# Molecular Epidemiological Study of Human Adenovirus in Children with Acute Flaccid Paralysis and Gastroenteritis in Pakistan


**By**

**Zainab Khattak**

**Department of Microbiology**

**Faculty of Biological Sciences**

**Quaid-i-Azam University**

**Islamabad, Pakistan**

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**By**

**Zainab Khattak**

**Department of Microbiology**

**Faculty of Biological Sciences**

**Quaid-i-Azam University**

**Islamabad, Pakistan**

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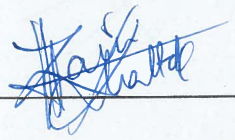
Student / Author Signature: 

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This is to certify that the research work presented in this thesis, entitled titled **"Molecular Epidemiological Study of Human Adenovirus in Children with Acute Flaccid Paralysis and Gastroenteritis in Pakistan"** was conducted by **Ms. Zainab Khattak** under the supervision of **Dr. Aamer Ali Shah.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

Student Name: **Ms. Zainab Khattak** Signature:_____

**Examination Committee:**

a) External Examiner 1: Signature:_____

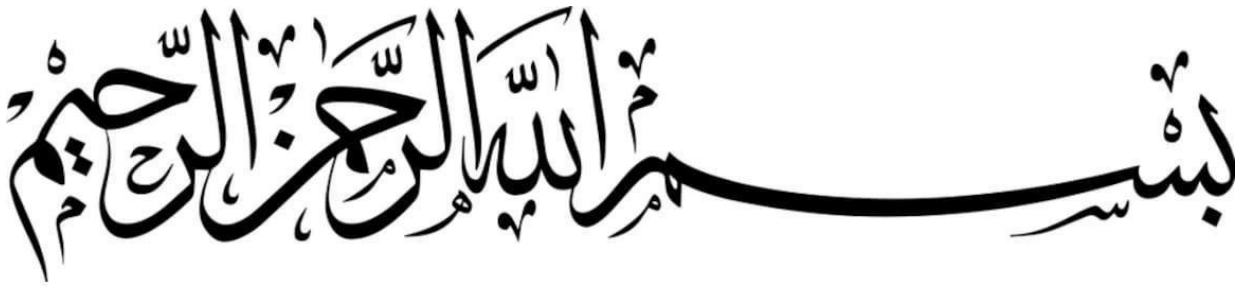
**Dr. Ghazala Kaukab Raja**
Professor & Director University,
Institute of Biochemistry & Biotechnology,
PMAS Arid Agriculture University,
Murree Road, Rawalpindi.

b) External Examiner 2: Signature:_____

**Dr. Allah Nawaz**
House No. 8 (F.F), Street No. 17-B
F-Block, Naval Anchroge, Islamabad

Supervisor Name: **Dr. Aamer Ali Shah** Signature:_____

Name of HOD: **Dr. Aamer Ali Shah** Signature:_____



IN THE NAME OF ALLAH, THE MOST GRACIOUS AND THE MOST MERCIFUL

# MY LORD!

# INCRESASE ME IN KNOWLEDGE

# (SURAH TA-HA, AYAH 114)

# DEDICATION

*I dedicate this thesis in the memory of*

***My late father and my mentor Mr. Ghazi Marjan Khattak,***

*A strongest person and gentle soul who taught me to trust in Allah (SWT), believe in hard work and that so much could be done with little. I love you and still miss you every day*

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| | |
|---|---|
| ANOVA | Analysis of Variance |
| BLAST | Basic Local Alignment Tool |
| Bp | Base Pair |
| CHL | Children Hospital Lahore |
| MHL | Mayo Hospital Lahore |
| KGH | Kharadar General Hospital |
| NICH | National Institute of Child Health |
| BBH | Benazir Bhutto Hospital |
| HAdV | Human Adenovirus |
| AFP | Acute Flaccid Paralysis |
| PB | Punjab |
| SD | Sindh |
| BA | Baluchistan |
| KPK | Khyber Pukhtunkhwa |
| GB | Gilgit Baltistan |
| AJK | Azad Jammu & Kashmir |
| FATA | Federally Administered Tribal Areas |
| HEP-2C | Human Epithelial Cell Line |
| MEM | Minimal Essential Medium |
| CPE | Cytopathic Effect |

| | |
|---|---|
| PCR | Polymerase Chain Reaction |
| L1 | LOOP 1 |
| L2 | LOOP 2 |
| NGS | Next-Generation Sequencing |
| qPCR | Quantitative PCR |
| EIA | Enzyme Immunoassay |
| PCF | Pharyngoconjuctival Fever |
| ARD | Acute Respiratory Disease |
| HCT | Hematopoietic Cell Transplantation |
| EKC | Epidemic Keratoconjunctivitis |
| CNS | Central Nervous System |
| WHO | World Health Organization |
| GPEI | Global Polio Eradication Initiative |
| EPI | Expanded Program on Immunization |
| DNA | Deoxyribo Nucleic Acid |
| dNTPs | Deoxy nucleoside tri phosphate |
| hrs | hours |
| MEGA | Molecular Evolutionary Genetic Analysis |

# MOLECULAR EPIDEMIOLOGICAL STUDY OF HUMAN ADENOVIRUS IN CHILDREN WITH ACUTE FLACCID PARALYSIS AND GASTROENTERITIS IN PAKISTAN

## SUMMARY

Infectious diseases are among the leading cause of disability and mortality in Pakistan. Epidemics of new and old infectious agents appear from time to time, having a negative impact on people and magnifying the burden on the economy. In Pakistan, among major causes of mortality in children less than five years, diarrhea contributes 11% to the total mortality rates. Before reaching age five, one in every eleven children in Pakistan dies. Post neonatal deaths are due to diarrhea, pneumonia, meningitis and sepsis. WHO collaborated rotavirus surveillance program screen the diarrheal samples for rotavirus but no system presents in country for other diarrhea causing viruses. In addition to this, Pakistan also harbors the highest numbers of poliomyelitis cases in the world, threatening global eradication goals. Surveillance for poliomyelitis relies on investigation and reporting of children who develop acute flaccid paralysis (AFP). However, the country making tremendous efforts for the eradication of polio with the help of its extensive AFP surveillance activities. But a recent report indicates the increased in number of non-polio AFP cases from 2018 (14 per 100,000 children aged < 15 years) to 2019 (16.2 per 100,000 children aged < 15 years) which highlighted the role of other infectious and non-infectious agents that may be responsible in causing AFP. Pakistan is still struggling to cope with AFP cases, which are caused by agents other than poliovirus, but among these agent's adenovirus is ignored. In our country, there is no system present for the screening of non-polio AFP causing adenoviruses while there is data present from other countries that focused on the human adenoviruses responsible for causing AFP. However, the infectious morbidity and mortality is huge in country, Pakistan had no formal training programs in infectious diseases, and a tremendous shortage of individuals with the requisite skills to conduct independent research in infectious diseases affecting Pakistani children. In light of above mentioned facts, investigation of adenovirus prevalence among children with AFP as well as in diarrheal samples was then conducted with the aim of testing its association with the disease.

Acute flaccid paralysis (AFP) is an infrequent clinical syndrome includes a sudden onset of flaccid paralysis in one or more limbs in children less than 15 years old. Infectious and non-infectious agents, (e.g. metabolic disorders), trauma and metal toxicity as well as post-infectious autoimmune conditions (e.g. Guillain-Barre Syndrome) may be involved in the establishment of this syndrome in humans. The recognized viral etiologies of AFP including poliovirus, nonpolio enteroviruses, flaviviruses such as West Nile, St Louis, and Japanese encephalitis viruses, herpesviruses, adenoviruses, and others. AFP includes the participation of spinal cord or anterior horn cell ("non-poliovirus" anterior myelitis) which is considered to happen rarely.

For the study of AFP, 172 stool samples were collected from all over Pakistan under the framework of AFP surveillance activities for polio eradication program during 2017. All of these samples were polio and enteroviruses negative. So these samples were screened for the presence of human adenoviruses. Several genotypes of human adenoviruses were isolated from stool samples. The results of current analysis showed the presence of HAdV in a substantial portion of AFP cases, but then again more research is required to elucidate the function of adenovirus, however, conduction of such studies provide the basis for future comprehensive analysis in order to provide health authorities about the causes and patterns of AFP along with the statistics needed to avoid contact to or evade risk factors comprised in AFP cases and prevalence.

According to results, total 89/172 (51.74%) samples were confirmed for adenoviruses. Mostly children with age below 5 years (n= 83/ 89, 93.25%) were affected with the infection. In the study the detection rate of adenoviruses in AFP cases were found throughout year with male (n= 52/89, 58.42%), more affected then females (n= 37/89, 41.57%). The prevalence rate of adenoviruses for different provinces as Punjab (54.11%), Sindh (52.94%), KPK (50%), Baluchistan (50%), FATA (57.14%), GB (25%) and AJK (33.33%). The results of our study showed that the adenovirus genotypes were present with the occurrence rate as HAdV-A31= 24.24%; HAdV-A18 = 3.03%; HAdV-B3 = 9.09%; HAdVB7 = 12.12%; HAdV-C1 = 6.06%; HAdV-D19 = 6.06%, and HAdVF41= 39.39%. The phylogenetic analysis of adenovirus strain showed viral distinct lineages close genetic relation with the global strains. To the best of our knowledge, this is the first work that provide the comprehensive outline on prevalence, genetic diversity and molecular epidemiology of human adenoviruses strains present in stool samples of AFP patients.

Second part of the study includes gastroenteritis and diarrhea infection which are a major public health problem resulting in significant morbidity and mortality each year with substantial economic losses. Internationally, the principal pathogens of pediatric diarrhea have been the enteric viruses, particularly rotavirus. Few other childhood diarrheas related enteric viruses includes norovirus, human adenovirus (HAdV), human astrovirus, and sapovirus. Understanding of the pathogens that are responsible in producing diarrhea is precarious for prevention and management of the diarrheal diseases. Pakistan is at high risk of epidemics because of overcrowded cities, unsafe drinking water, inadequate sanitation, poor socioeconomic conditions, low health awareness and inadequate vaccination coverage. The diarrheal diseases contribute to the major disease burden under five along with pneumonia in Pakistan. Unlike vaccine preventable diseases like Polio, measles and maternal & neonatal tetanus, Pakistan does not have any formal responsive surveillance system to monitor the burden of diarrheal diseases and to respond to the outbreaks. In order to evaluate the association of adenovirus infections in the acute gastroenteritis cases, this study was carried out during 2017-2018 at five main hospitals of the country; Benazir Bhutto Hospital, Rawalpindi (BBH), Mayo Hospital, Lahore (MHL), The Children's Hospital, Lahore, Kharadar General Hospital (KGH), Karachi and National Institute of Child Health (NICH), Karachi among hospitalized patients having age less than five years with acute gastroenteritis. In these areas to our information, no research study highlighting the association of adenovirus in causing gastroenteritis had been conducted. Diarrheal ailments aided to a substantial source of illness and death particularly in the developing countries like Pakistan however, the standard data regarding the presence of adenoviruses related with gastro-enteric infections is not presently obtainable for Pakistan. The objective of this study is to help in better understanding of the clinical and molecular epidemiology of enteric adenoviruses related with acute gastroenteritis in hospitalized children in Pakistan.

Total 204/1,118 (18.24%) samples were found positive for human adenovirus. The prevalence of adenovirus in BBH was 13.90% with females (64.51%) were more affected then males (35.48%). The higher detection (45.16%) was observed in children with age $\leq 9$ months without any seasonal peculiarity. In MHL, 18.84% infection was found with increased rate (42.30%) in children having age $\leq 9$ months. Mostly males (53.84%) were affected than females (46.15%). The virus was found mostly in summer months. In CHL the detection percentage for adenovirus was 32.07%). Mostly

children (68.62%) having age ≤ 12 months were affected with higher rate of detection found in winter. Males were more (70.58%) affected as compared to females (29.41%). In case of KGH, 12.08% were found positive for adenoviruses with females (54.54%) more infected than males (45.45%). Increased detection (81.81%) was observed in children with age ≤ 18 months without any seasonal peculiarity. In NICH, the prevalence rate for adenovirus was 17.8%. Increased detection (60.8%) was found in children with ≤9 months of age. Males (63.5%) were more infected with virus then females (36.5%). The detection of virus was found more in winter.

Similarly, the co-infection rate of adenovirus with rotavirus was also studied which was found as 26.76%. In this study, the incidence of viral co-infection was more in male cases comparative to females without any statistical difference present among them. According to our results, children with co-infection did not that much differ from those with mono-infection with regard to clinical parameters.

The molecular analysis of adenovirus positive cases revealed the presence of several genotypes in the diarrheal cases. Genetic characterization of adenoviruses indicated a huge diversity in Pakistani population. The prevalence of these diverse genotypes pose the great impact of the variants to cause disease severity in pediatrics population. The detected genotypes include HAdV-B3, B7, C2, D28, F40, and F41. Among these types, HAdV-F40 was isolated with increased frequency followed by HAdV-F41. The phylogenetic analysis of adenoviruses showed viral lineages with close genetic relation with the global strains.

Our findings confirm the earlier findings that Pakistan is among one the five countries where diarrheal morbidities is considerably high and advocate implementing robust surveillance system for gastro-enteric infections in Pakistan with the immediate introduction sound preventive measures. The presence of adenovirus in diarrheal samples other than rotavirus increases the importance of other viruses to be associated with diarrheal infection, which needs to be consider seriously on government level. Similarly, presence of adenoviruses in AFP cases which are poliovirus and other enterovirus negative must be taken into consideration and furthermore explore its presence in the samples with the association to the disease.

In conclusion, our results may describe the association of adenoviruses in AFP as well as diarrheal infections in Pakistan. The results of this study provide the epidemiological picture of adenoviruses in cases of AFP and gastroenteritis. These outcomes are constant with the reports of other studies that signifies the prevalence of adenoviruses in AFP and gastroenteritis. However, additional molecular and clinical analysis is needed.

## *Chapter 1* INTRODUCTION

Human adenoviruses (HAdVs) can produce range of ailments that includes the conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia. Young children having age less than 5 years are usually susceptible with mostly self-limiting and mild infections of gastroenteritis and respiratory infections or a combination of both (Walls *et al.*, 2003). Among immunocompromised individuals, adenoviruses have progressively been known as important viral pathogens with increased morbidity and mortality in the last years. This statement can be related with the progression of population of immunocompromised individuals, particularly in case of acquired immunodeficiencies and increased antagonistic interferences along with the advancement of further subtle diagnostic approaches, and with the enhanced alertness of this virus as a pathogen (Echavarría, 2008).

The immunocompromised individual can be defined as the one with incapability of developing a normal immune response against diseases. These incapibilities or immunodeficiencies may be due to congenital reasons and produced by defects in the gene, are hardly detected. These can be classified into humoral, cellular, or combined immunodeficiency. In comparison to congenital, the acquired immunodeficiencies are recurrent but they are not produced by inherent defects during the growth or function of T and B cells. This compromised immunological reaction is taken as an advantage by adenoviruses (AdVs) and hence produced the acute or constant infections, that directs to increased sickness or even deaths among patients (Echavarría, 2008).

For adenovirus infection, the risk factors include are the young age i.e. the child becomes 2 to 3.5 times more susceptible to be infected as compared to adults), allogeneic transplantation, T-cell reduction, distinct or HLA (human leukocyte antigens)-unmatched grafts, entire body radiation, and reduced T-cell count afterward transplantation (Van Tol *et al.*, 2005). As pediatric population are more probable to endure any primary infections or recurrence and have immature intestinal immunity, they are more exposed to attack by the virus (Echavarría, 2008).

Two main factors play role in increasing the attention in HAdVs. These includes the insistent HAdV infections in immunocompromised adults and children which are being progressively connected to their illness (Ghebremedhin, 2014); and use of HAdV as a vectors which are efficient gene transfer tools and are employed for clinical purposes of gene transferring (http://www.abedia.com/wiley/vectors.php). But the use of these vector is dangerous as the virus can possibly

undergone recombination with wild type virus, produce mobilization (in which the vector infected cell becomes replication competent HAdV infected cell that results in the intensification and dispersion of the replication-defective vector), hepatic lesions, thrombocytopenia, neutropenia, systemic inflammation, and fever (Harmon & Byrnes, 2017; Mennechet *et al.,* 2019).

Noteworthy, in overcrowded places, epidemics of HAdVs have been described universally (Lion, 2014). High rate of infection arise in winter and spring, however it persists as common during the whole year (Veltrop-Duits *et al.,* 2011). The normal incubation period of the virus lasts for 5–12 days, however the infectious session persists for weeks to months (Choudhry *et al.,* 2016; Kuschner *et al.,* 2013; Radin *et al.,* 2014). In particular area, the leading HAdV types undergone alteration in certain time (Lin *et al.,* 2004) and frequently the dissemination of novel types through continents occurs (Abbas *et al.,* 2013; Lynch *et al.,* 2011; Zhang *et al.,* 2016). In the view of seeing the dynamics of sero-prevalence, the worldwide dispersal of HAdVs is chiefly significant (Mennechet *et al.,* 2019).

In addition to causing the conjunctivitis, pneumonia, gastroenteritis, and hemorrhagic cystitis, few strains of adenovirus are responsible to infect the tissues of the central nervous system (CNS) infrequently, which in turn produce aseptic meningitis, meningoencephalitis, and encephalitis. There are reports present regarding the infrequent cases or minor epidemics of neurological ailments after adenovirus infection (Baldwin *et al.,* 2000; Carter *et al.,* 2002; Echavarria *et al.,* 2001; Lema *et al.,* 2005; Metzgar *et al.,* 2005; Mori *et al.,* 2005; Pereira Filho *et al.,* 2007). In present study we discussed about gastroenteritis and acute flaccid paralysis (AFP) diseases that may be contributed by HAdVs.

Acute gastroenteritis (AGE) can be describes with the inflammation of the stomach or intestines which includes symptoms like diarrhea, dehydration, presence or absence of nausea, vomiting, fever, fatigue, and abdominal cramps. Among these symptoms of contagious gastrogastroenteritis, major one is the acute diarrhea. The definition of diarrhea by World Health Organization (WHO) is the episodes of three or above three loose or watery stools in a day. AdV is the second principal cause of mortalities among children. Virtually, in life span of every individual, he/she will encounter as a minimum of one incident of acute diarrhea. In the initial 2 years of life, international estimation showed the mean ratio of severe diarrhea from 3.5 to 7 episodes. This is because the gastrointestinal (GI) tract is more susceptible organ for virus to be attack as this organ is in

continuous connection with the outside environment through the oral route. As a result of this contact the inflammation of the stomach and the intestines (gastroenteritis) occur which in turn produce nausea, vomiting and diarrhea. Usually children in unindustrialized countries undergone this diarrhea. However, the immuno-compromised people of the industrialized countries are similarly affected. Higher disease burden of diarrhea related viral infections are identified in the developing countries due to poor hygiene conditions, absence of safe drinking water, overcrowded population, and unplanned urbanization (Golding *et al.,* 1997).

In developing countries, due to suitable discarding of sewage and safe drinking water, bacterial and parasitic gastrointestinal contaminations are deteriorating but these countries still have increased viral gastroenteritis. In the industrialized world, the most common pathogens of producing diarrhea are the viruses. Though since 1901, viruses were described to cause diseases in human but in 1972, they were termed to be causative agent of diarrhea because of the first discovery of virus causing gastroenteritis (norovirus) which came into picture in the outbreak of diarrhea in Norwalk (California, United States). Then in a bit of this discovery, several other viruses responsible for gastroenteritis were revealed (Oude Munnink & Van der Hoek, 2016).

In respect of time period and etiology, diarrhea is also classified as:

- ***Acute diarrhea:*** The time period for acute diarrhea is below than 2 weeks i.e. 14 days.
- ***Persistent diarrhea:*** It is above 2 weeks.
- ***Chronic diarrhea***: It remains for above 30 days.

In the context of its pathophysiological mechanism diarrhea can further be categorized:

- ***Osmotic diarrhea***: Due to insufficient presence of lactase, if there is improper resorption of bile salts or disaccharides in the small intestine than it will be osmotic diarrhea.
- ***Secretory diarrhea***: Excessive stimulation of the intestinal secretion is the secretory diarrhea with bulky amounts of watery diarrhea.
- ***Exudative diarrhea:*** It can be describing by the mucosal injury because of inflammation and possess blood, pus and proteins along with accretion of water and electrolytes in the lumen secondary to the hydrostatic pressure in blood and lymph vessels. It is the result of the invasive enteric infections e.g. *Shigella* infection. Typically, motility disturbance diarrhea produced from

the elevated level of intestinal motility; however, it can also result from low intestinal motility with secondary bacterial overproduction.

In developing countries, the yearly mortality ratio in children < 5 years old has lessened from 4.5 million to 1.8 million in last 20 years. This is because of the beginning of oral rehydration therapy (ORT), but the morbidity ratio is still above i.e. 2/3 in these countries. Hence, attainment of the UN Millennium Development Goal 4 (MDG-4) and the elongated-period target of decreasing the death rate of children to 20 deaths or less per 1000 livebirths among all countries by 2035 will demand considerable reductions in death as of the diarrheal illness. Globally in newborns and young children, rotaviruses are thought to be a main reason of severe gastroenteritis between various other enteric diarrheal viruses. In research findings, the link of other enteric viruses, for example caliciviruses, astroviruses, and enteric AdVs, with diarrhea has been accounted. Universally, frequently in Africa and South Asia the diarrheal ailments produce 1/10 child deaths that accounts for 800000 deaths; but in comparison Asia possess higher proportion of deaths i.e. 31% to Africa with 25% due to diarrhea. According to one study, 32% of the annual 7.6 million mortalities in children below age of 5 years are more concerted in South Asia (Walker, Aryee, Boschi-Pinto, & Black, 2012). Nevertheless, for precise estimation of flawless disease burden and chief causal etiologies, the data from this area is too rare (You *et al.,* 2010).

The report from global health survey conducted in 2008 showed that five countries endure the increased burden of diarrhea associated mortalities in < 5 year children which includes Pakistan, India, Nigeria, Democratic Republic of the Congo and China. Similarly, the report from the global survey of childhood mortality revealed that between the 18 countries of Eastern Mediterranean region, Pakistan possessed increased rates of <5 mortalities that accounts 464886 deaths (You *et al.,* 2010). The etiologies of mortalities due to diarrhea among the six distinct geographical counties was compared by Black (2010). According to him, 17% of deaths occur in the Eastern Mediterranean region, 4% in Western Pacific region, 5% in European countries, 7% in the America, 12% in the South East Asia and 18% in the African region. Hence, the most significant sources of childhood deaths are communicable illnesses comprising diarrhea was highlighted (Black *et al.,* 2010).

In a report that include data taken from 187 countries was used to compared the < 5 mortalities which showed that the rate was found elevated in Pakistan (80.3/1000 children). This rate was matched to neighboring India (62.6/1000 children), Nepal (47.3/1000 children), Bhutan (54.1/1000 children), Bangladesh (55.9/1000 children) and China (15.4/1000 children) (Rajaratnam *et al.*, 2010).

Adenovirus subgenus F (AdV F), representing AdV 40 and 41, has been identified to be related with the acute gastroenteritis and produce 1 to 20% of the worldwide diarrheal ailments in both un-hospitalized and hospitalized children (Reis *et al.*, 2016). Less frequently, serotype 31 can also be related with gastroenteritis. Principally young children having age < 2 years are mostly affected by AdV40 and AdV-41, which produces infection during whole year. The infection produced have symptoms of watery diarrhea go along with vomiting, low-grade fever, and mild dehydration. Protracted diarrhea i.e. having 8.6 and 12.2 days, duration is a distinctive characteristic of AdV40 and AdV-41 infections. The incidence of enteric AdV infections in childhood is reported to be between 3.1% to 13.5% in studies performed in Europe, Asia, Northern and Southern America. Children predominantly those having age between 6 months to 2 years, who take part mostly ingroup care, are at higher risk. Reinfection can occur. Gastroenteritis can frequently last 10-14 days. The proportion of enteric adenovirus 40 and 41 differs both in developed countries (1–8%) as well as in developing countries (2–31%) but in immunocompromised individuals, the frequency is higher (Sanaei Dashti *et al.*, 2016).

WHO define AFP as the abrupt start of weakness and floppiness in any body part in a child under 15 years of age or if a clinician figure outs polio in any individual of some age except adults, spastic paralysis, old cases or cases having clear reasons as trauma. It is a clinical disorder with abrupt production of respiratory and bulbar weakness. It also has group of diagnostic potentials that can be the consequence of infectious or non-infectious agents e.g. metabolic disorders, trauma and metal toxicity along with post-infectious autoimmune conditions (e.g. Guillain-Barre Syndrome (GBS)). Typically, the paralysis involves merely one leg or one arm though this happens less frequently but involvement of both legs and both arms to be involved is not as much frequent. The identification that usually perplexed it with polio are GBS, transverse myelitis, traumatic paralysis and occasionally other neurotropic viruses like Rabies, Japanese encephalitis. Likewise, in assessment of AFP cases the following critical diseases must be considered e.g.

polyneuropathy, metabolic disorder like hypokalemic periodic paralysis, polymyositis, neuropathies, neuromuscular junction disorders and tumors. The reasons of producing AFP are due to however are not restricted to, enteroviruses (including poliovirus), adenoviruses, acute west nile virus infection, campylobacter spp., transverse myelitis, peripheral neuropathy, acute non-bacterial meningitis, brain abscess, China syndrome, post-polio sequelae, tick paralysis, myasthenia gravis, porphyria and botulism. Several agents that are accountable for diverse cases of AFP cannot be identified merely by their clinical symptoms. At present, AFP is analyze as a part of the Global Polio Laboratory Network (GPLN) program, in countries where poliovirus remains prevalent (Odoom *et al.*, 2012).

In patients having acute myelopathies, AFP neurological symptoms, adenoviruses have been identified in the spinal cord and cerebrospinal fluid (CSF). Several HAdV-B types (HAdV-B3 and HAdV-B7), have been detected in acute myelopathies. AFP caused by adenovirus 21 is through anterior horn cell damage or neuropathy of the brachial or lumbosacral plexus. In Malaysia, from clinical samples, which includes serum, CSF, throat, cardiac muscle, brain and lung, of the AFP children who expired in an outbreak of hand, foot and mouth disease principally related with enterovirus 71 in 1997, species B adenoviruses were detected. This indicates the co-circulation or connection of these two viral agents in the epidemic. After this outbreak in Malaysian Peninsula, five more mortalities were reported in young children with same clinical symptoms. Afterward from the epidemic of 1997 the isolates were additionally characterized via neutralization, DNA restriction digest and nucleotide sequence of part of the hexon gene and the complete fiber gene. This characterization showed that adenovirus 21 was the responsible agent of those AFP cases. According to few reports, adenovirus 7 infection have been associated with incidences of acute transverse myelopathy and acute encephalopathy (Haddad-Boubaker *et al.*, 2019).

Childhood mortality in Pakistan is a major cause of concern as out of every 10 children one child dies before reaching the age of five and one among, just after they are born. The mortality rate of child in Pakistan is higher than 100 deaths/ 100,000 live births with nearly 74,000 deaths each year (Kawai, O'Brien *et al.*, 2012). Regardless of these miserable numbers, the country lacks any proper nationwide surveillance system for monitoring the diarrheal infections load and related hazards. For addressing the MDG-4 on reduction of childhood mortality, there must be presence of suitable population-relied data on the proportions and etiologies of neonatal and childhood

mortality. For effectual control of diarrheal illnesses, a complete laboratory built investigation and monitoring system is mandatory. This system also aids in provision of proof-based data for vaccine initiation in the country routine immunization program to lessen the infection load. In Pakistan, the number of below 5 years of age children is above 20 million. Out of total annual mortalities in Pakistan, nearly 50% were described in below 5 year's age children. Likewise, among the total sick population in Pakistan, nearly 3/5 (large fraction) are provided by the children. Since, 1990, Pakistan has been capable to lessen the death proportion for children by 15%, however it is still included in the countries having increased deaths in children. Based on the latest available data, very limited progress was made between 2013 and 2014 in the use of proven diarrhea interventions in the 15 countries in which Pakistan is the one with the highest-burden of child mortality (Alam *et al.,* 2015).

According to WHO report, the neurological illnesses accounting 6% of total Disability Adjusted Life Years and was respectively greater than tuberculosis, HIV/AIDS, malignant neoplasms, ischaemic heart disease, respiratory disease and digestive diseases. In Pakistan, the load of neurological syndromes is approximately 4-5% however pediatric neurology data is scare in Pakistan (Awan *et al.,* 2016). In a study conducted by Zaheer (2009) on all pediatric admittances to a main tertiary hospital, about 6.7% of children were hospitalized due to neurological ailment e.g. inherited / perinatal /progressive neurological syndromes (20%), CNS illnesses (13%), neoplastic complaints of CNS (16.9%), febrile fits (15%), seizure ailments (10.7%), and cerebral palsy (9.8%). 17.6% of hospitalized children mortalities are accounted because of neurological illnesses (Zaheer *et al.,* 2009).

The etiologies of viral gastroenteritis cases in Pakistan is not well identified because of the laboratory limitations, financial burden of such studies and underreporting. Although there are few studies present that somehow revealed the viral causes for gastroenteritis but these reports mainly focused on the rotavirus. Likewise, on norovirus occurrence in children hospitalized to Civil Hospital, Karachi, single study was conducted two decades before. Furthermore, just two studies in the 1990s and in 2013 was conducted on the astrovirus that concentrating on gastroenteritis patients from Karachi and Rawalpindi, correspondingly. In Pakistan, there is single report that have defined AdV ailment in children having gastroenteritis (40, 44); however, no other detailed hospital-based reports are present. Therefore, this current study showed the first detailed

examination of clinical implication and molecular epidemiology of adenoviruses that are related with acute dehydrating gastroenteritis in the children below the age of 5 years from Pakistan and to perform a genetic analysis. This study also analyzed the presence of human adenovirus in AFP children, which deliver the origin for additional study and interference. More, national database development is mandatory and archives on behalf of the etiologies of viruses other than polio causing AFP in Pakistan.

This study will be helpful in providing important information on:

1. Adenovirus epidemiology in Pakistan

2. Adenovirus genetic diversity

3. Interventions for control of adenovirus infections

**Aim**

To evaluate the molecular epidemiological study of HAdV in children with acute flaccid paralysis and gastroenteritis in Pakistan.

**Objectives**

1. Burden of human adenoviruses in children affected with paralytic and diarrheal infections

2. Genotype identification and phylogenetic analysis to assess the extent of human adenoviruses diversity associated with paralysis and diarrheal infections

3. Clinical significance of human adenoviruses genotypes in terms of severity and duration of clinical illness.

4. Co-infection of human adenoviruses with rotavirus to determine the effect in virulence through disease severity.

# *Chapter 2* REVIEW OF LITERATURE

## 2.1 History

In 1953 with the intention of discovering the agent responsible for "common cold" Wallace Rowe and Robert Huebner cultivated human tonsils and adenoids in tissue culture. Rowe observed that from adenoids tissue the cells originated altered their morphology and deteriorated ultimately (Rowe *et al.,* 1953). For this, the etiological agent was thought to be a virus. In 1954, Hilleman and Werner instantaneously identified an agent that most probably was a virus, however unlike an influenza virus that produced acute respiratory illness in armed staff (Hllleman & Werner, 1954). Consequently, the relation among these viruses was exhibited (Hllleman & Werner, 1954). Based on cell type where they were originally identified, in 1956 it was suggested that this set of viruses must be generally termed as "adenoviruses" (Hllleman & Werner, 1954). Key events that took place since the discovery of adenovirus is shown in Figure 2.1.

In virological research, this was the beginning of an age with immense attainments and impressions in perceiving together essential virological incidences. These actions include viral structure and its replication in addition to comprehending cellular events and the interaction among virus and cell and eventually the consequence on the entire organism. For instance, contrary to previous belief, in 1977, Phillip Sharp and Richard Roberts revealed that genes were not just parts of an elongated continuous segment of DNA but could comprise of numerous minor sections of DNA (Berget *et al.,* 1977; Chow *et al.,* 1977). All of these discoveries were made by utilizing adenovirus by way of a model organization because its genes have vital resemblances to advanced entities, comprising human. In the field of adenovirus, another significant incident is the utilization of adenovirus in gene therapy that possesses both scientific inference and vast medical influence (Wirth *et al.,* 2013). The following reasons supported the use of adenoviruses as vectors for gene delivery:

i) Provide a high gene expression.

ii) Without incorporation of viral DNA into the host genome, adenovirus can cause disease equally in dividing and non-dividing cells.

iii) The virus whole biology is properly implicated (Thomas *et al.,* 2003).

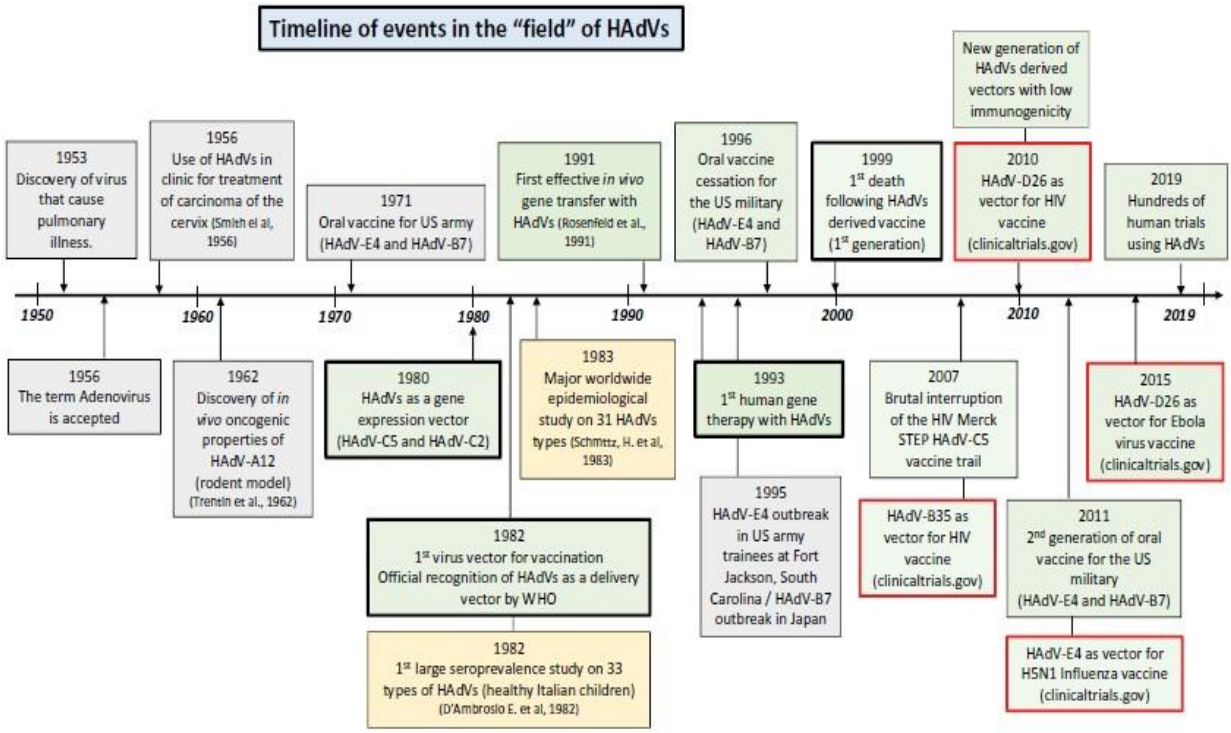



**Figure 2.1.** Key events in the adenovirus "field". The gray boxes indicate major findings on the etiology and pathogenicity. Green boxes indicate notable events involving adenovirus-derived vectors. The red boxes indicate the major epidemiological studies. The bold borders indicate important events or discoveries. The red bold borders indicate the initiation of some clinical trials involving vectors derived from "alternative or rare" adenovirus types.

## 2.2 Taxonomy and Classification

The International Committee on Taxonomy of Viruses (Robinson *et al.*, 2013) has categorized adenoviruses into the family *Adenoviridae*. The family is further grouped into five genera named as Atadenovirus (contains A and T-rich genomes and infects wide array of hosts), Aviadenovirus (causing disease in birds), Ichtadenovirus (at present just single class identified from sturgeon), Siadenovirus (causing disease in frogs and a few birds) and Mastadenovirus (ailing mammals). The virus is present in genus, Mastadenovirus. Initially the categorization of the various forms of virus was founded on its serological basis, hemagglutination arrangements and oncogenicity in rodents along with further biological aspects (Wirth *et al.*, 2013). Yet, there is debate on classification of adenoviruses that whether the forms must be categorized following whole-genome sequencing as an alternative. Various adenovirus types differ according to method of taxonomy (Robinson *et al.*, 2013).

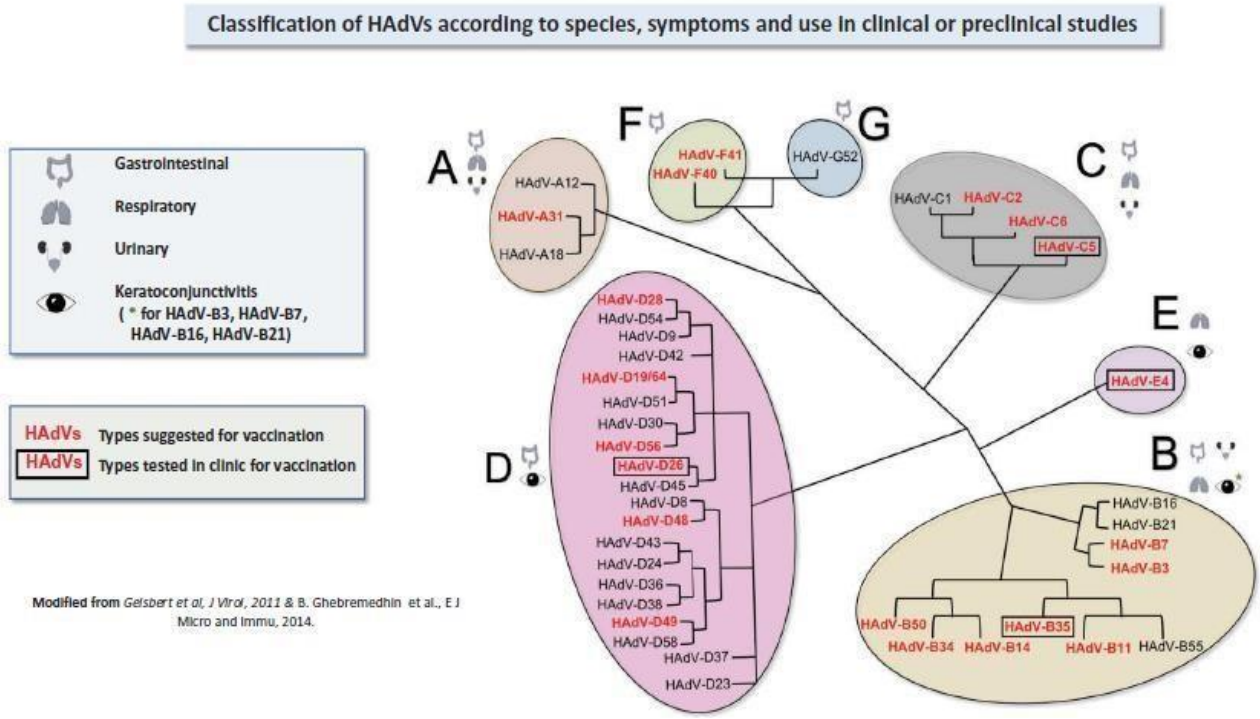



**Figure 2.2.** Adenovirus classification by species (A-G) and clinical symptoms. Types marked in red are vaccine vector candidates. Framed types have been tested in clinic.

## 2.3 Adenovirus Structure and Composition

Adenoviruses are non-enveloped, regular icosahedrons with 20 triangular surfaces and 12 vertices and is about 70-100 nm in diameter (Shenk, 2001; J Van Oostrum & Burnett, 1985; Jan van Oostrum *et al.,* 1987). Adenoviruses have 36 kbp genome (Richman *et al.,* 2009) and related proteins and for every 1000 nucleotides the virus has ~1 open reading frame (ORF). Viral DNA contains four internal core proteins these are Mu, VII, V, and terminal protein. By means of protein VII, protein V attaches the DNA to the capsid and the penton base. Protein μ is a minute protein that assists the DNA condensation and is firmly related with the DNA. The histone like protein (p) VII play role in protecting viral DNA from cellular DNA impairment reactions (Avgousti *et al.,* 2017; Karen & Hearing, 2011; Lischwe & Sung, 1977). The adenovirus capsid has 240 hexon capsomers and 12 penton capsomers, in conjunction with numerous minor capsid proteins (Figure 2.3). The minor capsid proteins comprise pVI, pIIIa, pVIII, and pIX that are vital for capsid constancy. The fiber knob attaches to one of the various host cell receptors in viral infection (Goosney & Nemerow, 2003; G. Nemerow *et al.,* 2009; G. R. Nemerow, 2000). Two hypervariable loops are present on penton base protein. In every penton base protein (five per penton base capsomer), the contact among fiber knob and host cell receptor causes secondary contact linking the hypervariable loop 2 (HVL2) arginine-glycine aspartic acid (RGD) motifs. These penton base proteins along with host cell integrin's avb3, avb5 and avb1 consecutively produce endocytosis of the virus (Li *et al.,* 1998; Li, Stupack *et al.,* 1998; Li *et al.,* 2000). The structural proteins of the virus performed several roles such as the minor capsid structural protein VI (pVI) which is important as a minimum in three diverse features of the viral "life" cycle. These are endosomal seepage access into the cell, nuclear assembly at the time of viral replication, and firmness of the integral, infective virus at host exterior (Moyer *et al.,* 2016; Moyer, Wiethoff *et al.,* 2011; Wiethoff *et al.,* 2005; Wodrich *et al.,* 2003). Three compactly connected hexon molecules made the hexon capsomere. Up till now adenovirus protease (adpol) is the last identified protein within the core and is important to make the virion infective (Weber, 1976). It furthermore performs important part in the ingress of the adenovirus into cells in addition to in the assemblage of new virion particles (Greber *et al.,* 1996; McGrath *et al.,* 2001).

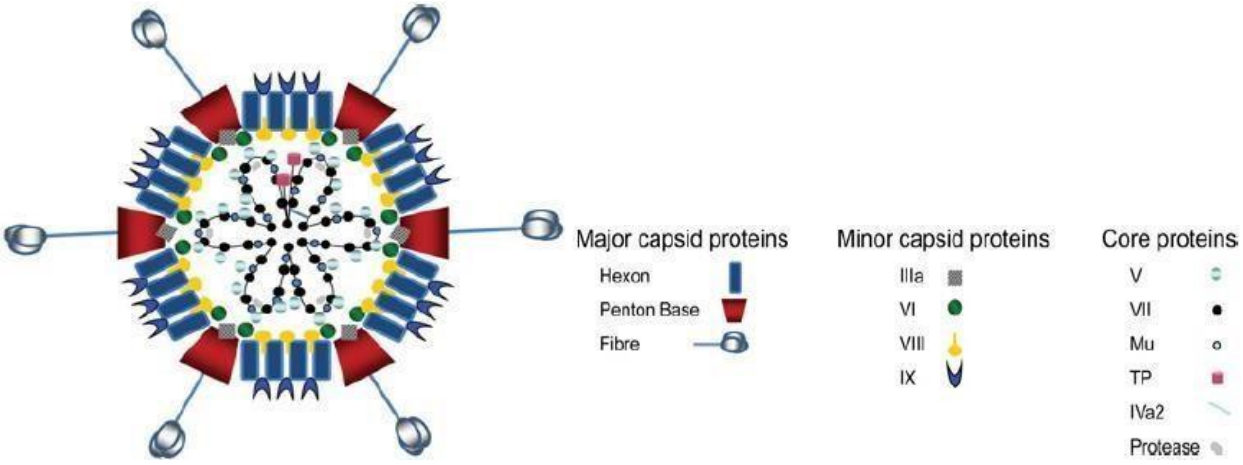



**Figure 2.3.** Capsid structure of human adenovirus

## 2.4 Capsid Proteins

### 2.4.1 Fiber

In 1965, the viral fiber was initially described in the AdV2 capsid (Valentine & Pereira, 1965). The fiber is made up of trimeric protein, and can be separated into a tail, a shaft and a head (knob) (Chroboczek *et al.*, 1995). Present one at each vertex of the virion, most of the mammalian adenoviruses contains 12 fiber trimers. Human adenoviruses belongs to subgroup F viruses (Ad 40 and Ad 41) have two fiber proteins. Fiber protein performs double well-implicit roles, that includes the structural function in the viral capsid and the contacts with cellular adenovirus receptors along with intracellular trafficking, endosomal discharge and virus maturation (Hong & Engler, 1991). The arrangement of the knob domain is like a trimer and encloses both conserved and variable domains (loops) (Van Raaij *et al.*, 1999) (Figure 2.4).

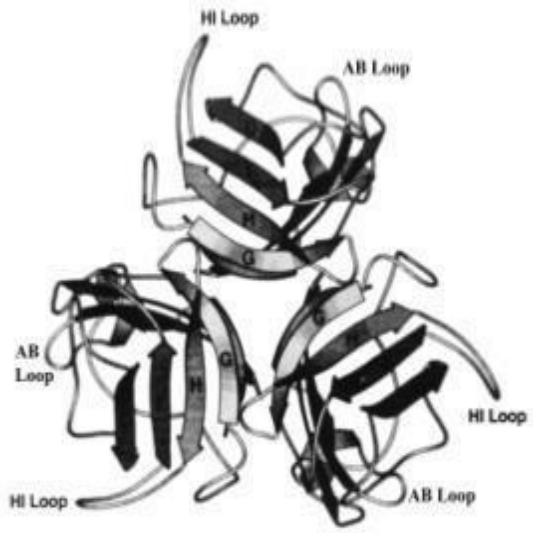



**Fig. 2.4.** Ribbon diagram of Adenovirus 5 knob viewed down the three-fold symmetry axis with the R sheet β-strands D, I, H and G faces the cellular receptor.

### *2.4.2 Penton Capsomere*

The penton is a heteromeric organization of a pentameric base and a trimeric fiber, made up of 5 subunits (Stewart *et al.,* 1991). The protein sequence of penton base is extremely conserved among AdVs of the identical subgroup, like between Ad2 and Ad5 of specie C have 98.6% homology (Neumann *et al.,* 1988). The characteristics of the penton base include two moieties with increase homology, which are divided by a non-homologous spacer part of different extension that encompasses the well-preserved RGD motif, liable for virus entrance (Belin & Boulanger, 1993).

### *2.4.3 Hexon*

The major capsid protein is the hexon protein having molecular mass of around 360 kDa with three similar subunits of app 120 kDa each (Cornick *et al.,* 1971). The general form of the hexon protein, which is trimeric protein, is atypical and is classified into pseudo-hexagonal base, which is affluent in β-structure, and a trilateral peak produced by three extensive loops having secondary structure (Athappily *et al.,* 1994). Among adenovirus serotypes the hexon exterior loops show the maximum alterations and have the majority of the type particular epitopes (Toogood *et al.,* 1989).

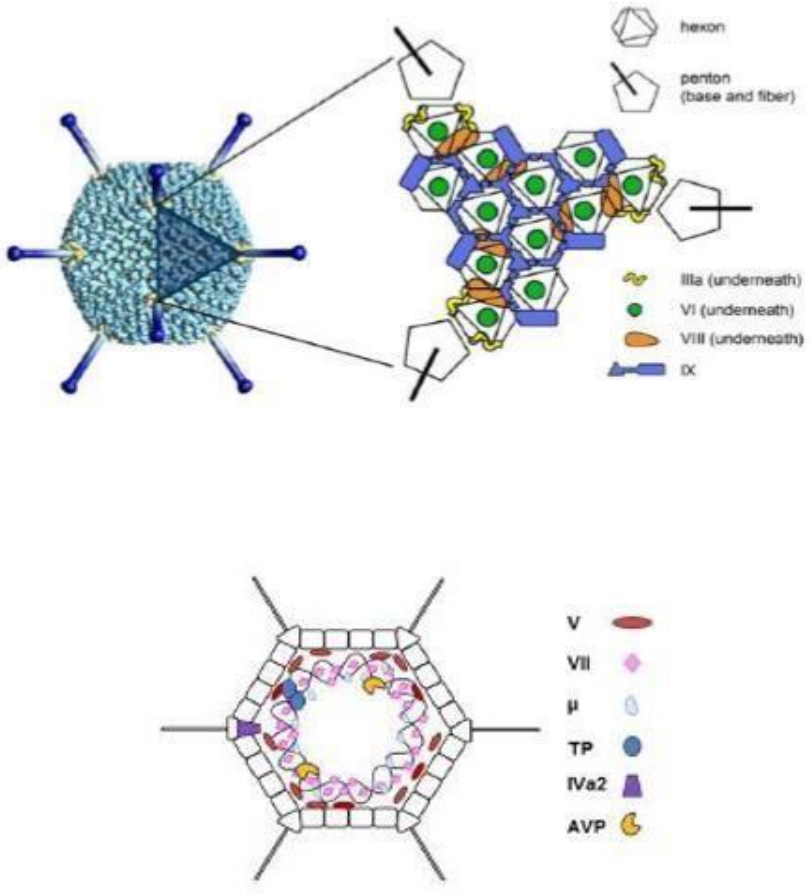



**Figure 2.5.** Cartoon diagram of HAdV virion. A) Enlarged diagram of one icosahedral facet (dark blue triangle), displaying the 12 hexon and 3 penton capsomers as well as the approximate locations of other minor structural proteins that support the major capsid proteins. B) Predicted locations and arrangement of structural proteins and DNA within the core of the capsid (TP = terminal protein; AVP = AdV protease).

