Final Technical Report

Med/69

PROJECT NO.PSF/C-NIH/MED(69).

F I R S T   A N N U A L   R E P O R T

F R O M

J U L Y ,   1981

T O

J U N E ,   1982

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STUDY OF VIRAL RESPIRATORY
DISEASES IN CHILDREN   IN
RAWALPINDI/ ISLAMABAD AREA

(DR. ABDUL GHAFOOR)
PRINCIPAL INVESTIGATOR

FIRST ANNUAL REPORT

PROJECT No.   PSF/C-NIH/MED(69).

PROJECT TITLE: Study of Viral Respiratory Diseases
                               in Children in Rawalpindi/Islamabad
                               Area.
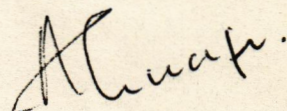
NAME OF THE INSTITUTION: National Institute of Health,
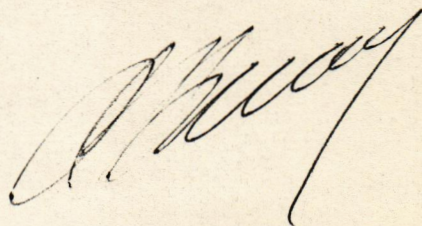   Islamabad.

TOTAL AMOUNT OF GRANT: Rs.1,54,360/-

PREVIOUS AMOUNT PAID: Rs.1,01,220/-

REPORT PERIOD: July, 1981 to June,1982.

Signature of Principal Investigator.

Signature of Institutional Head.

C O N T E N T S

S U M M A R Y

The study was carried principally to isolate &
identify various viral organisms associated with respiratory
diseases in children. Acute respiratory diseases are very
common throughout the year in our country and contribute
to morbidity and probably to mortality. Viruses of various
types are known to cause a wide range of acute respiratory
infections ranging from mild colds and catarrh to severe
bronchiolitis and pneumonia. Bacteria & fungi also cause
respiratory diseases including serious conditions such as
otitis media and pneumonia. In one year period of study 1354
specimens of acute respiratory disease (ARD) were collected
& processed in National Institute of Health. Most of the
specimens were collected from out patient ward & indoor ward.
Out of these 701 specimens were positive for bacterial agents
while 447 were positive for viral agents. Of bacterial agents
102 specimens were of B-haemolytic & of much importance.
These specimens were also tested for sensitivity to various
antibiotics, commonly prescribed in our clinics. Erythromycin,
Cloxallin, Cephalotin, Septran & Vibramycin found most effective
as none of the specimen was found resistant against these
drugs. On the other hand higher incidence of resistance was
observed against Streptomycin (41.67%) & Tetracycline (29.41%).
Of virus organisms, isolation of Influenza Virus Strain A/Bangkok/
2/79 H_3N_2 was of much significance as it has been isolated
for the first time in Pakistan. Its isolation was communicated
to WHO authorities & it has now been published in the report of
W.H.O. Other viral agents like Echo-11, Rhino viruses, Entero-
viruses & polioviruses were isolated from throat samples.
106 specimens are under process for virus isolations. Most
of the samples await their isolation on Primary Monkey Kidney
Cell cultures.

INTRODUCTION

Communicable diseases of the respiratory tract are a
major cause of morbidity and mortality all over the world.
According to WHO information it may be estimated that about
2.2 million deaths from acute respiratory diseases occur
throughlut the world every year (WHO Technical Report No.642,
1980). It was reported that acute respiratory diseases
accounted for 61% of the deaths associated with respiratory
diseases. If all causes of deaths are considered, then acute
respiratory diseases account for 6% of the total number
reported. Bacterial and viral pneumonias are by for the most
important causes of death, together accounting for 75% of all
deaths from acute respiratory diseases. If all cases of death
reported in the world are taken into account, pneumonia accounts
for about 5%.

If the child population (i.e. children aged 0-14 years)
is considered, ARD accounts for 20% of all deaths. However the
highest mortality for that group of diseases is reported for
infants below 1 year of age, the death rate in some countries
exceeding 2000 per 1,00,000 live births.

Even in developed countries morbidity from respiratory
diseases is less easy to measure than mortality and most
information has been derived from surveys of particular
populations.

