularly RSV as a substantial threat. The frequency of pathogen positive specimens and incidence of coinfections were significantly higher in under-one children, which makes it the most vulnerable age group for ARIs. Additionally, around 19.17% (23/26) of the RSV cases were detected in children less than one year of age. The frequency of RSV infections in this age group seems to be a consequence of multifactorial complex events, involving especially immunological factors. Maternal antibodies are a vital means of protection to infants against respiratory viruses as they have a relatively immature immune system to mount an effective immune response. RSV antibodies are found in all adults and children older than three years of age. At one year of age, 25-50% of children have antibodies against RSV, indicating the high frequency of this infection at a young age [61] and then the incidence of RSV infections decreases with age, probably because of the development of anti-RSV immunity which is boosted during each subsequent re-infection [62]. Currently, there are no effective antivirals or vaccines against RSV infections. Several RSV vaccines are being evaluated in clinical trials and have yet to be licensed [63].

The study identified 25 cases with viral-bacterial mixed infections where RSV and *S. pneumoniae* were the predominant viral and bacterial partners, respectively, followed by HRV and *K. pneumoniae* duo. Each of HRV, RSV, *S. pneumoniae,* and *K. pneumoniae* showed a seasonal peak in September and both *S. pneumoniae,* and *K. pneumoniae* had significant participation in mixed infections demonstrating that viral infection renders bacterial colonization. Higher rates of bacterial infections by *S. pneumoniae* and *K. pneumoniae* in synergy with RSV, HRV, and other viruses may indicate that predominant ARI viruses predispose their hosts to secondary infections by bacteria. The other major viral partners of bacterial mixed infections included HMPV and HPIV. The mechanisms by which viruses influence bacterial colonization and invasion are very diverse. It has been shown that viral infections may predispose to bacterial super-infections by favoring bacterial attachment sites on nasopharyngeal epithelial cells and through increased mucous production that promotes bacterial growth [64, 65]. The real clinical

significance of these infections has not yet been fully elucidated. Under the circumstances, emphasis should be given on deciphering the role of bacterial mixed infections in promoting disease pathology. It is also important to remember that the detection rate of mixed infection is dependent mostly on methodology and number of pathogens that are being screened. Despite differences in methodologies, the similarity in rates of hospitalized viral respiratory illness in different countries suggests that respiratory viruses indeed are important contributors of childhood hospitalization around the world.

There are several shortfalls of the study including (a) shorter study period, (b) low number of specimens, (c) exclusion of coronavirus from viral panel selected for RT-qPCR assay, (d) lack of some clinical information and follow-up study, (e) use of nasal swabs instead of nasopharyngeal swabs for specimen collection, (f) exclusion of ARI-causing fungal pathogen detection. Thirty three out of 150 specimens remained undetected of any viral or bacterial pathogens. It is highly likely that the inclusion of fungal pathogen detection system and the addition of coronavirus in the viral panel would increase the rate of pathogen detection to some extent. In addition, the detection rate of bacterial pathogens could have been higher if PCR or qPCR had been used instead of culture method as the former one has higher sensitivity. However, we wanted to isolate all types of possible organisms and the use of specific primers would have confined the range of organisms. For example, *E. agglomerans* which has been rarely reported as a causative agent of ARIs was isolated in 3 (9%) nasal swab specimens in this study. The nasopharyngeal aspirate (NPA) has been considered to be the best sampling technique, but the procedure of obtaining an NPA specimen is uncomfortable and often frightening for young children. It is also unpleasant for the medical staff, who have to carry out the procedure with a struggling, crying, and coughing child. However, pathogen detection sensitivity did not differ significantly between nasal swab and NPA approaches of specimen collection when sensitive real-time PCR method was used to detect pathogens [66, 67]. In the study, only those viruses that are frequently detected in Bangladesh were listed in the viral panel. Even with these limitations, our study provides important preliminary data that can be used for more focused surveys in a larger population. The frequency of viral infection should be taken into account by pediatricians to avoid the irrational use of antibiotics.

Conclusion:

In summary, ARI incidence, as well as pathogen detection rates, were higher during post-monsoon and winter in Bangladesh. HRV and RSV were the predominant viral causative agents in under-five ARIs. On the other hand, *S. pneumoniae* came out as the most prevalent bacterial causative agent. Children aged less than one year of age were most susceptible to infections including confections. CTX-M1 is an emerging multi drug resistance gene which is a serious issue in Bangladesh. This is the high time to take right policy of antibiotics uses and unnecessary antibiotics should be avoided.

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Appendices:

Appendix-I

Media composition

The composition of the media used in our study has been given below and media was autoclaved at 121°C and 15 Ib pressure for 15 minutes

MacConkey Agar (Difco™):

Approximate Formula* Per Liter	
Peptone	17.0 g
Proteose Peptone	3.0 g
Lactose	10.0 g
Bile Salts No. 3	1.5 g
Sodium Chloride	5.0 g
Agar	13.5 g
Neutral Red	0.03 g
Crystal Violet	1.0 mg

BD Columbia Agar with 5% Sheep Blood:

Approximate Formula* Per Liter	
Water Pancreatic Digest of Casein	12.0 g
Peptic Digest of Animal Tissue	5.0
Yeast Extract	3.0
Beef Extract	3.0
Corn Starch	1.0
Sodium Chloride	5.0
Agar	13.5
Sheep Blood, Defibrinated	5 %

1.0 Tripple Sugar Iron Agar

Ingredients	Amounts (g/l)
Lab Lemco poder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium Chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric thio Sulphate	0.3
Phenol red	0.024
Agar	12.0
Final P^H (at 25^0c)	7.4± 0

6.5 gms was suspended in 100 ml distilled water. It was boiled to dissolve the medium completely.