## 2.5 HAdV Genome

The genome of all types of adenoviruses comprises of about 30-38 kbp of linear, double-stranded DNA (Richman *et al.*, 2009), with ITRs at each terminus. In its every 5' terminus of linear dsDNA, there is a terminal protein (Sussenbach, 1984), which act as primers in viral replication process and give surety that whole genome is replicated (Srinivasan, 2015). Inverted repeats are the end sequences of every DNA strand exist on 5' and 3' ends (about 100kbps). There is variation in genomic size ranges from 34.1 kbp up to 35.9 kbp and are separated into early (E) and late (L)

genes. Proteins are articulated from six unlike transcription units such as E1A, E1B, E2A, E2B, E3 and E4 in the initial stage. Different from early genes, the late genes are transcribed from the MLP promoter (Ziff & Evans, 1978). The functions of E-genes encompass the regulation and virus replication (E1) (Flint & Shenk, 1989), replication (E2) (Hay *et al.,* 1995), dodging of the immune system (E3) (Wold *et al.,* 1995) and cell cycle management (E4) (Täuber & Dobner, 2001). The L-genes, which are transcribed afterward, instruct the structural proteins highlighted over (Figure 2.6). The E2 genes products are involved in the initiation of replication of the DNA. These proteins are the terminal binding protein (TP), the DNA polymerase (Adpol) and the DNA binding protein (DBP). The first protein is the section of the distinctive characteristic in replication process as the virus replicates through protein-priming method rather than a RNA/DNA single stranded primer method (Liu *et al.,* 2003). The second protein is the viral DNA polymerase that possesses a fundamental 3´- 5´ proofreading exonuclease function and relates to the Pol α family. The third or last expressed protein is the DBP that have likeness to single-stranded DNA and RNA in addition to double stranded DNA (Stuiver & van der Vliet, 1990). The adenovirus death protein (ADP) aids in lysis of the cell by means of contact with the mitotic spindle assembly checkpoint protein, MAD2B (Ying & Wold, 2003) this protein also lies between lytic and importunate adenovirus disease (Murali *et al.,* 2014).

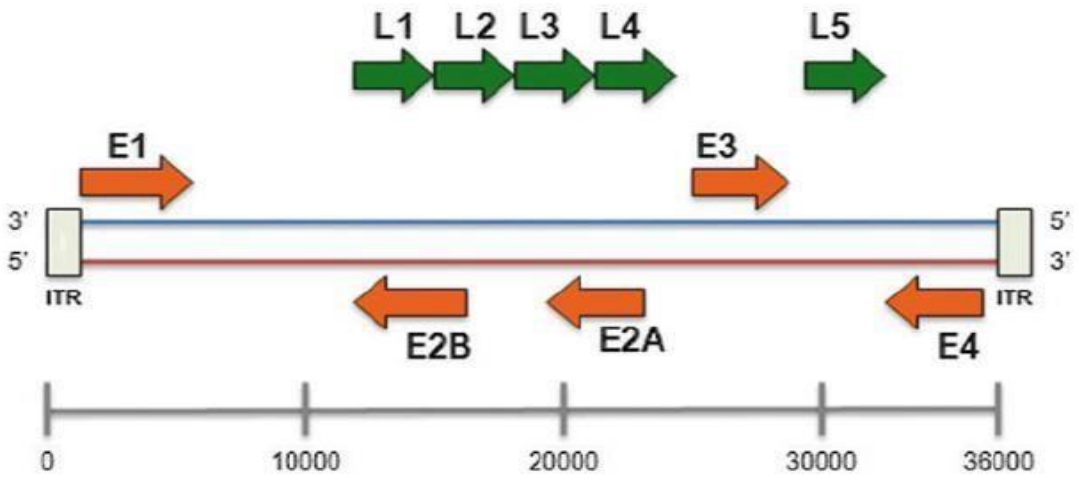



**Figure 2.6.** Schematic representation of the adenovirus genome. The organization of a typical adenovirus (adenovirus 5) genome including the early proteins E1–4, the late proteins L1–5, and the terminal repeats (ITR) are indicated.

## 2.6 Viral life cycle

### *2.6.1 Binding*

For the adenovirus type-definite tissue tropism, this first exposure is as well the critical aspect because various kinds possess various fibers to mark definite cell surface proteins (receptors). As adenovirus and coxsackie virus contribute to the identical receptor hence the protein was afterward called as coxsackie and adenovirus receptor (Bergelson *et al.,* 1997) which is a transmembrane firm-junction protein play role in cell-to-cell adherence and is copiously present in a broad range of tissues such as the intestine, prostate, heart, testis and pancreas. The fiber of adenovirus species from A and C-F except species B possess the attraction for CAR however just species C employed CAR as a functional receptor (Roelvink *et al.,* 1998; Tomko *et al.,* 1997). Numerous supplementary receptors have consequently been recognized, among which CD46, Desmoglein-2 (DSG-2) and the sialic acid-containing proteins are the major distinguished receptors (Fig 2.7). CD46 receptor is employed by adenovirus species B and species D (Ad37 and Ad49) (Gaggar *et al.,* 2003; Lemckert *et al.,* 2006; Marttila *et al.,* 2005; Persson *et al.,* 2009; Segerman, Atkinson, *et al.,* 2003; Wu *et al.,* 2004) while the cadherin protein, Desmoglein-2 was found to be utilized by species B (types 3, 7, 11 and 14) (Wang *et al.,* 2011) and is vital in cell bonding. As notified that based on the attraction

features of the contact, species B types can exploit above single receptor (Segerman *et al.*, 2003; Tuve *et al.*, 2006).

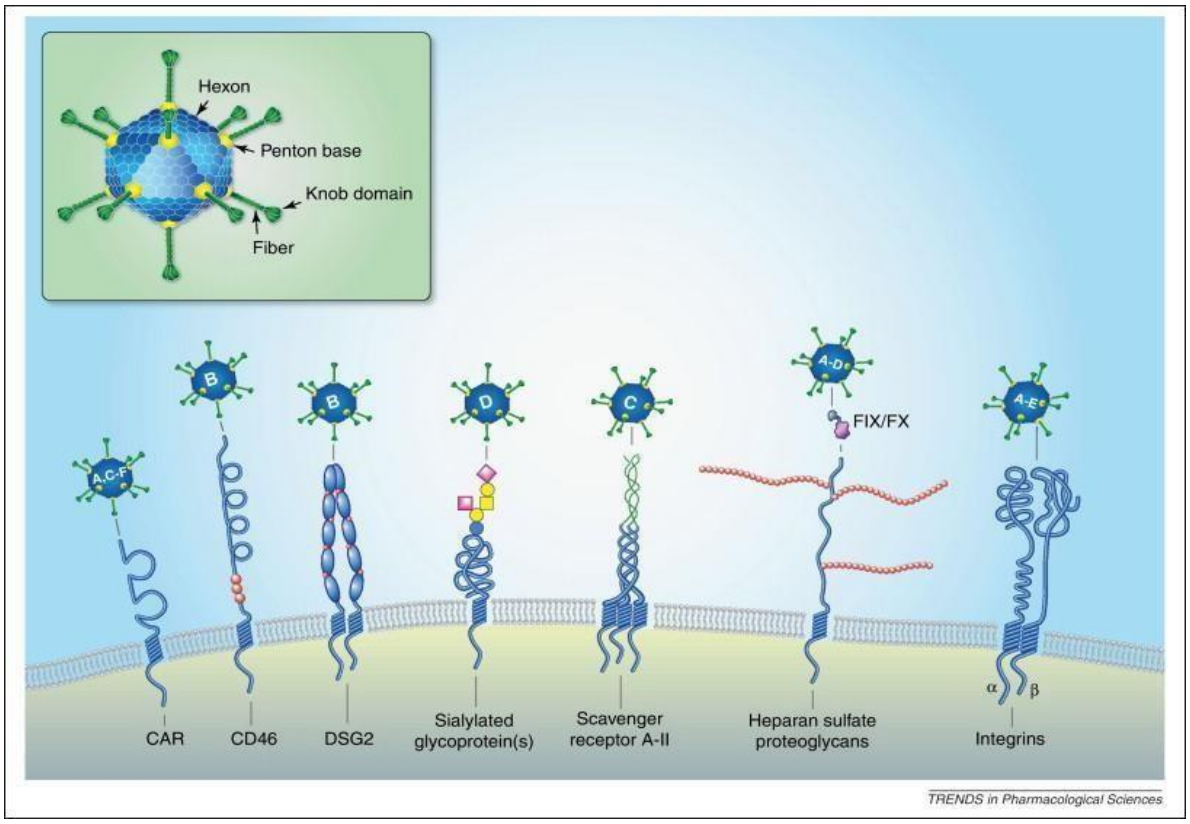



**Figure 2.7.** Receptors used by human adenoviruses.

### *2.6.2 Internalization*

Generally, when the virion binds to the receptors e.g. CAR, they require the interaction with a supplementary receptor for internalization into the cell (Fig 2.8) which are nominated co-receptors belongs to the integrin family. In addition to the cell to cell attachment like CAR, the integrins are too concerned in binding to the extracellular matrix. These are made up of two subunits i.e. α and β, and now a day's αvβ3, αvβ5, αvβ1, α5β1 and α3β1 integrins have been revealed to aid in cell entrance (G. R. Nemerow *et al.*, 1994; Wickham *et al.*, 1994). The penton base which owned a tripeptide amino acid sequence Arginine-Glycine-Aspartic acid (RGD), facilitates the virion contact with the integrins (Albinsson & Kidd, 1999). Subsequent to the early receptor binding, in not more than 5 minutes, adenovirus can be detected in endosomes (Fig. 2.8). Though, the most considered entrance passageway of adenovirus is the clathrin-mediated endocytosis but macropinocytosis

admission has also been defined. At the cellular periphery, macropinocytosis directs to the vacuole development and has been stated for species C2 and C5 and species B3 and B35 adenovirus (Meier *et al.,* 2002).

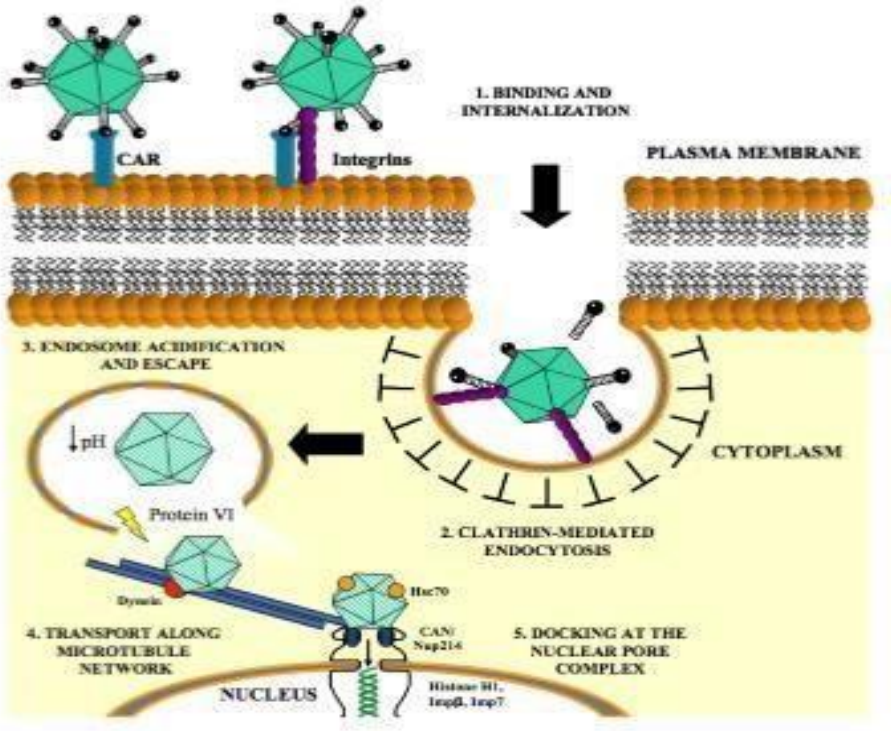



**Figure 2.8.** Illustration of adenovirus uptake and transport to nucleus.

At the time when the endocytosis begins, the stepwise disassembling of the virion also commence simultaneously. At the plasma membrane the fiber proteins begin to separate from the penton base trailed by a conformational variation in the penton base protein that reveals hydrophobic parts and deteriorates the shape of the capsid (Greber *et al.*, 1993; Nakano *et al.,* 2000) and then there is discharge of interior proteins, for example IIIa, V, VIII and VI (Puntener *et al.,* 2011). For initial endosome escape (Wiethoff *et al.,* 2005), Protein VI performs an important task. Later the early extracellular attachment in 15 minutes, the virion un-coating method and endosomal escape is very fast, and freely virions can be identified in the cytosol (Greber *et al.,* 1996). Though, the endosomal escape speed is different among different adenovirus strains (Miyazawa, Crystal, & Leopold, 2001). For the endosomal escape, the precise process is not identified, an acidic pH is recognized to be vital (Seth *et al.,* 1984). This acidification is supposed to trigger the adenovirus protease which in

turn splits the protein VI, therefore membrane lytic capability of VI is activated (Greber *et al.*, 1996).

### *2.6.3 Transport to nucleus*

Subsequently to the endosomal seepage to cytoplasm, the virion becomes partially dismantled with structural proteins hexon, a portion of penton base and protein IX. DNA condensed with protein V and VII in the capsid. The environment of the cytoplasm of the cell is a very congested and glutinous, producing the spontaneous dispersion to the nucleus difficult. This hindrance is overwhelming by the virion through active transport to the nucleus along the microtubules. Motor protein, called dynein assists this transport through "walks" on the microtubule on the way to the microtubule-organizing center (MTOC) immediate to the nucleus (Leopold *et al.*, 2000). Yet, motor protein kinesin on the other hand is headed far away from the nucleus, aids for the intracellular transport of species C adenovirus (Suomalainen *et al.*, 1999). After infection, the movement headed to the nucleus is fast i.e. in about 60 min the virions can be found surrounding the nucleus (Trotman *et al.*, 2001). Subsequent to the arrival of the capsid at the MTOC, close to the nucleus, the capsid is unable to enter into the nucleus because of the nuclear pores which are approximately about half of the size of the capsid, nonetheless docking to the nuclear pore is necessary (Ribbeck & Görlich, 2001). The interaction among capsid and the nuclear pore proteins CAN/Nup214 aids in the docking (Fig. 2.8). Thus, via nuclear pore the viral DNA can then easily diffuse into the nucleus (Trotman *et al.*, 2001).

### *2.6.4 Replication of Genome*

The replication process of the adenovirus is completed in two phases, one is early phase and another is late phase where viral DNA replication takes place. When the virus core moves into the host cell nucleus, uncoating occurs. Now the DNA of the virus is free and go through replication process. As a result, several copies of the virus are formed which then afterwards definite period of infection cause cell lysis and virus copies are then released (Srinivasan, 2015).

### *2.6.5 Assembly and egress*

Inside the nucleus, the assemblage of progeny virions occurs while in the cytoplasm, the protein translation takes place. It means that both processes as inward and outward transferring carried out

on the nuclear pore complex. It has been exposed that with the help of revealing nuclear localization signals (NLSs) and nuclear export signals adenovirus proteins employs the cellular pathways for nuclear transport. NLSs and NESs are tiny extensions of basic amino acid remains. Though, hexon protein lacks the NLS however protein VI is thought to transport the hexon proteins among the cytoplasm and nucleus (Wodrich *et al.,* 2003). Within the nucleus, trimerization of the hexon protein occur that results in the formation of capsomeres. These capsomeres along with the other capsid proteins arrange the vacant capsid structure. Viral DNA is supposed to be "injected" into the hollow capsids; however, it is also proposed that the capsid is grown encircling the DNA core (Ostapchuk & Hearing, 2005). Inside the infected cell, in competition to mature virions, a huge quantity of vacant capsids and incomplete DNA containing capsids are produced (Sundquist *et al.,* 1973). Deprived of any gene expression, these incomplete virions have the ability to produce inflammation (McCoy *et al.,* 1995). But, the exact mechanism of adenovirus produced lysis of the cell is not clearly implicited. So in the last step of the cell lysis, about 104 progeny virions were discharged from each cell along with a massive shunt of viral proteins and DNA without encapsidated (Green & Daesch, 1961)

## 2.7 Epidemiology

### *2.7.1 AFP Epidemiology*

Adenoviruses are circulated globally and its infections occur during the whole year irrespective of seasonality. HAdV type B predominantly types B3, B7, B16 (de Azevedo *et al.,* 2004) and B21 (Ooi *et al.,* 2003) has been the major explained strains in AFP patients, however types C, D and E have also been accounted (Ivanova *et al.,* 2012), along with the presence of HAdV-A31 in a considerable portion of AFP individuals (Haddad-Boubaker *et al.,* 2019).

After adenovirus infection, the intermittent incidences or small epidemics related to neurological ailment are reported. In 1997, the teenagers with acute flaccid paralysis (AFP) in Malaysia who expired in the period of an epidemic of hand, foot and mouth infection with enterovirus 71, species B21 adenoviruses were identified from their clinical specimens (serum, CSF, throat, cardiac muscle, brain and lung) which indicates the co-circulation or connection of both of these viruses in the epidemic (Ooi *et al.,* 2003). Reasons of producing acute transverse myelopathy and acute

encephalopathy associated with adenovirus C7 disease have also been documented. Together in children as well as in adults encompassing those that engage CNS, a broad range of sicknesses are caused by human adenoviruses (Wold & Horwitz, 2007). As human adenovirus is the typical resident of human lymphatic tissue (Garnett *et al.,* 2002; Roos *et al.,* 1972), it can be believable that an "opportunistic" adenovirus infection causes the encephalitis along with its relation with definite flaws in host resistances (Roos *et al.,* 1972). In the AFP monitoring in Brazil, Peru and Bolivia from the period of 1997 to 2002, adenoviruses reported for 1.87% among all viral agents that were obtained from 3420 stool samples and this ratio was in proximity to Sabin-related polioviruses (2.66%) obtained in the similar period (data not shown). The most common species identified in this surveillance was the species C which is very frequent agent and accounted for above 80% of human population infection at the start of life (de Azevedo *et al.,* 2004). Virus can set up an asymptomatic constant illness after the initial infection during which the contagious virions are removed in stool for numerous years (Garnett *et al.,* 2002). This could be explained with the increased rate of species C adenoviruses detection from stool as compared to the species B adenoviruses. In the spinal cord and cerebrospinal fluid of individuals with neurological symptoms adenoviruses have been identified (Faulkner & Van Rooyen, 1962) and have been accounted to produce encephalitis. Adenovirus was assumed to be accountable in causing CNS injure. Enterovirus 71 infection aids in the incursion of the adenovirus into the CNS (Cardosa *et al.,* 1999; Ooi *et al.,* 2003). Table 2.1 shows epidemiology of human adenoviruses in AFP patients from different countries.

**Table 2.1.** Epidemiology of human adenovirus in AFP patients from different countries

| Country | Percentage | Reference |
|---|---|---|
| Brazil | 10.28% | Azevedo *et al.*, 2004 |
| Hong Kong | 31.4% | Chan *et al.*, 2014 |
| USA | 0.08% | Dupuis *et al.*, 2011 |
| Malaysia | 100% | Ooi *et al.*, 2003 |
| South Asian children | 8.57 % | Victoria *et al.*, 2009 |
| Taiwan | 3.3% | Huang *et al.*, 2013 |
| Iran | 12.7% | Yousefi *et al.*, 2018 |
| Russia | 1.05% | Ivanova *et al.*, 2012 |
| Turkey | 2.3% | Bayrakdar *et al.*, 2016 |

### *2.7.2 Gastroenteritis Epidemiology*

Equally in industrialized and non-industrialized states, human adenoviruses 40 and 41 are common and producing gastroenteritis in newborns and in below 5 years old children. Globally the gastroenteritis because of adenoviruses has been accounted as of 1.5 to 12%. Among hospitalized children of industrialized countries, the incidence of enteric adenoviruses has been documented from 1.1 to 7.9%. According to Kotloff et al among infected children in USA the prevalence of Adenovirus F was 4.1% (Kotloff *et al.*, 1989). In California, USA, Norberto et al identified the enteric adenoviruses ratio as 0.8% among infected children by employing ELISA (Rodriguez-Baez *et al.*, 2002).

At the Royal Children's Hospital, Melbourne, Australia, stool samples were utilized from hospitalized children having acute gastroenteritis in the period 1981 to 1992. In these samples the isolation of adenoviruses 40 and 41 were brought out via techniques like electron microscopy and then monoclonal antibody enzyme immunoassay. According to this study, the fecal specimen's positive for enteric adenoviruses was 3.1%, among which adenovirus 40 was observed in 14% and

adenovirus 41 was 86% in infected children (Grimwood *et al.,* 1995). In children having < 5 years' age, the prevalence of Human Adenoviruses 40 and 41 triggering acute viral gastroenteritis has been stated as 1.55% in four cities of Brazil (Soares *et al.,* 2002). From Buenos Aires, Argentina, Berciartu *et al.,* 2002 also stated the occurrence of adenoviruses in AGE children to be 2% (Bereciartu *et al.,* 2002). Another report from Colombia in 2008 by Ospino *et al.,* described the occurrence percentage of enteric adenoviruses as 1.35% in children below five years having acute gastroenteritis (Ospino *et al.,* 2008).

14% occurrence rate of enteric adenoviruses was testified by a year study carried out among hospitalized children with acute gastroenteritis in Berlin, Germany (Oh *et al.,* 2003). Reports from UK in 1999-2001 presented the detection rate of enteric adenoviruses from fecal specimens of infected children was 7.9% (Simpson *et al.,* 2003). The incidence rate of adenovirus F from Denmark was reported to be 3.6% by Olesen *et al.,* 2005 in children with gastroenteritis (Olesen *et al.,* 2005). In Rouen, France for the finding of enteroviral pathogen a related study was carried out from hospitalized children with acute gastroenteritis which indicated the detection rate of enteric adenoviruses as 0.7% while for rotavirus it was 17.3% among infected children as the foremost reason of viral gastroenteritis (Cardine *et al.,* 2002).

According to one report, 5.4% of the patients were diseased by adenoviruses 40 and 41 analyzed by stool samples taken from 1 to 89 years of individuals in Austria (Huhulescu *et al.,* 2009). From Netherlands Maarseveen *et al,* (2010) stated the incidence frequency of enteric adenoviruses as 9.9% by investigating 486 stool samples from acute viral gastroenteritis patients (van Maarseveen *et al.,* 2010). In south-west, Hungary in diarrheic children a four-year study from 2003 to 2006 was took place, which revealed the total incidence rate of adenoviruses in case of childhood gastroenteritis was 8.1% with a steady reduction in finding rate from 11.7% in 2003 to 5.7% in 2006 (Bányai *et al.,* 2009). Likewise, in Greece, 4604 fecal specimens from acute gastroenteritis hospitalized children were taken in a six-year study period in order to investigate the role of enteric viruses, which showed occurrence proportion of enteric adenovirus F was 3.5% in North West Greece (Levidiotou *et al.,* 2009). A year study in Spain that encompassing 820 fecal samples from children with gastroenteritis were analyzed that revealed incidence rate as 60% for enteropathogenic bacteria, 31% for enteroviruses, 25% group A rotavirus and 3% for enteric adenoviruses (Román *et al.,* 2003). Similarly, several other studies were carried out on hospitalized children with acute

gastroenteritis in 2007-08 in Istanbul, Turkey (Akan *et al.,* 2009). According to them the frequency of enteric adenovirus was 8.9% in patients having diarrhea and (or) vomiting presenting a hospital while in Lanzhou China, 544 stool samples were analyzed during the year 2005-2007 by employing ELISA and PCR or RT-PCR techniques which showed occurrence of adenovirus F as 4.4% (Jin *et al.,* 2009). Detection of 19.8% of enteric adenoviruses was reported in northern Taiwan by investigation of 257 children with acute gastroenteritis (Chen *et al.,* 2007).

From July 2004 to June 2005, 337 stool samples were taken from children with acute gastroenteritis and investigated for the incidence of enteroviruses by RT-multiplex PCR in a study in Maizuru city of Japan. Among all the adenoviruses identified, adenovirus 41 was the most predominant serotype that reported for 85.2% (Shimizu *et al.,* 2007). Among South Korean children Huh et al, (2009), reported the incidence rate 2.6% from 10,028 stool samples (Huh *et al.,* 2009). Correspondingly, in Vietnam the fecal samples were included from 1,010 hospitalized children having acute gastroenteritis and were confirmed for the finding of enteroviral pathogen. In this finding, it was described that the incidence of adenovirus 40 and 41 was 3.2% among the diseased youngsters (Nguyen *et al.,* 2007). In a study conducted in 2010 for the investigation of the frequency of viral, bacterial and parasitic enteropathogens in various hospitals in Jeddah and Makkah cities of Saudi Arabia, fecal specimens were taken from 270 children with acute gastroenteritis below of five years' age. According to this study, the prevalence of adenovirus is 7% in gastroenteritis patient in Saudi Arabia (Johargy *et al.,* 2010). Fecal samples were taken from 872 Iranian children with gastroenteritis < seven years of age. It was reported that enteric adenoviruses were detected in 6.7% children with acute gastroenteritis (Saderi *et al.,* 2002). In order to determine the epidemiology of enteric adenoviruses in Dhaka City, Bangladesh, a total of 917 stool samples during the period 2004-05 were taken from youngsters having acute gastroenteritis. In this study, enteric adenoviruses were identified in 1.9% of stool subjects from infected children (Dey *et al.,* 2007). During a study for the detection of the viruses causing gastroenteritis in children in India, 439 stool samples were collected of infected children from western cities like Aurangabad, Nagpur and Pune in the years 2005-2007. This study indicated that the prevalence of enteric adenoviruses in Aurangabad, Nagpur and Pune was 7%, 7.5% and 9% respectively (Verma *et al.,* 2009). Table 2.2 shows the epidemiology of human adenovirus in gastroenteritis patients from different countries.

**Table 2.2.** Epidemiology of human adenovirus in gastroenteritis patients from different countries

| Country | Percentage | Reference |
|---|---|---|
| Egypt | 20% | Zaki and Kheir, 2017 |
| Iran | 5.18% | Dashti *et al.*, 2016 |
| Austria | 9% | Joshi *et al.*, 2016 |
| Iraq | 23.33 % | Al-Khoweledy, 2017 |
| Turkey | 8.6% | Biçer *et al.*, 2011 |
| Portugal | 12.4% | Ribeiro *et al.*, 2015 |
| China | 4.7% | Lu *et al.*, 2017 |
| Brazil | 43% | Costa *et al.*, 2017 |
| Saudi Arabia | 15.5% | Meqdam and Thwiny, 2007 |
| UK | 1.5% | Cunliffe *et al.*, 2010 |
| Germany | 2.9% | Karsten *et al.*, 2009 |
| India | 11.8% | Banerjee *et al.*, 2016 |
| Nepal | 13% | Joshi *et al.*, 2016 |
| Bangladesh | 10.7% | Afrad *et al.*, 2018 |

## 2.8 Status in Pakistan

Presently, regarding the viral causes of acute gastroenteritis, very inadequate information is present in Pakistan, which is a developing country, excluding some concentrated on rotavirus in the latest periods. Similarly, regarding the prevalence of enteric adenoviruses limited reports are present from Pakistan. Agboatwalla *et al.* (1995) reported from Karachi, Pakistan that the prevalence of enteric adenoviruses was 10% in 1992 in children with gastroenteritis. During this study ELISA technique was used for the detection of enteric adenoviruses and rotaviruses (Agboatwalla *et al.*, 1995). Another study conducted by Ahmed et al., (2016) from Peshawar, Pakistan that showed the prevalence of human adenovirus as 38.94%. This study was carried out for determination of the quality of water by finding the prevalence of different enteric viruses (rotavirus, adenovirus, hepatitis A virus and enterovirus) present in water samples that were gathered from various regions of Peshawar (Ahmad *et al.*, 2016).

While regarding the human adenovirus prevalence in AFP, no data is available from Pakistan. According to our best information, the detailed analysis on the epidemiology that concentrated on the occurrence of human adenovirus among hospitalized youngsters having acute gastroenteritis and in AFP have never been carried out in Pakistan. The present evaluation intended to evaluate the epidemiology of widespread HAdV genotypes related with acute gastroenteritis in hospitalized children and in AFP children and their genetic characterization.

## 2.9 Transmission

Dissemination of HAdV can mostly take place with the help of the aerosol drops, fecal–oral conduction, and unclean fomites and hardly by contact to cervical canal discharges at the time of birth and in solid organ transplants recipients. The virus is well viable as at 36 °C majority of the virus types are infective for one week to numerous weeks at room temperature (Mahl & Sadler, 1975). Additionally, for a number of months the virus can be stable on arid places. They are usually unaffected towards lipid antiseptics like soap however; the virus is susceptible to 70 % ethyl alcohol, heat, formaldehyde and chlorine (Rutala *et al.,* 2006).

## 2.10 Clinical presentation

### *2.10.1 Acute Flaccid Paralysis (AFP)*

Acute Flaccid Paralysis (AFP) can be described as the quick and abrupt inception of weakness of muscles in various body fractions, which includes foot, hand and infrequently muscles of respiration tracts and engulfing system. During the period starting from the birth to below 15 years of age, this abrupt weakness starts anytime and does not grow additionally subsequent to its enhancement via 4-5 days of symptoms (Abbasian *et al.,* 2012; Lo *et al.,* 2010; Poorolajal *et al.,* 2011). The symptoms encompassing is the fever, vomiting, constipation or occasionally diarrhea, headache, fatigue, rigidity in the neck in addition to pain in the limbs. Most detected genotype in AFP infection is the HAdV type B specifically subtypes with B3, B7, B14, B16, B21 and B35. In addition to them types C, D and E have been stated in studies (Haddad-Boubaker *et al.,* 2019).

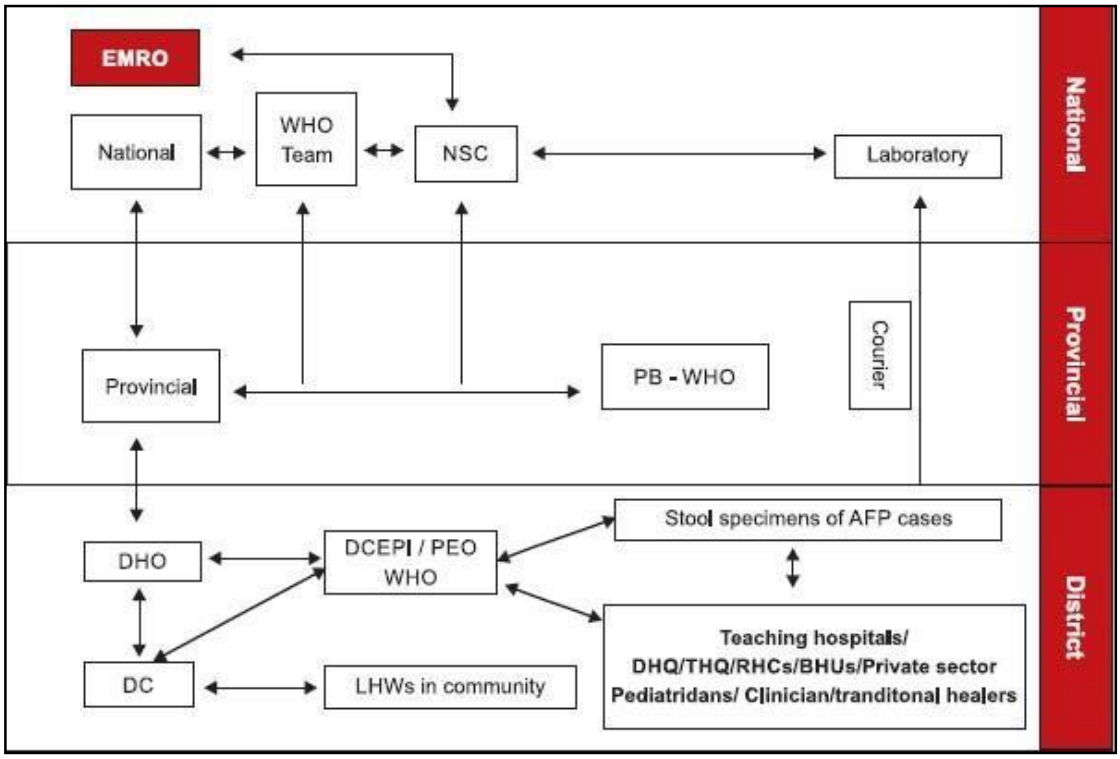



**Figure 2.9.** Organogram of AFP Surveillance System, Pakistan

Key: DC-District Coordinator, DHO-District Health Officer, EPI-Expanded Program on Immunization, PEO-Polio Eradication Officer, WHO- World Health Organization, LHW-Lady Health Worker, NSC-National Surveillance Cell, EMRO- Eastern Mediterranean Regional Office.

### 2.10.2 Disfunction of the central nervous system (CNS)

In case of CNS dysfunction, several forms of clinical presentation have been noted and among them both meningitis and encephalitis are regarded as the main symptoms. Adenovirus related general signs or indications of CNS disfunction were fits, changed condition of perception, headache, and optical illusions and the utmost frequent finding were pyretic attack, encephalitis/encephalopathy, afebrile attack and aseptic meningitis/meningismus along with pneumonia, tonsillitis, and transient encephalopathy. Several serotypes have been isolated from both brain and CSF, which includes adenovirus types 1, 2, 3, 5, 6, 7, 12, 26 and 32 in separated cases (Y.-C. Huang *et al.*, 2013).

### *2.10.3 Encephalitis and meningitis*

Meningitis can be described as the inflammation of the meninges along with temperature, headache, common malaise, drowsiness, and nausea while encephalitis is demonstrated as the inflammation of the brain parenchyma with clinical support of brain dysfunction like neurological manifestations and seizures (Dupuis *et al.,* 2011). In cases of encephalitis or meningoencephalitis HAdVs have also been isolated in cerebrospinal fluid (CSF) includes serotypes HAdV-A31; B1, B3, B7, B11, HAdV-C, C2, HAdV-D, D26, D49 (Haddad-Boubaker *et al.,* 2019).

### *2.10.4 Respiratory tract*

In young children having febrile respiratory infections, adenoviruses are the frequently identified viruses. Usually the time period of the disease ranges from 5 to 7 days, though its manifestations can be continuing for up to 14 days (Flomenberg *et al.,* 2019).

### *2.10.5 Pharyngitis and Coryza*

The widespread indications of adenovirus illness are pharyngitis and coryza. Pharyngitis includes the conjunctivitis, laryngotracheitis, bronchitis, or pneumonia, fever, malaise, headache, myalgia, abdominal ache, exudative tonsillitis and cervical adenopathy. In young children, adenoviruses are the major reason of tonsillitis (Flomenberg *et al.,* 2019).

### *2.10.6 Otitis media and bronchiolitis*

Particularly in kids less than 1 year, otitis media is an additional widespread symptom. However, in a small number of studies, the occurrence of adenovirus in middle ear fluid merely has been reported. Pertussis-resembling disease, bronchiolitis, or an exanthema can also be developing from adenoviruses (Flomenberg *et al.,* 2019).

### *2.10.7 Pneumonia*

Pneumonia has also been caused by several serotypes of adenoviruses like serotype 1, 2, 3, 4, 5, 7, 14, 21, and 35. As compared to adult children, the newborns have severe pneumonia in addition to lethargy, diarrhea, and vomiting. Rarely extrapulmonary difficulties can also take place, which

includes meningoencephalitis, hepatitis, myocarditis, nephritis, neutropenia, and disseminated intravascular coagulation. Chest radiography in case of adenoviral pneumonia shows disseminated bilateral pulmonary penetrates along with other pathological alterations like necrotizing bronchitis, bronchiolitis, and mononuclear cell infiltration, hyaline membranes, and necrosis. After adenoviral pneumonia in small children there is an increase prevalence of pulmonary sequelae which includes bronchiectasis and bronchiolitis obliterans.

### *2.10.8 Eyes*

The typical adenoviral disorder is a pharyngoconjunctival fever that comprises of a benign follicular conjunctivitis with febrile pharyngitis and cervical adenitis. The commonly identified serotypes are from adenovirus species B (serotypes 3 and 7) while numerous serotypes from species B, C, D, and E have also been associated. Species D (serotypes 8, 19, and 37) are mainly related with epidemic keratoconjunctivitis (EKC) which is a more serious disease. It carries serious ache, indistinct vision and brings major financial mislays in the place of work.

### *2.10.9 Gastrointestinal system*

In young children, subgroup F adenoviruses serotype 40 and 41 causes about 5 to 10 percent of acute diarrheal infections. Diarrhea is 8 to 12 days longer. Since one study of 14 cases of enteric adenovirus-associated diarrhea in admitted newborns, more than three-month duration recommended that enteric adenoviruses are possibly a source of nosocomial illness. Epidemiologic reports have not revealed an obvious association among the majority of further serotypes and gastroenteritis, contrasting serotypes 40 and 41. Yet, in a few studies, the species A serotype 31 has been related with juvenile diarrhea. Additionally, mesenteric adenitis, that mimic appendicitis and infrequently produce intussusceptions have been related with subordinate serotype of adenoviruses.

### *2.10.10 Hepatitis*

In immune-compromised hosts, in particular with species C serotype 5, hepatitis is a fineelaborated problem of adenovirus diseases. In pediatric liver transplant receivers, adenovirus hepatitis is a significant complication, which can be lethal in these receivers.

### *2.10.11 Genitourinary tract*

In children, adenovirus species B (serotypes 11 and 21) have been related with acute hemorrhagic cystitis. This is self-restricted infection and is more widespread in males but typically, it does not include the fever or hypertension. It is signifying with its possible mystification with other severe infections of the kidney for instance glomerulonephritis. In adults, adenovirus species D (serotypes 19 and 37) have been infrequently related with the disease urethritis while in case of immunocompromised patients, adenovirus species B (serotypes 11, 34, and 35) are related with hemorrhagic cystitis and tubule-interstitial nephritis.

### *2.10.12 Disseminated*

In both immune-compromised and immune-competent youngsters, circulated adenovirus illness has been accounted and in adults who have endure hematopoietic cell or solid organ transplantation. From disseminated infection general mortality rate was 73% (83% in immunocompromised and 60% in immunocompetent hosts).

### *2.10.13 Myocarditis*

Adenovirus was the highly widespread source of viral myocarditis in two succession of acute myocarditis in children that occur in 60% of polymerase chain reaction-confirmed cases of viral myocarditis.

### *2.10.14 Other*

Together with rhabdomyolysis, viral myositis has sporadically been related to adenovirus. Less than 10 reports of adeno-related arthritis have also been accounted (Flomenberg *et al.,* 2019).

### *2.10.15 Transplant recipients*

In patients who gone through organ or hematopoietic cell transplantation (HCT) the array of adenovirus illness can start from asymptomatic detaching to lethal circulated ailment. Disease can be produced from three aspects these are (i) primary infection, (ii) in the transplant recipient restart of dormant infection and (iii) restart of infection transported in the donated organ (Koneru *et al.,*

1990). In hospital-relied retrospective finding, adenoviral infections were contrasted among pediatric HCT and solid organ transplant recipients (de Mezerville *et al.,* 2006) and the results showed the occurrence at a mean of 1.6 months' post-transplantation. In HCT recipients all mortalities happened are solely from the result of adenoviral infection because of serious immune dysfunction. The reported common broad range of symptoms are pneumonia, colitis, hepatitis, hemorrhagic cystitis, tubulointerstitial nephritis, encephalitis, and dispersed infection. The more infrequent and predominance types found are isolates of species B serotypes 11, 34, and 35 (Flomenberg *et al.,* 2019).

### *2.10.16 Solid organ transplantation*

Among solid organ transplant receivers, adenovirus caused illnesses array from non-symptomatic to serious and dispersed by extended viral shedding and illness and death as well as related graft dysfunction and refusal. As compared to adults, adenovirus more often ails pediatric solid organ transplant recipients. There are possibilities of three clinical situations: (1) asymptomatic viremia or infection (2) adenovirus infection with the participation of definite organ and signs (eg, pneumonia, enteritis, and meningoencephalitis) and (3) disseminated disease, when two or more than two organs are associated. Between liver transplant receivers, the occurrence seems greater then it occurred in heart and kidney transplant receivers. Species B serotypes 11, 34, and 35 are nearly entirely related in this syndrome. For intrusive adenoviral illness in pediatric patients with liver transplantation, the furthermost-shared demonstration is the hepatitis. In this case, the adenovirus serotype 5 was the usually isolated while serotypes 1 and 2 were too detected (Flomenberg *et al.,* 2019).

### *2.10.17 Congenital immunodeficiency syndromes*

Prime adenoviral infections in children having immunodeficiency syndromes can cause serious illness that is normally lethal, for instance severe combined immunodeficiency disease (SCID). In SCID individual, types of adenovirus responsible include A31 and numerous species of B and C serotypes with lethal pneumonia and hepatitis (Johansson *et al.,* 1991).

### *2.10.18 AIDS*

From HIV-infected individuals several atypical adenovirus serotypes have been identified. The most frequent types species B serotypes identified from urine are 11, 34, and 35 with numerous intermediate strains. From fecal specimens the species D adenoviruses were the largest identifiers as well as 8 other serotypes, 43 to 49 and 51. In HIV-infected individuals regardless of the ratio of finding in fecal or urine samples, AdVs are an unusual source of illness or death (Hierholzer *et al.*, 1988).

### *2.10.19 Malignancy*

In children and adults with hematologic malignancies different symptoms of adenoviral infections together with viral triggered hemophagocytic lymphohistiocytosis (HLH), have been accounted (Joffe *et al.*, 2018).

### *2.10.20 Possible association with obesity*

Relationships among the occurrence of adenovirus serotype 36 antibodies and obesity has established in a few human findings (Shang *et al.*, 2014).

### *2.10.21 Adenovirus infections in immune-competent persons*

Adenovirus infections in immune-competent or else healthy individuals can be rigorous but is usually self-restrictive and can still be asymptomatic. In children, species C adenoviruses are frequently related with respiratory ailments and report for 5-10 % of all respiratory diseases. Adenovirus type 3, 7 and 14-cause pharyngoconjuctival fever (PCF) the clinical manifestation of which includes the fever, pharyngitis, and conjunctivitis. Acute respiratory disease (ARD) which is linked with fever, rhinorrhea, cough and sore throat are dominantly produced by species C, species B (type 7, 14) and species E (type 4). ARD usually continues for 3-5 days with tonsillitis and otitis media. Furthermore, gastrointestinal tract is affected by type 40 and 41 of the adenovirus and are accountable for producing 5-15 % of acute diarrheal infection in children. Urinary tract infection is caused by type 11 and 21 of species B, which produce haemorrhagic cystitis. The infrequently accounted symptoms of adenovirus infections are myocarditis, hepatitis and meningoencephalitis. Species D (type 8, 19 and 37) mostly caused the more widespread and extremely infectious eye

infection called epidemic keratoconjunctivitis (EKC) with symptoms of considerable morbidity such as photophobia, discomfort, red-eyes, tearing, and indistinct vision along with extensive weakened sight or sightlessness (Flomenberg *et al.,* 2019).

### 2.11 Immune response to adenovirus

In the majority of cases following adenovirus infection, type particular neutralizing antibodies may grow that give the protection against both in the present infection as well as in re-infections by the similar serotype (Berk, 2007).

#### 2.11.1 Immune reaction of the host

**1. Cellular immune reaction**

T-cells give cellular immunity with an effectual protection through both CD8+ cytotoxic (CTLs) and CD4+ helper cells. On the cell surface a complex with class I proteins of the MHC formed which is recognized by CTLs as virus antigen. Perforin discharges from this event which undergone cell lysis. Thus, former to virus assemblage and discharge, infected cells eliminated at the beginning. With the help of E3 gp19K to keep the MHC antigens in the ER adenoviruses can fight this cellular line of attack and thus interrupting the antigen identification practice (Kvist *et al.*, 1978). In stopping the T cell cytolysis function, the role of E4 gene has also been established (Kaplan *et al.*, 1999).

**2. Humoral immune response**

The foremost part of the host defense line of attack is the humoral reaction. It relies on the capability of B cells that express the surface immunoglobulins in order to identify the particular epitope on an unknown antigen. After the activation of the specific B cells, immunoglobulins are liberated as antibodies towards the plasma that cooperate with the antigens. In the adenoviral genome, adenovirus–counteracting antibodies worked against epitopes mainly on the fiber, penton base and hexon (Gahéry-Ségard *et al.*, 1998; Willcox & Mautner, 1976).

### *2.12 Techniques of identification*

The diagnostics techniques for the detection of adenoviruses depend on disease form and the specimen attained. Equally direct and indirect methods can be employed for the identification of adenoviruses. Direct methods include conventional and molecular methods like electron microscopy, detection of virus in cell culturing, antigen identification and the genome identification with or without amplification. But in scientific laboratories, electron microscopy is not regularly employed. Indirect diagnostic methods include serological procedures, but are restricted because of absence of sensitivity.

Laboratories can detect and type human adenoviruses using:

- Virus isolation
- Identification of the antigen
- Neutralization of the with type-specific antisera
- Molecular detection (e.g. PCR)
- Incomplete or complete sequencing of the genome

#### 2.12.1 Virus Isolation

For epidemiological findings and for an extensive validation for the records of hospital-born epidemics, detection of adenoviruses by species, serotypes, and strains is pertinent, however it is frequently not highlighted in clinical performances, given it the self-restricted infection course. Conventional serotyping techniques have now been progressively substituted by PCR methods. Yet for any disease, the gold standard continues to be the viral culture. In the adenovirus family except a small number of viruses all exhibit a distinctive cytopathic effect in epithelial cell lines such as HEp-2, Graham 293, A549, human embryonic kidney cell lines and HeLa from all types of clinical samples and produce cytopathic effect typically in 2 to 7 days though it can utilize 28 days and is characterized by cells rounding and clumping with intranuclear inclusion bodies (Y. T. Huang & Turchek, 2000).

### 2.12.2 Direct adenovirus antigen identification

Direct adenovirus antigen identification method is rapid and sensitive as compared to cell culturing and is used widely for diagnosis of gastrointestinal infection as well as respiratory infections. Most common approaches used for direct antigen detection are enzyme immunoassay used especially for fecal samples and immunofluorescence used especially for swabs, respiratory samples and biopsies. Other antigen detection techniques used are latex agglutination and immunochromatography and are specially used for fecal specimens, with which immunofluorescence may not be accomplished, or when few samples may be analyzed at same time (Fujimoto *et al.,* 2004).

### 2.12.3 Enzyme Immunoassay (EIA)

Enzyme Immunoassay (EIA) is based on the antigen recognition found in the testing sample by antibodies coated on wells of plastic plates. The antigen-antibody reaction is usually detected by a colorimetric reaction coming from an enzyme coupled to secondary antibodies. EIA is simple, rapid and high-throughput; moreover, sensitivity can be modulated increasing the enzyme concentration for magnification of the signal. EIA has proved to be extremely useful in surveillance studies mainly for its practicality. It has been suggested that EIA has lower sensitivity compared to other methods such as TEM and PCR. Nevertheless, the relatively lower sensitivity of these immunoassays could correspond to the lack of appropriate preparation of samples before screening (i.e. samples that have been frozen and thawed multiple times (Richards *et al.,* 2012).

### 2.12.4 Detection by Electron Microscopy

The electron microscopy technique is not used widely because electron microscope is limited to some institutes and is employed for the analysis of acute gastroenteritis. Due to the characteristic morphology, adenoviruses care be detected by electron microscopy (Echavarría, 2008).

### 2.12.5. Histopathology

Histopathological observations are mainly used for respiratory infections and are characterized by diffuse bronchiolitis with mononuclear cell infiltrates, necrosis of bronchial epithelial and interstitial pneumonitis. When adenovirus infect a cell its nucleus becomes enlarge and have

basophilic attachments encircled through a thin layer of cytoplasm. At this stage the cell are referred as smudge cells (Landry *et al.,* 1987).

### 2.12.6 Virus neutralization with type-specific antisera

With the help of the determination of the agglutination pattern of the viral isolate with the human and animal erythrocytes, various viral species can be identified. Hemagglutination inhibition or neutralization tests can be used for serotyping. Simplified micro-neutralization experiment performs fine with quickly producing, comparatively increased-Ad-titer viral isolates. Microneutralization assay form it advantageous for serotyping huge figures of isolates in laboratory built epidemiological investigation, epidemic surveys, and disease controller in scientific sites where hospital borne infection is supposed. In these sites, the fast reversal time of this assay directs to price savings, creating it a valued means.

### 2.12.7 Direct Fluorescent Assay

Formerly, there were difficulty in diagnosis of adenovirus infections and a number of infections were improperly treated with antibacterial agents. After the making of Direct Fluorescent Assay (DFA), in clinical assessment, production viral cultures were less frequently employed. The collection procedure for the specimens is mostly noninvasive because of the diverse samples like peripheral blood, stool, urine, bronchoalveolar fluid, nasopharyngeal aspirates or swabs. Therefore, in regular clinical analysis, these techniques are less frequently adopted and then favor goes towards the PCR-based practices, which are fast and quite consistent.

### 2.12.8 Molecular Method

PCR-based methods can be applied for the finding and recognition of viruses. These approaches are based on the obtaining of an amplification product using primers specific for the group of viruses under study. Therefore, the presence of conserved sequences for that group of viruses and the previous knowledge of those sequences are compulsory requirements. PCR-based methods have higher sensitivity than EIA. Moreover, PCR methods allow a wide range of applications. The specificity of the primers used in the PCR can be modulated to allow further classification of the group of viruses. Quantitative PCR (qPCR) is another modality of PCR. This technique allows the

quantification (and detection) of virus particles in the clinical sample, important feature when infectivity dose is being studied (Esona *et al.,* 2013).

Molecular methods arc widely used for recognition of adenovirus genome because of increased sensitivity and are especially used once viral load is quite small to be identified via cell culture or after noninfectious virus is present or once outcomes are required quickly. The uses of molecular methods for detection of viruses have amplified considerably in the previous years. Till 1997, just a limited polymerase chain reaction (PCR) approaches for fecal samples and eye swabs were obtainable for diagnostics purposes. But later on various generic and species specific PCR tests were established and are used for diverse clinical specimens. Conventional PCR is a qualitative method and the Real time PCR is both qualitative and quantitative assay. Since in Real time PCR, amplification of viral genome and identification of the amplified products carried out at the same time. A commercial real time PCR procedure (adenovirus r-gene PCR) was formed and can be used for the generic recognition of entire serotypes. But for real time PCR, several probes and over single set of primers are mandatory for the detection of all serotypes. In adenoviral diseases, increasing antibody titers exhibited in serological findings can also monitor treatment. The diagnosis is further difficult in immune-compromised individuals as persons can be asymptomatic carriers; possess subclinical illness or possesses complete infection with distributed characteristics. Therefore, for the verification of disease above single spot accompanies with quantitative approaches for example quantitative PCR and serological titers, clinical relationship turn out to be more dominant.