In studies of respiratory diseases seen by general
physicians in the U.K. it was found that these diseases
accounted for about one quarter of all consultations and
one half of all patients. Nearly one third of the patients
had colds, one third had upper respiratory disease, pharyngitis,
or tonsillitis and the rest had influenza, bronchitis or
pneumonia. Upper respiratory tract infections decreased with
age, whereas lower respiratory tract infections such as
pneumonia and bronchitis were particularly frequent in both
the young and the old. Absenteeism in the working population

through illness has also been monitored in the United Kingdom.
About one third of all absences from work were found to be
caused by respiratory diseases with higher figures in influenza
epidemic years. The consequent economic loss is enormous ,
both in lack of productivity and in the cost of medical care.
It has been calculated that about 10% of school children are
absent at any one time, respiratory infections being responsible
for about one third of the diseases keeping them at home.

In our hospitals and dispensaries in Pakistan respiratory
cases are usually registered as Pharyngitis, tonsillitis,
bronchitis or pneumonia but real etiological agents particularly
the viruses are seldom identified. Only some work in this
direction seem to have been done by Burney and Rehman (1963),
Burney & Malick (1965), Ahmad et al (1969), Ghafoor & Burney
(1981).

As the control of acute respiratory infections is more
complex than that of many other diseases due to involvement of
numerous pathogenic agents, the first research need for a
community control programme is a precise specification of the
nature and magnitude of the problem. This specification is
lacking in Pakistan. Hence it was necessary to collect and
analyze the data regarding different etiological agents.
Efforts have been made in this study to collect the data from
defined population.

LIST OF SCIENTISTS

Name:	Designation	%age of time
Dr. Abdul Ghafoor	Principal Investigator	25%
Dr. Sarfraz Ali	Co-Investigator	25%
Mr. M. Javed	A.R.O./Sr.Technical Asstt.	100%
Mr. Umar Farooq	Laboratory Technician	20%

EXPERIMENTAL PROCEDURES

Specimens were collected from the children upto five years of age attending the outpatient/indoor ward of Central Government Poly Clinic, Islamabad, Rawalpindi General Hospital and other hospitals. Specimens were also processed for viral and bacterial isolations referred to National Institute of Health. Two throat or nasal swabs were obtained from each patient. One was put into a Bijou bottle containing 2 ml of viral transport medium. The viral transport medium was Hank's balanced salt solution with 0.2% bovine albumin. It was also attempted to collect blood specimens from the patients and sera separated in NIH and these were either processed freshly or stored at -20°C.

Processing of throat/nasal swabs/secreations.

0.1 ml of specimen was inoculated as follow;

2 tubes containing cultures of Hela

2 tubes containing cultures of Hela(OHIO)

2 tubes containing cultures of Hep-2

2 tubes containing cultures of human lung fibroblasts (MRC5)

2 tubes containing cultures of BHK

2 tubes of MDCK with trypsin

(Recently cultures of primary monkey cells have also been included).

In case of isolation for Influenza viruses fertile hen's eggs 10-12 day and 12-15 days old were also inoculated. All the cultures were incubated at 35°C on roller drums for 48-72 hours and were observed upto 2 weeks before declaring them as negative & in some cases it was necessary to repassage.

Cultures were observed daily for CPE for RSV, adenoviruses, enteroviruses, rhinoviruses, berpesviruses, Myxo and paramyxo viruses.

Chick Embryo Technique:

As mentioned above this technique was used for isolation of myxoviruses (Influenza). After incubation at 33°C for 72 hours the eggs were chilled overnight in refrigerator and the allontoin and amniotic fluids were collected separately. Spot test for haemagglutination was performed for identification of Influenza virus. On tubes containing MDCK and Primary Monkey kidney cells haemagglutination was performed using 0.4% Guinea pig red blood cells.

Specimens showing cytopathogenic effect (CPE) on the various cells were preserved and a presumptive identification was made on the type of cytopathogenic effects. Methods recommended by Hsiung (1973) and Jackson & Muldon (1978) were adopted for identification of various viruses and these are as follow.