2.0 Simon Citrate Agar

Ingredients	Amounts(g/l)
Ammonium Dihydrogen phosphate	1.0
Dipottasium phosphate	1.0
Agar	15.0
Bromthymol Blue	0.08
Final pH	6.9 ±0.2

2.4 gms was suspended in 100 ml distilled water. It was boiled to dissolve the medium completely.

3.0 Bacteriological peptone

Ingredients	Amounts (% W/W)
Total nitrogen	14.0
Ammino nitrogen	2.6
Sodium chloride	1.6
Final pH (2% solution)	6.9 ±0.2

0.5 gms NaCl and Bacteriological peptone was suspended in 100 ml distilled water. It was boiled to dissolve the medium completely.

4.0 Agar Bacteriological

Ingredients	Amounts (% W/W)
Working strength	1.0%
Moisture	<7.5
Clarity	High
Mineral content	Very low
Final pH	6.9 ±0.2

5.0 Manitol Salt Agar

Ingredients	Amounts (g/l)
Bacteriological Agar	0.5
Nacl	0.5
Bacteriological peptone	1.0
pH	6.9 ±0.2
Distilled water	100 ml.

6.0 Urea Agar Base

Ingredients	Amounts (g/l)
Peptone	1.0
Glucose	1.0
Sodium chloride	5.0
Di sodium phosphate	1.2
Potasium dihydrogen phosphate	0.8
Phenol red	0.012
Agar	15.0
pH	6.8± 0.2 at 25^0c

2.0 gms Urae agar and 40% Urea solution was suspended in 100 ml distilled water. It was boiled to dissolve the medium completely.

8.0 6.5% NaCl Broth

Ingredients	Amounts (g/l)
Sodium Salt	6.5
Bacteriological peptone	1.0
pH	6.8± 0.2 at 25^0c

Distilled water 100ml

9.0 Bile Aesculin Agar

Ingredients	Amounts (g/l)
Peptone	8.0
Bile salt	20.0
Ferric citrate	0.5
Aesculin	1.0
Agar	15.0

4.5 gms Bile Aesculin Agar was suspended in 100 ml distilled water. It was boiled to dissolve the medium completely.

10.0 Mueller Hinton Agar

Ingredients	Amounts (g/l)
Beef; Dehydrated infurin from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.5
pH	7.4

38.0 gms Muller Hinton Agar was suspended in 1000 ml distilled water. It was boiled to dissolve the medium complet

Appendix-II:

Buffers and Reagents

Tris Boric Acid EDTA (TBE) Buffer (500 ml):

To 500 ml distilled water, 5.4 g Tris HCL powder, 2.75 g boric acid and 0.5M EDTA of 2 ml were dissolved. The pH of the buffer was adjusted to 8, autoclaved and stored at room temperature.

Phosphate Buffer Saline (PBS):

The composition along with amount is mentioned below:

1X PBS; 10 mMPhosphate (pH 7.2-7.4)

Reagents	For 100 ml
Sodium chloride(NaCl) (0.136M)	0.80
Di-sodium hydrogen Orthophosphate dodecahydrate Na2HPO4.12H2O	0.137

Potassium phosphate monobasic (KH_2PO_4) (2mM)	0.0275
Potassium Chloride (KCl) (2.68mM)	0.02

Appendix-III

Instruments:

The equipment used throughout the study are listed below:

Instruments	**Manufacturer**
Autoclave	WiseClave
Refrigerator	Electra, Samsung (+4°C)- to store bacteria; Vestfrost (+4°C)- to store bacterial medium
Freeze	Vestfrost (-20°C) to store stock antibiotics; ESCO (-80°C) to store stock bacteria.
Incubator	Memmert
Shaking Incubator	WiseCube
Oven	WiseVen
Water bath	WiseBath
Micropipette	(2-20µl)- Gilson and Costar® (20-200µl)- Gilson and Costar® (200-1000µl)- Gilson
Bio-Safety Cabinet	ESCO Class-II Type-A2 Labculture® Biological Safety Cabinet
Vortex Mixture Machine	WiseMix
Weighing Machine	OHAUS®
Weighing Paper	Fisherbrand®
Spectrophotometer	Eon™ BioTek®
96-Well Plate	Nunc™ 96F Microwell Plate
Centrifuge Machine	Thermo SCIENTIFIC
Light Microscope	OLYMPUS CX41
T 100 ™ thermal cycler	Bio-Rad
Gel documentation machine	Bio-Rad
CFX-96 Real Time	Bio-Red
Antibiotic disks	Oxoid