Molecular methods can also be used for typing purposes, for example, multiplex PCR and the size visualization of the amplified product or by applying amplified product trailed by hybridization with various species specific probes. Nowadays for various serotypes conformation and determination, sequencing technique is mostly used because it makes use of molecular equipment's and this facility is available in most laboratories. Typing can also be performed by combination of PCR, followed by restriction endonuclease digestion, which is a simple and sensitive method, but co infection may create problem with the results. The PCR and restriction endonuclease digestion methods can be used so cells infected by virus, purified virus or directly to stool samples from children with gastroenteritis (Allard *et al.,* 2001; Echavarría, 2008).

### 2.12.9 Sanger sequencing

A sequencing step (Sanger sequencing) is further performed in order to describe and analyze the detected regions. Advances in sequencing have led to the creation of metagenomics, the culturefree and unbiased sequence-independent identification of nucleic acids from environmental or clinical samples. The advantages of metagenomics over the other molecular methods are then selfexplainable. Furthermore, next-generation sequencing and Bioinformatics have made metagenomics a more sensible and high-throughput technique with the capacity of processing a high number of clinical samples and therefore approaching to a real time diagnosis and surveillance.

If characterizing a new species is the objective, sequencing is applied after PCR methods to identify the amplified products. In some cases, primers can be used to amplify conserved regions of the virus genome. Those fragments are then sequenced (Sanger sequencing) and assembled to reveal the identity of the virus. Sanger sequencing can also be applied in genotyping after the amplification of a region that has the purpose for that, either structural or non-structural regions (Muldrew, 2009). Purified DNA is sequenced by chain-termination method (Sanger sequencing). Reads are then computationally aligned and compared to available references in order to reveal the genotype of the virus (BLAST). In this case, similarities between the references and the sequence obtained from the experiment are a crucial step.

### 2.12.10 Next-generation sequencing and metagenomics

Next-generation sequencing overcomes the necessity of cloning by implementing efficient *in-vitro* amplification that produce shorter reads compared to Sanger sequencing (200-300 bp by Illumina, 250-400 bp by 454 Roche Applied Bioscience and 100 bp by Ion Torrent) but promoting a higher coverage. Being a sequence-independent method, NGS allows the detection and identification of virus without the need of previous knowledge of the target sequences. NGS is also a highthroughput method suggesting a potential application in surveillance. A Basic Local Alignment Search Tool (BLAST) of the sequencing data is further necessary in order to identify the virus having into consideration that the homology between the produced sequencing data and the available published data is sufficient for this task.

NGS has resulted in advances in metagenomics. Metagenomics refers to the analysis of all nucleic acids in environmental or clinical samples without the need of culturing methods or previous knowledge of the nature of the nucleic acids. In general, after nucleic acid extraction from the clinical samples, the genetic material coming from any organism living in the sample is fragmented, amplified and sequenced. After assembling of the sequencing reads, contigs can be assigned to the different microorganisms though similarity-based methods. In the case of clinical diagnosis, the capacity of the method in identifying a pathogenic microorganism from a given sample will depend on the abundance of the sequence reads and the similarities with available pathogenic sequences. The advantages of metagenomics together with NGS in clinical diagnosis then jump to the eye: a high-throughput method with the capacity of identifying even highly divergent microorganisms from a clinical sample (Capobianchi *et al.,* 2013).

**2.13 Management**

Usually the adenoviral infections are self-restricted. In matures and healthy children, HADV seldom produce severe disease but in case of immunocompromised hosts, neonates and newborns the virus can be critical. Thus the immune-compromised patients, chiefly allogenic transplant patients, those are at the higher threat of life risking diseases, the majority of the recommendations regarding treatment purposes are usually focused on them which includes the anti-pyretics, analgesics, anti-emetics, adequate hydration, and oxygen supplementation (Khanal *et al.,* 2018).

***2.14 Infection Control***

For hindrance of dissemination of the agent, the universal recommendations suggested via the Center for Disease Control (CDC) to the community comprises the proper hand washing with soap and water, particularly following interaction with a individual having any sort of flu-like infection that covers one's mouth and nose during coughing or sneezing. It also includes the avoidance of contacting one's eyes, nose, or mouth with dirty hands; prevent intimate exposure with ill people and residing at home by the people themselves at the time of illness. In swimming pools, it is suggested to maintain sufficient levels of chlorine in order to avoid the epidemics of conjunctivitis produced by adenoviruses.

### *2.15 Antiviral Therapy: Antiviral drugs used in the clinic*

In immune-competent and immune-compromised person's adenoviruses can be the reason of various illnesses that depends on adenovirus serotypes as gastroenteritis, conjunctivitis and respiratory tract ailment. Usually adenovirus disease symptoms are less severe and HAdV can produce treatable though serious illness as hepatitis and nephritis. However, in case of immunecompromised patients, adenoviral disease may cause mortality of the person. At present for the management of adenovirus, ailments there are no standard definite antiviral drugs exist. Though, for adenovirus treatment, cidofovir and ribavirin are employed off license as no other substitutes are exists. Cidofovir is a nucleotide analog of cytosine. It performs its function via selectively preventing the viral DNA polymerase, which in turn restrict the viral replication by competitive integration into the viral DNA chain of its active metabolite, cidofovir diphosphate, thus disrupts the synthesis and replication. In seriously immune-compromised individuals having adenovirus disease, the death ratio was revealed to have fallen to below 20 percent with treatment with two or more doses of cidofovir. Usually ganciclovir works by phosphorylation to the monophosphate structure by means of viral kinase and because adenoviruses are deficient in viral thymidine kinase, the efficiency of ganciclovir is then humble in contrast to cidofovir.

### *2.16 Vaccine status*

In children for enteric adenovirus treatment presently, there is no available vaccine. Yet, for a variety of other vaccines, adenoviruses are usually utilized as vectors. Just for the U.S. military adenovirus vaccine to types 4 and 7 is now suggested. These are live, oral, enteric-coated vaccines (Khanal *et al.,* 2018).

*Chapter 3* **MATERIALS AND METHODS**

The current study was divided into two parts. First part was based on isolation and identification of adenoviruses from acute flaccid paralysis cases, their molecular characterization and evolutionary relationships. The next part included the epidemiological and clinical analysis of adenoviruses in gastroenteritis patients hospitalized at five main tertiary care hospitals in three cities of Pakistan, Rawalpindi, Lahore and Karachi. The Internal Review Board of the National Institute of Health (NIH), Islamabad, Pakistan, approved the study concept and design.

## 3.1 Study Design

### *3.1.1 Acute Flaccid Paralysis (AFP) Cases*

In 2017, 172 stool samples (non-polio) from acute flaccid paralysis (AFP) cases were collected under the framework of an AFP surveillance program in Pakistan. Following the World Health Organization (WHO) guidelines, all the stool specimens were collected from the start of symptoms within 14 days and then were delivered to WHO Regional Reference Laboratory (RRL) for Polio Eradication Initiative, Virology Department, NIH Islamabad, Pakistan and stored at −20 °C.

### *3.1.2 Gastroenteritis Cases*

During 2017-2018, 1,117 fecal samples were taken from <5 children from hospitals; Benazir Bhutto Hospital, Rawalpindi (BBH), Mayo Hospital, Lahore (MHL), The Children's Hospital, Lahore (Li et al.), Kharadar General Hospital (KGH), Karachi and National Institute of Child Health (McCoy et al.), Karachi. WHO selected these sentinel sites on behalf of rotavirus surveillance activity in Pakistan because of high patient numbers from both rural and urban areas. In these regions to our information, no research study highlighting the prevalence of adenovirus in causing gastroenteritis had been conducted.

The laboratory work was conducted at the Department of Virology, NIH, Islamabad that assists as the WHO Collaborating Center for Research and Training in Viral Diagnosis. Before collection of stool specimens, informed written permission from the parents/guardians of the patients were taken. During sample collection, a pre-tested proper consented questionnaire form that includes

demographic and clinical information was also accomplished. Afterward samples were stocked in insulated carriers that were contained with frozen ice packs and delivered to Department of Virology, NIH in order to keep them at cold temperatures.

**Table 3.1.** Table showing total number of samples from respective sites and techniques used for the study.

| **S.No** | **Study Sites** | **No of Diarrheal Samples** | **Study Sites** | **No of AFP Samples** | **Lab Techniques** |
|---|---|---|---|---|---|
| 1 | KGH, Karachi | 182 | Punjab | 85 | Virus Isolation: Cell Culturing |
| 2 | NICH, Karachi | 416 | Sindh | 34 | |
| 3 | CHL, Lahore | 159 | KPK | 24 | Genotyping: Nested-PCR |
| 4 | MHL, Lahore | 138 | Baluchistan | 12 | |
| 5 | BBH, Rawalpindi | 223 | Gilgit Baltistan | 4 | Genetic Characterization: Sequence analysis |
| | | | FATA | 7 | |
| | | | AJK | 6 | |
| **Total** | | **1,118** | | **172** | |

### 3.2 Study Sites

The investigation for AFP analysis was carried out from AFP surveillance program under the polio eradication initiative in Pakistan. From stated AFP cases, entire fecal samples collected from all over Pakistan are investigated at the RRL for polio eradication in NIH, Islamabad. The investigation for gastroenteritis study was carried out from hospitals of Rawalpindi, Lahore and

Karachi of Punjab and Sindh province. From the capital city Islamabad, **Rawalpindi** is situated 14 kilometers south. The city covers 5286 sq.km area, having more than 3.3 million peoples with density of 636 individuals for every sq.km and is fourth most densely populated metropolitan city of the country. Between urban and rural regions, the people are divided nearly equal and 53.2% of residents live in the urban areas.

**Lahore** is the capital of Punjab province. It is the country's second-most populated city and is one of wealthiest city of Pakistan, with an expected GDP of $120 billion (PPP) as of 2017. It is the largest city and is the historic cultural center of the Punjab region. The city covers 1,772 $km^2$ (684 sq mi) area, having 11,126,285 populations with density of 6,300/$km^2$ (16,000/sq mi).

The capital of Sindh province of Pakistan is the Karachi. Karachi is the most populated cosmopolitan city in Pakistan while in the world; it is numbered as the $6^{th}$ populous city. It is located on the Arabian Sea, so it works like a transportation core, and is home to Pakistan's two major seaports, the Port of Karachi and Port Bin Qasim. The city covers 3,780 $km^2$ (1,460 sq mi), having 14,910,352 populations with density of 3,900/$km^2$ (10,000/sq mi) and is first most densely populated metropolitan city of the country.

## 3.3 Study Populations

### *3.3.1 Group 1*

This group includes stool specimens taken from children with the age below-15 having symptoms of headache, fever, vomiting and stiffness in limbs and neck with sudden onset of paralysis (absence of floppy and spasticity movements) either in upper and lower limbs under the framework of an AFP surveillance program in Pakistan. In identifying polio cases, the essential task is the AFP surveillance, or discovering and informing children with AFP. Towards the polio surveillance system, every case of AFP turns as a sign that polio might be flowing. In Pakistan, in 1997, AFP surveillance commenced however, in 2000 it gains dedicated attention. Presently, on national, provincial, and district levels, Pakistan devours a well-working and sensitive AFP surveillance network. For the confirmation of poliomyelitis identification, the paramount existing method is the detection and recognition of poliovirus from the feces.

### 3.3.2 Group 2

This group includes stool specimens taken from kids <5 years from hospitals; Benazir Bhutto Hospital, Rawalpindi (BBH), Mayo Hospital, Lahore (MHL), The Children's Hospital, Lahore (Li *et al.*), Kharadar General Hospital (KGH), Karachi and National Institute of Child Health (McCoy *et al.*), Karachi. These children were hospitalized having serious acute dehydrating gastroenteritis. **Benazir Bhutto Hospital, Rawalpindi** was came into being in 1962. It is the main teaching hospital for the upper Punjab and northern areas of Pakistan. **Mayo Hospital, Lahore** building was completed in 1870 and it began operating in 1871. It is one of the oldest and biggest hospitals in Lahore, Punjab, Pakistan. In 1984, in the yearly meeting of Pakistan Pediatrics Association the idea of the **Children's Hospital, Lahore** was offered and its foundation stone was commenced in 1990. The Hospital established in May 1995, is a public children's hospital located in Lahore, Punjab, Pakistan. From 1918 **Kharadar General Hospital, Karachi** has been delivering quality health amenities to the poor, socio-economical, and educationally low people of 4 million individuals of lowland regions of Karachi. In 1979, the president declared the school of pediatrics as the National Institute of Child Health (McCoy et al.). In Pakistan, it is the first, major and single hospital of the province of Sindh. This hospital is the key source of recognition for the country at national and international level.

## 3.4 Case Definition of Acute Flaccid Paralysis

WHO define AFP as the abrupt start of weakness and floppiness in any body part in children under 15 years of age. The word "flaccid" indicates the movements from loose and soft to submissive elongation whenever in the course of illness or it also indicates the disordered movements by means of motor neuron e.g. hyperflexia or extensor plantar reactions (Organization, 1998).

### 3.4.1 Inclusion Criteria

This includes

1. Children having age less than 15.
2. Signs of headache, fever, nausea and rigidity in each upper and lower limb along with neck with abrupt start of paralysis i.e. lack of floppy and spasticity actions.

### 3.4.2 Exclusion Criteria

This includes

1. Other reasons such as injury or shock.
2. Paralysis due to non-infectious reasons

## 3.5 Case Definition of Gastroenteritis

As per WHO rule an agreed description of diarrhea was taken up in order to make sure that the preferred case signifies a homogeneity that describe diarrhea as "the passage of three or more than three loose or watery stools in 24 hours. Though, this excludes the regular transition of formed or loose stools, or the "pasty" stools via breastfed children (WHO, 2017).

### *3.5.1 Inclusion Criteria*

1. Fever (above 38.5ºc or 101.5ºf) that lasts for above 3 days.
2. Abdominal or rectal ache.
3. Vomiting
4. Symptoms of dryness like recessed eyes, desiccation of skin and mouth.

### *3.5.2 Exclusion Criteria*

1. Non-infectious causes of gastroenteritis such as heavy metal toxicity, indigestions, food intolerance or diseases of the pancreas were not entitled.
2. Children more than 5 years.
3. Deficient information regarding demographic and medical report.

## 3.6 Laboratory based analysis for Detection of Adenoviruses

Entire subjects (AFP and diarrheal) were investigated for the recognition and genetic characterization of adenoviruses. All laboratory methods were carried out at Department of

Virology, NIH by follow the good biosafety measures that are planned by the Centers for Disease Control (CDC) and Prevention, Atlanta, Georgia, The United States of America (CDC, 2009).

### *3.6.1 Techniques Used for Adenovirus Diagnosis*

By employing nested polymerase chain reaction, the specimens taken were analyzed for virus. With the amplification of a characteristic genome section and phylogenetic analysis, genetically a set of positive specimens was differentiated with particulars (Table 3.1).

### *3.6.2 Sample Collection and Processing*

The samples from the study subject were taken in a sterilized screw-capped container and directly shifted to the Department of Virology, NIH, Islamabad. Prior to conduct any viral analysis, the stool specimens were processed for the removal of debris and organic contamination. For this purpose, by addition of 1-2 grams of stool into 1ml phosphate buffered saline (PBS) having calcium and magnesium chloride and 100µl chloroform in a 1.5 ml microcentrifuge tube, ten percent (10% w/v) stool suspensions were made. These tubes were then closed very safely and then vortexed [Vortex-Genei II, Scientific industries Inc., USA] vigorously for mixing purpose for maximum of 10 minutes and then centrifuged at 5000 rpm for 10 minutes at 4ºC. From each specimen, in a Class-II biosafety cabinet, one ml (1ml) clarified supernatant was securely shifted to a sterile 1.5ml microcentrifuge cryovial. In cryoboxes these aliquots were arranged and placed at -20ºC up to additional analysis. The original stool samples vials were secured by means of wrapping parafilm tape [Cat No. P7793, Sigma-Aldrich] on the tubes lid and placed at -80ºC.

### *3.6.3 Virus Isolation from Stool Suspensions*

Prior to begin any analysis, the cleared 10% fecal supernatant was maintained to room temperature. According to WHO standard techniques, all samples were analyzed. These stool suspensions were processed and inoculated into Hep-2C derived from human epithelial carcinoma cell lines. Human adenoviruses were isolated using continuous 96 well culture plate method. Hep-2C was grown in cell culture plate using Minimum Essential Media (MEM) as growth medium supplemented with 10 % fetal bovine serum. The culture plates were seeded with 500,000 cells/ml and placed the

plates at 37°C in $CO_2$ incubator. The confluency of the cells monolayer was observed on daily basis under microscope. As confluent monolayer developed, the growth medium was replaced with 9.6 ml of 2% maintenance medium. 0.4 ml supernatant of each specimen extract was inoculated into plate and placed the plates at 37°C in $CO_2$ incubator. While inoculating supernatant, last 8 wells were left as negative control (4 wells for 1 sample). Microscopic observations of each plate were done to see the appearance of cell morphology for 2-3 days and graded it in terms of cytopathic effect (CPE) varied from 1 to 4 depending upon the proportion of affected cells. 1+, 2+, 3+ and 4+ CPE presented that up to 25 %, 25-50 %, 50-75 % and 75-100 % cells respectively affected. Adenoviruses were provisionally recognized by characteristic grape-like CPE in HEp-2 cell line. On the basis of characteristic cytopathic effect early detection of adenoviruses delivered exceptional sensitivity (i.e., none of the CPE agents provisionally identified as enteroviruses were found to be adenoviruses) and specificity (mostly viruses selected by CPE characteristics were ascertained to be adenoviruses with the help of PCR). The typical adenovirus CPE was noted and the cell culture plate positive with 4+ CPE were stored at -20 °C till their further testing.

### 3.6.4 Molecular Detection and Nucleotide Sequencing

#### 1) DNA Extraction

The extraction of DNA directly from cell culture isolates was accomplished by making use of nucleospin DNA extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to company's protocols. This kit contains two lysis buffers (ST1 and ST2), one binding buffer (ST3), two washes buffers (ST4 and ST5) and an elution buffer (SE). In addition, NucleoSpin® Bead Tubes Type A, NucleoSpin® Inhibitor Removal Columns, NucleoSpin® DNA Stool Columns, and collection tubes (2 mL). The lysis buffer is employed for the lysis of the sample and deactivate the nucleases below the extremely denaturing settings. In short, 200 uL of cell culture fluids was transferred to a NucleoSpin® Bead Tube Type A. 850 µL of Buffer ST1 was added. NucleoSpin® Bead Tube was then shaked horizontally for 2–3 seconds to mix culture fluid and lysis buffer before putting it onto a heat incubator. NucleoSpin® Bead Tubes was incubated for 5

min at 70 °C. NucleoSpin® Bead Tube was then agitated in the MN Bead Tube Holder on a vortex [VortexGenei II, Scientific industries Inc., USA]. Samples were vortexed at full speed at room temperature (18–25 °C) for 10 minutes which were then centrifuged for 3 min at 13,000 x *g*. 600 µL of the supernatant was transferred to a fresh 2 mL microcentrifuge tube with lid (not provided). Then 100 µL of Buffer ST2 was added and vortexed for 5 s. Incubation was done for 5 min at 2–8 °C. Again centrifuged for 3 min at 13,000 x *g*. NucleoSpin® Inhibitor Removal Column (red ring) was placed in a collection Tube (2 mL, lid). Avoiding the pellet, 550 µL of the cleared lysate was transferred onto the NucleoSpin® Inhibitor Removal Column. Centrifugation was done for 1 min at 13,000 x *g*. NucleoSpin® Inhibitor Removal Column was discarded. 200 µL of Buffer ST3 was added and vortexed for 5 s. Place a NucleoSpin® DNA Stool Column (green ring) was placed in a Collection Tube (2 mL) and 700 µL sample was loaded onto the column. Again centrifuged for 1 min at 13,000 x *g*. The next step is the removal of flow-through and then putting back the column into the collection tube then 600 µL of Buffer ST3 was added to the NucleoSpin® DNA Stool Column. Centrifuged for 1 min at 13,000 x *g*. The next step is the removal of flow-through and then putting back the column into the collection tube then 550 µL of Buffer ST4 was added to the NucleoSpin® DNA Stool Column. Centrifuged for 1 min at 13,000 x *g*. The next step is the removal of flowthrough and then putting back the column into the collection tube then 700 µL of Buffer ST5 was added to the NucleoSpin® DNA Stool Column and vortexed for 2 s. Centrifuged for 1 min at 13,000 x *g*. The next step is the removal of flow-through and then putting back the column into the collection tube. Then 700 µL of Buffer ST5 was added to the NucleoSpin® DNA Stool Column and centrifuged for 1 min at 13,000 x *g*. The next step is the removal of flow-through and then putting back the column into the collection tube and centrifuged for 2 min at 13,000 x *g*. NucleoSpin® DNA Stool Column was placed into a new 1.5 mL microcentrifuge tube (not provided). 30 µL of Buffer SE was added to the column and was centrifuged for 1 min at 13,000 x *g*. NucleoSpin® DNA Stool Column was discarded and vortexed each microcentrifuge tube for 2 s. Till additional analysis, the eluted DNA was collected and kept at -70 ºC (Figure 3.1).

**Figure 3.1.** DNA extraction protocol

**NucleoSpin® DNA Stool**

| | | |
|---|---|---|
| **1 Prepare sample** |  | NucleoSpin® Bead Tube Type A<br>180–220 mg sample material<br>850 µL ST1, shake horizontally 2–3 s |
| **2 Lyse sample** |  | 70 °C, 5 min<br>Vortex 10 min at RT using MN Bead Tube Holder on Vortex-Genie® 2 at max. speed |
| **3 Precipitate contaminants** |   | 13,000 x *g*, 3 min<br>Transfer 600 µL supernatant<br>100 µL ST2<br>Vortex 5 s<br>5 min, 2–8 °C<br>13,000 x *g*, 3 min |
| **4 Filter lysate** |  | Transfer 550 µL cleared lysate on NucleoSpin® Inhibitor Removal Column<br>13,000 x *g*, 1 min |
| **5 Adjust binding conditions** |  | 200 µL ST3<br>Vortex 5 s |
| **6 Bind DNA** |   | Load 700 µL sample on NucleoSpin® DNA Stool Column<br>13,000 x *g*, 1 min |
| **7 Wash silica membrane** |   | 1st: 600 µL ST3 — 13,000 x *g*, 1 min<br>2nd: 550 µL ST4 — 13,000 x *g*, 1 min<br>3rd: 700 µL ST5 Vortex 2 s — 13,000 x *g*, 1 min<br>4th: 700 µL ST5 — 13,000 x *g*, 1 min |
| **8 Dry silica membrane** |   | 13,000 x *g*, 2 min |
| **9 Elute DNA** |   | 30–100 µL SE<br>13,000 x *g*, 1 min<br>Vortex 2 s |

**2) Nested PCR Assay for Adenovirus Screening in AFP Cases**

For the amplification, a nested touchdown PCR was employed (GeneAmp1PCR System 9700, Applied Biosystems, Warrington, UK) of partial region of hexon as described by Allard *et al*. The outer primer pair, hex1deg (5´-GCCSCARTGGKCWTACATGCACATC-3´) and hex2deg (5'CAGCACSCCICGRATGTCAAA-3'), which created a 301-bp product, and the nested primer pair, nehex3deg (5'-GCCCGYGCMACIGAIACSTACTTC-3') and nehex4deg (5'-CCYACRGCCAGIGTRWAICGMRCYTTGTA-3'), produced an amplimer of 171 bp were used for both sequencing and diagnosis. For first round of diagnostic PCR, 50µl reaction mixture was used containing 5ul of 10X PCR buffer, 6ul of 25mM of $MgC1_2$, 1ul 10mM of dNTPs, 2.5ul of each primer hex-1deg (10uM) and primer hex-2deg (10uM), 1ul 5 units of Taq DNA polymerase, 30ul template DNA and 2 ul PCR water was added for making the reaction volume up to 50ul (Table 3.2).

For second round of PCR, the reaction mix was identical as that in round 1 PCR except for using 10 ul of round 1 product as template and PCR water was then added accordingly (Table 3.3). The thermal profile for round 1 and round 2 PCR was same (Table 3.4) (Allard *et al.,* 2001).

**Table 3.2** Nested-PCR (round 1) reaction mix used for screening of human adenovirus

| **Round 1** | **Component** | **Volume (ul)** |
|---|---|---|
| Nested-PCR Master Mix (50 ul reaction) | 10X Taq PCR buffer, (PH 9, Applied Biosystems) | 5 |
| | 25mM $MgCl_2$ (Applied Biosystems) | 6 |
| | 10mM concentration of each deoxynucleoside triphosphate (Promega, Ref U1515) | 1 |
| | 10 uM of each primer of round 1 (forward and reverse) | 2.5 |
| | DNA polymerase (AmpliTaq @ DNA, 250 U, Cat: N808016) | 1 |
| | PCR water (DNase-RNase free) | 2 |
| | Nucleospin-extracted DNA | 30 |

**Table 3.3.** Nested-PCR (round 2) reaction mix used for screening of human adenovirus

| **Round 2** | **Component** | **Volume (ul)** |
|---|---|---|
| Nested-PCR Master Mix<br><br>(50 ul reaction) | 10X Taq PCR buffer, (PH 9, Applied Biosystems) | 5 |
| | 25mM $MgCl_2$ (Applied Biosystems) | 6 |
| | 10mM concentration of each deoxynucleoside triphosphate (Promega, Ref U1515) | 1 |
| | 10 uM of each primer of round 2 (forward and reverse) | 2.5 |
| | DNA polymerase (AmpliTaq @ DNA, 250 U, Cat: N808016) | 1 |
| | PCR water (DNase-RNase free) | 22 |
| | Round 1 PCR product | 10 |

**Table 3.4.** Thermal profile for Round 1 and Round 2

| **Temperature** | **Time** |
|---|---|
| 94°C | 3 Minutes |
| 94°C | 30 Seconds |
| 55°C | 30 Seconds |
| 72°C | 30 Seconds |
| | 35 cycles |
| 72°C | 5 Minutes |
| 4°C | ∞ |

**3) Nested PCR Assay for Adenovirus Screening in Gastroenteritis Cases**

For amplification of hexon loop 1 (L1) region, nested PCR was used by utilizing the primers described by Lu and Erdman. The external primers were AdhexF1 (5' TIC TTT GAC ATI CGI GGI GTI CTI GA3') and AdhexR1 (5' CTG TCI ACI GCC TGR TTCCAC A 3'). For internal amplification, internal primers AdhexF2 (5'GGY CCY AGY TTY AAR CCC TAY TC 3') and AdhexR2 (5' GGT TCT GTC ICC CAG AGA RTC IAGCA 3') were employed with 1 ul of the amplicons achieved from the above reaction in order to produce an amplicon of 820 bp (Table 3.5 and 3.6). Thermal profile for both the rounds is given in Table 3.7.

Primers HSAd1 (5' CTG ATG TAC TAC AACAGC ACT GGC AAC ATG GG 3') and HSAd2 (5' GCGTTG CGG TGG TGG TTA AAT GGG TTT ACG TTGTCC AT 3') were used to amplify (ABI genetic analyzer (Applied Biosystems, Foster City, California, USA)) the loop 2 (L2) region of the hexon gene generating an amplicon which size differs among 605 and 629 bp relied on the type of virus (Table 3.8). Thermal profile for L2 is given in Table 3.10 (Al Qurashi *et al.*, 2012).

**Loop 1**

**Table 3.5** Nested-PCR (round 1) reaction mix used for screening of human adenovirus

| **Round 1** | **Component** | **Volume (ul)** |
|---|---|---|
| Nested-PCR Master Mix (50 ul reaction) | 10X Taq PCR buffer, (PH 9, Applied Biosystems) | 5 |
| | 25mM $MgCl_2$ (Applied Biosystems) | 4 |
| | 10mM concentration of each deoxynucleoside triphosphate (Promega, Ref U1515) | 1 |
| | 10 uM of each primer of round 1 (forward and reverse) | 2.5 |
| | DNA polymerase (AmpliTaq @ DNA, 250 U, Cat: N808016) | 1 |
| | PCR water (DNase-RNase free) | 24 |
| | Nucleospin-extracted DNA | 10 |

**Table 3.6** Nested-PCR (round 2) reaction mix used for screening of human adenovirus

| **Round 2** | **Component** | **Volume (ul)** |
|---|---|---|
| Nested-PCR Master Mix (50 ul reaction) | 10X Taq PCR buffer, (PH 9, Applied Biosystems) | 5 |
| | 25mM $MgCl_2$ (Applied Biosystems) | 4 |
| | 10mM concentration of each deoxynucleoside triphosphate (Promega, Ref U1515) | 1 |
| | 10 uM of each primer of round 1 (forward and reverse) | 2.5 |
| | DNA polymerase (AmpliTaq @ DNA, 250 U, Cat: N808016) | 1 |
| | PCR water (DNase-RNase free) | 33 |
| | Nucleospin-extracted DNA | 1 |

**Table 3.7.** Thermal profile for Round 1 and Round 2

| **Temperature** | **Time** |
|---|---|
| 94°C | 2 Minutes |
| 94°C | 1 Minute |
| 45°C | 1 Minute |
| 72°C | 2 Minutes |
| | 35 cycles |
| 72°C | 5 Minutes |
| 4°C | ∞ |

**Loop 2**

**Table 3.8.** Single-round PCR reaction mix used for screening of human adenovirus

| | **Component** | **Volume (ul)** |
|---|---|---|
| Nested-PCR Master Mix (50 ul reaction) | 10X Taq PCR buffer, (PH 9, Applied Biosystems) | 5 |
| | 25mM $MgCl_2$ (Applied Biosystems) | 4 |
| | 10mM concentration of each deoxynucleoside triphosphate (Promega, Ref U1515) | 1 |
| | 10 uM of each primer (forward and reverse) | 2.5 |
| | DNA polymerase (AmpliTaq @ DNA, 250 U, Cat: N808016) | 1 |
| | PCR water (DNase-RNase free) | 24 |
| | Nucleospin-extracted DNA | 10 |

**Table 3.9.** Thermal profile for L2

| **Temperature** | **Time** |
|---|---|
| 95°C | 10 Minutes |
| 95°C | 1 Minute |
| 51°C | 1 Minute |
| 72°C | 1 Minute |
| | 40 cycles |
| 72°C | 5 Minutes |
| 4°C | ∞ |

**4) Gel Electrophoresis**

The nested-PCR products were resolved on 2% agarose gel comprising 0.5ug/ml Ethidium bromide (Bio-Rad, Part No. 161-0433, 10mg/ml) made in 1X TBE buffer (Tris-HCl Part No. Sigma-Aldrich RES3098TB701X-100G; Boric Acid Part No. Sigma-Aldrich 339067-100G and EDTA Part No. Sigma Aldrich E5134-100G). Briefly, in 100ml of 1X TBE buffer, 2 grams of agarose powder (Sigma-Aldrich part No. A9539-100gm) was mixed and liquefied in a microwave oven and kept in a water bath set at 55ºC. In a gel caster, 15 ul well combs were positioned and the molten agarose solution was decanted into the casting tray and chilled for about 1 and a half hours in order to solidify. In electrophoresis slot which comprising 100ml of newly made 1X TBE buffer, the gel was placed and then combs were detached securely.

From each sample, 5 ul of PCR amplicons was mixed with 3ul of 6X gel loading dye (Thermo Fisher Scientific, Part No. R0611 containing bromophenol blue and xylene cyanol) and loaded to relevant well of agarose gel. For every investigated sample, the gel loading order was noted. Molecular weight marker, negative and positive controls were loaded subsequent to the loading points. The next step was the electrophoresis of the gel at 121 volts for 45 minutes till the dye line was 80% down way of the gel. Then below a UV light source (Gel-Doc XR system Part No. 1708170, Bio-Rad Laboratories Inc., USA), the gel was then kept for visualization of the amplified DNA bands. The genotypes of the HAdV were then identified on the basis of the particular size of amplified product comparative to the 100bp molecular weight marker (BIO-RAD EZ LoadTM Molecular Rulers Part No. 170-8352 (100bp) loaded as 5ug/ul containing 500ng DNA per lane.

**5) Sequencing of Amplified PCR Products**

Numerous distinct constituents were present in the PCR reaction mix e.g. dNTPs, primers etc. in addition to undesired by-products that may amplify except anticipated DNA fragment. All of these contaminants are removed via this purification step, which is essential for obtaining high quality template for sequencing purposes. For cleaning the PCR products, a commercial purification kit was utilized with thorough procedure as defined below.

**a) Purification of Amplified PCR Products**

Following the manufacturer's guidelines, the PCR amplicons were purified by utilizing QIAquick PCR Purification kit (Qiagen, Part No. 28106). To the PCR cleaned product, the buffer PBI was poured in a 1:5 ratio and then mixed collectively in a 1.5ml microcentrifuge tube. Then onto QIAquick spin column, the mixture of the sample was poured and placed in a 2ml collection tube and were centrifuged for 1 minute at 14000rpm. The next step was the removal of flow-through and washing of the column with 750ul of wash buffer PE and centrifuged at 14000rpm for 1 minute. Again the filtrate was removed and the column was centrifuged at 14000rpm for 1 minute in order to eliminate any leftover ethanol present in PE buffer. To a fresh 1.5ml microcentrifuge tube, the column was transferred. Elution of the Purified DNA was done by the addition of 50ul of Buffer EB (10mM Tris-Cl, pH 8.5) on the middle of QIAquick membrane and were centrifuged for 1 minute at 8000 rpm and kept at -20ºC till additional investigation.

**6) Cycle Sequencing Reaction**

It is the type of reaction in which one primer is required for generation of the single stranded amplicons. Furthermore, fluorescent dyes (BigDye terminators) labeled dNTPs are added for the termination of the PCR reaction in order to generate PCR fragments of several lengths. The analyzation of these fragments are done by the laser integrated automated DNA sequencing machine. The sequencing of the purified PCR products were undergone bi-directionally for increased sequence accord guarantee and validation of sequence data. By employing ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Part No. 4390242), the cycle sequencing reaction was executed with 5-20ng of purified DNA according to manufacturer's guidelines. In a total volume of 10ul, the PCR reaction was commenced with 2ul of BigDye Terminator ready reaction mix (dideoxynucleoside triphosphates, Deoxynucleoside triphospohates, AmpliTaq DNA Polymerase, MgCl2, Tris-HCl buffer pH 9.0), 0.5 ul primer, 2 ul buffer and 3.5 ul of deionized water to compose the volume. The tubes were then placed in thermal cycler and preset to 25 cycles of amplification at 96ºC for 20 seconds, primer annealing at 60/55°C for 2 minutes and extension for 5 minutes at 72°C (Table 3.10). Prior to additional investigation, the amplified products were then cleaned

**Table 3.10.** Names and annealing temperature of primers used for cycle sequencing reaction

| S. No | Primer Name | Primer Sequence | Annealing Temperature |
|---|---|---|---|
| 1 | nehex3deg | 5′-GCC CGY GCM ACI GAI ACS TAC TTC-3′ | 60 ºC |
| 2 | nehex4deg | 5′-CCY ACR GCC AGI GTR WAI CGM RCY TTG TA-3′ | 60 ºC |
| 3 | AdhexF2 | 5'GGY CCY AGY TTY AAR CCC TAY TC 3' | 55 ºC |
| 4 | AdhexR2 | 5' GGT TCT GTC ICC CAG AGA RTC IAGCA 3' | 55 ºC |

**7) Purification and Removal of Big Dye Terminators**

To improve the concentration and quality of the sequence peak, the distinct BigDye terminators, salts and other charged molecules was discarded from the cycle sequencing reaction products.

**a) Ethanol/EDTA/Sodium Acetate Precipitation**

This is the step in which for attaining good signal in the following electrophoresis reaction on the genetic analyzer, the distinct terminators were discarded from cycle sequencing reaction. It includes the purification by mixing 10ul cycle sequencing reaction product, 1ul of 125mM EDTA, 1ul of 3M sodium acetate and 25ul of 100% ethanol. The tubes were wrapped and later these were incubating at room temperature for 15 minutes and then mixed by vortexing for 15 seconds. Then centrifugation occurred at 14000rpm for 20 minutes at 4ºC. The next step was careful removal of supernatant from every tube and then addition of 70ul of 70% ethanol and centrifugation at 14000rpm for 15 minutes at 4ºC was accomplished. Again, the supernatant was discarded from every tube. Purified DNA pellets containing tubes were placed at room temperature for 15 minutes

in order to discard any remaining ethanol. In 20ul deionized HiDi formamide (Applied Biosystems, Part No. 4311320), DNA was then dissolved and positioned to ABI Genetic Analyzer (Applied Biosystems, Foster City, California, USA) within PCR reaction plates (Applied Biosystems, Part No. 4306737).

**8) Editing of Sequence Data**

From automated Genetic Analyzer, the unrefined sequence data acquired which was then modified via Sequencher® version 4.9, Gene Codes Corporation, Ann Arbor, MI, USA (http://www.genecodes.com). This software was helpful in trimming of the sequence reads, contig assemblies along with the generation of the FASTA files of sequences. These FASTA files are then utilized for phylogenetic analysis.

**9) BLAST Analysis**

The FASTA files (the file format which is used for the comparison of the fast nucleotide or protein) of genomic sequences attained were proceeded over online BLAST (Basic Local Alignment Search Tool) analysis presented at [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. For adenoviruses along with its genotypes/serotypes, the contiguous matched and the reference strain sequences attained in this manner were downloaded in FASTA formats and saved. Then for phylogenetic and evolutionary purposes, these FASTA files were then utilized.

**10) Phylogenetic Tree**

In the form of phylogenetic trees, the evolutionary relations among virus types showed in this study and universal strains were investigated and were graphically represented. On the basis of mathematical statements, the tree is created that explained the evolution of nucleotides of representative genomes.

With the MEGA (Molecular Evolutionary Genetic Analysis) v.X64 software, the FASTA files of all reference and study sequences were tallied with one another. While with the help of CLUSTAL W program multiple sequence alignments were constructed which is integral characteristic of

MEGA software. The next step was the formation of phylogenetic trees by applying the maximum likelihood and neighbor-joining method accessible by default in MEGA software.

**11) Statistical Analyses**

By means of the Statistical Package for the GraphPad Prism software v8.2.0 (435) (GraphPad Software; San Diego, CA), the epidemiological and demographical information was investigated statistically. For age, gender, extent of symptoms and additional clinical and demographic variables, the detailed statistical tests like frequencies, percentages, ratio, mean, median and range were conducted. Between patients infected with various viruses and their subtypes, the analysis of variance (ANOVA) was performed for the comparing several demographic characteristics. The pvalue below 0.05 (two sided) were thought to be statistically significant. Chi-square test ($\chi 2$) and student's T-test were employed for determining the link among various serotypes/genotypes and disease severity.

In order to analyze the statistical importance of the phylogeny analysis bootstrap technique was used. The resampling method for reconstruction the trees by 100 or 1000 repetitions and analyzing if the identical nodes are produced is the Bootstrapping method. Bootstrap values $\geq 70$ were measured to be reliable that corresponds to the real clade with increased likelihood and was admitted as sign of good statistical backing as suggested by Hillis and Bull (Hillis & Bull, 1993)

*Chapter 4* **RESULTS**

The whole cross sectional evaluation was conducted at National Institute of Health, Islamabad, Pakistan. Totally 1, 290 stool samples during 2017-2018 were taken from acute flaccid paralysis and gastroenteritis patients to study the prevalence and characterization of human adenovirus. For acute flaccid paralysis, the study was conducted under the context of an AFP surveillance program in Pakistan and for gastroenteritis; it was hospital relied surveillance and intended at molecular diagnostic and epidemiology of human adenovirus related with gastro-enteric ailments in Pakistani children. For diarrheal samples, fecal specimens were taken from children from five tertiary care hospitals namely Benazir Bhutto Hospital, Rawalpindi (BBH), Mayo Hospital Lahore (MHL), Children's hospital and the Institute of Child Health (CHL), Lahore, Kharadar General Hospital (KGH) and National Institute of Child Health, Karachi (NICH) that serves as the sentinel sites of WHO for surveillance program of rotavirus in Pakistan and were transferred to the Department of Virology, National Institute of Health, Islamabad.

## ***4.1 Analysis of HAdV in Acute Flaccid Paralysis (AFP) cases***

For AFP, a total of 172 stool samples during the year 2017 (Jan-Dec) were collected from acute flaccid paralysis patients to study the prevalence and characterization of adenoviruses. These specimens were collected under the age of 15 years in the framework of an AFP surveillance program in Pakistan. All of these stool samples collected from all over Pakistan following World Health Organization (WHO) guidelines. These samples were collected within 14 days from the start of the symptoms and were transported to WHO Regional Reference Laboratory for Polio Eradication Initiative, Virology Department, National Institute of Health Islamabad, Pakistan and stored at −20 °C.

### 4.1.1 Demographic analysis of AFP patients

#### *i) Geographical Distribution*

The 172 stool specimens enrolled in this study were collected from various regions of Pakistan such as Punjab (PB), Khyber Pakhtunkhwa (KP), Sindh (SD), Baluchistan (BA), Federally Administered Tribal Areas (FATA), Azad Jammu & Kashmir (AJK) and Gilgit Baltistan (GB)

during 2017 (Figure 4.1). The highest percentage of AFP patients were present in PB province 49% (n=85) followed by SD province with 20% (n=34), and then KP, BA, FATA, AJK and GB with 14% (n=24), 7% (n=12), 4% (n=7), 4% (n=6) and 2% (n=4) respectively (Figure 4.2).

Among these AFP samples, 51.74% (n=89/172) were confirmed as HAdV by PCR analysis. From these positive samples, 51.68% (n=46/89) were isolated from PB province, then SD account for 20.22% (n=18/89), followed by KP, BA, FATA, AJK and GB with 13.48% (n=12/89), 6.74% (n=6/89), 4.49% (n=4/89), 2.24% (n=2/89) and 1.12% (n=1/89) respectively (Figure 4.3).

Furthermore, among all the districts (n=29) from Punjab (Muzaffargarh, Sheikhupura, Khanewal, Bahawalnagar, Chiniot, Bhakkar, Gujrat, Gujranwala, Multan, Dera Ghazi Khan, Sialkot, Sargodha, Kasur, Rawalpindi, Nankanasahib, Rajanpur, Chakwal, Attock, Lahore, Pakpattan, Narowal, Vehari, Mianwali, Rahim Yar khan, Faisalabad, Okara, Mandibahauddin, Talagang and Jhelum), the HAdV positive cases were isolated from districts (n= 24) such as Muzaffargarh, Sheikhupura, Khanewal, Bahawalnagar, Chiniot, Bhakkar, Gujrat, Gujranwala, Multan, Dera Ghazi Khan, Sialkot, Sargodha, Kasur, Rawalpindi, Nankanasahib, Rajanpur, Chakwal, Attock, Lahore, Pakpattan, Narowal, Vehari, Mianwali, and Jhelum. Among these districts the most detected percentage for HAdV was in Sialkot (10.86%), followed by Muzzafargarh (8.69%), Lahore (6.52%), Gujrat (4.34%) and Multan (2.17%) (Figure 4.4).

Similarly, the AFP cases were collected from the different districts (n=12) of Sindh which includes Liaqatabad, Binqasim, Jacobabad, Orangi Town, Mirpurkhas, Thatta, Larkana, Khairpur, Ghotki, Shikarpur, Tharparker and Umerkot. Among them, the HAdV was detected in districts (n=6): Liaqatabad, Binqasim, Jacobabad, Orangi Town, Mirpurkhas and Thatta. The most prevalent percentage of HAdV was isolated from Jacobabad (27.77%), followed by Mirpurkhas (22.22%), then Binqasim (16.66%), Liaqatabad, Orangi Town, and Thatta (11.11%) (Figure 4.5).

Also, in case of Khyber Pukhtunkhwa, the districts (n=9) includes were Bannu, Lower Dir, Mardan, Swabi, Charsada, Kohat, Abbottabad, Peshawar and Shangla. The positive HAdV samples were isolated from Bannu, Lower Dir, Mardan, Swabi, Charsada, Kohat, Abbottabad and Peshawar. The highest percentage was from Bannu, Mardan, Swabi, and Peshawar (16.66%), followed by Lower Dir, Charsada, Kohat and Abbottabad (8.33%) respectively (Figure 4.6).

Furthermore, Baluchistan districts (n=5) includes Pishin, Nasirabad, Quetta, Qillah Abdullah and Jafarabad. HAdV was isolated from Pishin, Nasirabad, Quetta and Qillah Abdullah, with highest percentage from Pishin and Qillah Abdullah (33.33%), followed by Nasirabad and Quetta (16.66%) respectively (Figure 4.7).

Similarly, from Federally Administrated Tribal Areas (FATA) 4 districts, from Azad & Jammu Kashmir (AJK) and Gilgit Baltistan (GB) only 1 district was included. The districts from FATA includes Orakzai Agency, Khyber Agency, Kurram Agency and South Waziristan. Only Orakzai Agency and Khyber Agency had HAdV cases with 50% each. Likewise, AJK includes only Muzafarabad with HAdV cases. Similar to AJK, in GB only Diamir was included with single sample, which was positive.