Adenoviruses:

Grape like clusters on Hela, Hep-2C were observed. Similar CPE also occured on Monkey kidney cultures. However CPE was delayed in MK cell cultures. Since adenoviruses are cell associated, both infected cultures and fluids were harvested. This was done by 2 to 3 cycles of freezing and thawing the infected cultures to release virus from infected cells. In some cases subcultures were also made by infecting Hep.2-C cells. Preliminary identification was done by haemagglutination using monkey and rat R.B.C. The test was done as follow:

1. Using the infected tissue culture fluid two fold serial dilution (1:10 to 1:640) in PBS was made. An amount of 0.4 ml was taken in tubes.

2. An amount of 0.4 ml of a 0.5% suspension of Rat R.B.C. was added to each dilution of virus fluid. R.B.C. were allowed to settle for 1-2 hours at 4°C or 22°C.

3. Virus titre was read by determining the highest dilution of tissue culture fluid capable of causing partial or complete haemagglutination(This represented one HA unit).

Complement Fixation Test:

All known adenovirus types have a common group antigen(s). Hence priliminary identification of adenoviruses was made with this test. Methods of standardization of reagents, including Veronal buffer, hemolysin and complement alongwith the procedure done are laid down as under:-

Preparation of Hemolysin dilutions and sensitization of Erythrocytes:

Sensitization was done by treatment of sheep RBC with the homologous antibody. The antibody (hemolysin) was prepared from rabbits immunized with sheep RBC. A stock solution of hemolysin was prepared at a 1:100 dilution by adding 2 ml of commercially made hemolysin in glycerol to 98 ml Veronal buffer (formula of veronal buffer is given below). The stock was kept at $-20^{o}C$ in small aliquotis.

1. From the stock 1:100 solution of hemolysin a dilution of 1:1000 was made, then serial dilutions of hemolysin were made by using Veronal buffer.

2. An equal volume of a 2% washed RBC suspension was added to each dilution of hemolysin.

3. Mixture was incubated at $37^{o}C$ for 30 minutes for sensitization of RBC.

Determination of Complement Units.

Normal g.pig sera was used as a source of complement. A 1:10 stock solution of complement was prepared in (VB) & was kept in ice bath during period of test.

1. From a stock solution of 1:10 of complement a dilution of 1:50 was made and then serial dilutions were prepared.

2. To each 0.2 ml of the various complement dilutions, an amount of 0.2 ml of sensitized RBC was added either with 2 units of hemolysin or with a serial dilution of hemolysin starting at 1:1000.

3. 0.2 ml of cold VB was added to each mixture and incubated for one hour at 37°C or overnight in the refrigerator.

4. The highest dilution of complement giving 100% hemolysis was considered as a full unit.

For identifying the viruses 2 full units of complements were used in the test.

Procedure of complement fixation test:

1. 2 fold serial dilutions of tissue culture fluid were made (1:2 to 1:64).

2. Similarly two fold serial dilutions of human antiserum in VB (1:16 to 1:128) were made (All sera were heat inactivated at 56°C for 30 minutes. Sera kept for too long were pretreated with kaolin as recommended by Spence (1960) & Hierbolzer (1969).

3. 0.1 ml of various dilutions of tissue culture fluid (antigen) were mixed with 0.1 ml serum (various dilutions of antiserum) and 0.2 ml complement containing 2 units.
Controls used were as follow:-

   0.1 ml antigen & 0.2 ml complement

    For serum control: 0.1 ml VB+

   0.1 ml serum + 0.2 ml complement

    For complement control: 0.2 ml VB+

   0.2 ml complement, with a back titration of the complement solution starting with 2 units of complement.

4. All mixtures were incubated in refrigerator at 4°C overnight.

5. On the following day these were kept at room temperature before the addition of sensitized RBC.

6. In the mean time sheep RBC were sensitized for indicator system by adding 2 units of hemolysin (1:4000) to equal amounts of sheep RBC (2%) and mixed thoroughly. After these were incubated at 37°C for 30 minutes.

7. 0.2 ml of the sensitized sheep RBC were added to each of the antigen antiserum mixtures, antigen control, serum control and complement control & incubated at 37°C for one hour.

8. Results were recorded as 0 to 4. 4$^+$ means no hemolysis.

   O-fixation indicated complete hemolysis.

Formula of veronal buffer (VB) solution:

Solution A.