**Figure 4.1.** Map of Pakistan showing provincial boundaries and samples collected from different provinces of Pakistan

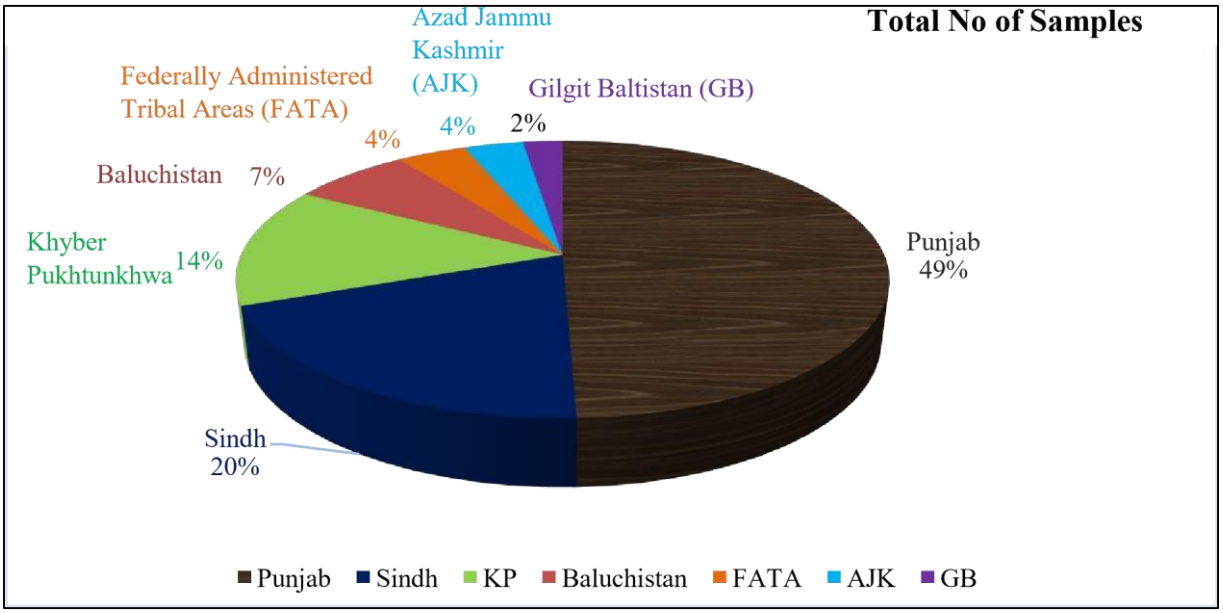



**Figure 4.2.** Distribution of total number of samples among AFP cases in Pakistan during 2017

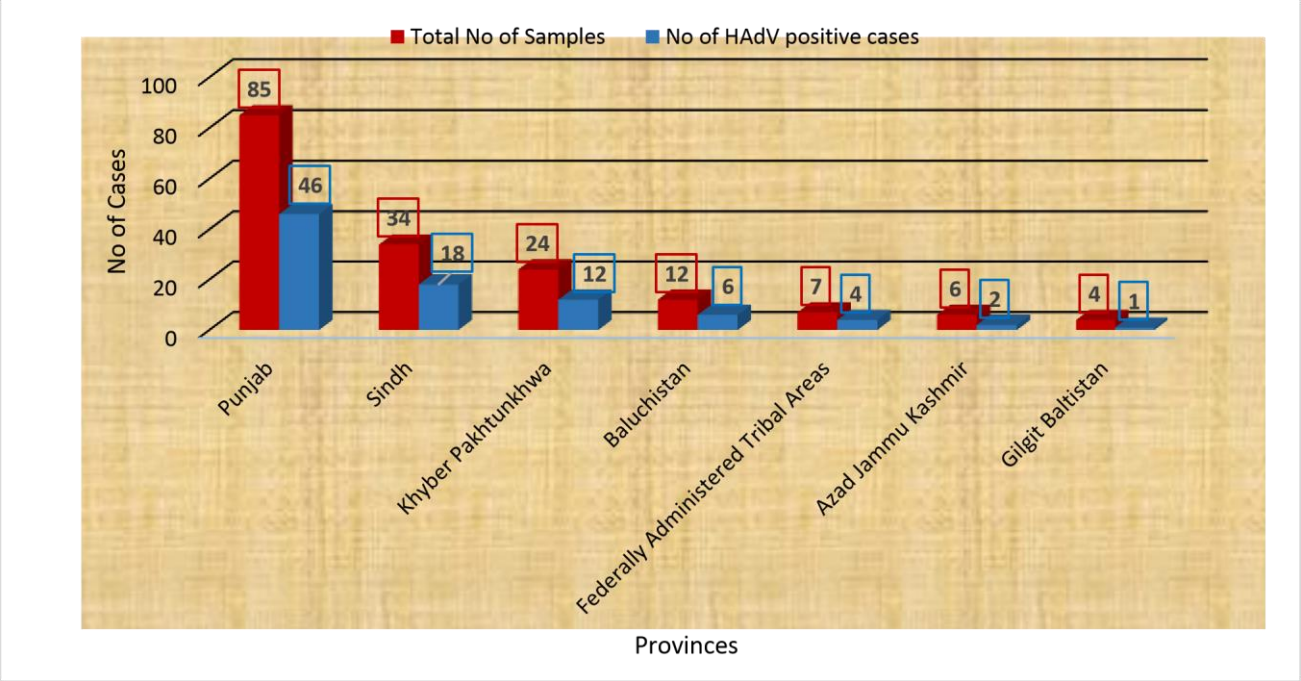



**Figure 4.3.** Province wise distribution of HAdV positive cases among AFP cases in Pakistan during 2017

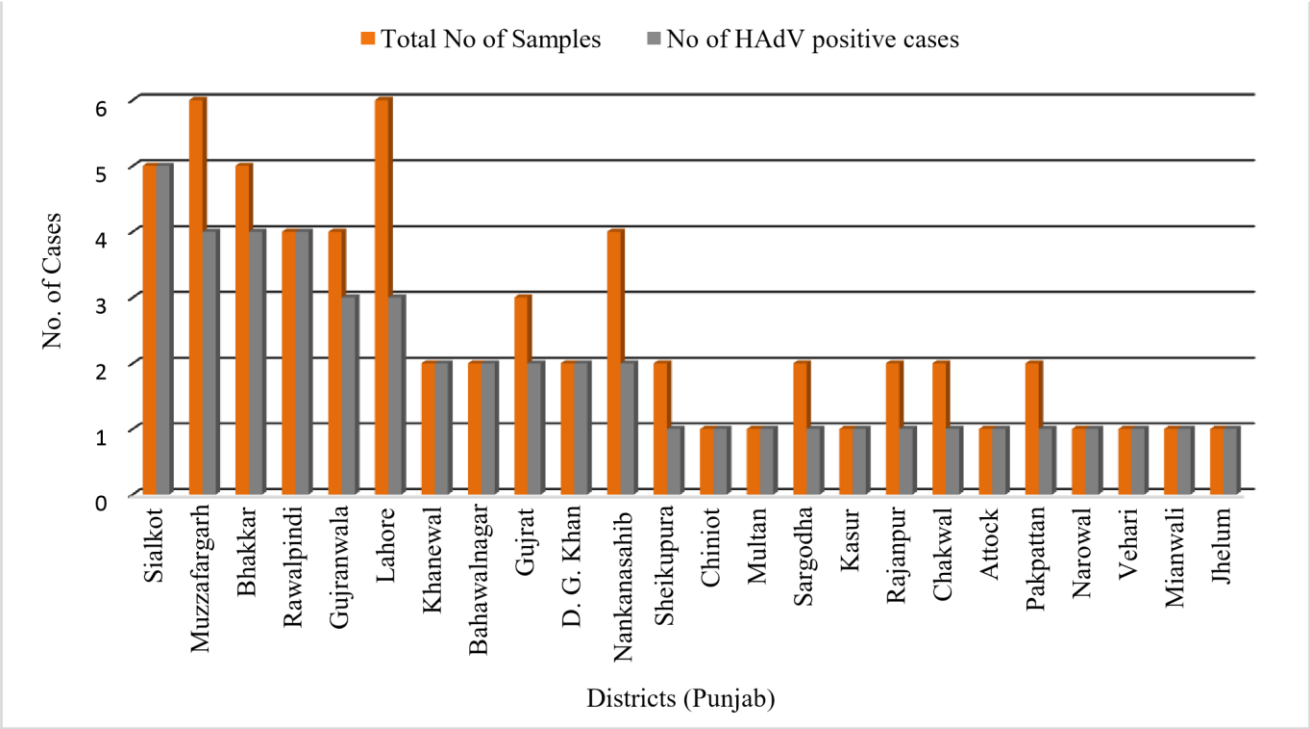



**Figure 4.4.** Distribution of HAdV positive cases among different district of Punjab during 2017

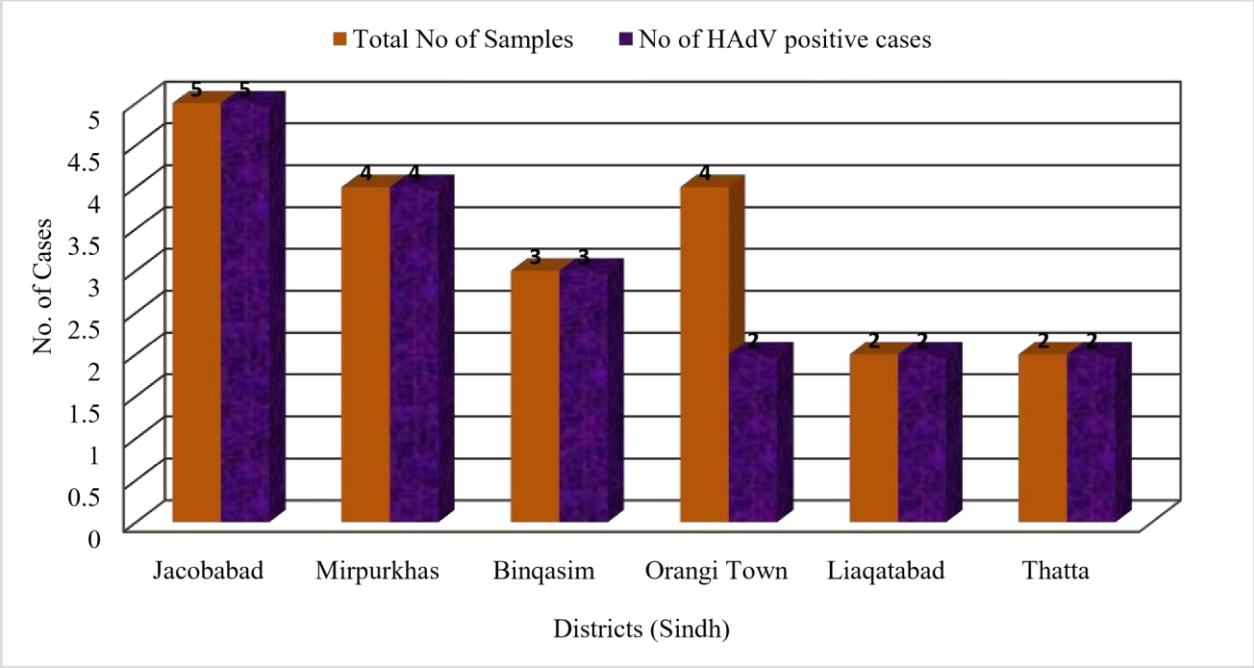
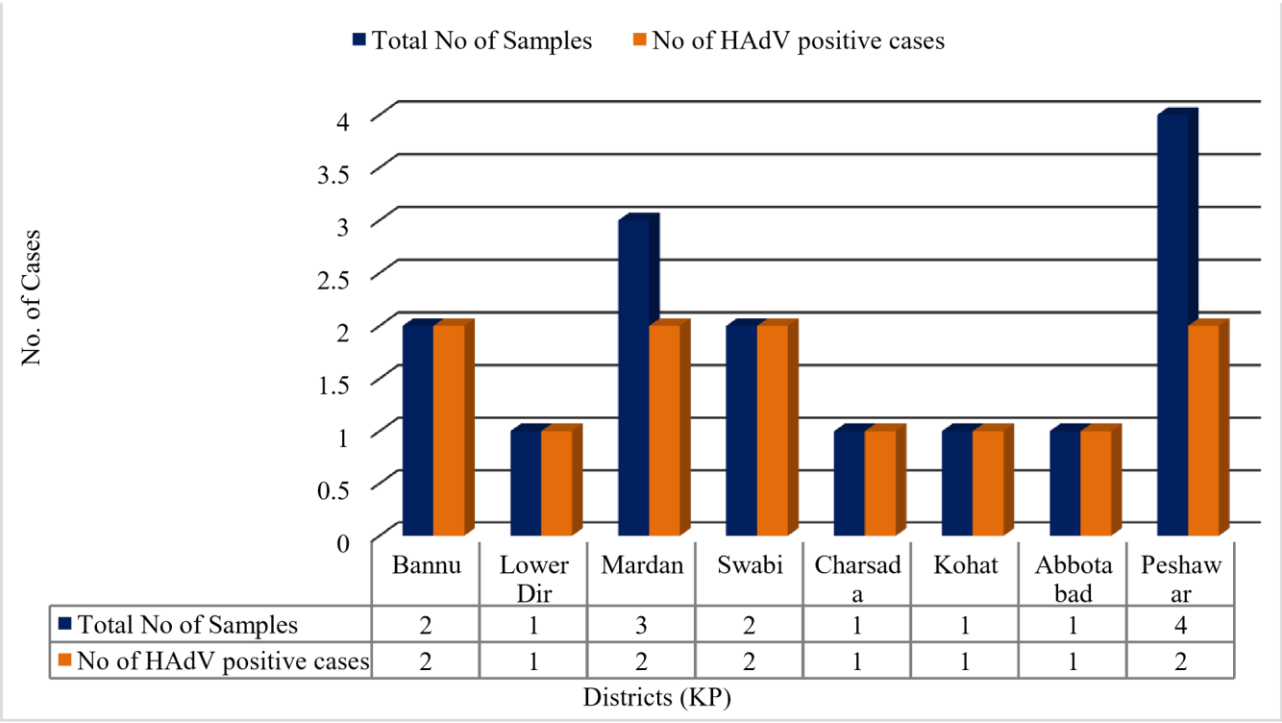



**Figure 4.5.** Distribution of HAdV positive cases among different district of Sindh during 2017




| | Bannu | Lower Dir | Mardan | Swabi | Charsada | Kohat | Abbotabad | Peshawar |
|---|---|---|---|---|---|---|---|---|
| Total No of Samples | 2 | 1 | 3 | 2 | 1 | 1 | 1 | 4 |
| No of HAdV positive cases | 2 | 1 | 2 | 2 | 1 | 1 | 1 | 2 |

**Figure 4.6.** Distribution of HAdV positive cases among different district of KP during 2017

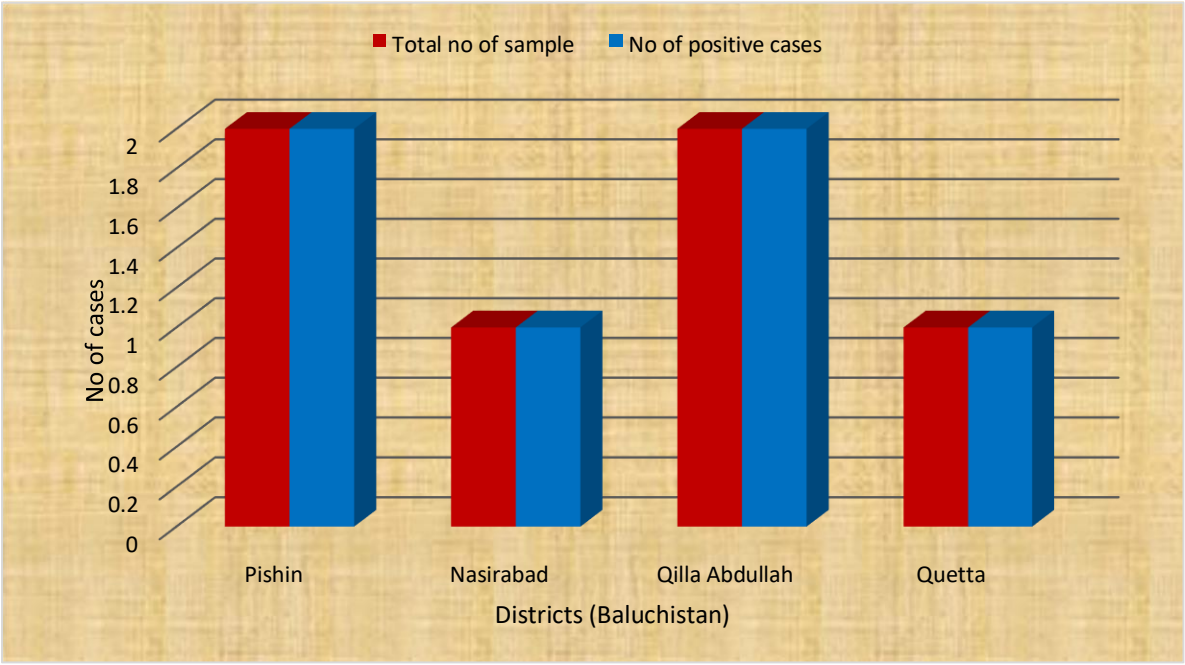
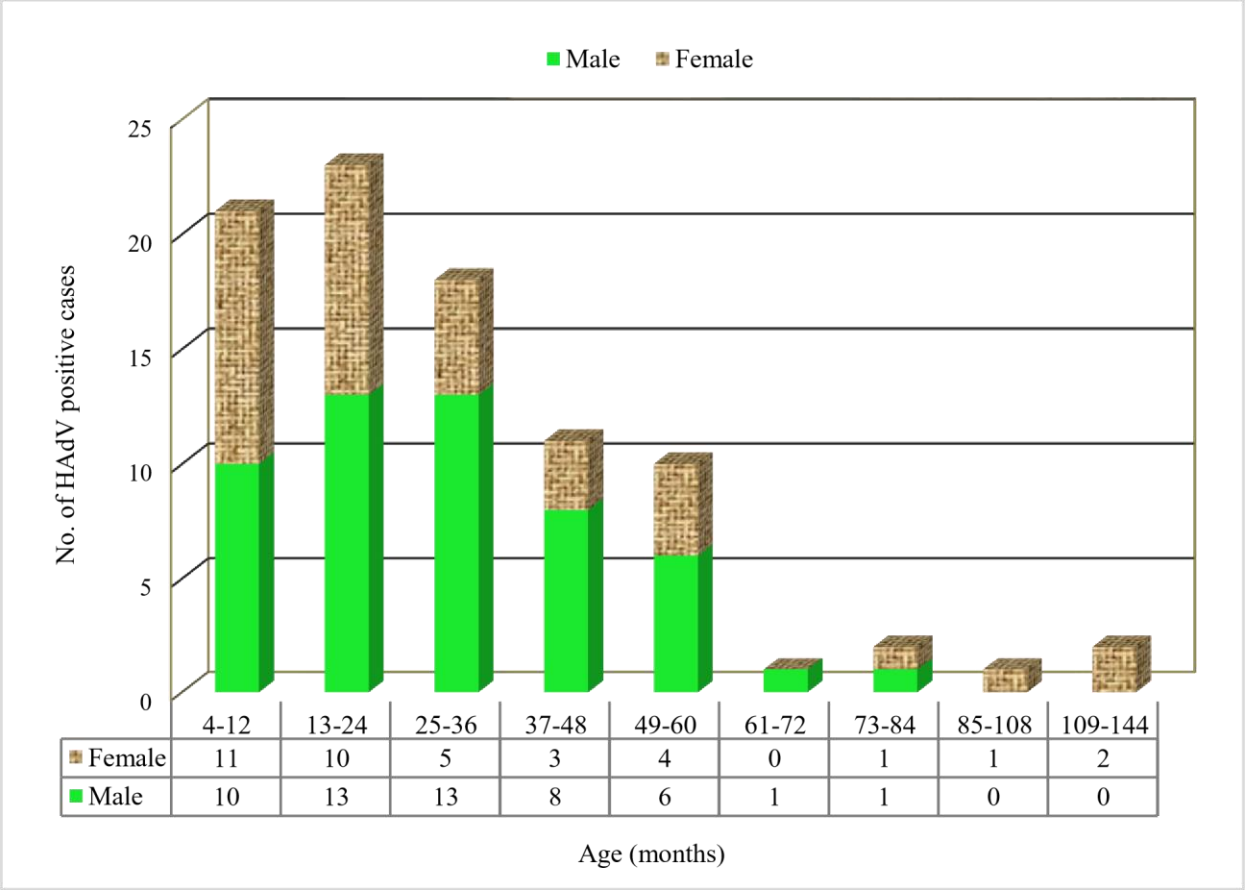



**Figure 4.7.** Distribution of HAdV positive cases among different district of Baluchistan during 2017

### *ii) Gender and Age-wise Distribution*

Among positive samples 52 (58.42%) were male while 37 (41.57%) were females with 1:1.40 ratio (Figure 4.8). The ages of these children ranges from 4 months to 144 months (mean age=31.99, median age=26). According to our results, the highest proportion (n=23, 25.84%) was observed among 13-24 months old followed by 4-12 months (n=21, 23.59%) and then 25-36 (n=18, 20.22%) old. Likewise, the lower proportion was found in 73-84 months and 109-144 months (n=2, 2.24%) followed by 61-72 months and 85-108 months (n=1, 1.12%) respectively (Figure 4.9).




| | 4-12 | 13-24 | 25-36 | 37-48 | 49-60 | 61-72 | 73-84 | 85-108 | 109-144 |
|---|---|---|---|---|---|---|---|---|---|
| Female | 11 | 10 | 5 | 3 | 4 | 0 | 1 | 1 | 2 |
| Male | 10 | 13 | 13 | 8 | 6 | 1 | 1 | 0 | 0 |

**Figure 4.8.** Gender and age-wise distribution of HAdV among AFP patients during 2017

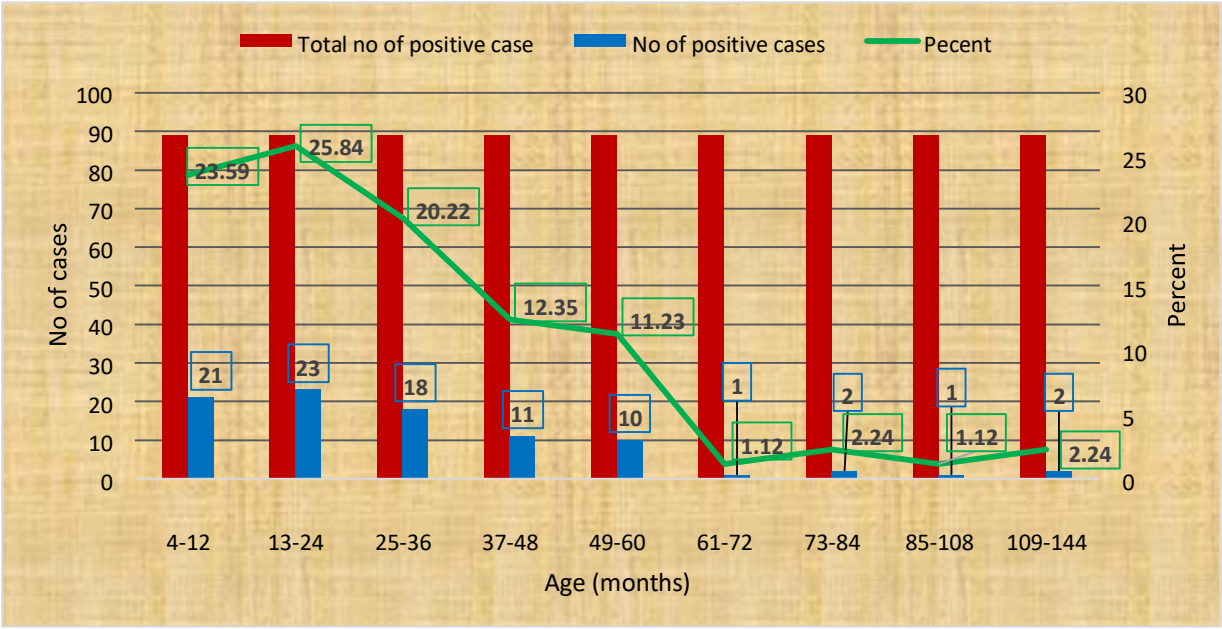



**Figure 4.9.** Age-wise distribution of positive cases and their percentage out of total positive cases (n=89) among AFP patients during 2017

### *iii) Month-wise Distribution*

According to this study, HAdV positive cases were found throughout the year as shown in Figure 4.10. However, the seasonality of HAdV positive cases were high in spring (March-April) 37.07% (n=33) and in winter (Dec-Feb) 43.82% (n=39). The highest peak for HAdV positive cases was found in March (n=21) and December (n=17). The peak was then followed by February (n=14) and April (n=12). In summer season (May-August), the rate of HAdV isolation was less (n=14) as compared to winter season (December-February, n=39). The lowest ratio was detected in autumn season (September-November, n=3). This shows that HAdV was found more in winter-spring (December-March) season (Figure 4.11).

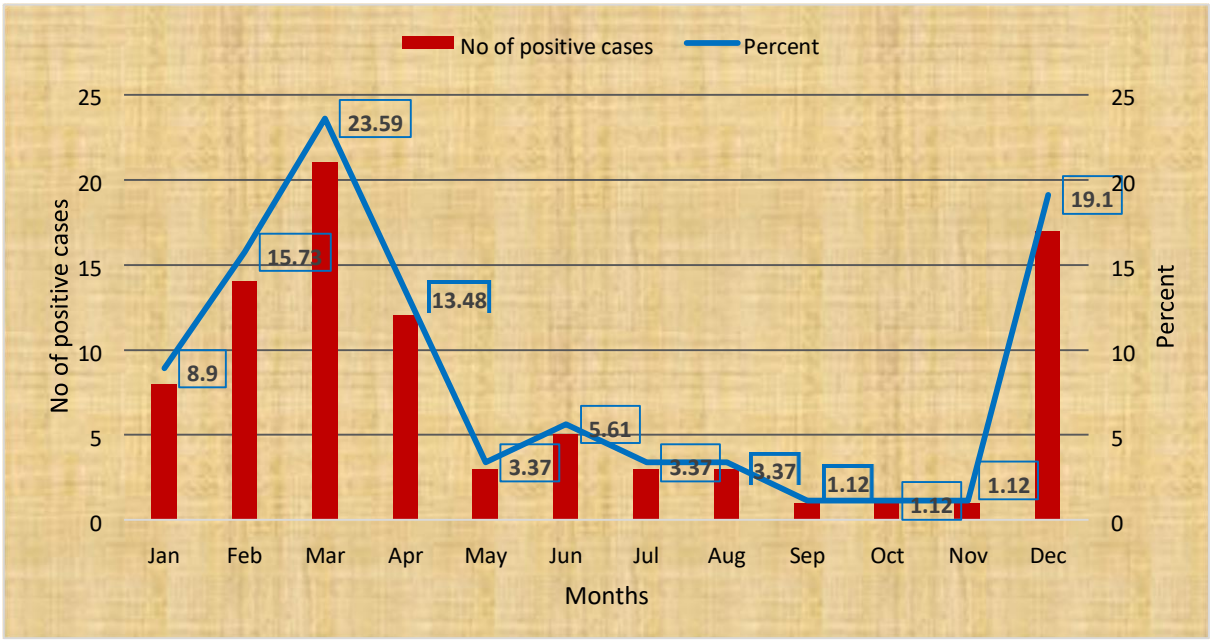



**Figure 4.10.** Month-wise distribution of HAdV among AFP patients during 2017

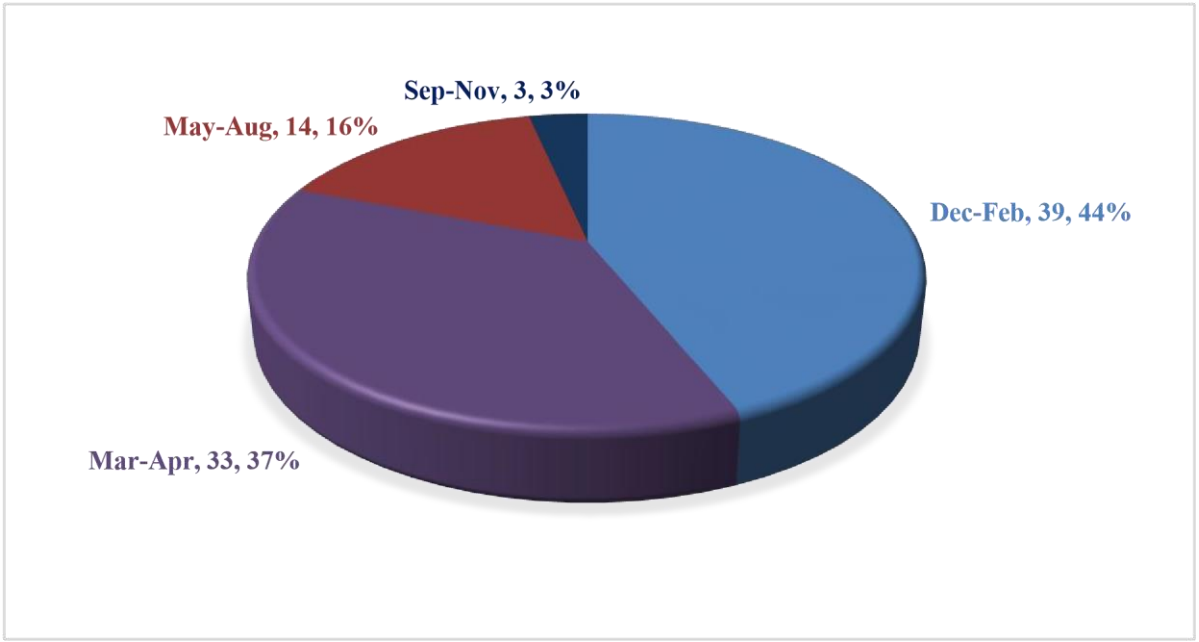
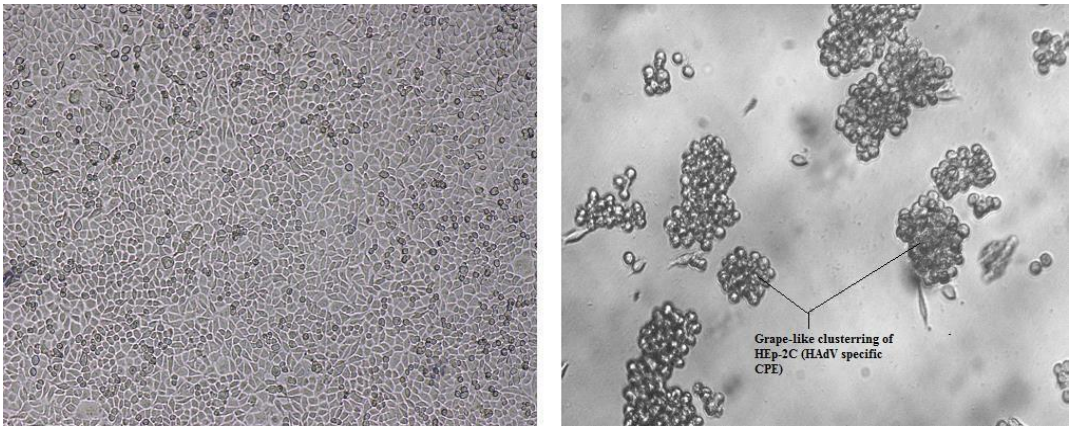



**Figure 4.11.** Quarterly distribution of HAdV among AFP patients during 2017

### 4.1.2 Human Adenovirus Isolation in Cell Culture

All 172 stool samples were processed and inoculated in cell line HEp-2C. The cell lines were observed daily for characteristic adenovirus cytopathic effect (Figure 4.12). The samples that showed 4+ CPE were isolated and were kept at -20˚C till additional testing. The samples that were negative on cell line were observed more for maximum of 10 days. According to the CPE, 94/172 (54.65%) samples were culture positive and were labelled as HAdVs. On the other hand, 78 samples were failed in producing CPE on HEp-2C cell lines and were labelled as negative for human adenoviruses (Table 4.1).




**A** Non-infected (control) HEp-2c cells **B** HAdV infected HEp-2c cells

**Figure 4.12. Microscopic photograph of non-infected (control) and HAdV infected HEp-2C cells.** A) Monolayer of normal HEp-2C cells in culture. B) HEp-2C cells with 4+ cytopathic effect with characteristic adenovirus infection.

**Table 4.1.** No of HAdVs isolated from AFP patients in HEp-2C cell line

| S. No | Cell Line | No of Samples | Total |
|---|---|---|---|
| 1 | Hep-2C + | 94 | 172 |
| 2 | Hep-2C - | 78 | |

### 4.1.3 Human adenovirus detection by Nested-PCR

All the cell culture supernatants (n=94) that were found positive in HEp-2C cells were further confirmed as HAdV by nested-PCR using their extracted genomic DNA for primer sets specified for hexon gene amplification. According to PCR results 51.74% (n=89/172) samples were confirmed as HAdV (Figure 4.13).

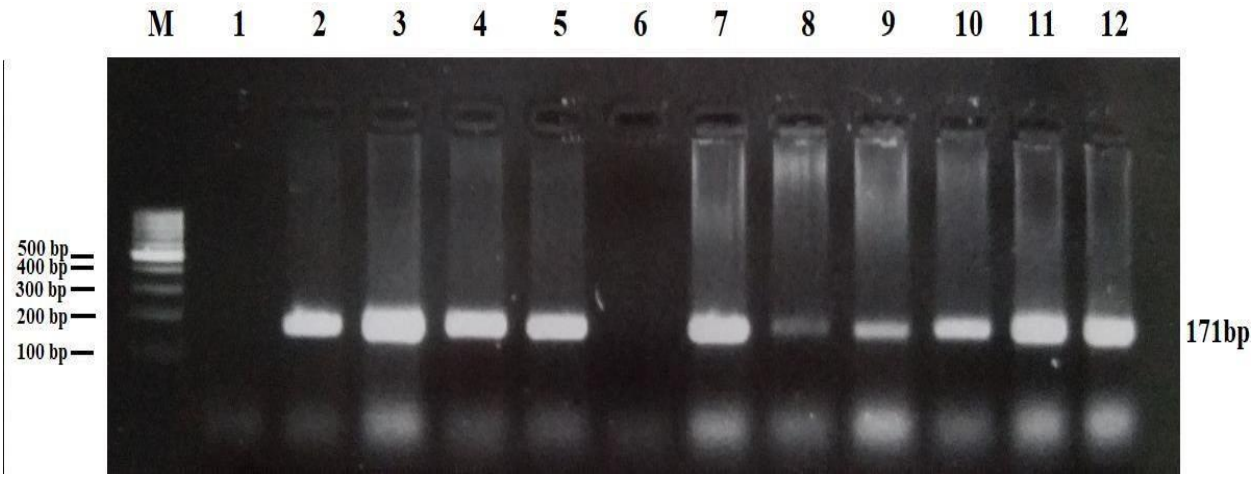



**Figure 4.13. SYBR safe dye-stained agarose gel showing PCR amplification products using a nested PCR primer pairs of adenovirus (Allard *et al.*, 2001).** Lanes 2-5, 8-12: positive samples (171bp), lane 6: negative control, lane 7: positive control (AdB7 DNA). M: molecular marker 100 bp DNA ladder, Life technologies).

### 4.1.4 Molecular analysis of human adenovirus strains, Acute Flaccid Paralysis cases

Screening of total 172 samples collected during 2017 for the presence of adenovirus antigen, cell culture yielded 94 (54.6%) samples positive for human adenovirus infections. This positive subset of 94 samples was further screened for the presence of adenovirus DNA through nested-PCR. The nested-PCR yielded 89 (51.74%) samples positive for major capsid protein (hexon) common to all recognized adenovirus genotypes and were kept at -80°C for further analysis.

**1) Phylogenetic analysis on the basis of hexon gene segments of adenovirus**

The results of our study that the adenovirus genotypes were present with the occurrence as HAdVA31= 8 (24.24%); HAdV-A18 = 1 (3.03%); HAdV-B3 = 3(9.09%); HAdV-B7 = 4(12.12%); HAdV-C1 = 2(6.06%); HAdV-D19 = 2 (6.06%), and HAdV-F41= 13(39.39%).

**2) Genotypes of adenovirus from AFP samples**

Molecular typing analysis successfully characterized 33 study isolates (37.07%) into 7 different genotypes of human adenoviruses including HAdV-A31, A18, B3, B7, C1, C6, D19, D37 and F41. The most detected genotype of adenovirus was HAdV-F41 with prevalence rate of 39.39% (n=13) followed by HAdV-A31 with detection rate of 24.24% (n=8) respectively. The descriptive analysis on molecular epidemiology, evolutionary relationships and its phylogeny of investigated strains into each specific genotypes are described below.

***a) Human adenovirus A***

According to this study, total 9 isolates from AFP cases were successfully characterize for Human adenovirus A specie with overall rate of 27.27% among positive isolates. The partial nucleotide sequence of hexon gene of all HAdV-A subject strain were subjected to phylogenetic investigation along with their related relatives and prototype sequences recovered from GeneBank in order to get insight into their evolution and epidemiology. Amino acid substitution is given in table 4.2.

***b) Human adenovirus A31***

Total 8 isolates were classified as HAdV-A31 in adenovirus A specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.14).

### c) Human adenovirus A18

Single isolate of HAdV-A18 was detected by sequence analysis of hexon gene. Pairwise distance score of study isolates were assessed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.14).

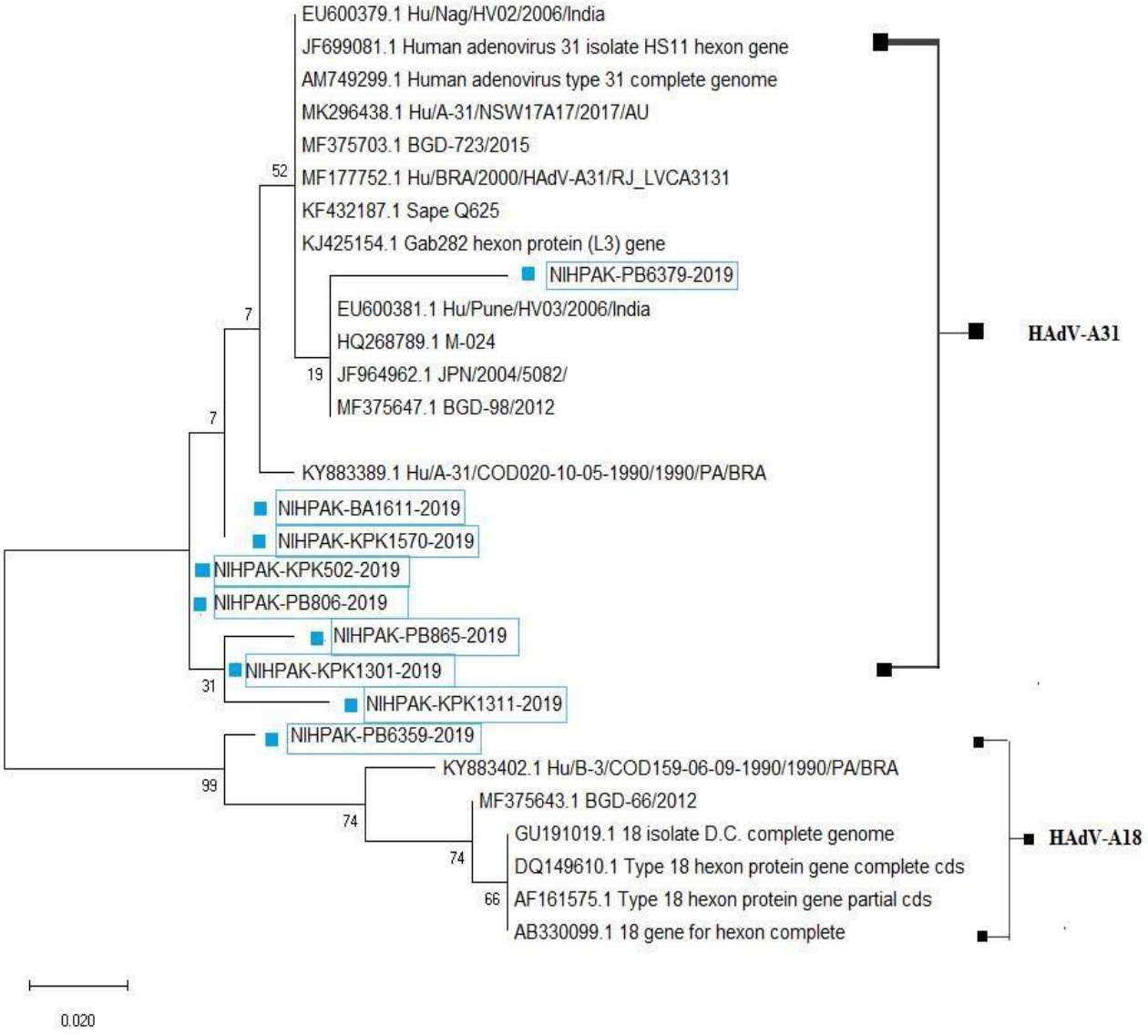



**Figure 4.14.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA X6. The substitution method used was Kimura 2-parameter using maximum likelihood model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**Table 4.2.** Amino acid substitutions in HAdV-A31 isolate a compared with reference strain EU600381.1.

| | AMINO ACID POSITION | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1 | | 36 | 37 | 39 | 46 | 50 | 66 | 84 |
| 2 | EU600381.1 | **D** | **T** | **F** | **R** | **V** | **L** | **F** |
| 3 | NIHPAK-KPK502 | **E** | **.** | **.** | **.** | **.** | **.** | **.** |
| 4 | NIHPAK-PB806 | **E** | **.** | **.** | **.** | **.** | **.** | **.** |
| 5 | NIHPAK-PB865 | **E** | **.** | **.** | **.** | **.** | **.** | **.** |
| 6 | NIHPAK-KPK1301 | **E** | **.** | **.** | **.** | **.** | **.** | **.** |
| 7 | NIHPAK-KPK1311 | **K** | **A** | **.** | **K** | **.** | **V** | **.** |
| 8 | NIHPAK-KPK1570 | **E** | **.** | **.** | **.** | **.** | **.** | **.** |
| 9 | NIHPAK-PB6379 | **K** | **.** | **L** | **.** | **G** | **.** | **Y** |
| 10 | NIHPAK-PB1161 | **E** | **.** | **.** | **.** | **.** | **.** | **.** |

### *d) Phylogenetic Analysis*

Partial (171bp) hexon gene sequences of HAdV-A strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. Two distinct monophyletic groups strains (HAdV-A31 and HAdV-A18) of all isolates were their respective reference strains were found (Figure 4. 14). The genetic relationship within and between groups were further analyzed.

HAdV-A31 comprised of eight isolates detected in 2017 from KPK (n=4), PB (n=3) and BA (n=1) provinces and exhibited similarity 99.98% similarity with their reference strains from the countries

India, Japan, Bangladesh and Brazil (Accession Nos. EU600381.1, HQ268789.1, JF964962.1, MF375647.1 and KY883389.1). Similarly, one isolated of HAdV-A18 was detected from PB province and showed 99.98% similarity with prototype strain (Accession No. GU191019.1) (Figure 4.14). The pairwise mean distance between the groups and reference strain are given in Table 4.3.

**Table 4.3.** Table showing pairwise mean distance of nucleotide and amino acids among human adenovirus A isolates with reference strain.

| **GROUP** | **Nucleotide (% Similarity)** | **Amino Acid (% Similarity)** |
|---|---|---|
| | **EU600381.1** | **EU600381.1** |
| NIHPAK-KPK502-2019 | 95.2 | 98 |
| NIHPAK-PB806-2019 | 95.2 | 98 |
| NIHPAK-PB865-2019 | 94.6 | 98 |
| NIHPAK-KPK1301-2019 | 95.2 | 98 |
| NIHPAK-KPK1311-2019 | 91.9 | 93 |
| NIHPAK-KPK1570-2019 | 95.8 | 98 |
| NIHPAK-PB6379-2019 | 93.3 | 93 |
| NIHPAK-PB1161-2019 | 89 | 98 |

**e) Human adenovirus B**

According to this study, total 7 isolates from AFP cases were successfully characterize for Human Adenovirus B specie with overall rate of 21.21%. The partial nucleotide sequence of hexon gene of all

HAdV-B study strains were subjected to phylogenetic investigation along with their related relatives and prototype sequences recovered from GeneBank in order to get insight into their evolution and epidemiology.

### *f) Human adenovirus B3*

Total 3 isolates were classified as HAdV-B3 in adenovirus B specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.15).

### *g) Human adenovirus B7*

4 isolates of HAdV-B7 was detected by sequence analysis of hexon gene. Pairwise distance score of study isolates were assessed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.15).

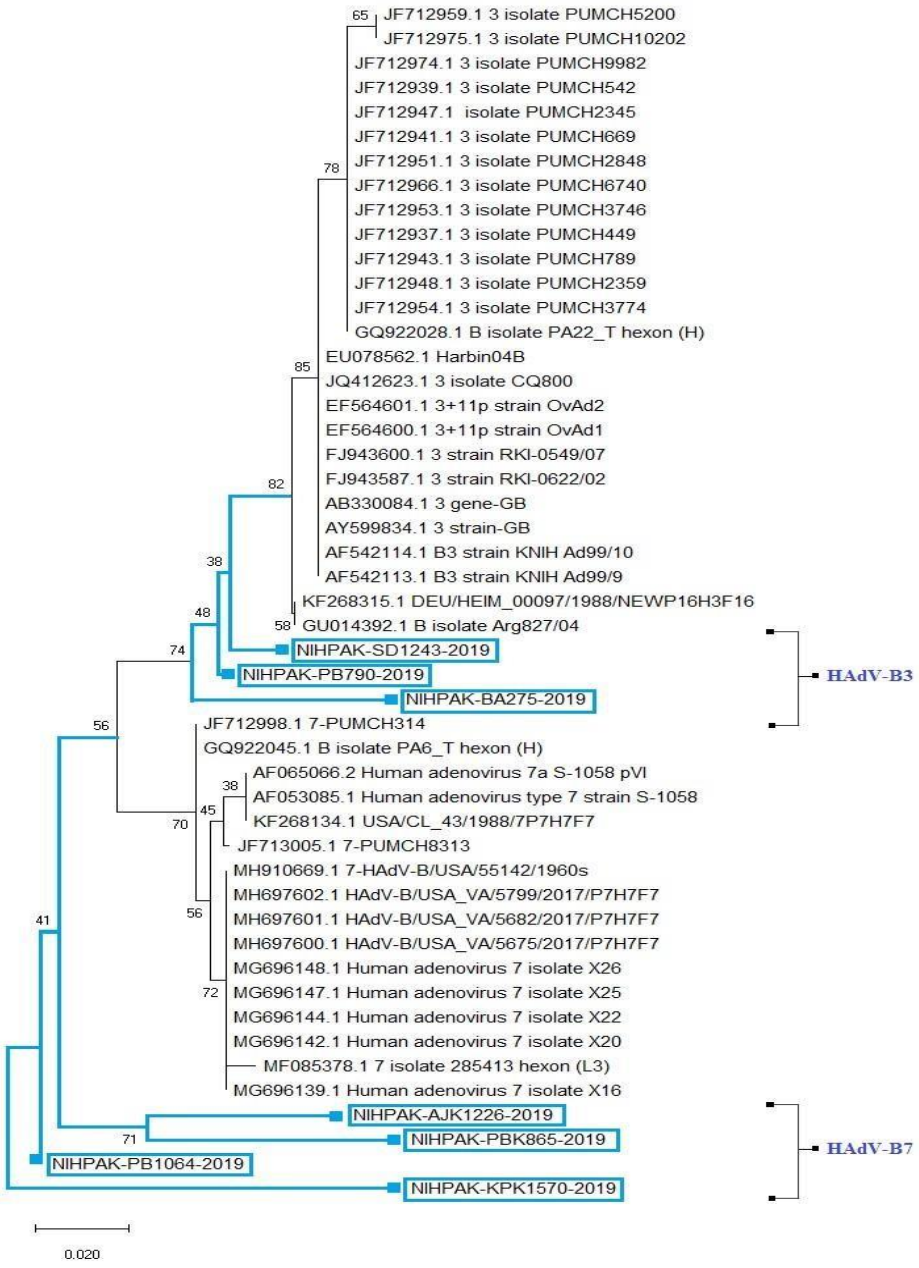



**Figure 4.15.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA X6. The substitution method used was Kimura 2-parameter using neighborjoining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**h) Phylogenetic Analysis**

Partial (171bp) hexon gene sequences of HAdV-B strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. Two distinct monophyletic groups strains (HAdV-B3 and HAdV-B7) of all isolates were their respective reference strains were found (Figure 4. 15). The genetic relationship within and between groups were further analyzed. HAdV-B3 comprised of three isolates detected in 2017 from SD, PB and BA provinces and exhibited similarity 99.93% similarity with prototype strain from the USA (Accession Nos. KF268315.1). Similarly, four isolates of HAdV-B7 was detected from PB (N=2), AJK (N=1) AND KPK (N=1) provinces and showed 99.83% similarity with prototype strain (Accession No. MH910669.1) (Figure 4.15). The pairwise mean distance within and between the groups are given in Table 4.4.

**Table 4.4.** Table showing pairwise mean distance of nucleotide and amino acids among human adenovirus B isolates

| **GROUP** | **Nucleotide (% Similarity)** | **Amino Acid (% Similarity)** |
|---|---|---|
| | **KF268315** | **KF268315** |
| NIHPAK-SD1243 | 96.5 | 91 |
| NIHPAK-PB790 | 94.5 | 89 |
| NIHPAK-BA275 | 90 | 85 |

### *i) Human adenovirus C*

According to this study, total 2 isolates from AFP cases were successfully characterize for Human adenovirus C specie with overall rate of 6.06%. The partial nucleotide sequence of hexon gene of all HAdV-C subject strains were subjected to phylogenetic investigation along with their related relatives and prototype sequences recovered from GeneBank in order to get insight into their evolution and epidemiology.

### *j) Human adenovirus C1*

Two isolate was classified as HAdV-C1 in adenovirus C specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.16).

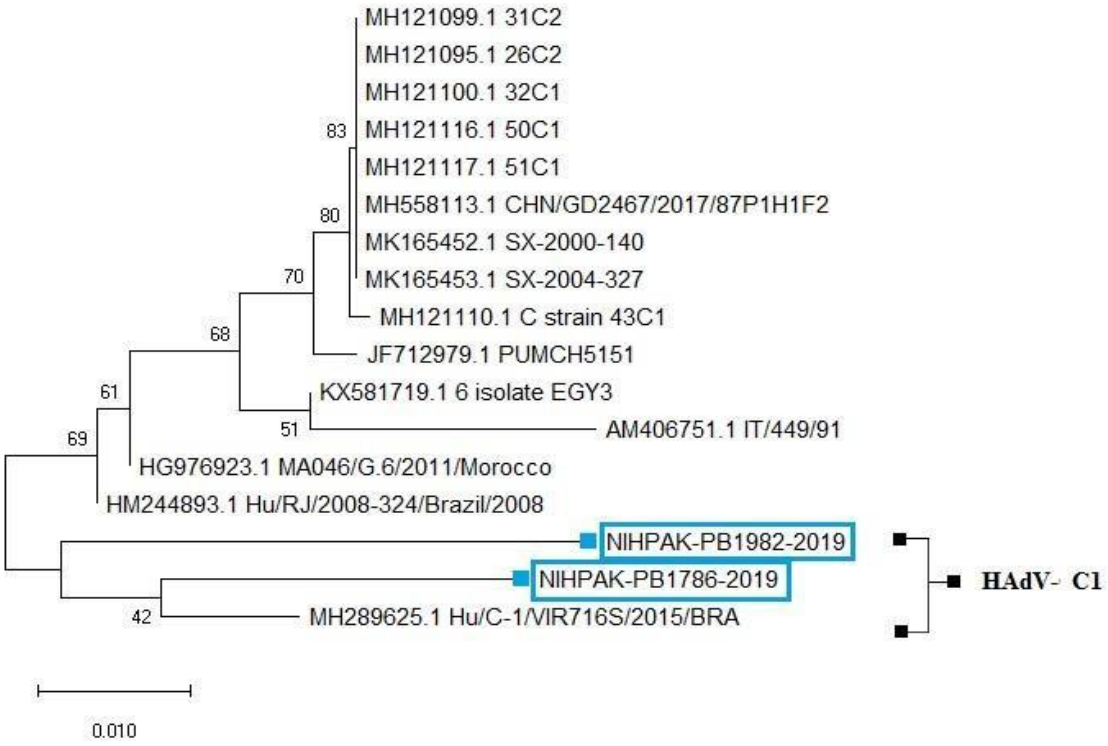



**Figure 4.16.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA X6. The substitution method used was Kimura 2-parameter using neighborjoining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**k) Phylogenetic Analysis**

Partial (171bp) hexon gene sequences of HAdV-C strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. Single distinct monophyletic group strains (HAdV-C1) of all isolates were their respective reference strains were found (Figure 4. 16). The genetic relationship within and between groups were further analyzed. HAdV-C1 comprised of two isolates detected in 2017 from PB province and exhibited similarity 99.92% similarity with reference strain from the Brazil (Accession No. MH289625.1) (Figure 4.16). The pairwise mean distance within and between the groups are given in Table 4.5.

**Table 4.5.** Table showing pairwise nucleotide and amino acids comparison among human adenovirus C isolates

| **GROUP** | **Nucleotide (% Similarity)** | **Amino Acid (% Similarity)** |
|---|---|---|
| NIHPAK-PB1786-2019 with MH289625.1 | 99.97 | 99.92 |
| NIHPAK-PB1786-2019 with NIHP AK-PB1982-2019 | 99.96 | 99.9 |
| NIHPAK-PB1982-2019 with MH289625.1 | 99.96 | 99.9 |

### *l) Human adenovirus D*

According to this study, total 2 isolates from AFP cases were successfully characterize for Human adenovirus D specie with overall rate of 6.06%. The partial nucleotide sequence of hexon gene of all HAdV-D subject strains were subjected to phylogenetic investigation along with their related relatives and prototype sequences recovered from GeneBank in order to get insight into their evolution and epidemiology.

### *m) Human adenovirus D19*

Two isolates were as classified as HAdV-D19 in adenovirus D specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.17).

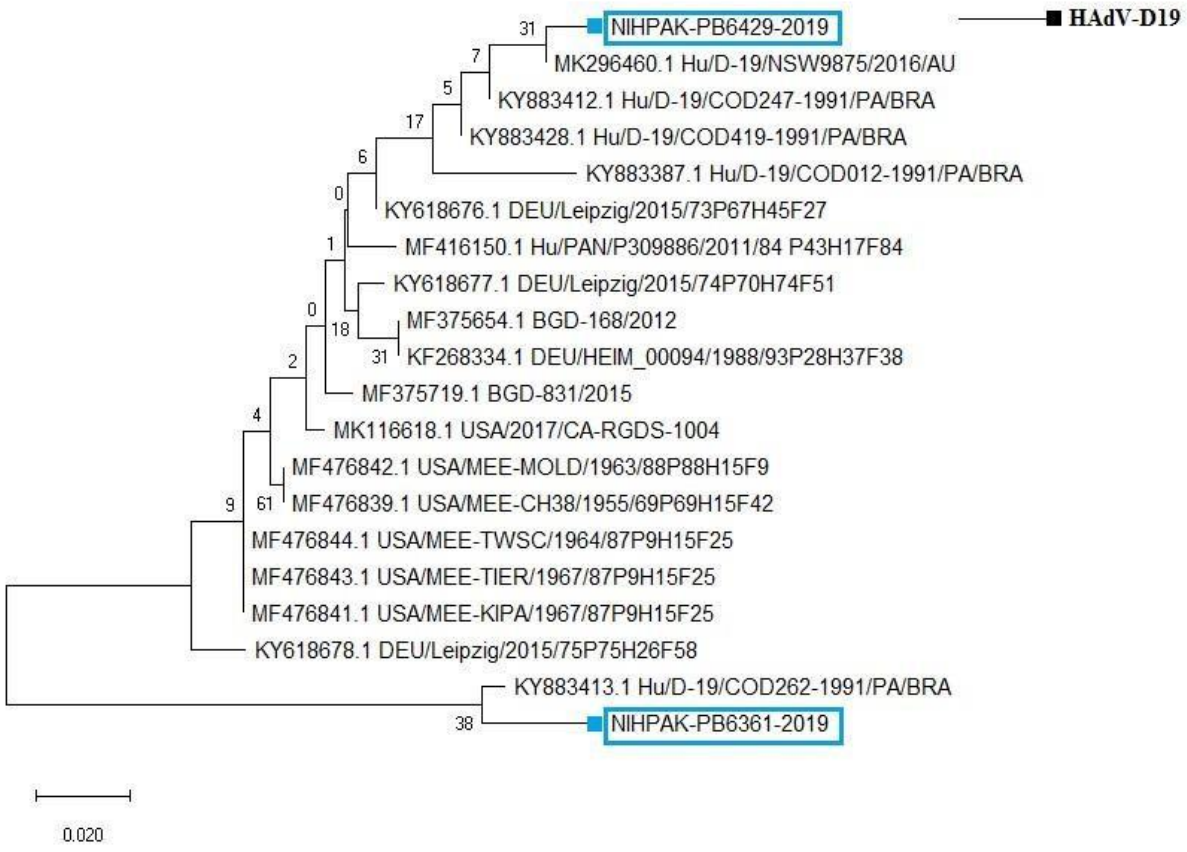



**Figure 4.17.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA X6. The substitution method used was Kimura 2-parameter using maximum likelihood model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**n) Phylogenetic Analysis**

Partial (171bp) hexon gene sequences of HAdV-D strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. Single distinct monophyletic group strains (HAdVD19) of all isolates were their respective reference strains were found (Figure 4. 17). The genetic relationship within and between groups were further analyzed. HAdV-D19 comprised of two isolates detected in 2017 from PB province and exhibited similarity 99.99% similarity with

reference strains from the Brazil and Australia (Accession No. MK296460.1 and KY883413.1) (Figure 4.17). The overall mean distance within and between the groups are given in Table 4.6.

**Table 4.6.** Table showing pairwise nucleotide and amino acids comparison among human adenovirus D isolates

| **GROUP** | **Nucleotide (% Similarity)** | **Amino Acid (% Similarity)** |
|---|---|---|
| NIHPAK-PB6429-2019 with MK296460.1 | 99.2 | 99.96 |
| NIHPAK-PB6429-2019 with NIHP AK-PB6361-2019 | 99.99 | 99.97 |
| NIHPAK-PB6361-2019 with MK296460.1 | 99.9 | 99.96 |
| NIHPAK-PB6429-2019 with KY883413.1 | 99.99 | 99.96 |
| NIHPAK-PB6361-2019 with KY883413.1 | 99.2 | 99.96 |

**o) Human adenovirus F**

According to this study, total 13 isolates from AFP cases were successfully characterize for Human adenovirus F specie with overall rate of 39.39%. The partial nucleotide sequence of hexon gene of all HAdV-F subject strains were subjected to phylogenetic investigation along with their related relatives and prototype sequences recovered from GeneBank in order to get insight into their evolution and epidemiology.

**p) Human adenovirus F41**

Total 13 isolates were classified as HAdV-F41 in adenovirus F specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed.

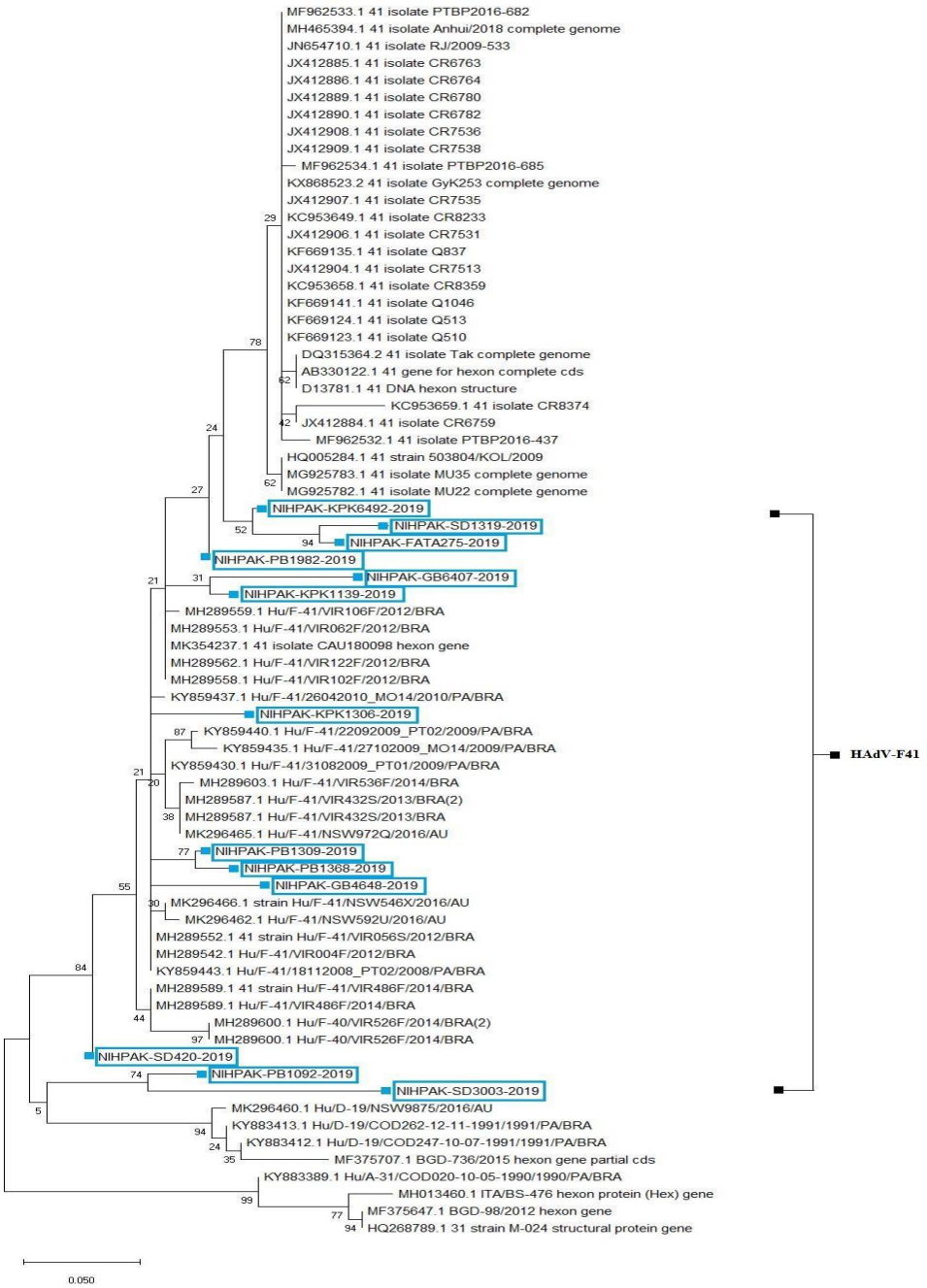



**Figure 4.18.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA X6. The substitution method used was Kimura 2-parameter using maximum likelihood model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

### *q) Phylogenetic Analysis*

Partial (171bp) hexon gene sequences of HAdV-F strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. Single distinct monophyletic group strains (HAdVF41) of all isolates were their respective reference strains were found (Figure 4. 18). The genetic relationship within and between groups were further analyzed.

HAdV-F41 comprised of thirteen isolates detected in 2017 from PB (n=4), KPK (n=3), SD (n=3), GB (n=1) and FATA (n=2) regions and exhibited similarity 99.7% similarity with prototype strain from the Netherland (Accession No. DQ315364) (Figure 4.18). The overall mean distance within and between the groups are given in Table 4.7.

**Table 4.7.** Table showing overall mean distance of nucleotide and amino acids among human adenovirus F isolates

| **GROUP** | **Nucleotide (% Similarity)** | **Amino Acid (% Similarity)** |
|---|---|---|
| HAdV-F within group | 99.89 | 99.92 |
| HAdV-F with reference strains | 99.94 | 99.98 |

## 4.2 Analysis of HAdV in Gastroenteritis Cases

For diarrheal patients, 1,118 fecal samples were taken from children ≤ five years of age from five tertiary care hospitals namely Benazir Bhutto Hospital, Rawalpindi, Mayo Hospital Lahore, Children's hospital Lahore, Kharadar General Hospital and National Institute of Child Health, Karachi, in January 2017 to December 2018 who were hospitalized because of acute gastroenteritis. These samples were then transported to the Department of Virology, National Institute of Health, Islamabad. All of the laboratory work were conducted in virology lab for human enteric adenovirus infections in gastroenteritis patients.

### 4.2.1 Detailed statistics of samples obtained from the Benazir Bhutto Hospital, Rawalpindi

From BBH, total 223 fecal specimens from admitted children having age ≤5 years were obtained during January 2017 to August 2017 and delivered to Department of Virology, National Institute of Health, Islamabad. Overall, the percentage of positive samples was 13.90% (n=31) for adenovirus infections respectively.

#### *1) Month-wise distribution of BBH samples*

Among total 223 admissions in this study, during the May-August months, 44.39% (n=99) of patients were reported whereas during January-April, 55.60% (n=124) patients were admitted. The month-wise number and percentage of total samples obtained from diarrheal patients is shown in Figure 4.19.

#### *2) Gender and Age-wise distribution of BBH samples*

Among 223 samples, 110 (49.32%) patients were male and 113 (50.67%) were females with ratio 1: 1.02 (mean= 1.507 ± 0.5011). The samples of the children investigated in this study had a mean age of 14.83± 15.68 months having range from 1 to 60 months. For study, the investigated subjects were grouped into 7 age groups with the gap of 9 months between their ages. Mostly they belong to ≤ 9 months of age (n= 118, 52.91%), followed by 10-18 months of age (n=48, 21.52%) whereas

19-27 and 28-36 months of age were came after (n=23, 10.31% and n=14, 6.27%) them. However, children having age more than 3 years were 20 in number as shown in Figure 4.20.

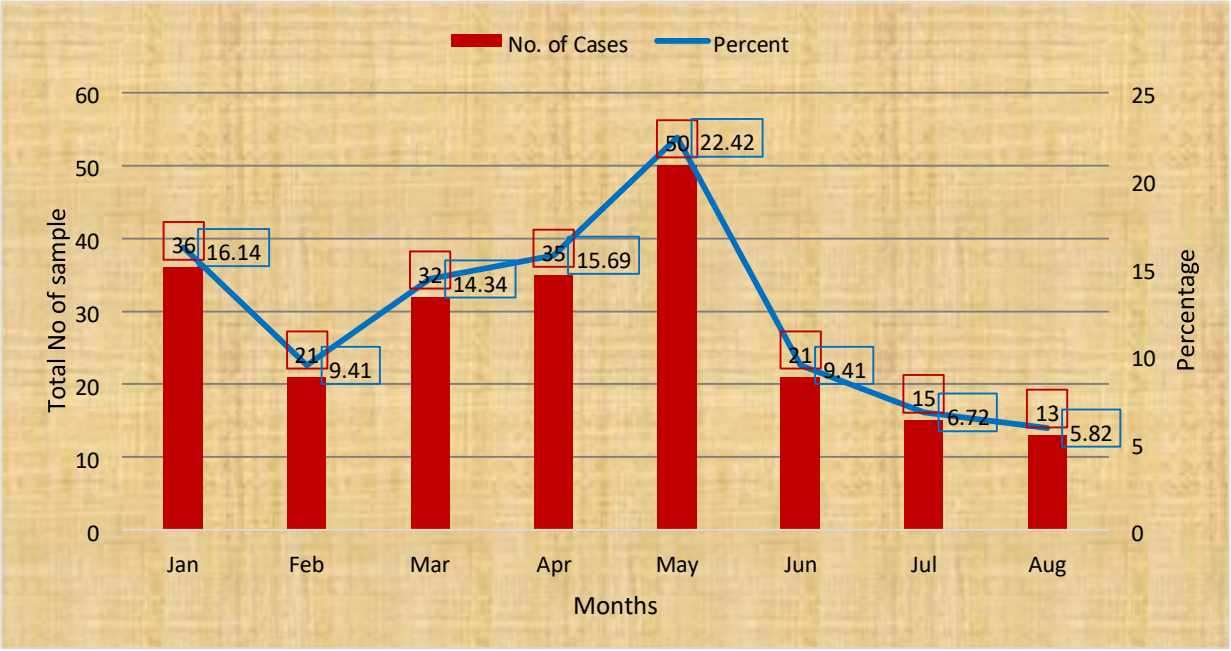



**Figure 4.19.** Month-wise distribution of samples collected and their percentage out of total number of samples (n=223) at BBH during Jan-Aug 2017

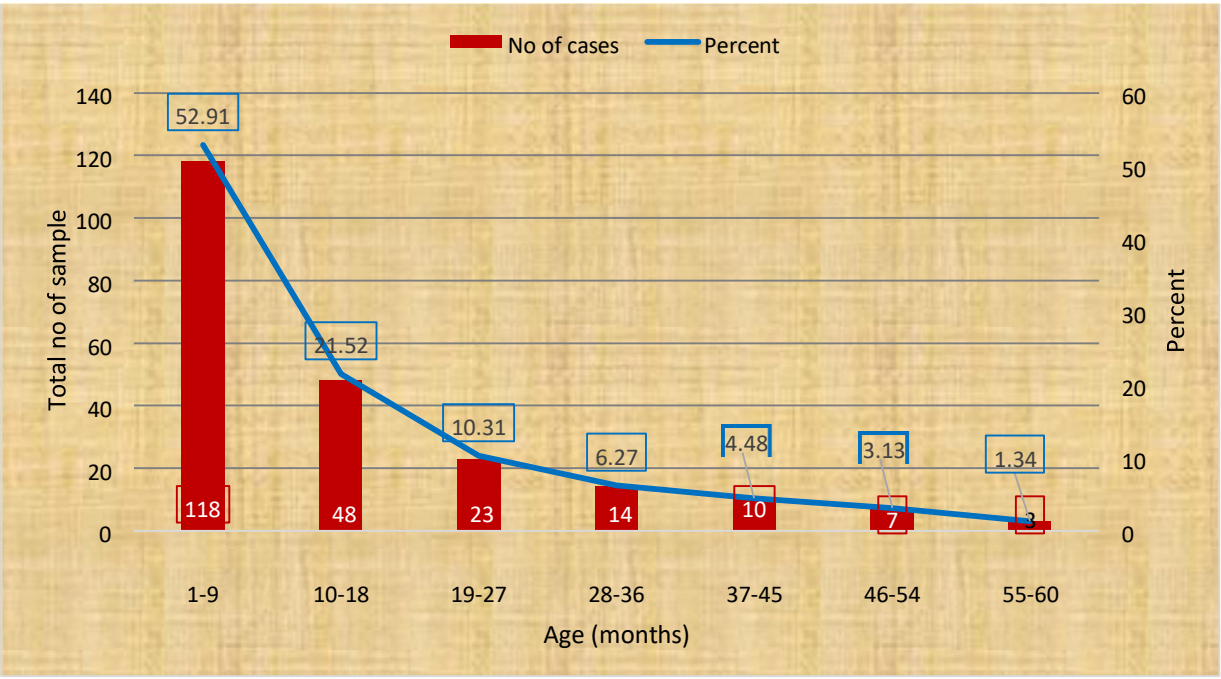



**Figure 4.20.** Age wise distribution of samples collected and their percentage out of total number of samples (n=223) tested at BBH during 2017

### 4.2.2 Adenovirus analysis at BBH

Among total number of samples i.e. 223, 31 (13.90%) samples were found positive for human adenoviruses whereas 192 (86.09%) samples were found completely negative. These HAdV positive patients were regarded as responsible for "enteric viral" infections or diarrhea.

#### *1) Monthly dispersal of BBH positive samples during 2017*

Among 31 positive samples, the monthly-based dispersion graph is shown in Figure 4.21, which showed that in 2017, the frequency of positive isolates for human adeno viruses remained found throughout the investigated months of 2017 without any peculiar seasonality between summer (May-August) and winter (Jan-April) months. For illustration, increased infections were found in May, July (monsoon month) and January.

### *2) Age and gender based division of BBH positive samples during 2017*

Based on five age groups, the rate of positive samples was assessed with a gap of 9 months. The end group i.e. fifth one contained samples from children with age 37 months and above. In these groups of different ages, more positive samples (45.16%; n=14/31) were gone with the children having age below 9 months which was then followed by children having age 10-18 months (38.70%; n=12/31) as shown in Figure 4.22. Among positive cases, HAdV were found to be more common in females as compared to males (64.51% vs 35.48%; n=20 vs 11) with female to male ratio of 1.8:1.

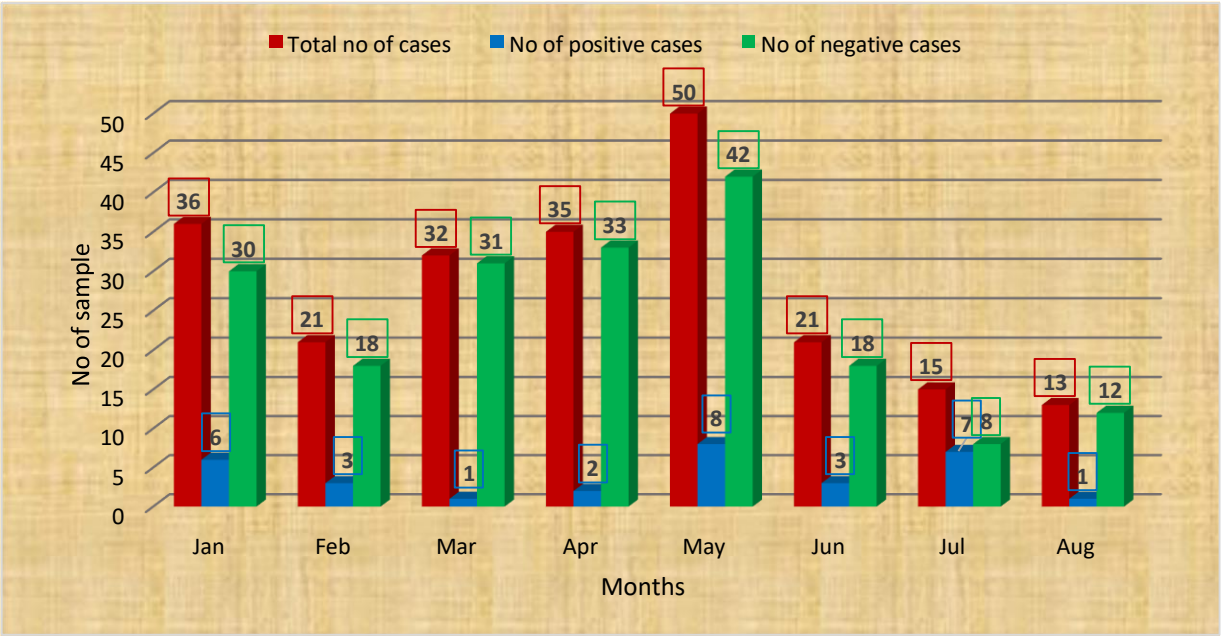



**Figure 4.21.** Month wise distribution of total number of samples (red bar), positive (blue bar) and negative (green bar) samples tested for adenovirus infections in BBH during 2017.

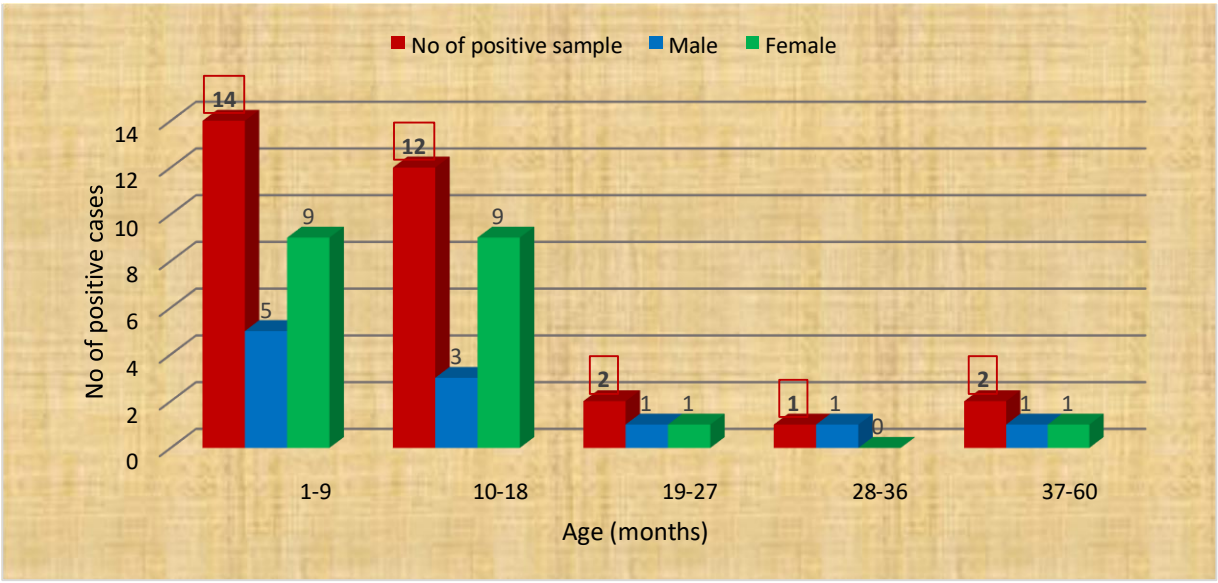



**Figure 4.22.** Age wise distribution of total positive samples (red bar), male positive (blue bar) and female positive (green bar) at BBH during 2017.

### 4.2.3 Assessment of demographic and clinical parameters among diarrheal patients in BBH

The enrolled subjects at BBH were analyzed for the assessment of demographic and clinical parameters between the positive and negative patients for adenovirus infections. Presence of adenovirus in samples were regarded as positive while those without adenovirus were termed as negative. With the help of chi-square test, the qualitative parameters were evaluated while the quantitative variables were evaluated by employing student's *t*-Test.

**1) Evaluation of demographic and clinical data among positive and negative cases**

Evaluation of the clinical characteristics like fever, diarrhea, vomiting, the factor which was found significant ($p$=0.0012) was vomiting when assessed among positive and negative groups for adenovirus infections (Table 4.8). Out of 31 patients positive for adenoviruses, vomiting was recorded in 25 (80.6%) patients whereas 6 (19.3%) had no vomiting. The insignificant factor found was fever and diarrhea among positive and negative cases ($p$=0.6757 and $p$=0.3273 respectively). Similarly, the factor age was also found to be insignificant among positive and negative cases

($p$=0.1551) though most of infections (n= 26; 83.9%) were establish in children having age below 18 months.

**2) Evaluation of clinical data among positive and negative cases**

Statistical interpretation was performed for the quantitative variables by employing student's *t* Test (Table 4.9). Among the quantitative variables, the mean number of diarrheal episodes was found as a significant factor ($p$=0.0102). These variables were compared between the patients who were found positive 4.033±1.033 and negative 4.632±1.196 for adenovirus infections. The mean duration of diarrhea (2.600±1.940 days), mean number of vomiting episodes (3.280±1.948) and mean duration of vomiting (2.440±1.781) were found insignificant with $p$-values 0.7341, 0.3816 and 0.7824 correspondingly.

**Table 4.8.** Chi square analysis of qualitative variables of patients with adenovirus infections

| Total No. of Cases = 223 | Positive (n= 31) n (% age) | Negative (n= 192) n (% age) | *P-value* |
|---|---|---|---|
| **Gender** | | | |
| Male (n=110) | 11 (35.5) | 99 (51.6) | $0.0966^{NS}$ |
| Female (n=113) | 20 (64.5) | 93 (48.4) | |
| **Age groups (months)** | | | |
| 1-9 (n=118) | 14 (12.9) | 104 (54.2) | $0.1551^{NS}$ |
| 10-18 (n=48) | 12 (38.7) | 36 (18.75) | |
| 19-27 (n=23) | 2 (6.45) | 21 (10.9) | |
| 28-36 (n=14) | 1 (3.22) | 13 (6.7) | |
| 37-60 (n=20) | 2 (6.45) | 18 (9.37) | |
| **Clinical signs** | | | |
| **Fever** | | | |
| Present (n=129) | 19 (61.3) | 110 (57.3) | $0.6757^{NS}$ |
| Absent (n=94) | 12 (38.7) | 82 (42.7) | |
| **Vomit** | | | |
| Yes (n=209) | 25 (80.6) | 184 (95.8) | **$0.0012^{S}$** |
| No (n=14) | 6 (19.3) | 8 (4.17) | |
| **Diarrhea** | | | |
| Yes (n=220) | 30 (90.7) | 190 (99) | $0.3273^{NS}$ |
| No (n=3) | 1 (3.2) | 2 (1) | |

S= Significant *p*-value; NS= non-significant *p*-value,

% ages are given in Adenovirus positive and negative groups according to the variables such as gender, age groups, fever, vomiting and diarrhea

**Table 4.9.** Comparison of clinical parameters (quantitative variables) between patients found positive and negative for adenovirus infections using Student t-test

| **Clinical Symptoms** | **No. of Samples** | **Mean** | **Std. Deviation (±)** | **Std. Error Mean** | ***p*-value** |
|---|---|---|---|---|---|
| Diarrhea Episodes/24hrs | Positive (n=30) | 4.033 | 1.033 | 0.1887 | **0.0102$^{S}$** |
| | Negative (n=190) | 4.632 | 1.196 | 0.0867 | |
| Diarrhea duration (days) | Positive (n=30) | 2.600 | 1.940 | 0.3543 | 0.7341$^{NS}$ |
| | Negative (n=190) | 2.484 | 1.699 | 0.1232 | |
| Vomiting Episodes/24hrs | Positive (n=25) | 3.280 | 1.948 | 0.3895 | 0.3816$^{NS}$ |
| | Negative (n=184) | 3.652 | 1.997 | 0.1472 | |
| Vomit duration (days) | Positive (n=25) | 2.440 | 1.781 | 0.1292 | 0.7824$^{NS}$ |
| | Negative (n=184) | 2.543 | 1.752 | 0.3563 | |
| Age (months) | Positive (n=31) | 13.55 | 12.99 | 2.334 | 0.6240$^{NS}$ |
| | Negative (n=192) | 15.04 | 16.09 | 1.161 | |

NS= non-significant *p*-value <0.05

Table 4.10. Comparison of clinical parameters among subjects with adenovirus and rotavirus co-infection using ANOVA

| Clinical Parameters | Infection (No. of cases) | Range | Mean±SD | 95% Confidence Interval Lower | Upper | *P-Value* |
|---|---|---|---|---|---|---|
| **Diarrhea episodes/ 24 hrs** | HAdV+ve (n=30) | 2-6 | 4.065±1.031 | 3.686 | 4.443 | **0.0493** $^{S}$ |
| | Rotavirus+ve (n=45) | 2-7 | 4.422±1.177 | 4.069 | 4.776 | |
| | Rotavirus+HAdV (n=5) | 4-7 | 5.400±1.140 | 3.984 | 6.816 | |
| | Negative (n=143) | 2-7 | 4.570±1.193 | 4.372 | 4.768 | |
| **Diarrhea duration (days)** | HAdV+ve (n=30) | 1-6 | 2.645±1.924 | 1.939 | 3.351 | **0.0324** $^{S}$ |
| | Rotavirus+ve (n=45) | 1-6 | 2.356±1.681 | 1.851 | 2.861 | |
| | Rotavirus+HAdV (n=5) | 3-6 | 4.800±1.304 | 3.181 | 6.419 | |
| | Negative (n=143) | 1-6 | 2.599±1.722 | 2.313 | 2.884 | |
| **Vomiting episodes/24 hrs** | HAdV+ve(n=25) | 1-8 | 3.280±1.948 | 2.476 | 4.084 | **0.0153** $^{S}$ |
| | Rotavirus+ve (n=45) | 1-8 | 3.289±1.674 | 2.786 | 3.792 | |
| | Rotavirus+HAdV (n=5) | 4-9 | 6.200±2.168 | 3.508 | 8.892 | |
| | Negative (n=148) | 1-8 | 3.547±1.998 | 3.223 | 3.872 | |
| **Vomiting duration (days)** | HAdV+ve(n=25) | 1-6 | 2.440±1.781 | 1.705 | 3.175 | 0.0744 $^{NS}$ |
| | Rotavirus+ve (n=45) | 1-6 | 2.489±1.804 | 1.947 | 3.031 | |
| | Rotavirus+HAdV (n=5) | 3-6 | 4.600±1.140 | 3.184 | 6.016 | |
| | Negative (n=148) | 1-6 | 2.581±1.726 | 2.301 | 2.861 | |
| **Age (months)** | HAdV+ve(n=31) | 1-60 | 13.29±12.86 | 8.574 | 18.01 | 0.5871 $^{NS}$ |
| | Rotavirus+ve (n=45) | 1-60 | 15.62±13.86 | 11.46 | 19.79 | |
| | Rotavirus+HAdV (n=5) | 2-24 | 12.20±8.497 | 1.650 | 22.75 | |
| | Negative (n=142) | 1-60 | 17.25±17.76 | 14.31 | 20.20 | |

Significant *p*-values ($\leq 0.05$) are given in bold

### 4.2.4 Investigation of tendencies in demographic and clinical parameters among gastroenteritis patients

Among HAdV positive and negative patients, the tendencies in demographic aspects of gastroenteritis patients enrolled at BBH during 2017 were contrasted and evaluated. The chi-square and student's *t*-Test were employed for analysis of qualitative and quantitative parameters of the patients.

#### 1) Age and gender wise analysis

For an adenovirus infection, the age distribution of positive cases was determined to evaluate the most vulnerable age group between the hospitalized patients. Likewise, based on gender the difference between positive and negative patients was assessed in order to recognize the gender based difference and male to female ratio between the investigated positive patients.

The proportion of positive HAdV cases were observed during 2017 was 31/223 (13.90%). Dispersal of adenovirus infection among positive cases (n=31) was evaluated on the basis of five age groups. It was detected that in children below 18 months of age the adenovirus detection rate were more focused as compared to those having above 18 months (83.9% vs 16.12%; n=26 vs 5 respectively). The maximum incidence of adenovirus positive cases were detected in females contrasted to males (64.5% and 35.5% correspondingly) regardless of their age group. Generally, in adenovirus positive incidences, male to female ratio was observed as 1:1.8.

#### 2) Monthly based evaluation of enteric adenovirus infection in BBH samples

The dispersion of positive isolates for viral infections during 2017 was inspected to discover any seasonal array of enteric adenovirus infections. No atypical seasonal changes of adenovirus infections detected in 2017. The highest proportion of adenovirus positive samples was found in May (25.80%), July (22.6%) and January (19.3%) as shown in Figure 4.23. Overall, adenovirus infections were observed throughout the year with an increased infection tendency in the start summer and late winter months.

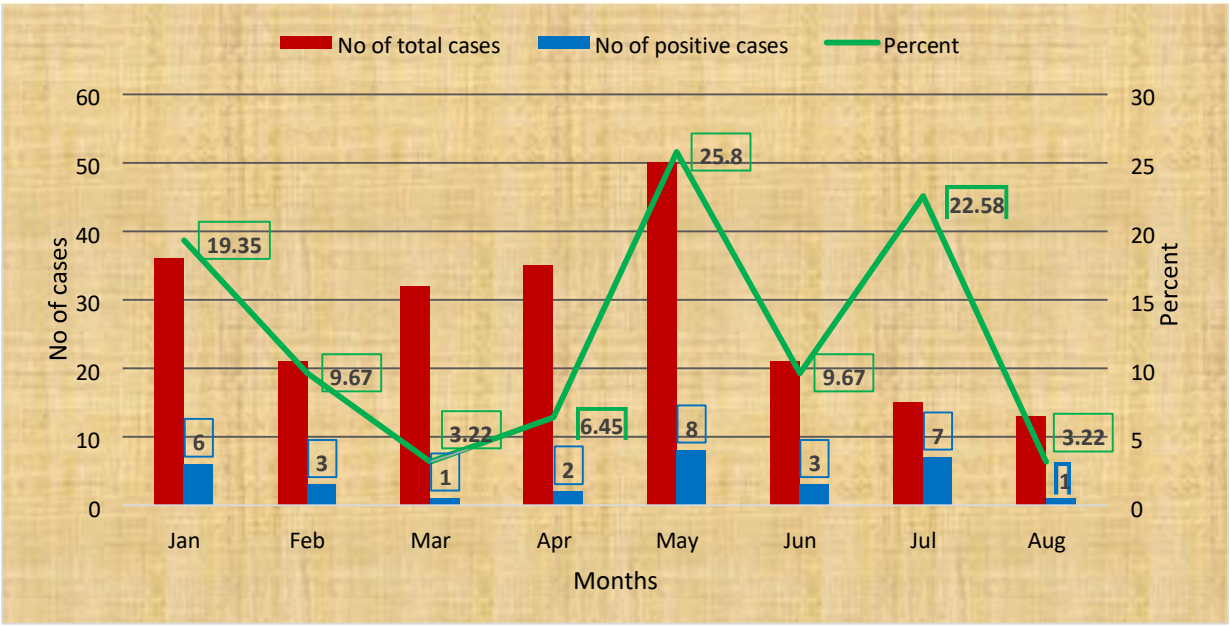



**Figure 4.23.** Month wise distribution of samples positive for adenovirus during 2017. Total number of samples (red bar), positive samples (blue bar) and their percentages (green line) is plotted on Y-axis and the months are given on X-axis.

### 4.2.5 Relation of qualitative clinical factors with adenovirus infections

Quantitative variables were evaluated in order to discover the co-relation of these factors with the positive or negative position of infection against adenovirus. These variables include age, duration and number of diarrhea and vomiting episodes, for which student's t-test was performed as presented in Table 4.8 and 4.9.

Five clinical parameters were evaluated which includes age, diarrhea episodes per 24 hours, duration of diarrhea, vomiting episodes per 24 hours, duration of vomiting. Among them only episodes of diarrhea were observed to be significantly related with adenovirus infections with *p*values of 0.0102 respectively. The mean episodes of diarrhea of these patients remained 4.033±1.033.

### 4.2.6 Evaluation of clinical parameters for determination of clinical effect of co-infection of adenovirus with rotavirus

In this study, the samples taken from gastroenteritis patients were previously analyzed for rotavirus, which is worldwide the most commonly, stated viral source for diarrhea. We, therefore, analyzed the significance of co-infection via the comparison of clinical signs and symptoms of patients infected with adenovirus to those co-infected with rotavirus by putting on the statistical test called ANOVA (Analysis of Variance).

#### *1) Comparative analysis of quantitative clinical parameters among adenovirus positive patients with rotavirus co-infections*

Data presented in Tables 4.10 demonstrate the outcomes of ANOVA test, used to deduce the importance of co-infection of adenovirus with rotavirus. It was detected that patients with double infection of adeno-rotavirus and rotavirus showed more serious clinical indications and illness therefore describing their clinical importance in gastroenteritis cases.

Data exhibited in Table 4.10 shows that the number of diarrheal episodes per 24 hours, diarrhea duration (days) and number of vomiting episodes per 24 hours were significantly associated to disease in patients co-infected with adenovirus and rotavirus (*p*-value 0.0493, 0.0324 and 0.0153 respectively). The mean number of diarrheal episode per 24 hours, diarrhea duration (days) and number of vomiting episodes per 24 hours of HAdV-rotavirus co-infection was 5.400±1.140, 4.800±1.304 and 6.200±2.168.

### 4.2.7 Detailed statistics of samples obtained from the Mayo Hospital, Lahore 2017-2018

From MHL, total 138 fecal specimens from admitted children having age ≤4 years were obtained during January 2017 to December 2018 and delivered to Department of Virology, National Institute of Health, Islamabad. Overall, the percentage of positive samples was 18.84% (n=26) for adenovirus infections respectively.

#### *1) Month-wise distribution of MHL samples*

Among total 138 admissions in this study, during the April-August months, 45.65% (n=63) of patients were reported whereas during September-March, 54.34% (n=75) patients were admitted. The month-wise number and percentage of total samples obtained from diarrheal patients is shown in Figure 4.24.

#### *2) Gender and Age-wise distribution of MHL samples*

Among 138 samples, 78 (56.52%) patients were male and 60 (43.47%) were females with ratio 1: 1.3 (mean= 1.435±0.4975). The mean age of the children investigated in this study was 10.86± 8.469 months having range from 2 to 48 months. For study, the investigated subjects were grouped into 5 age groups with the gap of 9 months between their ages. Mostly they belong to ≤ 9 months of age (n= 73, 52.891%), followed by 10-18 months of age (n=47, 34.05%) whereas 19-27, 28-36 and 37-48 months of age were coming after (n=12, 8.69%, n=3, 2.17% and n=3, 2.17%) them as shown in Figure 4.25.

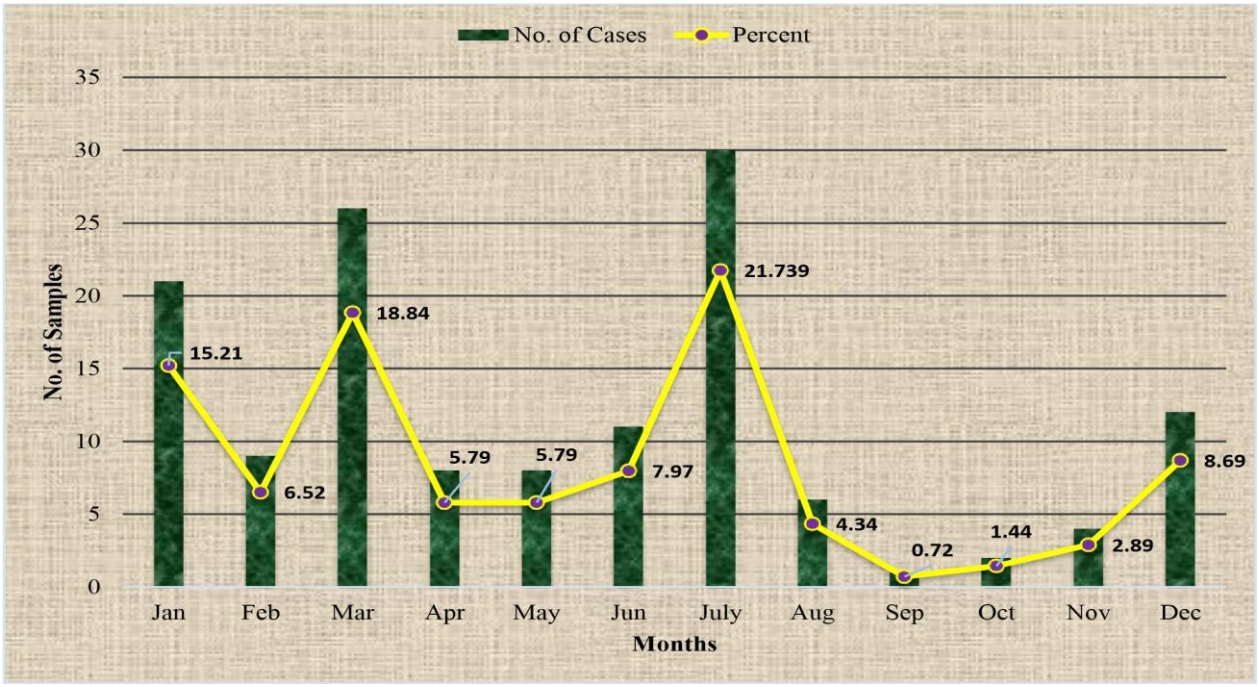



**Figure 4.24.** Frequency and Percentage of total samples collected at MHL during 2017-2018

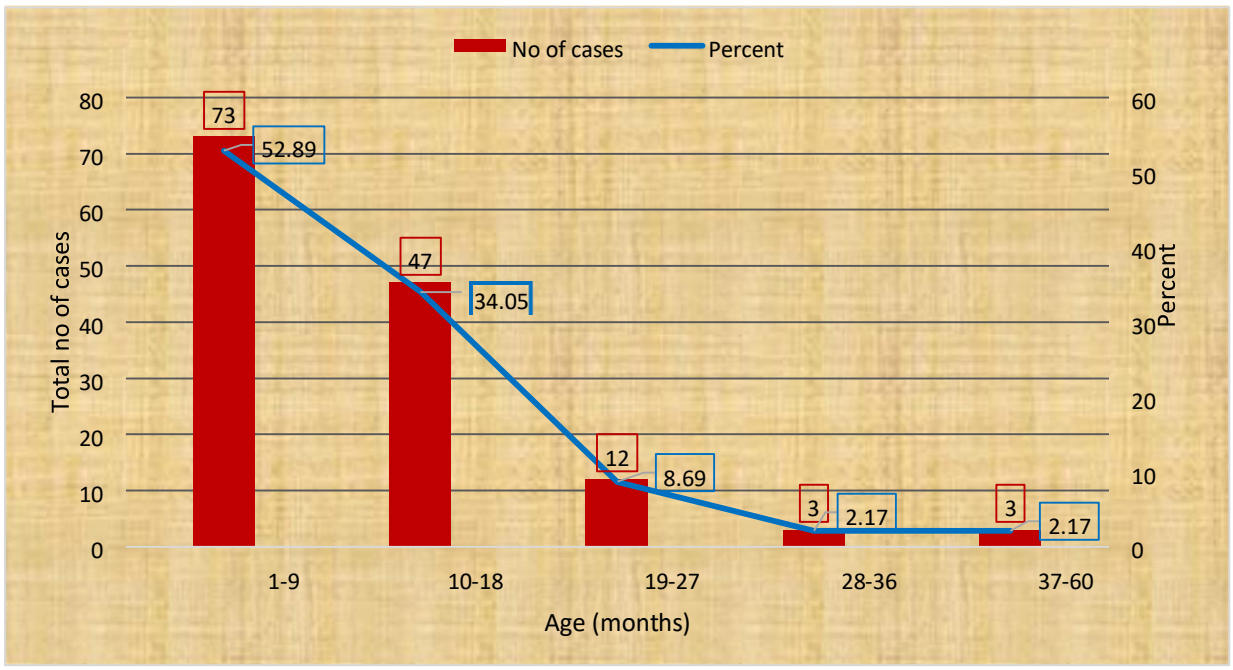



**Figure 4.25.** Age wise distribution of total number of samples and their percentage tested at MHL during 2017-2018

### 4.2.8 Adenovirus analysis at MHL

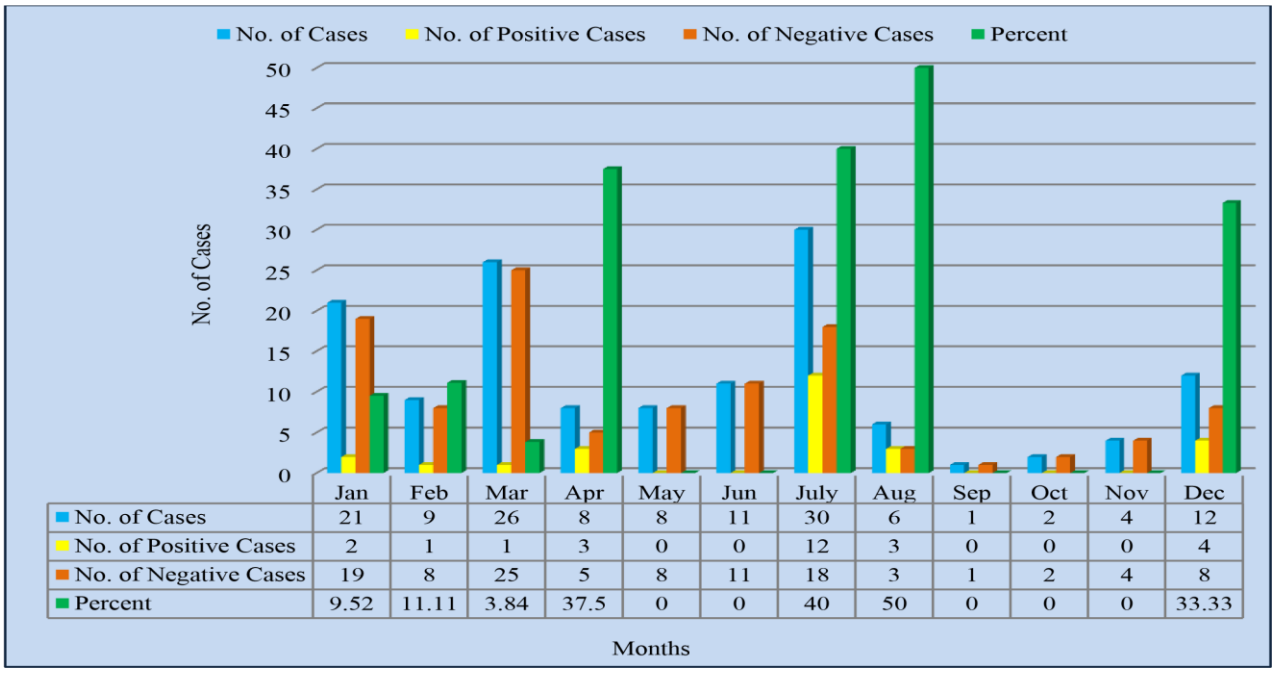
Among total number of samples i.e. 138, 26 (18.84%) samples were found positive for human adenoviruses whereas 112 (81.15%) samples were found completely negative. These HAdV positive patients were regarded as responsible for "enteric viral" infections or diarrhea.

#### *1) Monthly dispersal of MHL positive samples during 2017-2018*

Among 26 positive samples, the monthly-based dispersion graph is shown in Figure 4.26, which showed that in 2017-2018, the proportion of samples positive for human adeno viruses were found mostly in summer months. For illustration, increased infections were found in during July (monsoon month) and December as shown in Figure 4.26

#### *2) Age and gender based division of MHL positive samples during 2017 -2018*

Based on four age groups, the rate of positive samples was assessed with a gap of 9 months. The last group i.e. fourth one contained samples from children with age 28 months and above. In these groups of different ages, more positive samples (42.30%; n=11/26) were gone with the children having age below 9 months which was then followed by children having age 10-18 months (38.46%; n=10/26) as shown in Figure 4.27. Among positive cases, HAdV were found to be more common in males as compared to females (53.84% vs 46.15%; n=14 vs 12) with female to male ratio of 1.16:1.




| | Jan | Feb | Mar | Apr | May | Jun | July | Aug | Sep | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of Cases | 21 | 9 | 26 | 8 | 8 | 11 | 30 | 6 | 1 | 2 | 4 | 12 |
| No. of Positive Cases | 2 | 1 | 1 | 3 | 0 | 0 | 12 | 3 | 0 | 0 | 0 | 4 |
| No. of Negative Cases | 19 | 8 | 25 | 5 | 8 | 11 | 18 | 3 | 1 | 2 | 4 | 8 |
| Percent | 9.52 | 11.11 | 3.84 | 37.5 | 0 | 0 | 40 | 50 | 0 | 0 | 0 | 33.33 |

**Figure 4.26.** Month wise distribution of positive (yellow bar) and negative (orange bar) samples tested for adenovirus infections in this study. The total number of samples is also plotted and indicated by blue bar. The percentage of positive samples with adenovirus detected is given as green bar.

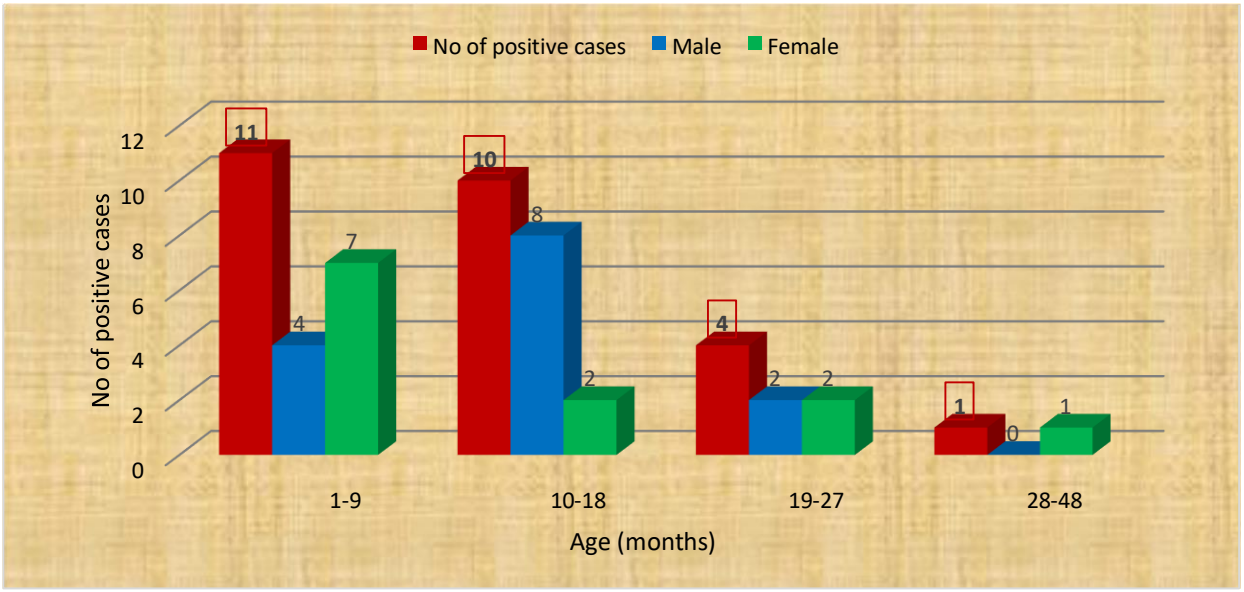



**Figure 4.27.** Total number of samples positive for human adenovirus and their distribution among male and female subjects at MHL during 2017-2018

### 4.2.9 Assessment of demographic and clinical parameters among diarrheal patients in MHL

The enrolled subjects at MHL were analyzed for the assessment of demographic and clinical parameters between the positive and negative patients for adenovirus infections. Presence of adenovirus in samples were regarded as positive while those without adenovirus were termed as negative. With the help of chi-square test, the qualitative parameters were evaluated while the quantitative variables were evaluated by employing student's *t*-Test.