NaCl	83.80 gm
NaHCO$_3$	2.52 gm
Sodium barbital	3.00 gm
D.Water	1000 ml

Solution B.

Barbital	4.60 gm
Mg Cl$_2$6H$_2$O	1.00 gm
CaCl$_2$ 2H$_2$O	0.20 gm
Hot d.water	500 ml.

After cooling solution A is added to B to make 2000 ml (5 x). It is sterilized by filteration.
To 200 ml of mixture added D.W to 1000 (1:5 dilution). Final identification of adenovirus was done by neutralization test. In this test type specific serum & Hep-2 cell culrures were used, as recommended by Lennette & Schmidt (1979).

Enteroviruses:

Enteroviruses were recognized by observing the cytopathogenic effect on Hep-2C & Hela cell culture. Hela (OHIO) are specially susceptible to rhinoviruses while Monkey Kidney tissue cultures are highly susceptible to polioviruses. New borne mice were also inoculated (1/p) for

for isolation of Cexsackie viruses. (However coxsackie viruses could not be isolated during the period).

Rhinoviruses are inactivated when incubated at 37°C for 3-4 hours at pH 3. Acid lability is the principal characteristic that distinguishes rhinoviruses from enteroviruses ((Tyrrell,1960, Dimmock & Tyrrell,1962, Kisch et al,1964).

For identification of rhinoviruses pH stability test was done. The method was as follow:

1. To 5 ml of 0.2 M Tris buffer solution(pH 7.4) a few drops of concentrated HCl were added so as to reach a pH of 3.0. The solution was diluted to a final volume of 20 ml with Distilled H$_2$0.
2. 0.2 ml of virus suspension (specimen under process) was added to 1.8 ml of diluted buffer, pH 3.0. As a control, 0.2 ml of the virus suspension was added to 1.8 ml of stock Tris buffer, pH 7.4.
3. Mixtures were kept at room temperature for 3 hours.
4. A serial 10 fold dilution of the two mixtures was made & inoculated each into 2-3 culture tubes of Hela cells.

As the rhinoviruses are not stable at an acid pH, identification was made with the above mentioned test and the infectivity titres of a rhinovirus suspension treated at pH 3.0 were 2 to 4 logs less than virus suspension kept at pH 7.4. In contrast to rhino-viruses, the enteroviruses are stable at the low pH. Since the rhinoviruses are ether-resistant, test for ether stability was also performed.

The enteroviruses showing pH susceptibility were classed as rhinoviruses and no further attempt was made to classify as this is a highly specialised work which is beyond the scope of this project.

The enteroviruses resistant to acid pH were identified as possible ECHO. Further typing has not yet been done because the neutralizing sera were not available and have recently been received through WHO.

Myxoviruses:

Mainly Influenza A & B were expected. These were identified by Haemagglutination-Inhibition method.

Haemagglutination - Inhibition test:

The HA antigen titration was done to know optimally reactive pH and approximate titre of the preparation. The sample was then tested against known antisera.

1. 2 fold dilutions of serum were prepared.
2. HA antigen was diluted to contain 4 units per 0.2ml. The dilution necessary to give this number of units was calculated from the titre at pH showing the highest HA activity.
3. 0.2 ml of the virus antigen containing 4 HA units was added to 0.2 ml of each dilution of serum. It was incubated at room temperature for one hour.
4. A 10% suspension of 1 day old chicken erythrocytes was diluted to 1:20 in the adjusting diluent that yielded optimal pH for HA activity. 0.4 ml of diluted RBC suspension (0.5%) was added to each virus antibody mixture.
5. Three controls were prepared as follow:
    Serum control: Serum plus diluent & RBC,
    Cell control:- Diluent plus RBC
    Antigen control: Titration of the diluted HA preparation was done for verifying the exact number of units employed.
6. All the controls & test & hamologens titrations were incubated at 37°C for 60 minutes. The end point titres were read as the highest dilution of serum completely inhibiting the agglutination of 4HA units.

Pseudomyxoviruses(Paramyxoviruses).

Respiratory syncytial virus: The bronchial secre-tions/ throat swabs were pressed in VTM/PBS & centrifuged. The deposit was taken and put into slide and Indirect Immunoflorescence ~~was taken and put into slide and Indirect Immune~~ was performed using the sera and respective conjugates and paramyxoviruses.