**i) Evaluation of demographic and clinical data among positive and negative cases**

Evaluation of the clinical characteristics like fever, diarrhea, vomiting, the factor which was found significant ($p$=0.0362) was fever when assessed among positive and negative groups for adenovirus infections (Table 4.11). Out of 26 patients positive for adenoviruses, fever was recorded in 18 (69.2%) patients whereas 8 (30.7%) had no fever. The insignificant factor found was vomiting and diarrhea among positive and negative cases ($p$=0.3887 and $p$=0.4925 respectively). Similarly, the factor age was also found to be insignificant among positive and negative cases ($p$=0.4701) though most of infections (n= 21; 80.76%) were establish in children having age below 18 months.

**ii) Evaluation of clinical data among positive and negative cases**

Statistical interpretation was performed for the quantitative variables by employing student's *t* Test (Table 4.12). Among the quantitative variables, all the clinical parameters e.g. the mean number of diarrheal episodes (4.308±1.123), mean duration of diarrhea (2.192±1.625 days), mean number of vomiting episodes (3.480±2.002) and mean duration of vomiting (2.240±1.640 days) were found insignificant with *p*-values 0.6799, 0.6039, 0.5384 and 0.4665 respectively. The age was also found insignificant (*p*-value=0.1208) with mean value 13.21±10.14.

**Table 4.11.** Chi square analysis of qualitative variables of patients with adenovirus infections

| Total No. of Cases = 138 | Positive (n= 26) n (% age) | Negative (n= 112) n (% age) | *P-value* |
|---|---|---|---|
| **Gender** | | | |
| Male (n=78) | 14 (53.8) | 64 (57.1) | $0.7600^{NS}$ |
| Female (n=60) | 12 (46.1) | 48 (42.9) | |
| **Age groups (months)** | | | |
| 1-9 (n=73) | 11 (42.3) | 62 (55.3) | |
| 10-18 (n=47) | 10 (38.4) | 37 (33) | $0.4701^{NS}$ |
| 19-27 (n=12) | 4 (15.3) | 8 (7.14) | |
| 28-48 (n=6) | 1 (3.8) | 5 (4.46) | |
| **Clinical signs** | | | |
| **Fever** | | | |
| Present (n=70) | 18 (69.2) | 52 (46.4) | $\mathbf{0.0362^{S}}$ |
| Absent (n=68) | 8 (30.7) | 60 (53.6) | |
| **Vomit** | | | |
| Yes (n=127) | 25 (96.1) | 102 (91.1) | $0.3887^{NS}$ |
| No (n=11) | 1 (3.9) | 10 (9) | |
| **Diarrhea** | | | |
| Yes (n=136) | 26 (19.1) | 110 (98.2) | $0.4925^{NS}$ |
| No (n=2) | 0 (0) | 2 (1.8) | |

S= Significant *p*-value; NS= non-significant *p*-value

% ages are given in Adenovirus positive and negative groups according to the variables such as gender, age groups, fever, vomiting and diarrhea

**Table 4.12.** Comparison of clinical parameters (quantitative variables) between patients found positive and negative for adenovirus infections using Student t-test

| **Clinical Symptoms** | **No. of Samples** | **Mean** | **Std. Deviation (±)** | **Std. Error Mean** | ***p*-value** |
|---|---|---|---|---|---|
| Diarrhea Episodes/24hrs | Positive (n=26) | 4.308 | 1.123 | 0.2203 | $0.6799^{NS}$ |
| | Negative (n=110) | 4.200 | 1.210 | 0.1154 | |
| Diarrhea duration (days) | Positive (n=26) | 2.192 | 1.625 | 0.3187 | $0.6039^{NS}$ |
| | Negative (n=110) | 2.018 | 1.514 | 0.1444 | |
| Vomiting Episodes/24hrs | Positive (n=25) | 3.480 | 2.002 | 0.4005 | $0.5384^{NS}$ |
| | Negative (n=102) | 3.765 | 2.083 | 0.2063 | |
| Vomit duration (days) | Positive (n=25) | 2.240 | 1.640 | 0.3280 | $0.4665^{NS}$ |
| | Negative (n=102) | 1.990 | 1.506 | 0.1491 | |
| Age (months) | Positive (n=26) | 13.21 | 10.14 | 1.989 | $0.1208^{NS}$ |
| | Negative (n=112) | 10.35 | 7.963 | 0.7524 | |

NS= non-significant *p*-value<0.05

### 4.2.10 Investigation of tendencies in demographic and clinical parameters among gastroenteritis patients

Among HAdV positive and negative patients, the tendencies in demographic aspects of gastroenteritis patients enrolled at MHL during 2017-2018 were contrasted and evaluated. The chisquare and student's *t*-Test were employed for analysis of qualitative and quantitative parameters of the patients.

#### 1) Age and gender wise analysis

For an adenovirus infection, the age distribution of positive cases was determined to evaluate the most vulnerable age group between the hospitalized patients. Likewise, on the basis of gender the difference between positive and negative patients was assessed in order to recognize the gender based difference and male to female ratio between the investigated positive patients.

The proportion of positive HAdV cases were observed during 2017-2018 was 26/138 (18.84%). Dispersal of adenovirus infection among positive cases (n=26) was evaluated on the basis of four age groups. It was detected that in children below 18 months of age the adenovirus detection rate were more focused as compared to those having above 18 months (80.76% vs 19.23%; n=21 vs 5 respectively) (Figure 4.28). The maximum incidence of adenovirus positive cases ware detected in males contrasted to females (53.84% and 46.15% correspondingly) regardless of their age group. Generally, in adenovirus positive incidences, male to female ratio was observed as 1.6:1.

#### 2) Monthly based evaluation of enteric adenovirus infection in MHL samples

The dispersion of positive isolates for viral infections during 2017-2018 was inspected to discover any seasonal array of enteric adenovirus infections. The highest proportion of adenovirus positive samples was found in August (50%), July (40%), and April (37.5%) as shown in Figure 4.29. Overall, adenovirus infections were found most in summer then in winter months.

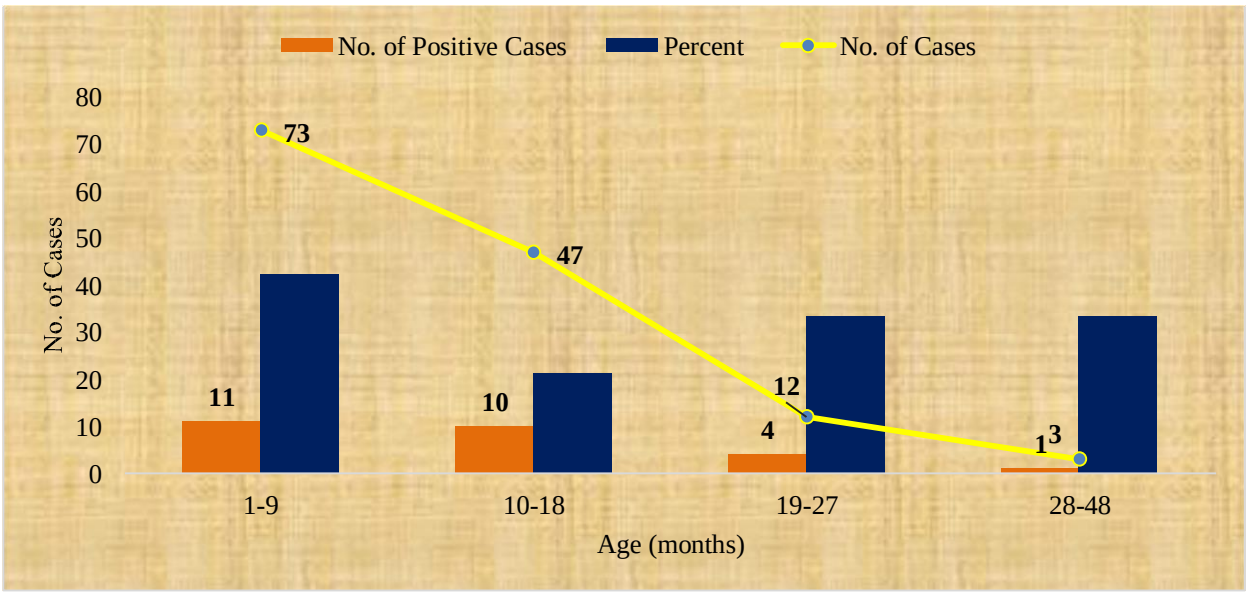



**Figure 4.28.** Total number of samples and those tested positive for human adenovirus infections with percent are given

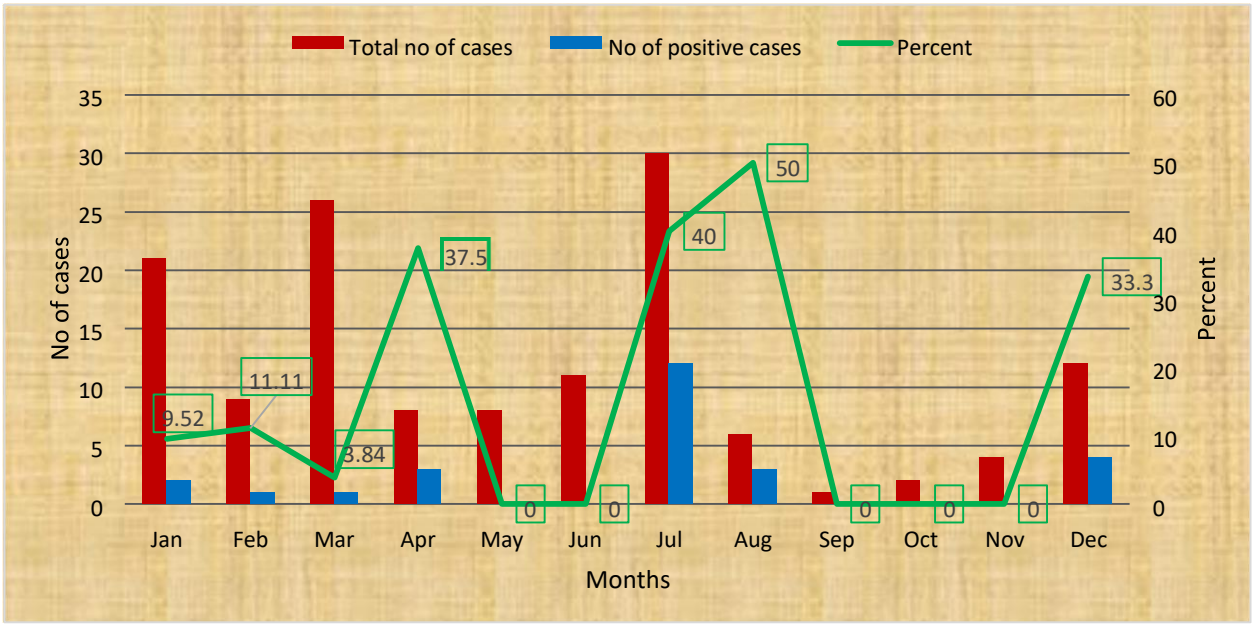



**Figure 4.29.** Month wise distribution of total number of samples (red bar), positive cases (blue bar) and their percentage (green line) tested for adenovirus.

### 4.2.11 Evaluation of clinical parameters for determination of clinical effect of co-infection of adenovirus with rotavirus

In this study, the samples taken from gastroenteritis patients were previously analyzed for rotavirus. We, therefore, analyzed the significance of co-infection via the comparison of clinical signs and symptoms of patients infected with adenovirus to those co-infected with rotavirus by putting on the statistical test called ANOVA (Analysis of Variance).

#### *1) Comparative analysis of quantitative clinical parameters among adenovirus positive patients with rotavirus co-infections*

Data presented in Tables 4.13 demonstrate the outcomes of ANOVA test, used to deduce the importance of co-infection of adenovirus with rotavirus. It was detected that patients with double infection of adeno-rotavirus and rotavirus showed more serious clinical indications and illness therefore describing their clinical importance in gastroenteritis cases.

Data exhibited in Table 4.13 shows that the number of diarrheal episodes per 24 hours, diarrhea duration (days) and number of vomiting episodes per 24 hours and vomiting duration (days) were significantly associated to disease in patients co-infected with adenovirus and rotavirus (*p*-value 0.0321, 0.0297, 0.0038 and 0.0001 respectively). The mean number of diarrheal episode per 24 hours, diarrhea duration (days) and number of vomiting episodes per 24 hours and vomiting duration (days) of HAdV-rotavirus co-infection was 5.071±0.9972, 3.143±1.406, 5.714±1.978 and 4.357±1.151.

Table 4.13. Comparison of clinical data among subjects with AdV and RV co-infection using ANOVA (Significant *p*-values (≤ 0.05) are given in bold)

| Clinical Parameters | Infection (No. of cases) | Range | Mean±SD | 95% Confidence Interval | | *P-Value* |
|---|---|---|---|---|---|---|
| | | | | Lower | Upper | |
| **Diarrhea episodes/ 24 hrs** | HAdV+ve (n=12) | 2-6 | 4.000±1.128 | 3.283 | 4.717 | **0.0321**[S] |
| | Rotavirus+ve (n=53) | 2-7 | 4.321±1.221 | 3.984 | 4.657 | |
| | Rotavirus+HAdV (n=14) | 3-7 | 5.071±0.9972 | 4.496 | 5.647 | |
| | Negative (n=59) | 2-7 | 4.051±1.209 | 3.736 | 4.366 | |
| **Diarrhea duration (days)** | HAdV+ve (n=12) | 1-6 | 3.167±1.850 | 1.991 | 4.342 | **0.0297**[S] |
| | Rotavirus+ve (n=53) | 1-6 | 2.132±1.606 | 1.689 | 2.575 | |
| | Rotavirus+HAdV (n=14) | 2-6 | 3.143±1.406 | 2.331 | 3.955 | |
| | Negative (n=59) | 1-6 | 2.153±1.412 | 1.785 | 2.520 | |
| **Vomiting episodes/24 hrs** | HAdV+ve(n=11) | 1-6 | 3.364±1.502 | 2.355 | 4.372 | **0.0038**[S] |
| | Rotavirus+ve (n=53) | 1-9 | 3.679±2.045 | 3.115 | 4.243 | |
| | Rotavirus+HAdV (n=14) | 3-9 | 5.714±1.978 | 4.572 | 6.856 | |
| | Negative (n=60) | 1-9 | 3.650±1.999 | 3.134 | 4.166 | |
| **Vomiting duration (days)** | HAdV+ve(n=11) | 1-6 | 2.909±2.023 | 1.550 | 4.268 | **0.0001**[S] |
| | Rotavirus+ve (n=53) | 1-6 | 2.566±1.658 | 2.109 | 3.023 | |
| | Rotavirus+HAdV (n=14) | 3-6 | 4.357±1.151 | 3.693 | 5.022 | |
| | Negative (n=60) | 1-6 | 2.133±1.546 | 1.734 | 2.533 | |
| **Age (months)** | HAdV+ve(n=13) | 3-48 | 15.85±11.94 | 8.629 | 23.06 | 0.0990[NS] |
| | Rotavirus+ve (n=53) | 2-41 | 11.34±8.875 | 8.893 | 13.79 | |
| | Rotavirus+HAdV (n=14) | 2-24 | 10.68±7.237 | 6.500 | 14.86 | |
| | Negative (n=58) | 2-24 | 9.762±5.807 | 8.235 | 11.29 | |

### 4.2.12 Detailed statistics of samples obtained from the Children Hospital, Lahore

From CHL, total 159 fecal specimens from admitted children having age ≤3 years were obtained during 2017-2018 and delivered to Department of Virology, National Institute of Health, Islamabad. Overall, the percentage of positive samples was 32.07% (n=51) for adenovirus infections respectively.

#### 1) Month-wise distribution of CHL samples

Among total 159 admissions in this study, during the May-August months, 31.44% (n=50) of patients were reported whereas during November-February, 40.88% (n=65) patients were admitted. The month-wise number and percentage of total samples obtained from diarrheal patients is shown in Figure 4.30.

#### 2) Gender and Age-wise distribution of CHL samples

Among 159 samples, 94 (59.11%) patients were male and 65 (40.88%) were females with ratio 1.4:1 (mean=1.415±0.4943). The samples of the children investigated in this study had a mean age of 11.03±6.812 months having range from 1 to 36 months. For study, the investigated subjects were grouped into 6 age groups with the gap of 6 months between their ages. Mostly they belong to ≤ 12 months of age (n= 111), followed by 13-18 and 19-24 months of age (n=22 and n=22) whereas 25-30 and 31-36 months of age were coming after (n=3 and n=1) them as shown in Figure 4.31.

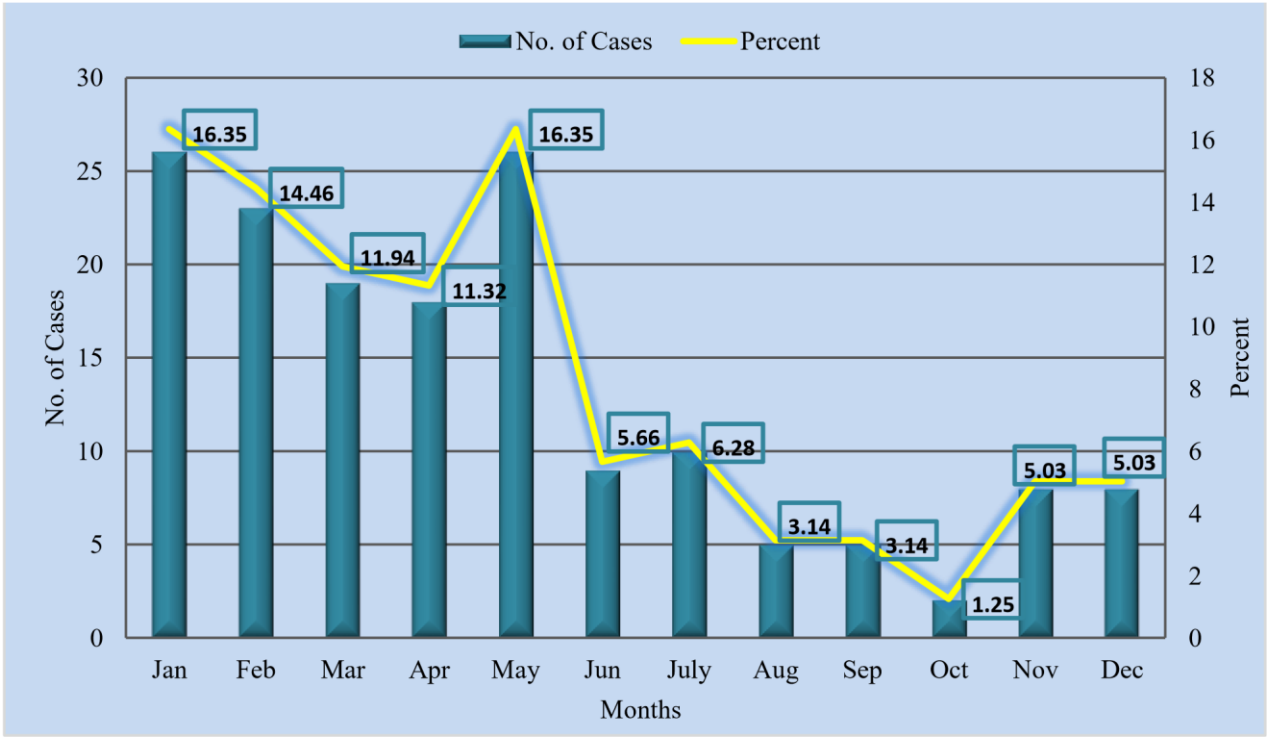



**Figure 4.30.** Frequency and Percentage of total samples collected during 2017-2018

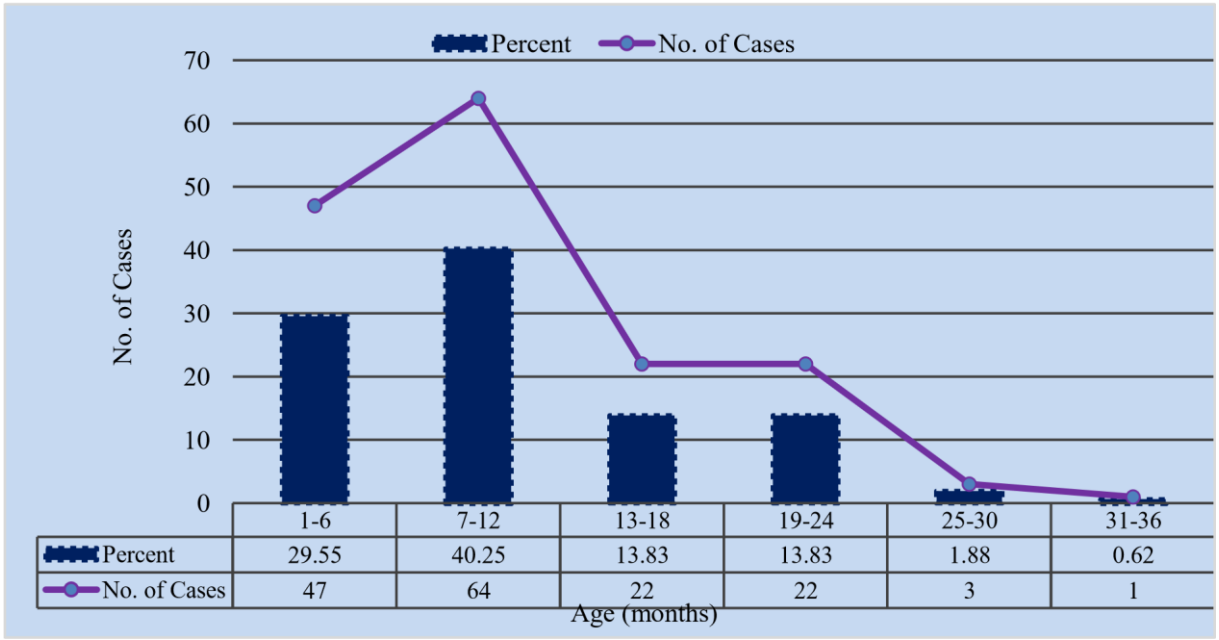



**Figure 4.31.** Age wise distribution of total number of samples tested during 2017-2018

### 4.2.13 Adenovirus analysis at CHL

Among total number of samples i.e. 159, 51 (32.07%) samples were found positive for human adenoviruses whereas 108 (67.92%) samples were found completely negative. These HAdV positive patients were regarded as responsible for "enteric viral" infections or diarrhea.

#### 1) Monthly dispersal of CHL positive samples during 2017-2018

Among 51 positive samples, the monthly-based dispersion graph is shown in Figure 4.32, which showed that in 2017-2018, the frequency of positive isolates for human adeno viruses were mostly found in winter (November-February, n=36) as compared to summer (May-August, n=7). For illustration, increased infections were found in January (n=20) followed by February (n=11) as shown in Figure 4.32.

#### 2) Age and gender based division of CHL positive samples during 2017-2018

Based on five age groups, the rate of positive samples was assessed with a gap of 6 months. The end group i.e. fifth one contained samples from children with age 25 months and above. In these groups of different ages, more positive samples (68.62%; n=35/51) were gone with the children having age below 12 months which was then followed by children having age 13-18 months of age (21.56%; n=11/51) as shown in Figure 4.33. Among positive cases, HAdV were found to be more common in males as compared to females (70.58% vs 29.41%; n=36 vs 15) with male to female ratio of 2.4:1.

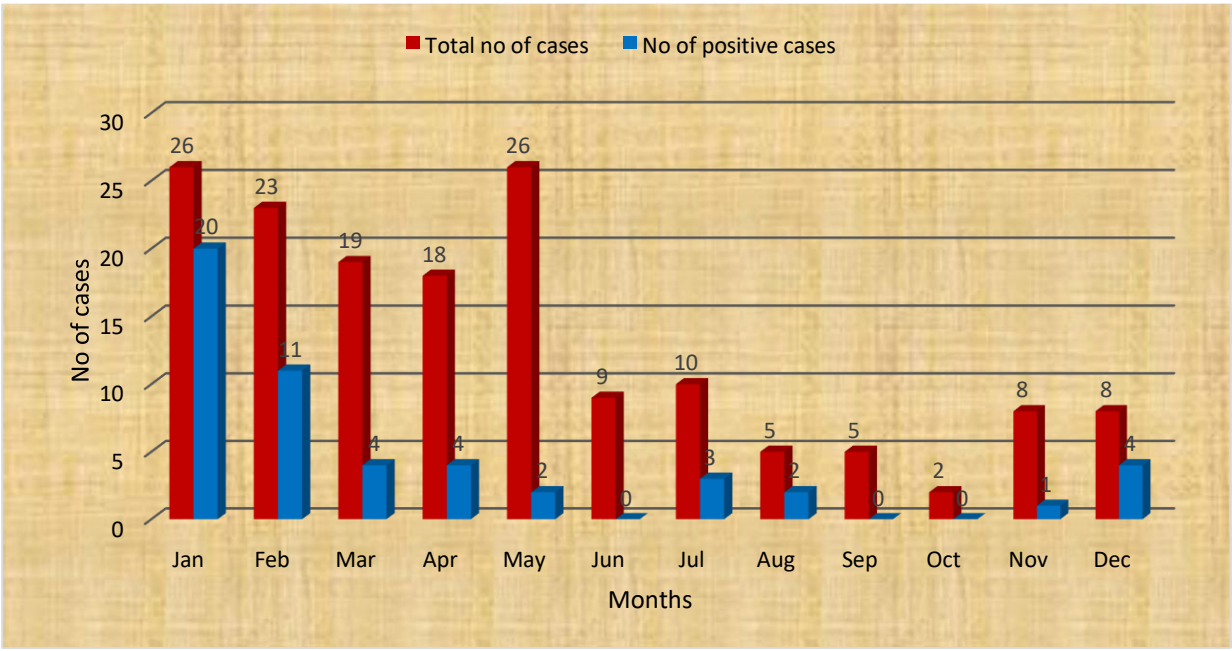



**Figure 4.32.** Frequency of total and positive samples during 2017-2018

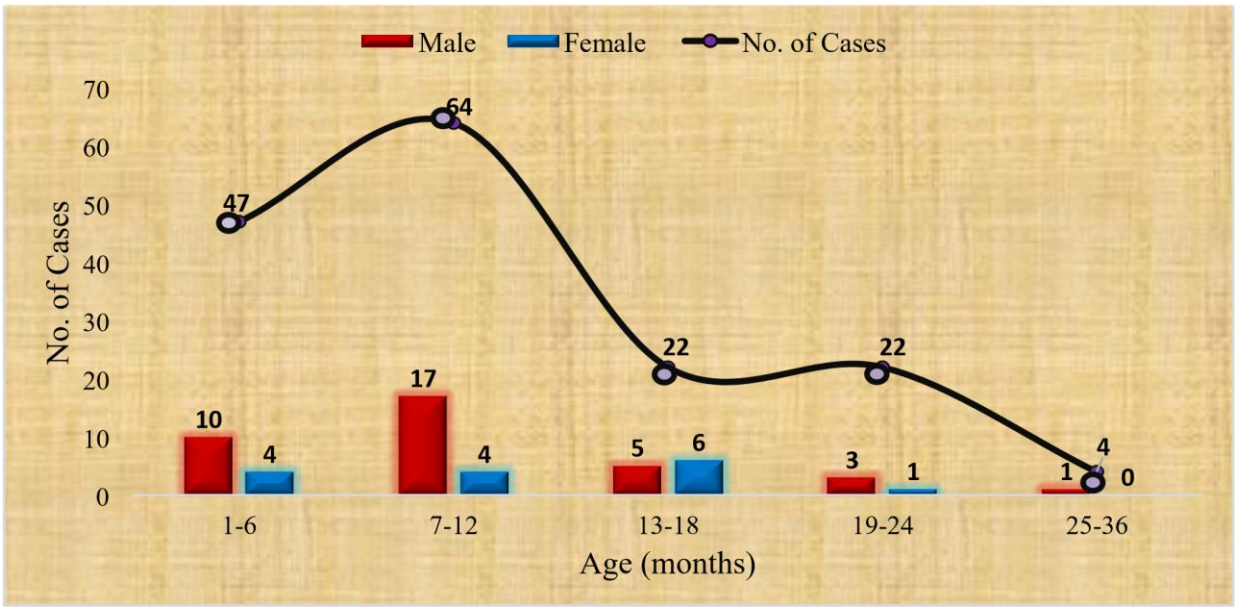



**Figure 4.33.** Age wise distribution of samples positive for adenovirus during 2017-2018. Number of male and female cases is plotted on Y-axis and the age groups are given on X-axis. Total number of samples is given in the form of black line.

### 4.2.14 Assessment of demographic and clinical parameters among diarrheal patients in CHL

The enrolled subjects at CHL were analyzed for the assessment of demographic and clinical parameters between the positive and negative patients for adenovirus infections. Presence of adenovirus in samples were regarded as positive while those without adenovirus were termed as negative. With the help of chi-square test, the qualitative parameters were evaluated while the quantitative variables were evaluated by employing student's *t*-Test.

**1) Evaluation of demographic and clinical data among positive and negative cases**

Evaluation of the demographic characteristics, the factor which was found significant ($p$=0.0432) was gender when assessed among positive and negative groups for adenovirus infections (Table 4.14). Out of 51 patients positive for adenoviruses, 36 (70.6%) males and 15 (29.4%) females were found whereas 58 (53.7%) males and 50 (46.3%) females were found negative. The insignificant factor found was fever, vomiting and diarrhea among positive and negative cases ($p$=0.7747, $p$=0.4366 and $p$=0.5847 respectively). Similarly, the factor age was also found to be insignificant among positive and negative cases ($p$=0.2474) though most of infections (n= 35) were establish in children having age below 12 months.

**2) Evaluation of clinical data among positive and negative cases**

Statistical interpretation were performed for the quantitative variables by employing student's *t*Test (Table 4.15). Among the quantitative variables, the mean number of diarrheal episodes (4.620±0.9234), the mean duration of diarrhea (2.120±1.466 days), mean number of vomiting episodes (3.580±1.642) and mean duration of vomiting (2.122±1.509 days) were found insignificant with *p*-values 0.9366, 0.1125, 0.7230 and 0.0850 correspondingly.

**Table 4.14.** Chi square analysis of qualitative variables of patients with adenovirus infections

| Total No. of Cases = 159 | Positive (n= 51) n (% age) | Negative (n= 108) n (% age) | *P-value* |
|---|---|---|---|
| **Gender** | | | |
| Male (n=94) | 36 (70.6) | 58 (53.7) | **0.0432$^{S}$** |
| Female (n=65) | 15 (29.4) | 50 (46.3) | |
| **Age groups (months)** | | | |
| 1-6 (n=47) | 14 (27.4) | 33 (30.5) | 0.2474$^{NS}$ |
| 7-12 (n=64) | 21 (41.1) | 43 (39.8) | |
| 13-18 (n=22) | 11 (21.5) | 11 (10.2) | |
| 19-24 (n=22) | 4 (7.8) | 18 (16.6) | |
| 25-36 (n=4) | 1 (1.9) | 3 (2.7) | |
| **Clinical signs** | | | |
| **Fever** | | | |
| Present (n=93) | 29 (56.8) | 64 (59.2) | 0.7747$^{NS}$ |
| Absent (n=66) | 22 (43.1) | 44 (40.7) | |
| **Vomit** | | | |
| Yes (n=155) | 49 (96.1) | 106 (98.1) | 0.4366$^{NS}$ |
| No (n=4) | 2 (3.9) | 2 (1.8) | |
| **Diarrhea** | | | |
| Yes (n=157) | 50 (98) | 107 (99.1) | 0.5847$^{NS}$ |
| No (n=2) | 1 (1.9) | 1 (0.9) | |

S= Significant *p*-value; NS= non-significant *p*-value,

% ages are given in Adenovirus positive and negative groups according to the variables such as gender, age groups, fever, vomiting and diarrhea

**Table 4.15.** Comparison of clinical parameters (quantitative variables) between patients found positive and negative for adenovirus infections using Student t-test.

| **Clinical Symptoms** | **No. of Samples** | **Mean** | **Std. Deviation (±)** | **Std. Error Mean** | ***p*-value** |
|---|---|---|---|---|---|
| Diarrhea Episodes/24hrs | Positive (n=50) | 4.620 | 0.9234 | 0.1306 | $0.9366^{NS}$ |
| | Negative (n=107) | 4.636 | 1.224 | 0.1183 | |
| Diarrhea duration (days) | Positive (n=50) | 2.120 | 1.466 | 0.2073 | $0.1125^{NS}$ |
| | Negative (n=107) | 2.604 | 1.891 | 0.1836 | |
| Vomiting Episodes/24hrs | Positive (n=49) | 3.580 | 1.642 | 0.2323 | $0.7230^{NS}$ |
| | Negative (n=106) | 3.701 | 2.129 | 0.2058 | |
| Vomit duration (days) | Positive (n=49) | 2.122 | 1.509 | 0.2155 | $0.0858^{NS}$ |
| | Negative (n=106) | 2.654 | 1.894 | 0.1831 | |
| Age (months) | Positive (n=51) | 10.77 | 6.276 | 0.8789 | $0.7763^{NS}$ |
| | Negative (n=108) | 10.48 | 6.099 | 0.5868 | |

NS= non-significant *p*-value<0.05

### 4.2.15 Investigation of tendencies in demographic and clinical parameters among gastroenteritis patients

Among HAdV positive and negative patients, the tendencies in demographic aspects of gastroenteritis patients enrolled at CHL during 2017-2018 were contrasted and evaluated. The chisquare and student's *t*-Test were employed for analysis of qualitative and quantitative parameters of the patients.

#### 1) Age and gender wise analysis

For an adenovirus infection, the age distribution of positive cases was determined to evaluate the most vulnerable age group between the hospitalized patients. Likewise, on the basis of gender the difference between positive and negative patients was assessed in order to recognize the gender based difference and male to female ratio between the investigated positive patients.

The proportion of positive HAdV cases were observed during 2017-2018 was 51/159 (32.07%). Dispersal of adenovirus infection among positive cases (n=51) was evaluated on the basis of five age groups. It was detected that in children below 12 months of age the adenovirus detection rate were more focused as compared to those having above 12 months (68.6% vs 31.3%; n=35 vs 16 respectively) (Figure 4.34). The maximum incidence of adenovirus positive cases were detected in males contrasted to females (70.6% and 29.4% correspondingly) regardless of their age group. Generally, in adenovirus positive incidences, male to female ratio was observed as 2.4:1.

#### *2) Monthly based evaluation of enteric adenovirus infection in CHL samples*

The dispersion of positive isolates for viral infections during 2017-2018 was inspected to discover any seasonal array of enteric adenovirus infections. The highest proportion of adenovirus positive samples was found in January (n=20) as shown in Figure 4.35. Overall, adenovirus infections were observed with an increased infection rate during the winter then in summer.

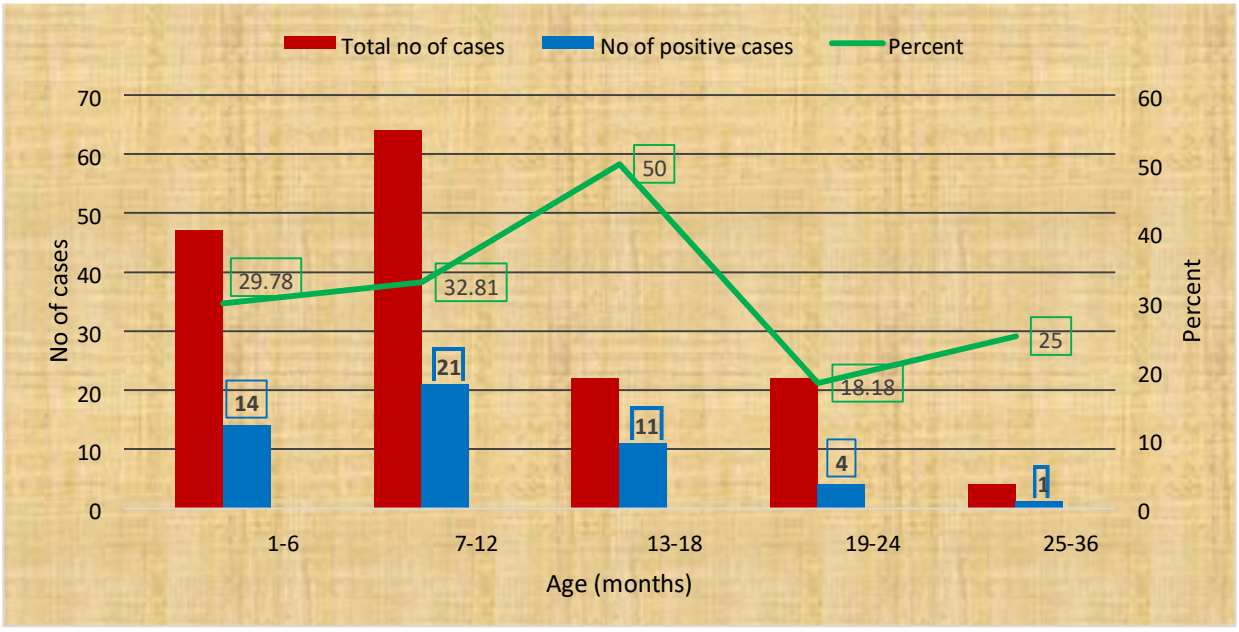



**Figure 4.34.** Age-wise distribution of total number of samples (red bar), positive samples (blue bar) and its percentage (green line) detected in CHL during 2017-2018.

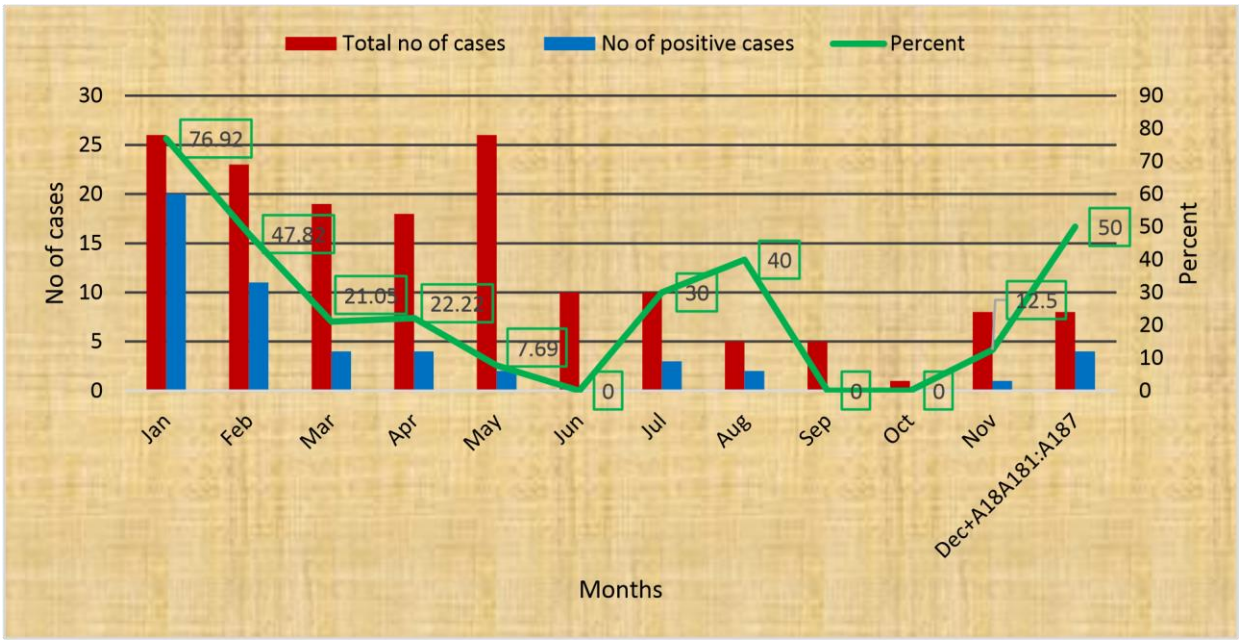



**Figure 4.35.** Month wise distribution of total number of samples (red bar), samples positive for adenovirus (blue bar) and its percentage (green line) at CHL during 2017-2018.

### 4.2.16 Evaluation of clinical parameters for determination of clinical effect of co-infection of adenovirus with rotavirus

In this study, the samples taken from gastroenteritis patients were previously analyzed for rotavirus. We, therefore, analyzed the significance of co-infection via the comparison of clinical signs and symptoms of patients infected with adenovirus to those co-infected with rotavirus by putting on the statistical test called ANOVA (Analysis of Variance).

#### *1) Comparative analysis of quantitative clinical parameters among adenovirus positive patients with rotavirus co-infections*

Data presented in Tables 4.16 demonstrate the outcomes of ANOVA test, used to deduce the importance of co-infection of adenovirus with rotavirus. It was detected that patients with double infection of adeno-rotavirus and rotavirus showed more serious clinical indications and illness therefore describing their clinical importance in gastroenteritis cases.

Data exhibited in Table 4.16 shows that the number of diarrheal duration (days) and number of vomiting episodes per 24 hours and vomiting duration (days) were significantly associated to disease in patients co-infected with adenovirus and rotavirus (*p*-value 0.0001, 0.0007 and 0.0001 respectively). The mean number of diarrheal duration (days) and number of vomiting episodes per 24 hours and vomiting duration (days) of HAdV-rotavirus co-infection was 2.346±1.764, 3.440±1.474, and 2.480±1.531 correspondingly.

Table 4.16. Comparison of clinical parameters among subjects with adenovirus and rotavirus co-infection using ANOVA

| Clinical Parameters | Infection (No. of cases) | Range | Mean±SD | 95% Confidence Interval | | *P-Value* |
|---|---|---|---|---|---|---|
| | | | | Lower | Upper | |
| **Diarrhea episodes/ 24 hrs** | HAdV+ve (n=26) | 3-6 | 4.692±0.8376 | 4.354 | 5.031 | 0.3169[NS] |
| | Rotavirus+ve (n=21) | 4-7 | 5.190±1.078 | 4.700 | 5.681 | |
| | Rotavirus+HAdV (n=24) | 4-7 | 5.083±1.213 | 4.571 | 5.595 | |
| | Negative (n=88) | 2-7 | 4.784±1.227 | 4.524 | 5.044 | |
| **Diarrhea duration (days)** | HAdV+ve (n=26) | 1-6 | 2.346±1.441 | 1.764 | 2.928 | **0.0001[S]** |
| | Rotavirus+ve (n=21) | 2-6 | 4.050±1.701 | 3.254 | 4.846 | |
| | Rotavirus+HAdV (n=24) | 2-6 | 4.292±1.517 | 3.651 | 4.932 | |
| | Negative (n=88) | 1-6 | 2.614±1.914 | 2.208 | 3.019 | |
| **Vomiting episodes/24 hrs** | HAdV+ve(n=25) | 1-8 | 3.440±1.474 | 2.831 | 4.049 | **0.0007[S]** |
| | Rotavirus+ve (n=21) | 1-8 | 4.708±2.236 | 3.764 | 5.652 | |
| | Rotavirus+HAdV (n=24) | 4-9 | 6.000±1.842 | 5.222 | 6.778 | |
| | Negative (n=89) | 1-8 | 4.138±2.571 | 3.590 | 4.686 | |
| **Vomiting duration (days)** | HAdV+ve(n=25) | 1-6 | 2.480±1.531 | 1.848 | 3.112 | **0.0001[S]** |
| | Rotavirus+ve (n=21) | 3-6 | 4.667±1.197 | 4.122 | 5.212 | |
| | Rotavirus+HAdV (n=24) | 3-6 | 4.875±1.035 | 4.438 | 5.312 | |
| | Negative (n=89) | 1-6 | 2.809±1.930 | 2.402 | 3.216 | |
| **Age (months)** | HAdV+ve(n=27) | 1-25 | 10.20±7.003 | 7.433 | 12.97 | 0.8519[NS] |
| | Rotavirus+ve (n=21) | 2-24 | 11.14±5.868 | 8.472 | 13.81 | |
| | Rotavirus+HAdV (n=24) | 1-24 | 9.604±6.439 | 6.885 | 12.32 | |
| | Negative (n=87) | 1-32 | 10.86±7.608 | 9.241 | 12.48 | |

Significant *p*-values ($\leq 0.05$) are given in bold

### 4.2.17 Detailed statistics of samples obtained from the Karachi General Hospital, Karachi

From KGH, total 182 fecal specimens from admitted children having age ≤5 years were obtained during January 2017 to December 2018 and delivered to Department of Virology, National Institute of Health, Islamabad. Overall, the percentage of positive samples was 12.08% (n=22) for adenovirus infections respectively.

#### *1) Month-wise distribution of KGH samples*

Among total 182 admissions in this study, during the May-August months, 46.70% (n=85) of patients were reported whereas during December-April, 42.85% (n=78) patients were admitted. The month-wise number and percentage of total samples obtained from diarrheal patients is shown in Figure 4.36.

#### *2) Gender and Age-wise distribution of KGH samples*

Among 182 samples, 101 (55.49%) patients were male and 81 (44.50%) were females with ratio 1.2:1 (mean= 1.446 ± 0.4988). The samples of the children investigated in this study had a mean age of 12.10± 9.846 months having range from 1 to 60 months. For study, the investigated subjects were grouped into 7 age groups with the gap of 9 months between their ages. Mostly they belong to ≤ 9 months of age (n= 82, 45.05%), followed by 10-18 months of age (n=58, 31.86%) whereas 19-27 and 28-36 months of age were came after (n=26, 14.28% and n=11, 6.04%) them. However, children having age more than 3 years were 5 in number as shown in Figure 4.37.

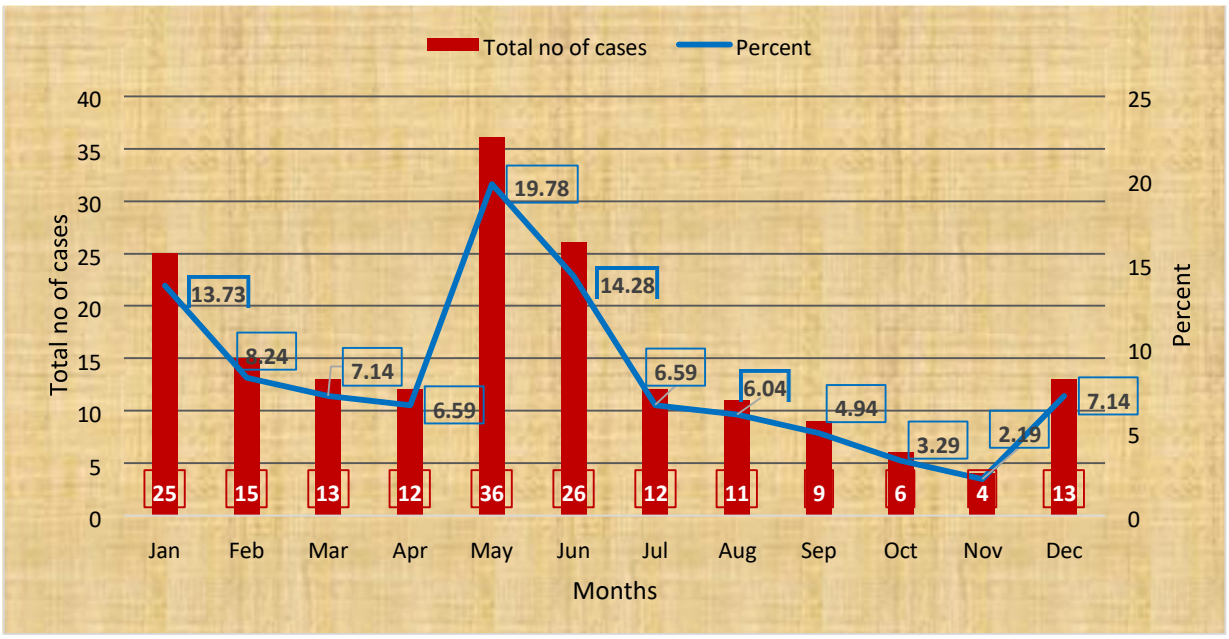



**Figure 4.36.** Month-wise distribution total number of samples and their percentage collected at KGH during 2017-2018

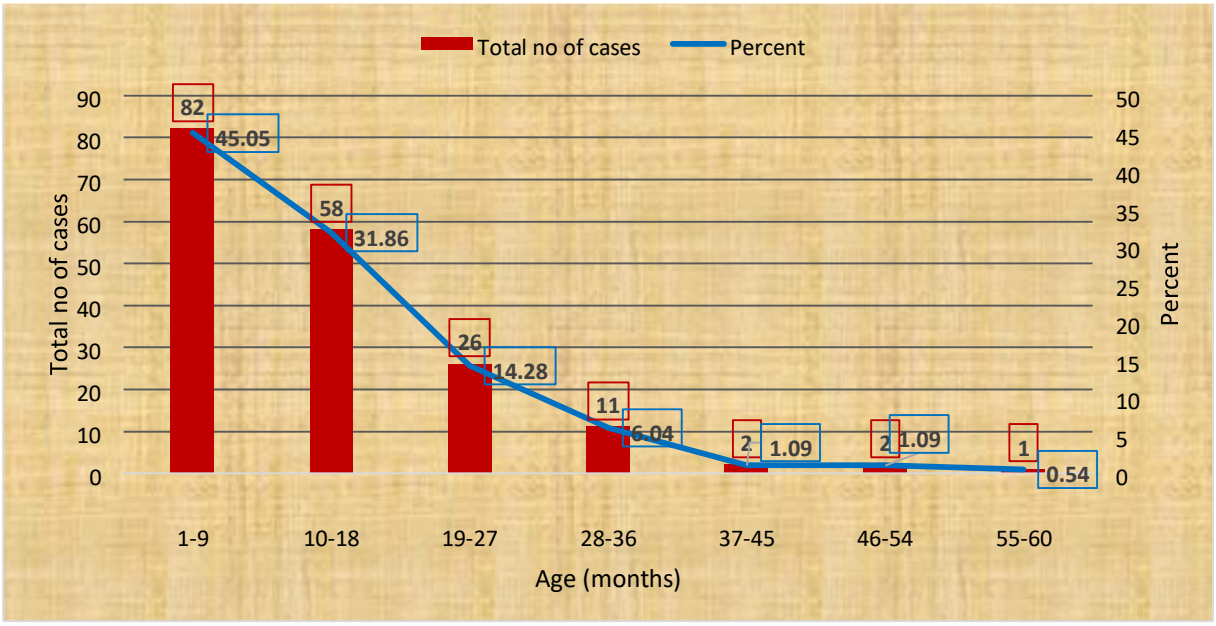



**Figure 4.37.** Age wise distribution of total number of samples and their percentage in respective age group tested at KGH during 2017-2018

### 4.2.18 Adenovirus analysis at KGH

Among total number of samples i.e. 182, 22 (12.08%) samples were found positive for human adenoviruses whereas 160 (87.91%) samples were found completely negative. These HAdV positive patients were regarded as responsible for "enteric viral" infections or diarrhea.

#### 1) Monthly dispersal of KGH positive samples during 2017-2018

Among 22 positive samples, the monthly-based dispersion graph is shown in Figure 4.38, which showed that in 2017-2018, the frequency of positive isolates for human adeno viruses between summer (May-October) and winter (Nov-April) months was 10% and 14.63% correspondingly. Although there was no obvious difference between the two seasons but for illustration, increased infections were found in winter (Nov-April) then summer (May-October) as shown in Figure 4.38.

#### 2) Age and gender based division of KGH positive samples during 2017-2018

Based on four age groups, the rate of positive samples was assessed with a gap of 9 months. The end group i.e. fourth one contained samples from children with age 28 months and above. In these groups of different ages, more positive samples (81.81%; n=18/22) were gone with the children having age below 18 months which was then followed by children having age 19-27 months of age (13.63%; n=3/22) as shown in Figure 4.39. Among positive cases, HAdV were found to be more common in females as compared to males (54.54% vs 45.45%; n=12 vs 10) with male to female ratio of 1.2:1.

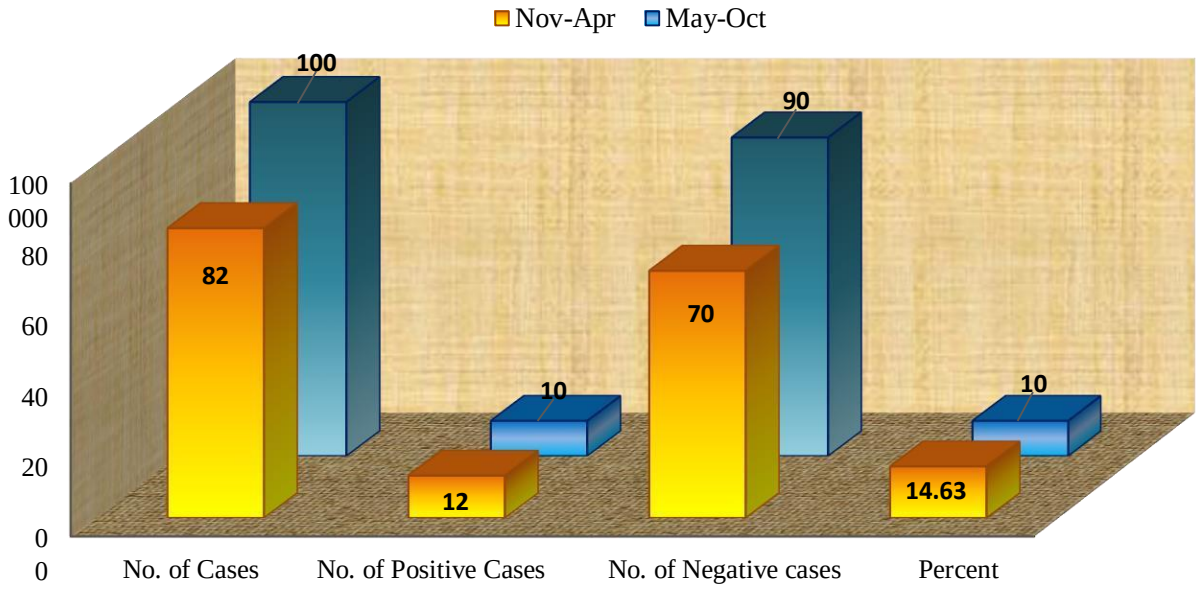



**Figure 4.38.** Month wise distribution of positive and negative samples tested for adenovirus infections in this study. The total number of samples and percentage of positive samples with adenovirus detected is given.

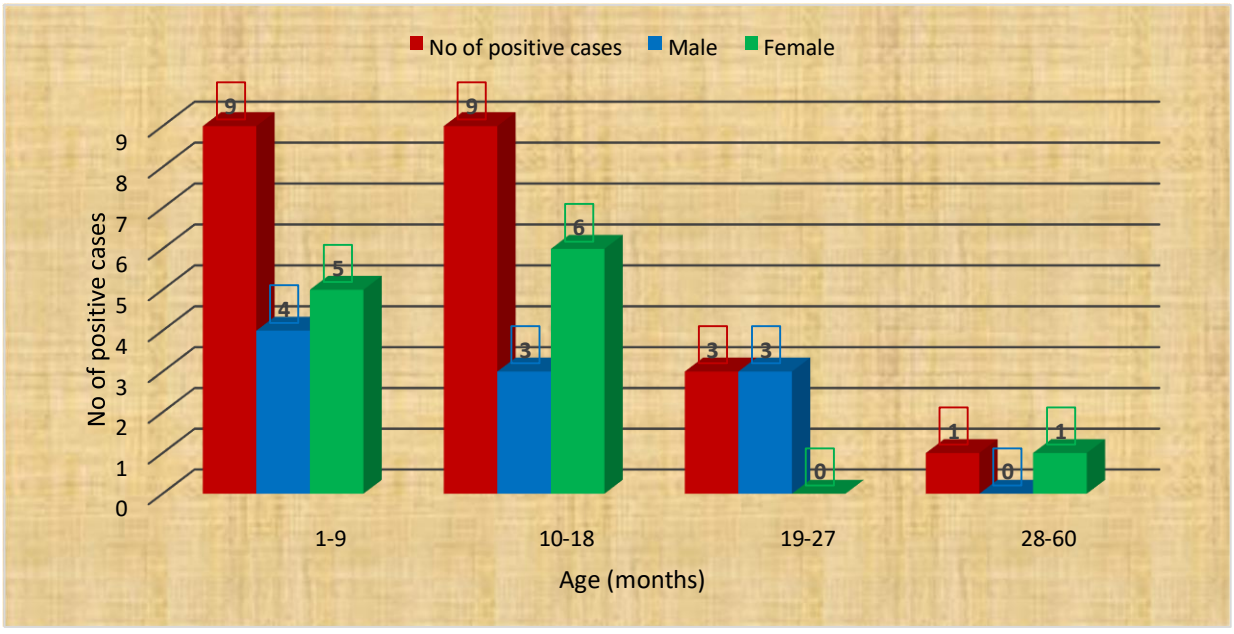



**Figure 4.39.** Age wise distribution of positive samples among male and female at KGH during 2017-2018.

### 4.2.19 Assessment of demographic and clinical parameters among diarrheal patients in KGH

The enrolled subjects at KGH were analyzed for the assessment of demographic and clinical parameters between the positive and negative patients for adenovirus infections. Presence of adenovirus in samples were regarded as positive while those without adenovirus were termed as negative. With the help of chi-square test, the qualitative parameters were evaluated while the quantitative variables were evaluated by employing student's *t*-Test.

*1)* ***Evaluation of demographic and clinical data among positive and negative cases***

Evaluation of the clinical characteristics like fever, diarrhea, vomiting, all of them were found insignificant ($p$=0.1094, $p$=0.3388 and $p$=0.4231 correspondingly) (Table 4.17). Similarly, the factor age was also found to be insignificant among positive and negative cases ($p$=0.7392) though most of infections (n= 18) were establish in children having age ≤18 months.

***2) Evaluation of clinical data among positive and negative cases***

Statistical interpretation was performed for the quantitative variables by employing student's *t* Test (Table 4.18). Among the quantitative variables, the mean number of diarrheal episodes (4.524±1.030), the mean duration of diarrhea (2.429±1.805 days), mean number of vomiting episodes (3.200±1.673) and mean duration of vomiting (2.250±1.650 days) were found insignificant with *p*-values 0.8701, 0.8065, 0.1721 and 0.4459 correspondingly.

**Table 4.17.** Chi square analysis of qualitative variables of patients with adenovirus infections

| Total No. of Cases = 182 | Positive (n= 22) n (% age) | Negative (n= 160) n (% age) | *P-value* |
|---|---|---|---|
| **Gender** | | | |
| Male (n=101 | 10 (45.4) | 91 (56.9) | $0.3122^{NS}$ |
| Female (n=81) | 12 (54.5) | 69 (43.1) | |
| **Age groups (months)** | | | |
| 1-9 (n=82) | 9 (40.9) | 73 (45.6) | $0.7392^{NS}$ |
| 10-18 (n=58) | 9 (40.9) | 49 (30.6) | |
| 19-27 (n=26) | 3 (13.6) | 23 (14.3) | |
| 28-60 (n=16) | 1 (4.5) | 15 (9.3) | |
| **Clinical signs** | | | |
| **Fever** | | | |
| Present (n=95) | 15 (68.1) | 80 (50) | $0.1094^{NS}$ |
| Absent (n=87) | 7 (31.8) | 80 (50) | |
| **Vomit** | | | |
| Yes (n=173) | 20 (90.9) | 153 (95.6) | $0.3388^{NS}$ |
| No (n=9) | 2 (9.1) | 7 (4.4) | |
| **Diarrhea** | | | |
| Yes (n=178) | 21 (95.4) | 157 (98.1) | $0.4231^{NS}$ |
| No (n=4) | 1 (4.6) | 3 (1.9) | |

S= Significant *p*-value; NS= non-significant *p*-value

% ages are given in Adenovirus positive and negative groups according to the variables such as gender, age groups, fever, vomiting and diarrhea

**Table 4.18.** Comparison of clinical parameters (quantitative variables) between patients found positive and negative for adenovirus infections using Student t-test.

| **Clinical Symptoms** | **No. of Samples** | **Mean** | **Std. Deviation (±)** | **Std. Error Mean** | ***p*-value** |
|---|---|---|---|---|---|
| Diarrhea Episodes/24hrs | Positive (n=21) | 4.524 | 1.030 | 0.2249 | $0.8701^{NS}$ |
| | Negative (n=157) | 4.478 | 1.233 | 0.09841 | |
| Diarrhea duration (days) | Positive (n=21) | 2.429 | 1.805 | 0.3938 | $0.8065^{NS}$ |
| | Negative (n=157) | 2.535 | 1.876 | 0.1497 | |
| Vomiting Episodes/24hrs | Positive (n=20) | 3.200 | 1.673 | 0.3742 | $0.1721^{NS}$ |
| | Negative (n=153) | 3.869 | 2.095 | 0.1694 | |
| Vomit duration (days) | Positive (n=20) | 2.250 | 1.650 | 0.3690 | $0.4459^{NS}$ |
| | Negative (n=153) | 2.588 | 1.887 | 0.1525 | |
| Age (months) | Positive (n=22) | 12.14 | 8.876 | 1.892 | $0.7345^{NS}$ |
| | Negative (n=160) | 12.94 | 10.64 | 0.8415 | |

NS= non-significant *p*-value<0.05

### 4.2.20 Evaluation of clinical parameters for determination of clinical effect of co-infection of adenovirus with rotavirus

In this study, the samples taken from gastroenteritis patients were previously analyzed for rotavirus. We, therefore, analyzed the significance of co-infection via the comparison of clinical signs and symptoms of patients infected with adenovirus to those co-infected with rotavirus by putting on the statistical test called ANOVA (Analysis of Variance).

#### *1) Comparative analysis of quantitative clinical parameters among adenovirus positive patients with rotavirus co-infections*

Data presented in Tables 4.19 demonstrate the outcomes of ANOVA test, used to deduce the importance of co-infection of adenovirus with rotavirus. It was detected that patients with double infection of adeno-rotavirus and rotavirus showed more serious clinical indications and illness therefore describing their clinical importance in gastroenteritis cases.

Data exhibited in Table 4.19 shows that the number of diarrheal episodes per 24 hours, diarrhea duration (days), number of vomiting episodes per 24 hours vomiting duration (days) were significantly associated to disease in patients co-infected with adenovirus and rotavirus (*p*-value 0.0001, 0.0002, <0.0001and 0.0005 correspondingly). The mean number of diarrheal episode per 24 hours, diarrhea duration (days) and number of vomiting episodes per 24 hours vomiting duration (days) of HAdV-rotavirus co-infection was 5.833±1.169, 5.500±1.049, 6.167±1.941 and 5.167±1.169 correspondingly.

Table 4.19. Comparison of clinical parameters among subjects with adenovirus and rotavirus co-infection using ANOVA

| Clinical Parameters | Infection (No. of cases) | Range | Mean±SD | 95% Confidence Interval Lower | Upper | *P-Value* |
|---|---|---|---|---|---|---|
| **Diarrhea episodes/ 24 hrs** | HAdV+ve (n=15) | 3-6 | 4.600±0.9856 | 4.054 | 5.146 | **0.0001**[S] |
| | Rotavirus+ve (n=51) | 2-7 | 4.961±1.199 | 4.623 | 5.298 | |
| | Rotavirus+HAdV (n=6) | 4-7 | 5.833±1.169 | 4.606 | 7.060 | |
| | Negative (n=106) | 2-7 | 4.217±1.179 | 3.990 | 4.444 | |
| **Diarrhea duration (days)** | HAdV+ve (n=15) | 1-5 | 1.933±1.280 | 1.225 | 2.642 | **0.0002**[S] |
| | Rotavirus+ve (n=51) | 1-6 | 2.216±1.701 | 1.737 | 2.694 | |
| | Rotavirus+HAdV (n=6) | 4-7 | 5.500±1.049 | 4.399 | 6.601 | |
| | Negative (n=106) | 1-6 | 2.717±1.901 | 2.351 | 3.083 | |
| **Vomiting episodes/24 hrs** | HAdV+ve (n=14) | 1-6 | 3.000±1.710 | 2.013 | 3.987 | **<0.0001**[S] |
| | Rotavirus+ve (n=51) | 2-8 | 4.706±1.847 | 4.186 | 5.225 | |
| | Rotavirus+HAdV (n=6) | 4-9 | 6.167±1.941 | 4.130 | 8.203 | |
| | Negative (n=102) | 1-9 | 3.647±1.881 | 3.278 | 4.016 | |
| **Vomiting duration (days)** | HAdV+ve (n=14) | 1-5 | 1.929±1.328 | 1.162 | 2.695 | **0.0005**[S] |
| | Rotavirus+ve (n=51) | 2-6 | 3.314±1.503 | 2.891 | 3.737 | |
| | Rotavirus+HAdV(n=6) | 4-7 | 5.167±1.169 | 3.940 | 6.394 | |
| | Negative (n=102) | 1-6 | 2.735±1.903 | 2.361 | 3.109 | |
| **Age (months)** | HAdV+ve (n=16) | 1-36 | 13.44±9.906 | 8.159 | 18.72 | 0.2496[NS] |
| | Rotavirus+ve (n=51) | 2-36 | 9.608±6.759 | 7.707 | 11.51 | |
| | Rotavirus+HAdV (n=6) | 3-12 | 8.667±4.082 | 4.382 | 12.95 | |
| | Negative (n=109) | 2-60 | 12.02±9.495 | 10.22 | 13.82 | |

Significant *p*-values (≤ 0.05) are given in bold

### 4.2.21 Descriptive statistics of samples collected from the National Institute of Child's Health, Karachi

From NICH, a total of 416 stool samples from hospitalized children ≤5 years of age were collected during January 2017 to December 2018 and transported to Department of Virology, National Institute of Health, Islamabad. All samples were tested for human adenoviruses. In total, 74 (17.8%) samples were found positive for adenovirus infections respectively.

#### *1) Month-wise distribution of NICH samples*

Out of total 416 enrollments in this study, 30.04% (n=125) of patients were reported during the May-August months whereas 37.7% (n=157) patients were admitted during November-March. Month wise distribution (number and percentage) of total samples collected from these patients is represented in Figure 4.40.

#### *2) Gender and Age-wise distribution of NICH samples*

Among 416 samples, 231 (55.5%) patients were male and 185 (44.5%) were females with ratio 1.2:1 (mean= 1.427 ± 0.4952). The samples of the children investigated in this study had a mean age of 11.11± 10.21 months with a range from 1 to 60 months. For study, the investigated subjects were categorized into 7 age groups with a difference of 9 months between their ages. Most of them were belongs to ≤ 9 months of age (n= 226, 54.32%), followed by 10-18 months of age (n=118, 28.5236%) whereas 19-27 and 28-36 months of age were came after (n=41, 9.85% and n=16, 3.84%) them. However, children having age more than 3 years were 15 in number as shown in Figure 4.41.

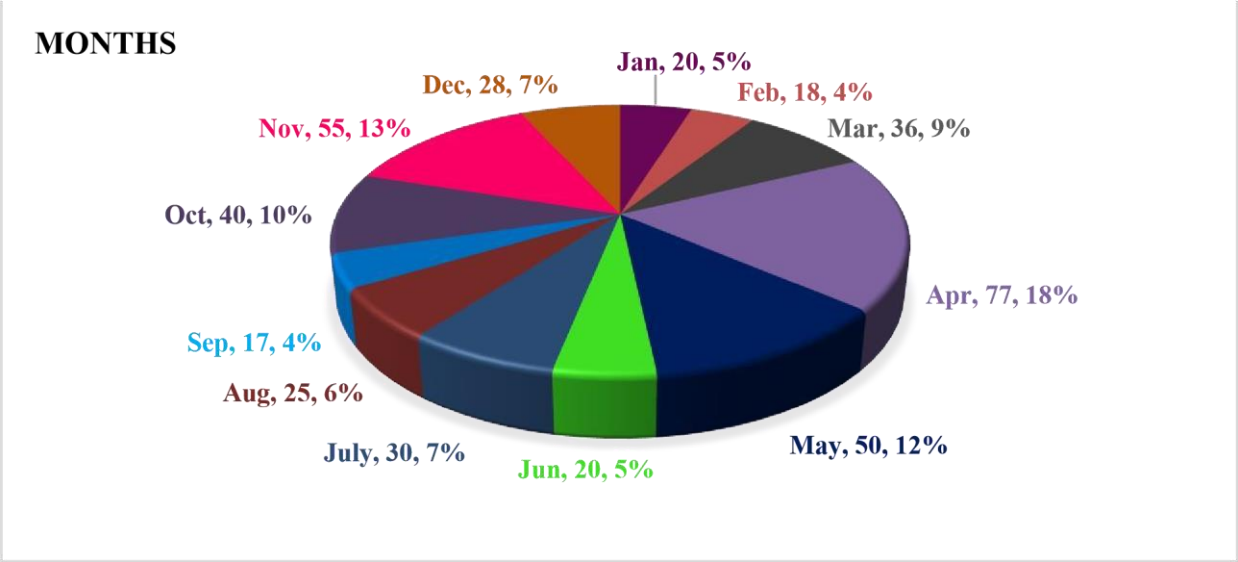



**Figure 4.40.** Frequency and Percentage of total samples collected during 2017-2018

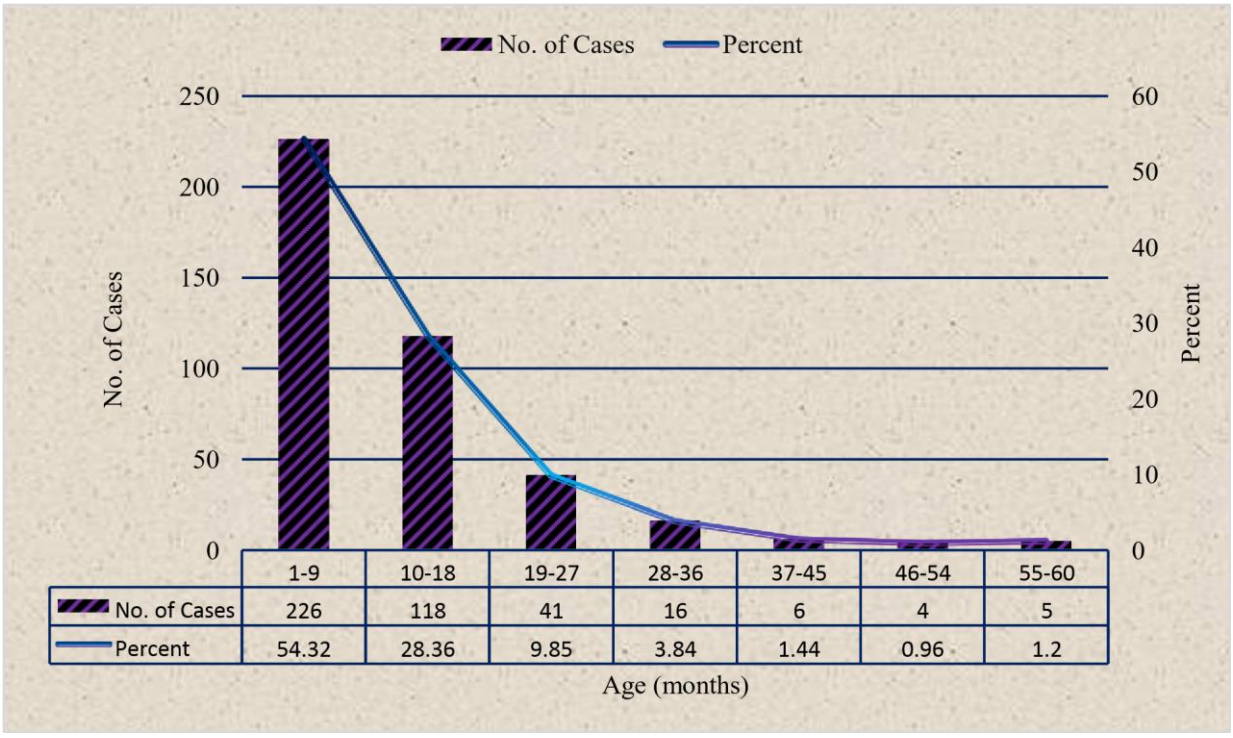



**Figure 4.41.** Age wise distribution of total number of samples tested during 2017-2018

### 4.2.22 Adenovirus analysis at NICH

Among total number of samples i.e. 416, 74 (17.8%) samples were found positive for human adenoviruses whereas 342 (82.2%) samples were found completely negative. These HAdV positive patients were regarded as responsible for "enteric viral" infections or diarrhea.

#### *1) Monthly dispersal of NICH positive samples during 2017-2018*

Among 74 positive samples, the monthly-based dispersion graph is shown in Figure 4.42, which showed that in 2017-2018, the frequency of positive isolates for human adenoviruses between summer (May-August) and winter (November-March) months was n=7 and n=45. For illustration, increased infections were found in December, January and April as shown in Figure 4.42.

#### *2) Age and gender based division of NICH positive samples during 2017-2018*

Based on four age groups, the rate of positive samples was assessed with a gap of 9 months. The end group i.e. fourth one contained samples from children with age 28 months and above. In these groups of different ages, more positive samples (60.8%; n=45/74) were gone with the children having age below 9 months which was then followed by children having age 10-18 months (32.4%; n=24/74) as shown in Figure 4.43. Among positive cases, HAdV were found to be more common in males as compared to females (63.5% vs 36.5%; n=47 vs 27) with male to female ratio of 1.7:1.

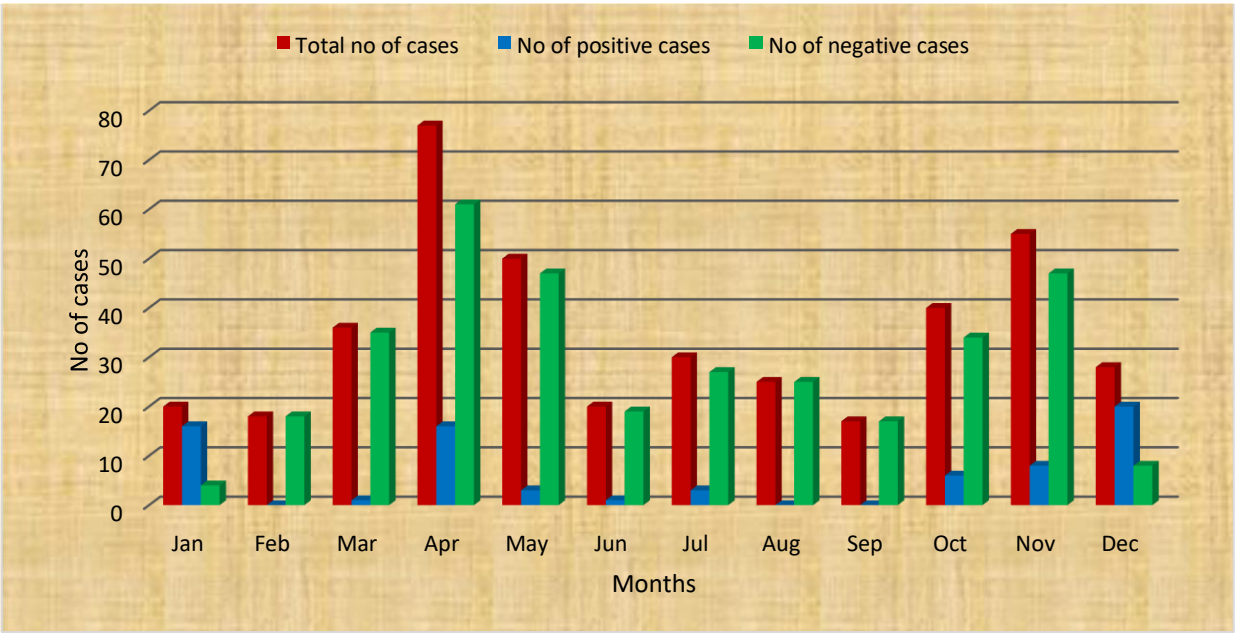



**Figure 4.42.** Month wise distribution of total, positive and negative samples tested for adenovirus infections at NICH during 2017-2018.

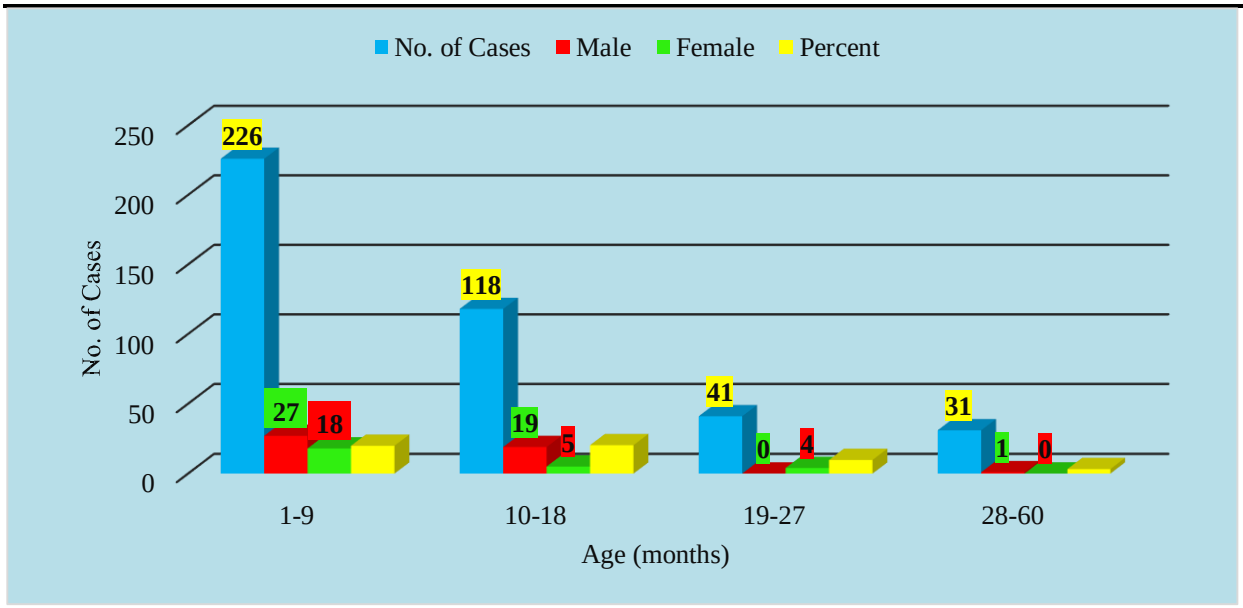



**Figure 4.43.** Age wise distribution of samples positive for adenovirus during 2017-2018. Number of male and female cases is plotted on Y-axis and the age groups are given on X-axis. Total number of samples is given in the form of blue bars.

### 4.2.23 Assessment of demographic and clinical parameters among diarrheal patients in NICH

The enrolled subjects at NICH were analyzed for the assessment of demographic and clinical parameters between the positive and negative patients for adenovirus infections. Presence of adenovirus in samples were regarded as positive while those without adenovirus were termed as negative. With the help of chi-square test, the qualitative parameters were evaluated while the quantitative variables were evaluated by employing student's *t*-Test.

#### *1) Evaluation of demographic and clinical data among positive and negative cases*

Evaluation of the clinical characteristics like fever, diarrhea, the factor which was found significant ($p$=<0.0001) was vomiting when assessed among positive and negative groups for adenovirus infections (Table 4.20). Out of 74 patients positive for adenoviruses, vomiting was recorded in 46 (62.1%) patients whereas 28 (37.8%) had no vomiting. The insignificant factor found were fever and diarrhea among positive and negative cases ($p$=0.0915 and $p$=0.2324 correspondingly). Similarly, the factor age was also found to be insignificant among positive and negative cases ($p$=0.0569) though most of infections (n= 69; 93.2%) were establish in children having age below 18 months.

#### *2) Evaluation of clinical data among positive and negative cases*

Statistical interpretation was performed for the quantitative variables by employing student's *t* Test (Table 4.21). Among the quantitative variables, the mean number of diarrheal episodes per 24 hours was found as a significant factor ($p$=0.0010). These variables were compared between the patients who were found positive 6.096±1.511 and negative 5.370±1.738 for adenovirus infections. The mean duration of diarrhea (3.301±1.151 days), mean number of vomiting episodes (4.957±1.897) and mean duration of vomiting (3.870±1.572 days) were found insignificant with *p*-values 0.3665, 0.2884 and 0.5798 correspondingly.

**Table 4.20.** Chi square analysis of qualitative variables of patients with adenovirus infections

| Total No. of Cases = 416 | Positive (n= 74) n (% age) | Negative (n= 342) n (% age) | *P-value* |
|---|---|---|---|
| **Gender** | | | |
| Male (n=231) | 47 (63.5) | 184 (53.8) | $0.1274^{NS}$ |
| Female (n=185) | 27 (36.5) | 158 (41.2) | |
| **Age groups (months)** | | | |
| 1-9 (n=226) | 45 (60.8) | 181 (52.9) | |
| 10-18 (n=118) | 24 (32.4) | 94 (27.4) | |
| 19-27 (n=41) | 4 (5.4) | 37 (10.89) | $0.0569^{NS}$ |
| 28-60 (n=31) | 1 (1.3) | 30 (8.7) | |
| **Clinical signs** | | | |
| **Fever** | | | |
| Present (n=200) | 29 (39.2) | 171 (50) | $0.0915^{NS}$ |
| Absent (n=216) | 45 (60.8) | 171 (50) | |
| **Vomit** | | | |
| Yes (n=342) | 46 (62.1) | 296 (86.5) | $\mathbf{<0.0001^{S}}$ |
| No (n=74) | 28 (37.8) | 46 (13.4) | |
| **Diarrhea** | | | |
| Yes (n=414) | 73 (98.6) | 341 (99.7) | $0.2324^{NS}$ |
| No (n=2) | 1 (1.4) | 1 (0.3) | |

S= Significant *p*-value; NS= non-significant *p*-value,

% ages are given in Adenovirus positive and negative groups according to the variables such as gender, age groups, fever, vomiting and diarrhea

**Table 4.21.** Comparison of clinical parameters (quantitative variables) between patients found positive and negative for adenovirus infections using Student t-test

| **Clinical Symptoms** | **No. of Samples** | **Mean** | **Std. Deviation (±)** | **Std. Error Mean** | ***p*-value** |
|---|---|---|---|---|---|
| Diarrhea Episodes/24hrs | Positive (n=73) | 6.096 | 1.511 | 0.1768 | **0.0010$^{S}$** |
| | Negative (n=341) | 5.370 | 1.738 | 0.09414 | |
| Diarrhea duration (days) | Positive (n=73) | 3.301 | 1.151 | 0.1347 | 0.3665$^{NS}$ |
| | Negative (n=341) | 3.510 | 1.900 | 0.1029 | |
| Vomiting Episodes/24hrs | Positive (n=46) | 4.957 | 1.897 | 0.2797 | 0.2884$^{NS}$ |
| | Negative (n=296) | 4.628 | 1.955 | 0.1136 | |
| Vomit duration (days) | Positive (n=46) | 3.870 | 1.572 | 0.2318 | 0.5798$^{NS}$ |
| | Negative (n=296) | 3.703 | 1.945 | 0.1130 | |
| Age (months) | Positive (n=74) | 9.399 | 6.256 | 0.7273 | 0.1195$^{NS}$ |
| | Negative (n=342) | 11.39 | 10.60 | 0.5730 | |

NS= non-significant *p*-value<0.05

### 4.2.24 Evaluation of clinical parameters for determination of clinical effect of co-infection of adenovirus with rotavirus

In this study, the samples taken from gastroenteritis patients were previously analyzed for rotavirus. We, therefore, analyzed the significance of co-infection via the comparison of clinical signs and symptoms of patients infected with adenovirus to those co-infected with rotavirus by putting on the statistical test called ANOVA (Analysis of Variance).

***1) Comparative analysis of quantitative clinical parameters among adenovirus positive patients with rotavirus co-infections***

Data presented in Tables 4.22 demonstrate the outcomes of ANOVA test, used to deduce the importance of co-infection of adenovirus with rotavirus. It was detected that patients with double infection of adeno-rotavirus and rotavirus showed more serious clinical indications and illness therefore describing their clinical importance in gastroenteritis cases.

Data exhibited in Table 4.22 shows that the number of diarrheal episodes per 24 hours, diarrhea duration (days) and number of vomiting episodes per 24 hours vomiting duration (days) were significantly associated to disease in patients co-infected with adenovirus and rotavirus *p*-value <0.0001, <0.0001, <0.0001 and 0.0082 correspondingly). The mean number of diarrheal episode per 24 hours, diarrhea duration (days) and number of vomiting episodes per 24 hours vomiting duration (days) of HAdV-rotavirus co-infection was 6.818±2.136, 5.909±2.212, 7.000±1.673 and 5.364±2.203 correspondingly. Similarly, the age was also found significantly associated to disease in patients co-infected with adenovirus and rotavirus with *p*-value 0.0159.

Table 4.22. Comparison of clinical parameters among subjects with AdV and RV co-infection using ANOVA (Significant *p*-values (≤ 0.05) are given in bold)

| Clinical Parameters | Infection (No. of cases) | Range | Mean±SD | 95% Confidence Interval | | *P-Value* |
|---|---|---|---|---|---|---|
| | | | | Lower | Upper | |
| **Diarrhea episodes/ 24 hrs** | HAdV+ve (n=62) | 3-10 | 6.032±1.578 | 5.631 | 6.433 | **<0.0001[S]** |
| | Rotavirus+ve (n=85) | 3-10 | 6.082±1.521 | 5.754 | 6.411 | |
| | Rotavirus+HAdV (n=11) | 4-10 | 6.818±2.136 | 5.383 | 8.253 | |
| | Negative (n=256) | 1-9 | 5.094±1.751 | 4.878 | 5.309 | |
| **Diarrhea duration (days)** | HAdV+ve (n=62) | 1-6 | 3.306±1.154 | 3.013 | 3.599 | **<0.0001[S]** |
| | Rotavirus+ve (n=85) | 1-6 | 3.271±1.169 | 3.018 | 3.523 | |
| | Rotavirus+HAdV (n=11) | 2-9 | 5.909±2.212 | 4.423 | 7.395 | |
| | Negative (n=256) | 1-8 | 3.633±2.088 | 3.376 | 3.890 | |
| **Vomiting episodes/24 hrs** | HAdV+ve(n=35) | 1-9 | 5.200±1.982 | 4.519 | 5.881 | **<0.0001[S]** |
| | Rotavirus+ve (n=85) | 1-9 | 5.376±1.908 | 4.965 | 5.788 | |
| | Rotavirus+HAdV (n=11) | 5-9 | 7.000±1.673 | 5.876 | 8.124 | |
| | Negative (n=154) | 1-9 | 3.695±1.780 | 3.411 | 3.978 | |
| **Vomiting duration (days)** | HAdV+ve(n=35) | 1-6 | 4.086±1.541 | 3.556 | 4.615 | **0.0082[S]** |
| | Rotavirus+ve (n=85) | 1-9 | 3.882±1.636 | 3.529 | 4.235 | |
| | Rotavirus+HAdV (n=11) | 2-9 | 5.364±2.203 | 3.883 | 6.844 | |
| | Negative (n=154) | 1-8 | 4.584±1.983 | 4.269 | 4.900 | |
| **Age (months)** | HAdV+ve(n=63) | 1-30 | 8.992±6.275 | 7.412 | 10.57 | **0.0159[S]** |
| | Rotavirus+ve (n=85) | 1-59 | 9.224±9.372 | 7.202 | 11.24 | |
| | Rotavirus+HAdV (n=11) | 1-24 | 10.36±7.311 | 5.452 | 15.28 | |
| | Negative (n=257) | 1-60 | 12.47±11.11 | 11.11 | 13.84 | |

### 4.2.25 Human Adenovirus Isolation in Cell Culture

All 1,118 stool samples were processed and inoculated in cell line HEp-2C. The cell lines were observed daily for characteristic adenovirus cytopathic effect. The samples that showed 4+ CPE were isolated and were kept at -20˚C till additional investigation. The samples that were negative on cell line were observed more for maximum of 10 days. According to the CPE, 496/1,118 (44.40%) samples were culture positive and were labelled as HAdVs. On the other hand, 621 samples were failed in producing CPE on HEp-2C cell lines and were labelled as negative for human adenoviruses (Table 4.23).

**Table 4.23.** No of HAdVs isolated from diarrheal patients in HEp-2C cell line

| S. No | Cell Line | No of Samples | Total |
|---|---|---|---|
| 1 | Hep-2C + | 496 | 1,118 |
| 2 | Hep-2C - | 612 | |

### 4.2.26 Human adenovirus detection by Nested-PCR

All the cell culture supernatants (n=496) that were found positive in HEp-2C cells were further confirmed as HAdV by nested-PCR using their extracted genomic DNA for primer sets specified for hexon gene amplification. According to nested-PCR (L1) results 18.26% (n=204/1,118) samples were confirmed as HAdV (Figure 4.44). Not a single sample was positive for L2 PCR.

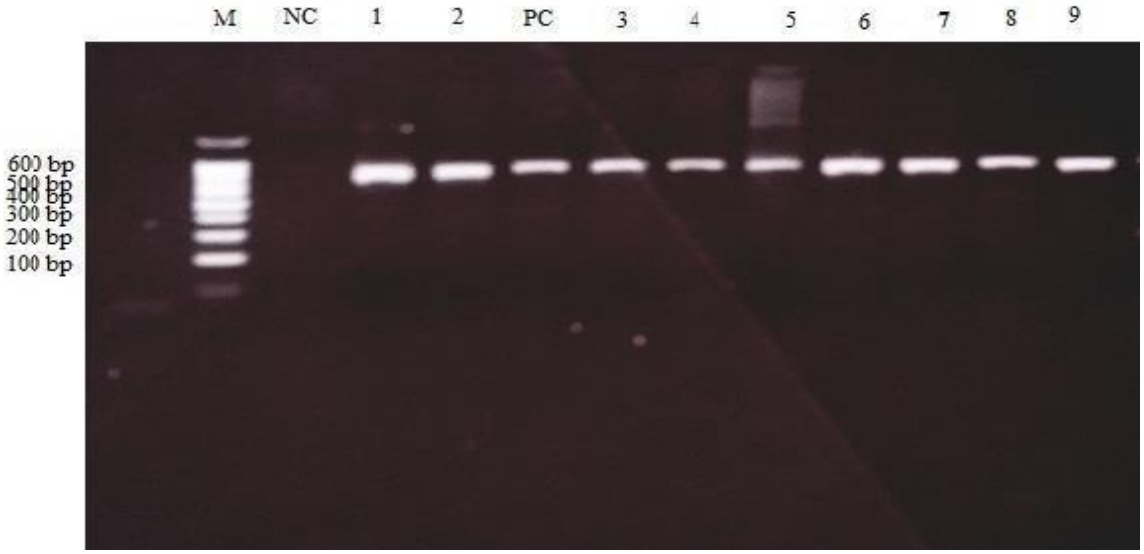



**Figure 4.44.** SYBR safe dye-stained agarose gel showing PCR amplification products using a nested PCR primer pairs of adenovirus (Qurashi *et al.*, 2012). Lanes 1-2, 3-9: positive samples (600bp), lane 1: negative control, lane 3: positive control (AdB7 DNA). M: molecular marker 100 bp DNA ladder, Life technologies).

### 4.2.27 Molecular analysis of human adenovirus strains, gastroenteritis cases

Screening of total 1,118 samples collected during 2017-2018 for the presence of adenovirus antigen, cell culture yielded 496 (44.3%) samples positive for human adenovirus infections. This positive subset of 496 samples was further screened for the presence of adenovirus DNA through nested-PCR. The nested-PCR yielded 204 (18.26%) samples positive for major capsid protein (hexon) common to all recognized adenovirus genotypes and were kept at -80°C for further analysis.

***1) Phylogenetic analysis on the basis of hexon gene segments of adenovirus***

The results of our study showed that the adenovirus genotypes were present with the occurrence rate as HAdV-B3 = 3 (6.6%); HAdV-B7 = 4 (8.8%); HAdV-C2 = 3 (6.6%); HAdV-D28 = 4 (8.8%), HAdV-F40= 18 (40%) and HAdV-F41= 13 (28.8%).

**2) Genotypes of adenovirus from diarrheal samples**

Molecular typing analysis successfully characterized 45 study isolates (22%) into 6 different genotypes of human adenoviruses including HAdV-B3, B7, C2, C6, D28, F40 and F41. The most detected genotype of adenovirus was HAdV-F40 with prevalence rate of 40% (n=18) followed by HAdV-F41 with detection rate of 28.8% (n=13) respectively. The descriptive analysis on molecular epidemiology, evolutionary relationships and its phylogeny of investigated strains into each specific genotypes are described below.

**a) Human adenovirus B**

According to this study, total 7 isolates from diarrheal cases were successfully characterize for Human adenovirus B specie with overall rate of 15.5% among positive isolates. The partial nucleotide sequence of hexon gene of all HAdV-B subject strains were subjected to phylogenetic investigation along with their related relatives recovered from GeneBank in order to get insight into their evolution and epidemiology.

**b) Human adenovirus B3**

Total 3 isolates were classified as HAdV-B3 in adenovirus B specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.45).

**c) Human adenovirus B7**

4 isolate of HAdV-B7 was detected by sequence analysis of hexon gene. Pairwise distance score of study isolates were assessed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.46).

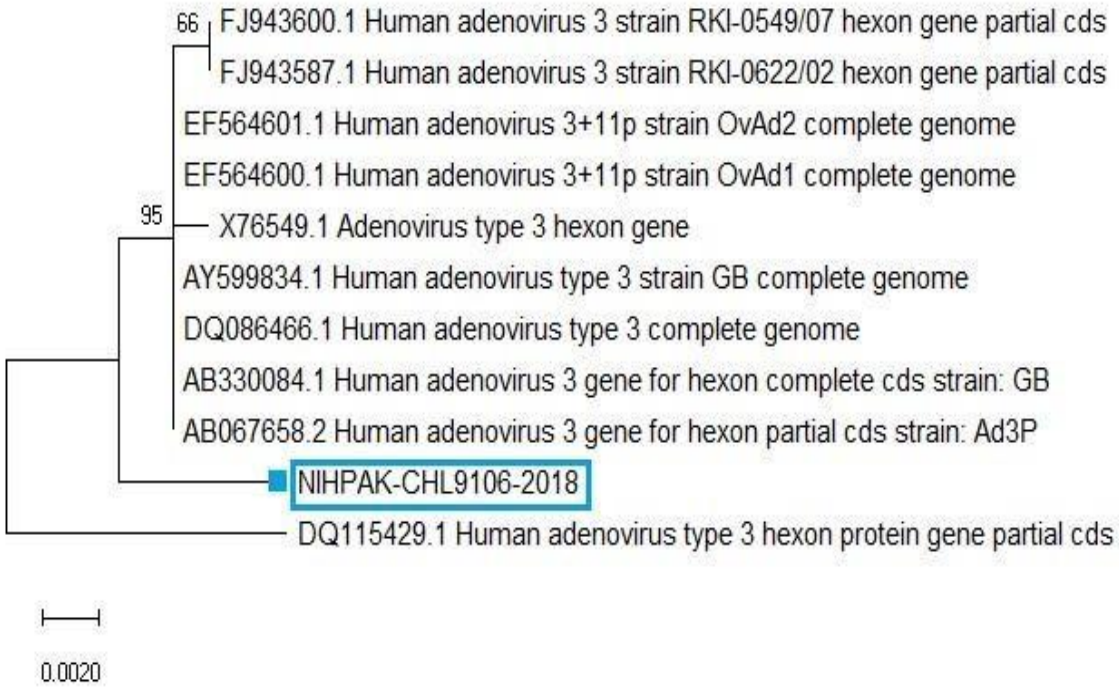



**Figure 4.45.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA X6. The substitution method used was Kimura 2-parameter using neighborjoining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**Table 4.24.** Amino acid substitutions in NIHPAK-CHL9106-2018 isolate a compared with DQ086466.1.

| | AMINO ACID POSITION | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | | 6414 | 6439 | 6443 | 6453 | 6482 | 6574 |
| 2 | NIHPAK-CHL9106 | R | E | D | E | N | D |
| 3 | DQ086466.1 | G | D | E | D | D | G |


**Figure 4.46.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA X6. The substitution method used was Kimura 2-parameter using neighborjoining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

### *d) Phylogenetic Analysis*

Partial (820bp) hexon gene sequences of HAdV-B strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. Two distinct monophyletic groups strains (HAdV-B3 and HAdV-B7) of all isolates were their respective reference strains were found (Figure 4. 45). The tree was constructed for the representative strains of HAdV-B3 and B7. The genetic relationship within and between groups were further analyzed.

HAdV-B3 comprised of three isolates detected in 2017-2018 from BBH (n=1), KGH (n=1) and CHL (n=1) and exhibited similarity 99% similarity with their reference strains from the Switzerland (Accession No. DQ086466.1). Similarly, four isolated of HAdV-B7 was detected from CHL (n=1), NICH (n=2) AND KGH (n=1) and showed 99% similarity with reference strain from Japan (Accession No. AF053085) (Figure 4.46). The pairwise mean distance within and between the groups are given in Table 4.25.

**Table 4.25.** Table showing pairwise nucleotide and amino acids comparison among human adenovirus B isolates with reference strain.

| **GROUP** | **Nucleotide (% Similarity)** | **Amino Acid (% Similarity)** |
|---|---|---|
| HAdV-B3 with DQ086466 | 99% | 99% |
| HAdV-B7 with AF053085 | 99% | 100% |

### *e) Human adenovirus C*

According to this study, total 3 isolates from diarrheal cases were successfully characterize for Human adenovirus C specie with overall rate of 1.4% among positive isolates. The partial nucleotide sequence of hexon gene of all HAdV-C subject strains were subjected to phylogenetic

investigation along with their related relatives recovered from GeneBank in order to get insight into their evolution and epidemiology.

**f) Human adenovirus C2**

Total 3 isolates were classified as HAdV-C2 in adenovirus C specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree for representative strains as built using hexon gene nucleotide sequences (Figure 4.47).