The technique has recently been introduced and it needs special precautions for the collection of specimens.

Sero-diagnosis: Where acute and convalescent phase sera and the antigens of ECHO, R.S., adeno & paramyxoviruses were available, Micro CFT were attempted.

R E S U L T S

Table - 1

Specimens collected from July,1981 to June,1982.

Total Number of specimen collected	=	1354
Positive for Viral agents	=	447 (33.01%)
Positive for bacterial agents	=	701 (51.77%)
Specimens under process	=	206 (15.21%)

TABLE -2

Various viral organisms isolated.

Name of Viruses	No. Isolated	%age
ECHO 11	11	4.92%
Rhinoviruses	48	30.18%
Adenoviruses	41(to be typed)	25.78%
Influenza virus (strain A/Bangkok/2/79 H_3N_2)	24	15.09%
Polioviruses	4	2.51%
Paramyxoviruses (Respiratory Syncytial Virus)	26 (to be typed)	16.35%
Herpes virus	5	3.14%
Unidentified Enteroviruses (and others in process of identification)	288	64.42%

During the period under study 1354 specimens were collected. Table 1 & Fig. 1 shows various collections. Most of the samples were inoculated into various media in a fresh state while only few were preserved in VTM. 447 specimens were processed for viral organisms. Out of these 159 isolates have been identified which are given in table 2. Polioviruses were possibly a contamination from vaccine, however it remains to be established whether they were vaccine strains or not. Only 5 isolates of Herpesvirus were identified. Clinical symptoms associated with prevalent viral respiratory diseases are given in table 3. Some specimens were preserved and isolations shall be made on arrival of antiseras from WHO Collaborating Laboratories & availability of Primary Monkey Kidney Cultures.(A few antiseras have been received and monkey kidney is also under culture).
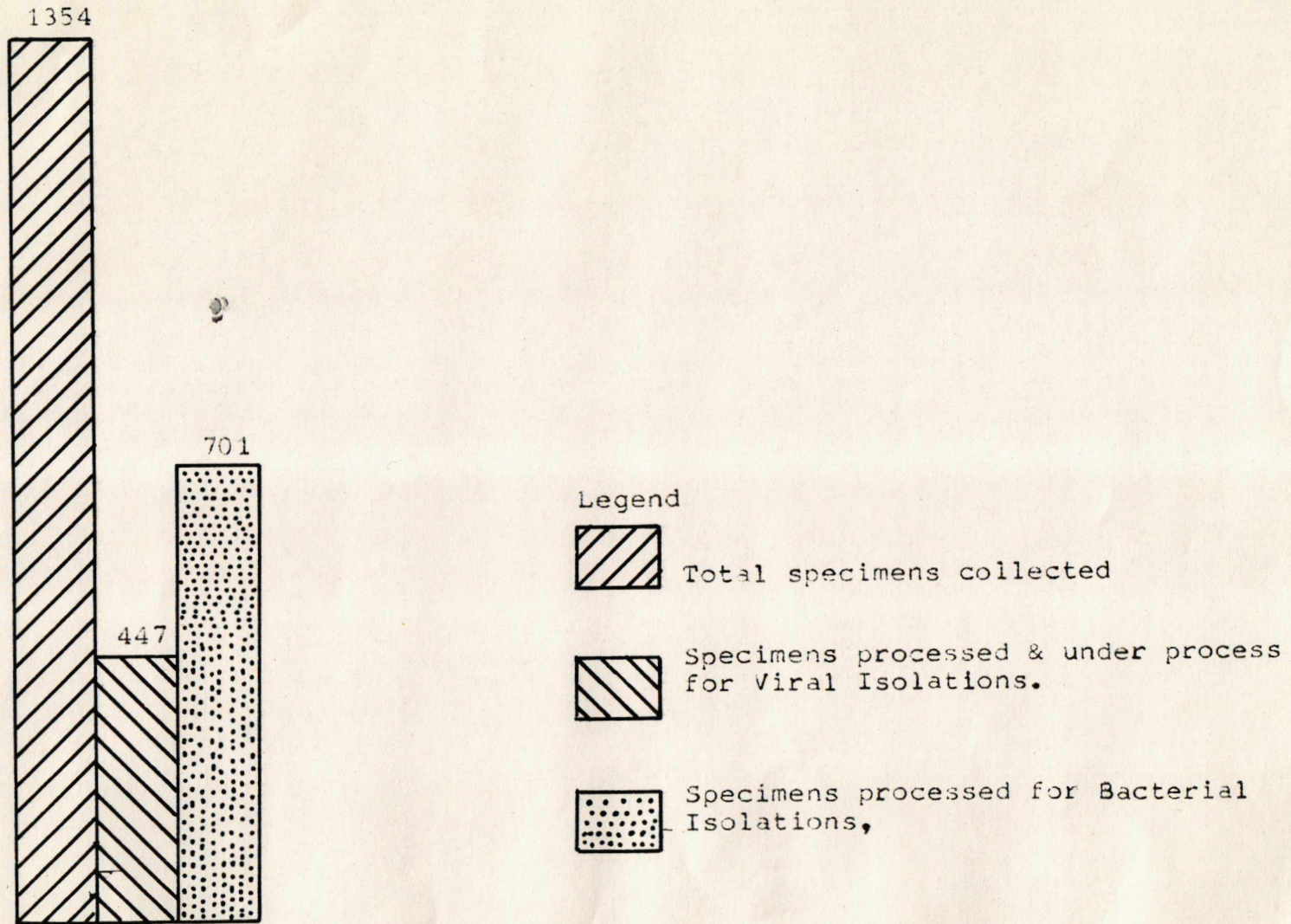
Fig. 1.   Specimens collected & processed.

TABLE - 3

Some Clinical Symptoms Associated with Respiratory Viruses

Name of Viruses	Symptoms				
	Common cold	Sore Throat	Croup	Bronchitis	Pneumonia
Influenza	+	++			
Rhinoviruses	+++			+	
Echo 11		++	++		
Enteroviruses		++			
R.S. Viruses		+	+	+	+
Adenoviruses	+	++		+	+

TABLE - 4

Bacterial Agents Isolated

Name of Bacterial Agent		No. isolated	%age
B-haemolytic streptococci	Group (A)	52	7.41
	Group (B)	18	2.56
	Group (C)	16	2.28
	Group(G)	16	2.28
	Total:	102	14.55%
∝-haemolytic streptococci		405	57.77
Staph coagulase+ve		69	9.84
Klebsiella species		29	4.13
Pneumococci		87	12.41
H. influenza		5	0.71
Proteus species		2	0.28
Candida species		2	0.28

TABLE - 5

Drug sensitivity test against B-haemolytic streptococci.

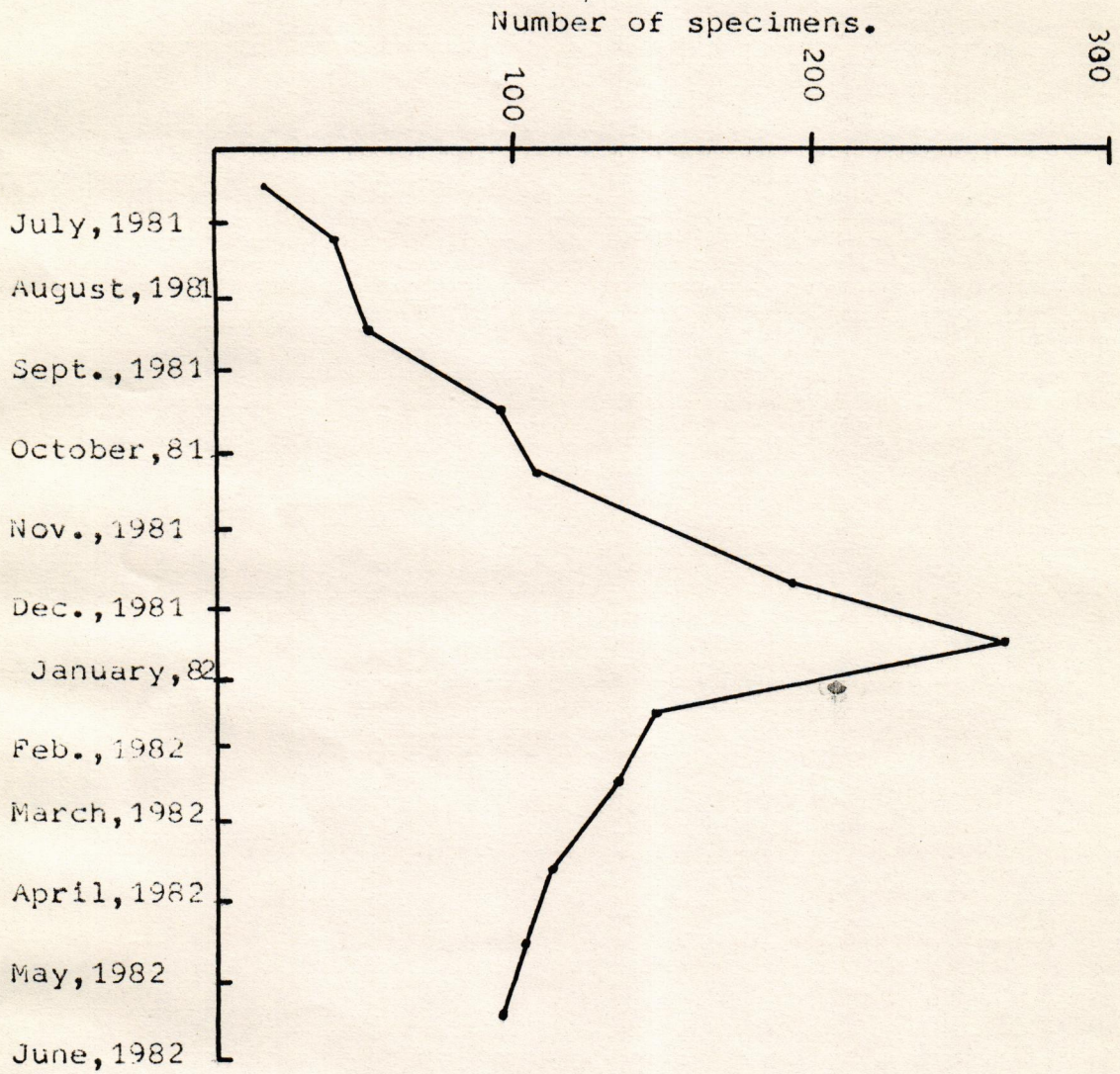| Name of Drug	Specimens tested	Specimens showing sensitivity	%age	Specimens showing Resistance	%age
Amoxil	100	95	95%	5	5%
Ampicilline	90	85	94.44%	5	5.56%
Ampiclox	95	90	94.74%	5	5.26%
Cephalotin	10	10	100%	-	0.0%
Cloxallin	15	15	100%	-	0.0%
Erythromycin	100	100	100%	-	0.0%
Lincocine	55	45	81.82%	10	18.18%
Septran	65	65	100%	-	0.0%
Streptomycin	60	35	81.82%	25	41.67%
Tetracycline	85	60	70.59%	25	29.41?
Vibramycin	60	60	100%	-	0.0%

TABLE - 6

Monthwise isolation of respiratory specimens.

Number of isolation in July,1981 = 16

August,1981 = 34

September,1981 = 50

October, 1981 = 97
November, 1981 = 114
December,1981 = 189

January,1982 = 266

February,1982 = 144

March, 1982 = 131

April,1982 = 114

May, 1982 = 104

June,1982 = 95

701 specimens were processed for bacterial isolations. Different bacterial agents possibly complicating a primary viral infection are given in table 4. Most prevalent bacterial organisms found were $\times$- haemolytic streptococci. The incidence was 57.77%. There were 52 isolates (7.41%) of B. haemolytic group (A). These are highly pathogenic and responsible for Rheumatic fever. B-haemolytic group (B), group (C) & group (G) ranged 2.28%, 2.28% & 2.56% respectively in prevalance. In all there were 102 isolates of B-haemolytic streptococci (14.55%). These isolates were also tested for drug sensitivity. The results are given in table 5. Erythromycin, Cloxallin and Vibramycin showed 100% sensitivity while tetracycline and streptomycin were found resistant by 29.41% and 41.67% respectively.

Table 6 shows monthwise collection of specimens while fig. 2 represents seasonal trend of respiratory affections in Rawalpindi/Islamabad area.

Number of specimens.

100

200

300

July,1981

August,1981

Sept.,1981

October,81

Nov.,1981

Dec.,1981

January,82

Feb.,1982

March,1982

April,1982

May,1982

June,1982

DISCUSSION

Acute respiratory disease is not a simple problem. It is not merely a viral, bacterial social or environmental problem but at various times & in different populations various factors interact in various ways. Data collection is the basic necessity and for this a standard set of virological and bacteriological techniques should be available to assist in identifying the role of the common viral and bacterial pathogens in the community. In this study various techniques successfully adopted have been described. While in this study some clinical data was also collected, the laboratory data is encourging and has formed a baseline for further studies with various techniques given in the report. Rhinovirus infections (common colds), Respiratory syncytial virus & Adeno-viruses are the most common acute infections recorded in this study. There were a few isolates of Echo-11 & Influenza virus. The strain of Influenza Virus (strain A/Bangkok/2/79 H_3N_2) was isolated for the first time & hence is of much significance. There were only 4 isolates of poliovirus which were suspected to be contamination of vaccine. As very judicious antibiotics are used in all respiratory illnesses due to lack of laboratory facilities the present study may help in identifying the respiratory affections & recommending appropriate antibiotics in respiratory illnesses particularly where bacterial complications have started. It will not be out of place to mention here that Influenza is the main example of respiratory disease for which effective vaccines have been produced and are currently available. In theory, at least mass immunization against influenza should prevent epedemics or halt their spread. Immunization on such a scale has never been achieved. The nearest approach to this objective was the compaign in 1976 against swine influenza virus in the USA. This programme was the largest mass vaccination programme ever undertaken in the USA (Cooper & Miller 1976, Voller & Friedman 1978). The more

or less continuous process of antigenic drift, which is the gradual alteration in the antigenic constitution of a viral strain and the occassional appearance through antigenic shift, of a mutant constituting an essentially new virus, make it necessary to keep influenza vaccines upto date through incorporation of currently active viruses. A number of field trials have shown that vaccines so constituted are highly effective (70-90%) in protecting against disease (WHO Technical Report Series No.642). So far such vaccines have not been used a large scale. However for doing any such attempt prevalent strains must be known and this study seems to provide maximum information about the prevalent respiratory viruses. Although at this state vaccine production in this field is not recommended but in late stage the baseline date will prove to be much benificial in this direction.

There is multiplicity of rhinovirus immunotypes. At present there are 110 distinct immuno-types(Jackson & Maldoon 1975). These may give rise to the common cold syndromes of rhinorrhoea, obstruction of the nasal passages, sneezing, pharyngeal discomfort and cough. The problems involved in preparing a vaccine containing all known immunotypes seem xixkwa virtually insurmountable, since data derived from virus isolations and serological studies indicate that rhinoviruses have a cosmopolitan distribution (WHO Technical Report No.642, 1980). In this study it was also found highly prevalent.

To help solve the problem of specific prophylaxis it has been assumed that certain immunotypes may be present, or may circulate, within given geographical areas. These would be candidates for incorporation into a vaccine. Hence significance of such isolation in our country is evident for future attempts for making a vaccine.

As regards bacterial isolations useful   study has been done on drug sensitivity. Drugs which can safely be used against B.haem. streptococci infection are Erythromycine, Cloxallin, Vibramycin etc.

C O N C L U S I O N

Throat and nasal swabs from 1354 cases were collected in the period under study. Out of these 701 were positive for bacterial agents while 447 were found positive for viral agents. Paramyxoviruses, adeno & Rhinoviruses were prevalent. Influenza virus strain A/Bangkok/2/79 H_3N_2 was isolated.

Drug sensitivity test was also done for B-haemolytic Streptococci.

F U T U R E   P L A N

In addition to collection of samples from various hospitals, survey will also be carried in selected schools to assess the extent of absenteeism & isolation of various viral & bacterial agents.

PUBLICATIONS

Paper under preparation

GRADUATE DEGREES.

NOT APPLICABLE.

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