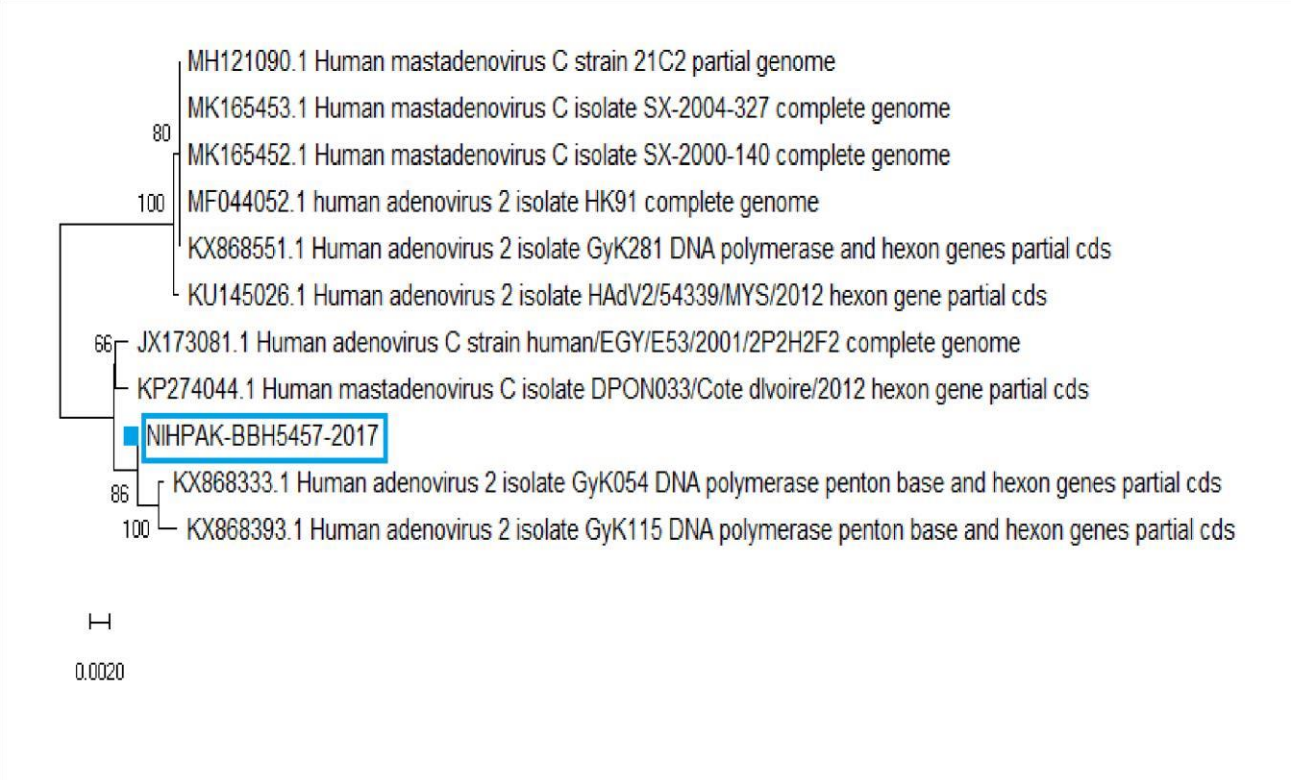



**Figure 4.47.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA version X6.0.

The substitution method used was Kimura 2-parameter using neighbor-joining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

### *g) Phylogenetic Analysis*

Partial (820bp) hexon gene sequences of HAdV-C strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. The monophyletic groups C strains (HAdV-C2) with their respective reference strains were found (Figure 4. 47). The genetic relationship within and between groups were further analyzed.

HAdV-C2 comprised of three isolates detected in 2017-2018 from BBH (n=2) and MHL (n=1) and exhibited similarity 96% similarity with their reference strains from the Germany (Accession No. KP274044.1) (Figure 4.47). The pairwise mean distance within and between the groups are given in Table 4.26.

**Table 4.26.** Table showing pairwise nucleotide and amino acids comparison among human adenovirus C isolates with reference strain.

| **GROUP** | **Nucleotide (% Similarity)** | **Amino Acid (% Similarity)** |
|---|---|---|
| HAdV-C2 with KP274044 | 96% | 98.7% |

### *h) Human adenovirus D*

According to this study, total 4 isolates from diarrheal cases were successfully characterize for Human adenovirus D specie with overall rate of 1.9% among positive isolates. The partial nucleotide sequence of hexon gene of all HAdV-D subject strains were subjected to phylogenetic investigation along with their related relatives recovered from GeneBank in order to get insight into their evolution and epidemiology.

### *i) Human adenovirus D28*

Total 4 isolates were classified as HAdV-D28 in adenovirus D specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.48).

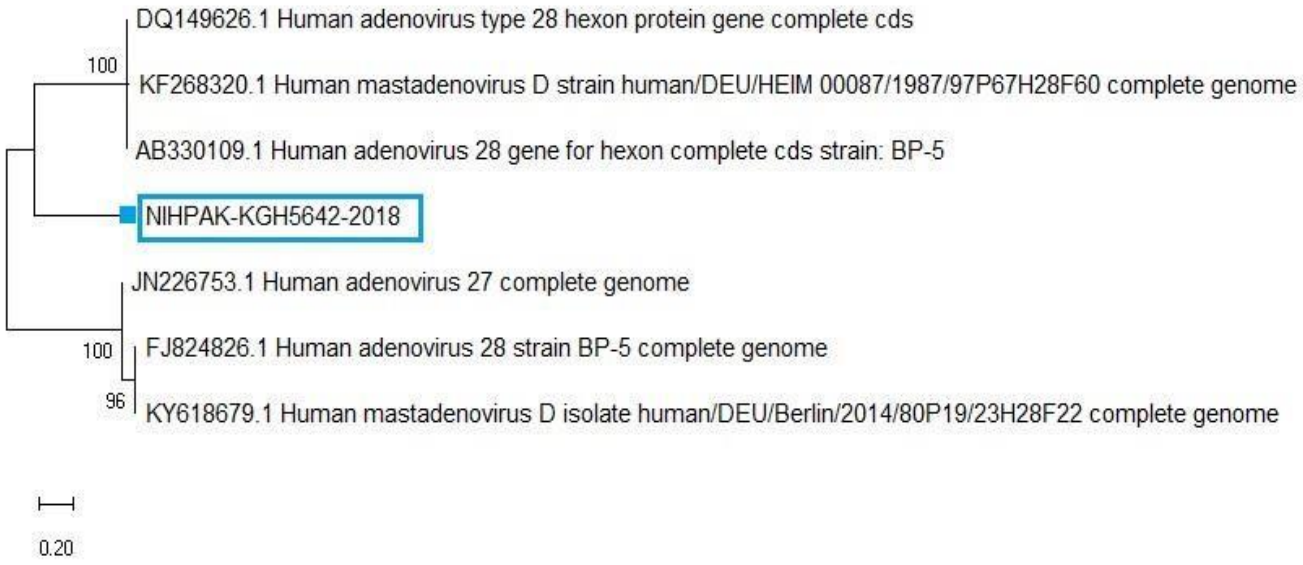



**Figure 4.48.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA version X6.0.

The substitution method used was Kimura 2-parameter using neighbor-joining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**Table 4.27.** Amino acid substitutions in NIHPAK-KGH5642-2018 isolate a compared with AB330109.1.1.

| AMINO ACID POSITION | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 123 | 124 | 125 | 127 | 128 | 129 | 130 | 132 | 133 | 135 | 136 | 137 | 138 | 139 | 140 | 188 | 191 | 356 |
| AB330109.1 | S | L | A | K | G | A | P | P | V | G | L | L | K | K | I | P | K | G |
| NIHPAK-KGH5642 | R | P | T | V | S | G | L | K | E | R | T | V | E | E | Q | Q | E | A |

### *j) Phylogenetic Analysis*

Partial (820bp) hexon gene sequences of HAdV-D strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. The monophyletic groups D strains (HAdV-D28) with their respective reference strains were found (Figure 4. 48). The genetic relationship within and between groups were further analyzed.

HAdV-D28 comprised of four isolates detected in 2017-2018 from KGH (n=1), NICH (n=1), CHL (n=1) and MHL (n=1) and exhibited similarity 94.3% similarity with their reference strains from Japan (Accession No. AB330109.1) (Figure 4.48). The pairwise mean distance within and between the groups are given in Table 4.28.

**Table 4.28.** Table showing pairwise nucleotide and amino acids comparison among human adenovirus D isolates with reference strain.

| GROUP | Nucleotide (% Similarity) | Amino Acid (% Similarity) |
|---|---|---|
| HAdV-D28 with AB330109 | 94.3% | 94.2% |

### *k) Human adenovirus F*

According to this study, total 31 isolates from diarrheal cases were successfully characterize for Human adenovirus F specie with overall rate of 15.1% among positive isolates. The partial nucleotide sequence of hexon gene of all HAdV-F subject strains were subjected to phylogenetic investigation along with their related relatives recovered from GeneBank in order to get insight into their evolution and epidemiology.

### *l) Human adenovirus F40*

Total 18 isolates were classified as HAdV-F40 in adenovirus F specie via sequencing of hexon gene. Pairwise distance scores of study isolates with the closely related and references sequences obtained from GeneBank were assessed and analyzed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.49).

### *m) Human adenovirus F41*

13 isolate of HAdV-F41 was detected by sequence analysis of hexon gene. Pairwise distance score of study isolates were assessed. Evolutionary tree was built using hexon gene nucleotide sequences (Figure 4.50).

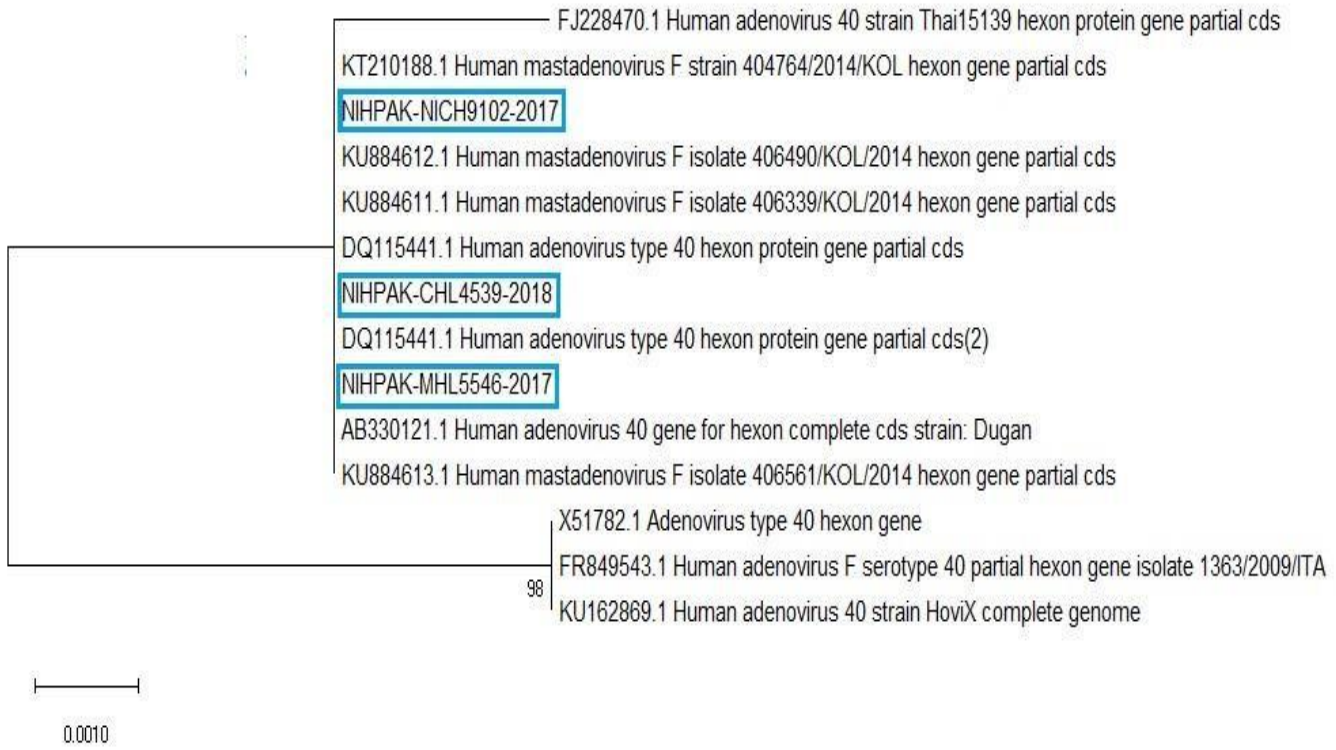



**Figure 4.49.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA version X6.0.

The substitution method used was Kimura 2-parameter using neighbor-joining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**Table 4.29.** Amino acid substitutions in NIHPAK-CHL9106-2018 isolate a compared with DQ086466.1.

| | AMINO ACID POSITION | |
|---|---|---|
| 1 | | 6043 |
| 2 | DQ115441.1 | L |
| 3 | NIHPAK-NICH9102 | S |
| 4 | NIHPAK-CHL4539 | S |
| 5 | NIHPAK-MHL5546 | S |

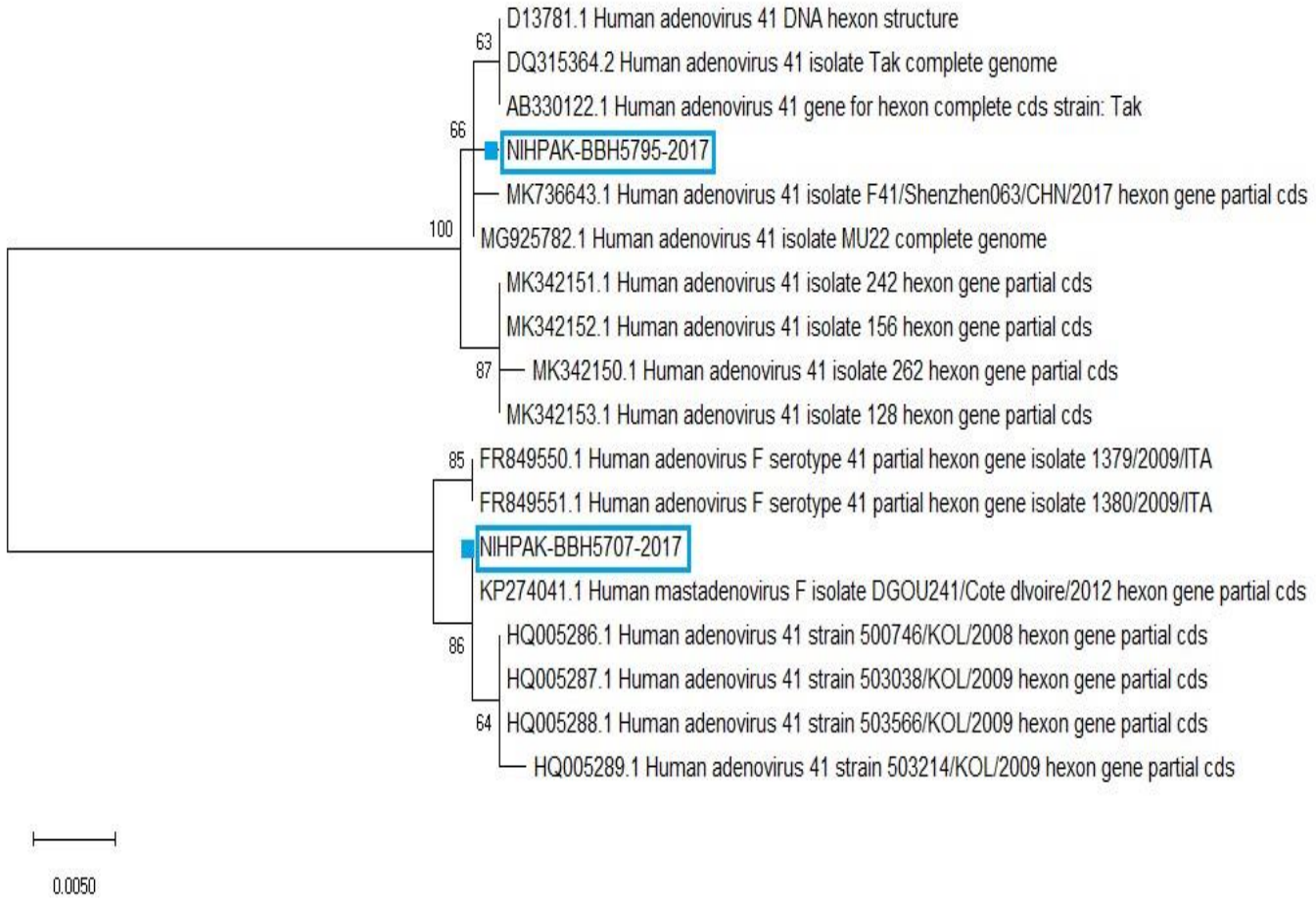



**Figure 4.50.** Phylogenetic tree reconstructed on the basis of partial hexon sequence of adenovirus strains using MEGA version X6.0. The substitution method used was Kimura 2-parameter using neighbor-joining model for phylogenetic reconstruction of tree. The HAdV strains from Pakistan are highlighted within blue box with blue color block.

**Table 4.30.** Amino acid substitutions in NIHPAK-BBH5707-2017 isolate a compared with AB330122.1.

| | AMINO ACID POSITION | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 114 | 115 | 123 | 149 | 155 | 166 | 195 | 196 | 228 | 250 | 251 | 255 | 266 | 285 |
| 2 | NIHPAK-BBH5707 | K | P | S | F | N | T | A | Q | S | P | S | E | S | I |
| 3 | AB330122.1 | Q | A | Y | L | D | A | T | D | N | A | N | V | T | V |

***n) Phylogenetic Analysis***

Partial (820bp) hexon gene sequences of HAdV-F strains were used for construction of phylogenetic tree. For determination of phylogenetic construction among isolates and genetic similarity, the tree analysis was employed. Two distinct monophyletic groups strains (HAdV-F40 and HAdV-F41) of all isolates were their respective reference strains were found (Figure 4. 49). The genetic relationship within and between groups were further analyzed.

HAdV-F40 comprised of eighteen isolates detected in 2017-2018 from BBH (n=3), KGH (n=5), NICH (n=2), MHL (n=4) and CHL (n=4) and exhibited similarity 100% similarity with their reference strains from USA (Accession No. DQ115441.1) Similarly, thirteen isolated of HAdVF41 was detected from BBH (n=2), KGH (n=3), NICH (n=1), MHL (n=4) and CHL (n=3 and showed 99.4-100% similarity with reference strain (Accession No. AB330122 and KP274041) from Japan and Germany (Figure 4.50). The pairwise mean distance within and between the groups are given in Table 4.31.

**Table 4.31.** Table showing pairwise nucleotide and amino acids comparison among human adenovirus B isolates with reference strain.

| GROUP | Nucleotide (% Similarity) | Amino Acid (% Similarity) |
|---|---|---|
| HAdV-F40 (3) with DQ115441 | 100% | 100% |
| HAdV-F41 with AB330122 | 99.4% | 99.4% |
| HAdV-F41 with KP274041 | 100% | 100% |

### 4.2.28 Overall analysis of AFP samples

#### i) Gender and age-wise Distribution

Among total samples 98/172 (56.972%) were male while 74/172 (43.02%) were females (Figure 4.51). The ages of these children ranges from 4 months to 144 months (mean age=38.489, median=30). Among total positive cases, 52/89 (58.42%) were male and 37/89 (41.57 %) were female. The highest proportion (84.61%) was observed among 37-48 months old followed by 4960 months (62.5%) and then 25-36 (56.25%) old. Likewise, the proportion was followed by 13-24 months (53.43%), 4-12 months (51.21%) respectively (Figure 4.51).

#### ii) Month-wise Distribution

According to this study, HAdV positive cases were found throughout the year as shown in Figure 4.52. However, the seasonality of HAdV positive cases were high in spring (March-April) and in winter season (Dec-Feb) having highest peak in December (89.47%) followed by March (80.76%). In summer season (May-Aug) and autumn season, the rate of HAdV isolation was low with lowest peak in October (5%) followed by November (7.14%). This shows that HAdV was found more in winter-spring (December-March) season (Figure 4.53).

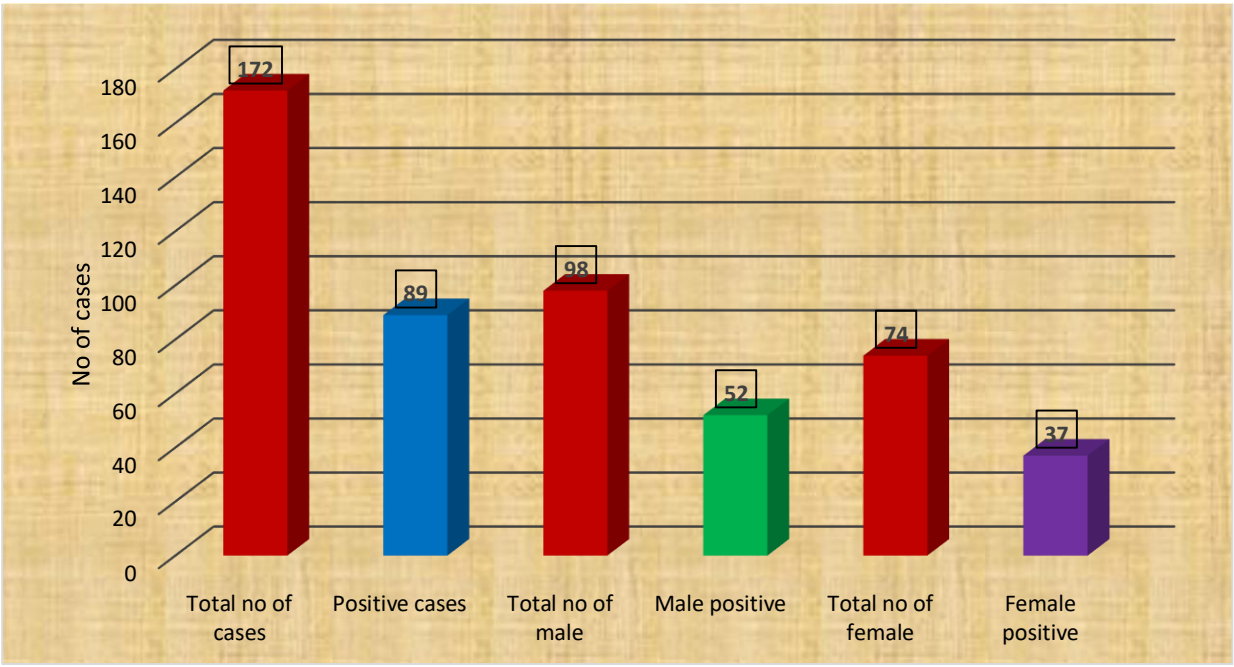



**Figure 4.51.** Gender-wise distribution of HAdV among AFP patients during 2017

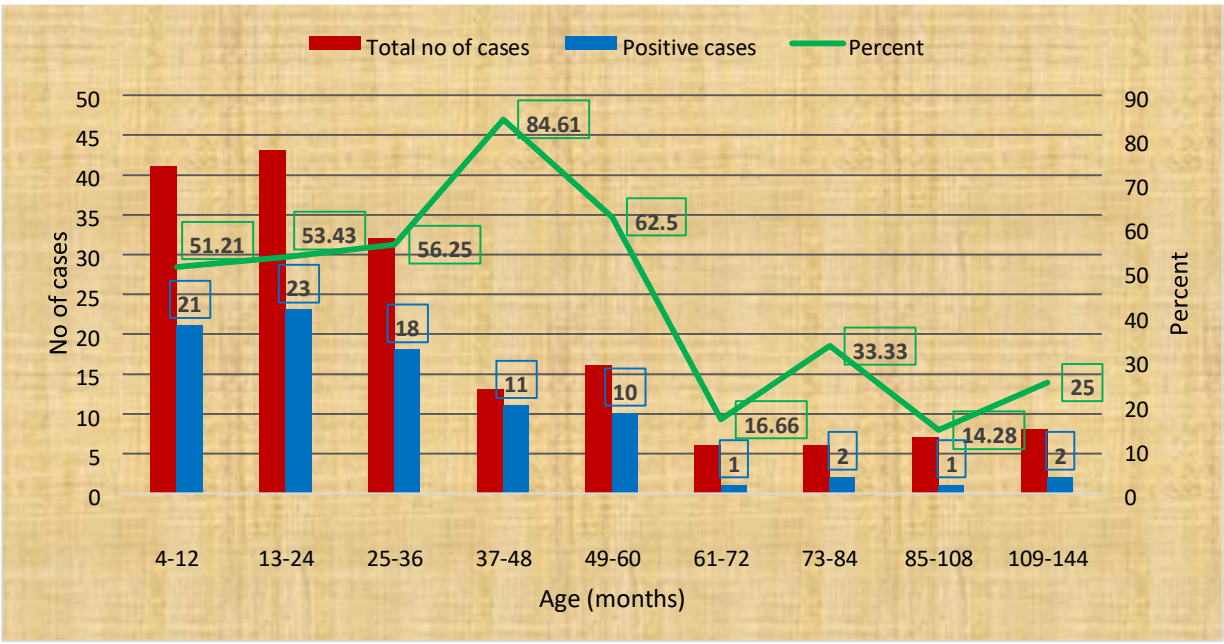



**Figure 4.52.** Age-wise distribution of HAdV among AFP patients during 2017

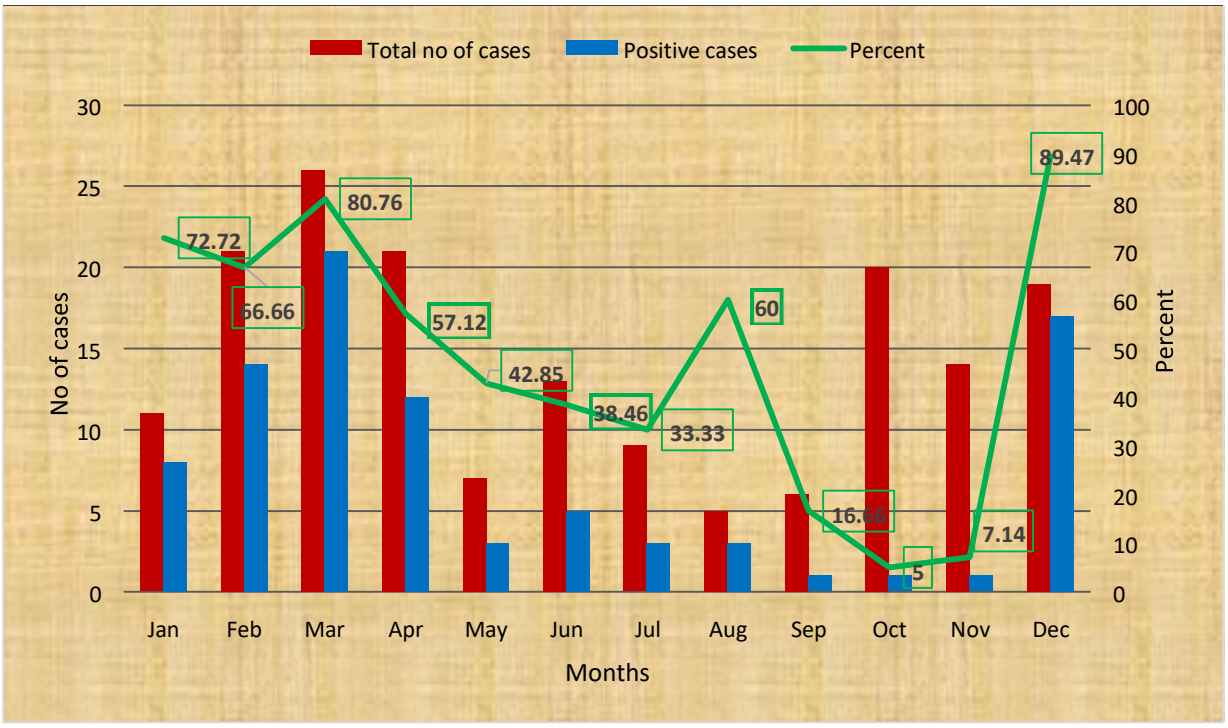



**Figure 4.53.** Month-wise distribution of HAdV among AFP patients during 2017

### 4.2.29 Overall analysis of Gastroenteritis samples

#### i) Gender and Age-wise distribution of Gastroenteritis samples

Among total samples, 614 (54.91%) patients were male and 504 (45.08%) were females. According to results, 118/204 (57.84%) were male positive and 86/204 (42.15%) were female positive (Figure 4.54). The samples of the children investigated in this study had a mean age of 11.44± 10.16 months having range from 1 to 60 months. For study, the investigated subjects were grouped into 5 age groups with the gap of 12 months between their ages. Among adenovirus positive cases, mostly of them belongs to 1-12 months of age (n= 93), followed by 13-24 months of age (n=76), then 25-36 and 37-48 months of age (n=24 and n=8) with last one 49-60 (n=3) as shown in Figure 4.55.

**ii) Month-wise distribution of Gastroenteritis samples**

Among 204 positive samples, the monthly-based dispersion graph is shown in Figure 4.56, which showed the frequency of positive isolates for human adeno viruses remained found throughout the year but having peak in winter season i.e. in November (49.18%) followed by January (35.93%).

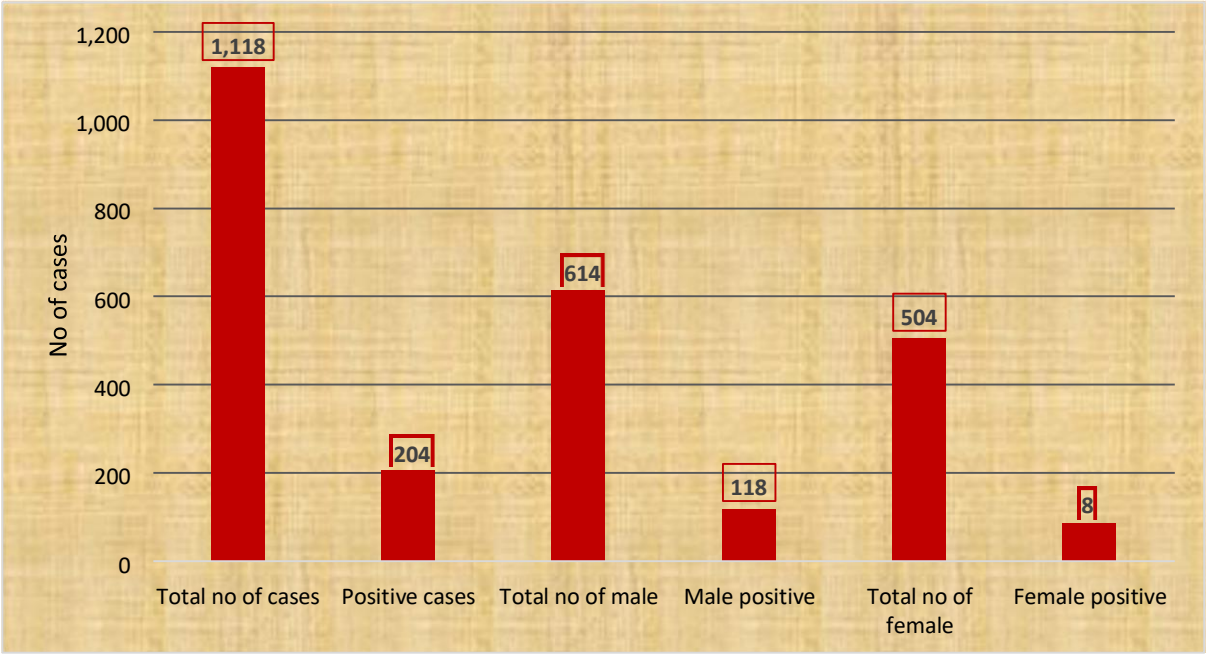



**Figure 4.54.** Gender-wise distribution of total and positive samples for adenovirus during 20172018

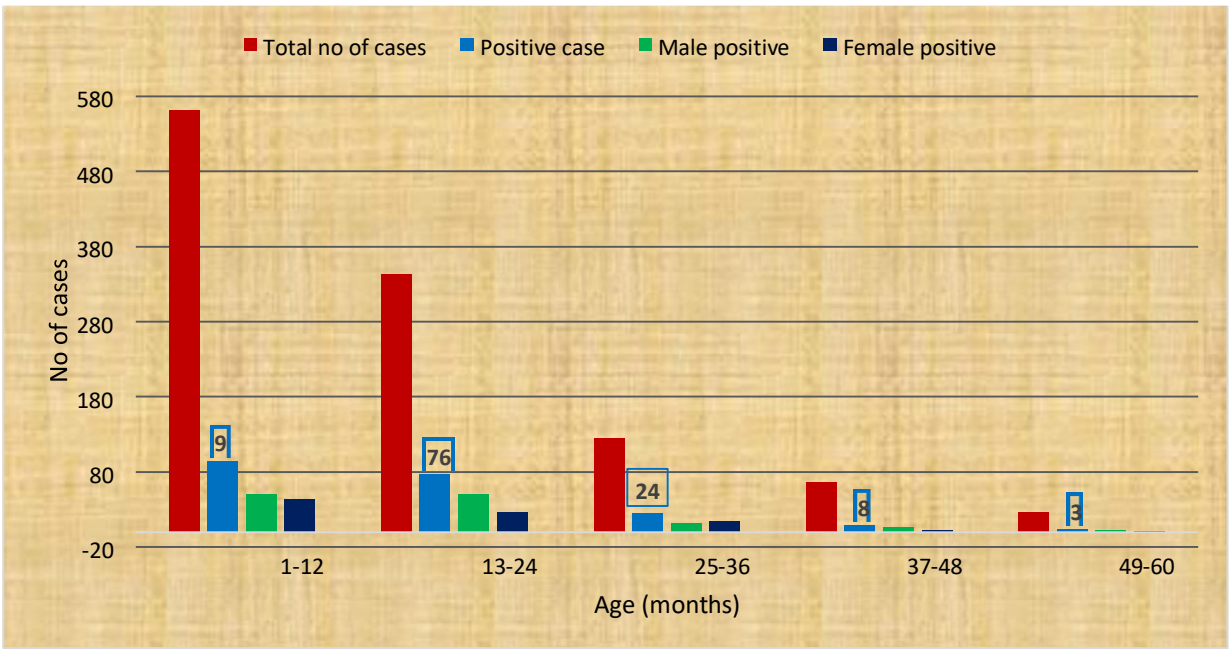



**Figure 4.55.** Age-wise distribution of total and positive samples for adenovirus during 2017-2018

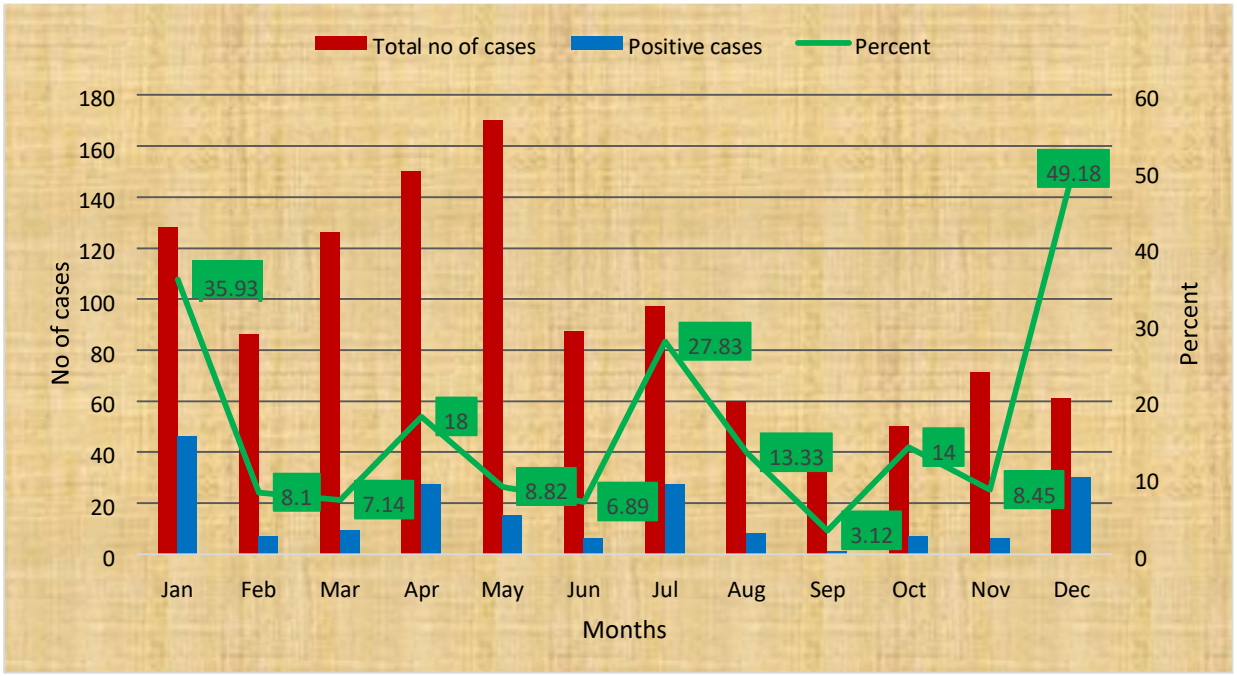



**Figure 4.56.** Month-wise distribution of total and positive samples for adenovirus during 2017-2018

*Chapter 5* **DISCUSSION**

Infectious diseases are among the leading cause of disability and mortality in Pakistan. Epidemics of new and old infectious agents appear from time to time, having a negative impact on people and magnifying the burden on the economy. In Pakistan, among major causes of mortality in children less than five years, diarrhea contributes 11% to the total mortality rates. Before reaching age five, one in every eleven children in Pakistan dies. Post neonatal deaths are due to diarrhea, pneumonia, meningitis and sepsis. WHO collaborated rotavirus surveillance program screen the diarrheal samples for rotavirus but no system presents in country for other diarrhea causing viruses. In addition to this, Pakistan also harbors the highest numbers of poliomyelitis cases in the world, threatening global eradication goals. Surveillance for poliomyelitis relies on investigation and reporting of children who develop acute flaccid paralysis (AFP). However, the country making tremendous efforts for the eradication of polio with the help of its extensive AFP surveillance activities. But a recent report indicates the increased in number of non-polio AFP cases from 2018 (14 per 100,000 children aged < 15 years) to 2019 (16.2 per 100,000 children aged < 15 years) which highlighted the role of other infectious and non-infectious agents that may be responsible in causing AFP. Pakistan is still struggling to cope with AFP cases, which are caused by agents other than poliovirus, but among these agent's adenovirus is ignored. In our country, there is no system present for the screening of non-polio AFP causing adenoviruses while there is data present from other countries that focused on the human adenoviruses responsible for causing AFP. However, the infectious morbidity and mortality is huge in country, Pakistan had no formal training programs in infectious diseases, and a tremendous shortage of individuals with the requisite skills to conduct independent research in infectious diseases affecting Pakistani children. In light of above mentioned facts, investigation of adenovirus prevalence among children with AFP as well as in diarrheal samples was then conducted with the aim of testing its association with the disease. In laboratory, the recurrent occurrence of adenoviruses from stool specimens that were derived from acute flaccid paralysis (AFP) cases were merged with the information's regarding the contribution of these agents in infections encompassing the central nervous system increased our concern about the probable association of human adenovirus in causing AFP that were resembled with the ones produced by the wild polioviruses. In addition to this, the reporting of adenoviruses from AFP cases, poor hygienic groundwork and viral diagnostic means of the country attracted our attention

towards the concerned genotypes responsible for causing AFP cases. Hence, this study highlighted the prevalence, genetic diversity and molecular epidemiology of human adenoviruses in AFP patients as well as diarrheal samples in Pakistan. To the best of our knowledge, this is the first work that provide the comprehensive outline on prevalence, genetic diversity and molecular epidemiology of human adenoviruses strains present in stool samples of AFP patients and diarrheal patients. This study was performed by means of developing the association among the existence of HAdV with AFP and diarrhea.

Acute flaccid paralysis (AFP) is an infrequent clinical syndrome includes a sudden onset of flaccid paralysis in one or more limbs in children less than 15 years old. Infectious and non-infectious agents, (e.g. metabolic disorders), trauma and metal toxicity as well as post-infectious autoimmune conditions (e.g. Guillain-Barre Syndrome) may be involved in the establishment of this syndrome in humans. The recognized viral etiologies of AFP including poliovirus, nonpolio enteroviruses, flaviviruses such as West Nile, St Louis, and Japanese encephalitis viruses, herpesviruses, adenoviruses, and others. AFP includes the participation of spinal cord or anterior horn cell ("nonpoliovirus" anterior myelitis) which is considered to happen rarely. (Sejvar *et al.,* 2005; Solomon *et al.,* 1998; Solomon & Willison, 2003). There are no distinctive clinical indications for the differentiation among different cases of AFP.

For acute flaccid paralysis, surveillance is the keystone of the poliomyelitis elimination program after thorough analysis of every case, since neurological lesions can possess different causes except poliomyelitis. Certainly, to a variety of therapeutic states or an anonymous reason, a main portion of AFP cases is ascribed. Through documented neuropathogenic potential adenoviruses, particularly agents of HAdV-B species might be accountable for a part of these incidences. Although the presence of adenoviruses in cerebrospinal fluid may helpful the contribution of these viruses in AFP, however more attempts would be required in this regard to confirm the function of these viruses in causing disease pathogenesis (Ivanova *et al.,* 2012). Concerning neurological ailments produced by HadVs, few reports are present. Most of these reports are related to encephalitis (Lema *et al.,* 2005). Although the responsible types of HAdV for neurological ailments (encephalitis, meningoencephalitis, and AFP) are well stated in various reports

(Dubberke *et al.,* 2006; Frange *et al.,* 2011; Haddad-Boubaker *et al.,* 2019; Hovi & Stenvik, 2000; Nagasawa *et al.,* 2006; Ohtsuki *et al.,* 2000; Ooi et al., 2003; Schnurr *et al.,* 1995). Most detected genotype in AFP infection is the HAdV type B specifically subtypes with B3, B7 (de Azevedo *et al.,* 2004), B14 (Straussberg *et al.,* 2001), B16 (de Azevedo *et al.,* 2004) B21 (Ooi et al., 2003) and B35 (Straussberg *et al.,* 2001). In addition to them types C, D and E have been stated in studies (Ivanova et al., 2012). A report from Borneo, Malaysia showed the presence of adenovirus in the course of a fatal epidemic of enterovirus 71. In this report adenovirus was detected in 10 patients that were died and also from 5 patients with increased disease severity by acute flaccid paralysis.7. These authors revealed that adenovirus was the responsible source of mortalities and morbidities of the patients. The initial reports of adenoviruses in regard of CNS disease was showed in France in 1956 (Kajon, Suárez, Avendano, Hortal, & Wadell, 1993). Onwards from this report, numerous other reports were accounted that highlighted the role of adenoviruses in CNS disease (Avellón *et al.,* 2001; Bereciartu, Bok, & Gómez, 2002; Carballal *et al.,* 2001; Casas *et al.,* 1995; Kumar *et al.,* 1994; Thompson *et al.,* 1997; Weissenbacber *et al.,* 1990). Modification of Koch's postulates by Fredricks and Relman (Fredericks & Relman, 1996) stated that for the sequence-based isolation of microbial pathogens, there must be presence of microbial nucleic acids in the infected organs or gross anatomic sites. Hence, if we had identified the HAdV genome in CSF of the patients, the linkage would have been robust (Haddad-Boubaker *et al.,* 2019).

In the tropics, with the reduction in the figure of incidences of polio, concentration has diverted towards the other reasons of acute flaccid paralysis (AFP)**.** AFP surveillance system helps in the examination of the epidemiology, clinical appearance and virological investigation consequences for pediatric AFP cases. However, based on cytopathic effect in cell culture, majority of the stated HAdVs were isolated in stool specimens. Most of these published strains were found in our study and their clinical picture showed the association of theses strains with the disease.

In 1995, AFP surveillance was established in Pakistan, and workers in all provinces were qualified in AFP surveillance by 1998. To the office of Expanded Program on Immunization (EPI) they were delivering case reports on monthly basis. In 1994 in Pakistan, the Polio Eradication Initiative

commenced their work with unusual success as in 1997 the quantity of confirmed wild polio cases gone down from 1015 to 32 cases. AFP surveillance was widened in 1997 and reinforced all over the country and developed an electronic research center database at National Institute of Health (NIH) Islamabad and AFP surveillance database in Islamabad office and Provinces. With the help of the AFP surveillance system, one can examine the epidemiology, clinical appearance and virological analysis outcomes for pediatric AFP cases. AFP surveillance has improved the poliomyelitis elimination campaign.

On the basis of cell-culturing, WHO suggested instructions were utilized for poliovirus surveillance which are extremely sensitive for enterovirus identification however these guidelines are not appropriate for isolation of other viruses that may be responsible in causing the AFP ailment (Haddad-Boubaker *et al.*, 2019). In our study, the presence of HAdV was higher (51.74%), probably because of the increased sensitivity of the "nested" PCR method employed. This rate is higher than prevalence rate reported in Hong Kong, which is 31.4% (Kong, 2014). In one study from Brazil, the prevalence rate for HAdV in AFP patients was 68.4% (Ferreira *et al.*, 2014), which is higher rate of detection like our study. This increased rate highlighted the association of adenoviruses with AFP. However, the detection rate is fluctuating in different regions like according to another study from Brazil the detection rate was 10.2% (de Azevedo *et al.*, 2004), from Taiwan it was 3.3% (Huang *et al.*, 2013), 12.7% from Iran (Yousefi *et al.*, 2018) and 17.3% from Tunisia (Haddad-Boubaker *et al.*, 2019). As the rate of polio is decreasing in Pakistan but still the AFP cases are high and also the absence of data in our country regarding the adenovirus presence in AFP cases and then the presence of this increased detection rate, all of these factors cause an alarming situation for the health authorities in order to cope with the paralysis cases.

The detection of adenoviruses concerning patient age was also evaluated. According to our results, mostly children with age below 5 years (93.25%) were more affected with the infection. The prevalence rate among under-five children was higher than the report from Taiwan (76%) (Huang *et al.*, 2013). Another study from Hong Kong showed the highest rate (45%) of infection in underfive children (Kong, 2014). During the outbreak of in Malaysia, the detected samples

positive for adenovirus were all having age less than five years of age (Ooi *et al.*, 2003). A study from Cuba showed the increased prevalence rate (53%) (Belsy *et al.,* 2009). In Iran, the highest rate of detection was observed (Yousefi *et al.,* 2018). In unindustrialized countries, the age based division of HAdV seems to be identical to that in industrialized countries (Cheng *et al.*, 2008). The reason of low rate of infection in adults and older persons can be described as the presence of transplacentally attained through maternal antibody in young infants and the improvement of neutralizing antibody against the most common strains of HAdV in the most of children having age >5 years. In this study the detection rate of adenoviruses in AFP cases were found throughout year as Belsy et al., detected adenovirus throughout the year in pediatric population (Belsy *et al.,* 2009).

This is the second report regarding the detection of HAdV-A31 genotype in AFP cases. Before this, a study from Tunisia showed the presence of A31 in AFP cases. HAdV-A18 were also found in accordance with the same report conducted in Tunisia (Haddad-Boubaker *et al.,* 2019).

Species of HAdV-B were also isolated in the present study. These includes the HAdV-B3 and B7. There are some reports present that showed the role of HAdV-B species as having neuropathogenic potential to cause a part of AFP cases (Haddad-Boubaker *et al.,* 2019). Accordingly, in report from Finland showed that 8/ 32 neonates have adenovirus type 7 infection in an outbreak with symptoms of meningitis or meningoencephalitis. Along with meningitis or meningoencephalitis, the infection of 7 children with adenovirus type 3 having characteristic symptoms of adenoviral infection like pneumonia, tonsillitis, which then grown into transitory encephalopathy (Bayrakdar *et al.,* 2016).

In initial phase of life, species C adenoviruses comes out as common agents which produce infection in above 80% of human population. The virus is accountable in establishing the asymptomatic consistent infection after primary infection during which the infectious virions are discharge in stool for numerous years (Garnett *et al.,* 2002). In this investigated study, molecular analysis of analyzed subjects showed the presence of type C adenoviruses. In AFP cases, Ivanova

et al. and Haddad-Boubaker et al also isolated specie C of adenoviruses (Haddad-Boubaker *et al.,* 2019; Ivanova *et al.,* 2012).

Identification of HAdV-D in the current study related with AFP cases may symbolize the occurrence of genomic variants that possess the capability to disseminate universally. Nevertheless, additional investigation regarding this typing with other molecular typing approaches are compulsory. According to these outcomes, the surveillance, serotyping and molecular characterization techniques necessary to be enhanced to recognize developing adenovirus variants in AFP cases. Alternatively, from patients with encephalitis, AFP and meningoencephalitis, HAdV-D was detected in a set of patients having acute febrile syndrome. After adenovirus infection, several periodic as well as small epidemics of neurological illness are well stated. Conversely, in formerly healthy people serotypes of HAdV-D could be contained in the causes of neurological syndromes (Belsy *et al.,* 2009).

HAdV-F41 were also detected in this study similar to the study conducted by huang et al. The clinical detection of adenoviruses in AFP ailments occurred as mild infection but this cannot decrease the importance of adenovirus infection because it is the most common agent in children (Huang *et al.,* 2013). However, in respect to CNS ailments, adenoviruses were implicated in patients but most of these information's were restricted to case studies (Bayrakdar *et al.,* 2016).

Because of the huge difference of environmental health settings, geographical localities, viral outbreaks in the certain countries, the presence of various genotypes seems to be logical. These features then affect the infection patterns of virus among various countries. Hence the conduction of such studies similar to the present study provide the basis for future widespread analysis in order to provide health authorities about the causes and patterns of AFP along with the statistics needed to avoid contact to or evade risk factors comprised in AFP cases and pervasiveness (Yousefi *et al.,* 2018). The upshots of current analysis showed the presence of HAdV in a substantial portion of AFP incidences, but then again more research is required to elucidate the function of adenovirus (Haddad-Boubaker *et al.,* 2019).

In spite of the having, significant development in children's community health globally, diarrhea is yet the second most predominant reason of mortalities in children with below-five years. Internationally, the principal pathogens of pediatric diarrhea have been the enteric viruses, particularly rotavirus. Few other childhood diarrhea related enteric viruses includes norovirus, human adenovirus (HAdV), human astrovirus, and sapovirus. Understanding of the pathogens that are responsible in producing diarrhea is precarious for prevention and management of the diarrheal diseases. Internationally, most common infection and cause of the illnesses and deaths is the diarrhea. Specifically, in children through their first years of life, viruses are known as significant source of diarrhea. Several viral agents are linked with diarrheal illness in youngsters (Qiu et al., 2018).

Pakistan is at high risk of epidemics because of overcrowded cities, unsafe drinking water, inadequate sanitation, poor socioeconomic conditions, low health awareness and inadequate vaccination coverage. The diarrheal diseases contribute to the major disease burden under five along with pneumonia in Pakistan. Unlike vaccine preventable diseases like Polio, measles and maternal & neonatal tetanus, Pakistan does not have any formal responsive surveillance system to monitor the burden of diarrheal diseases and to respond to the outbreaks. Previously, the technical assistance used to be provided by Disease Early Warning System (DEWS WHO) to the outbreaks. The Disease Early Warning System (DEWS), implemented by the World Health Organization (WHO) in collaboration with the Ministry of Health and National Institute of Health, was the main national surveillance system to detect and respond to infectious disease epidemics in Pakistan. Reporting and quality of the data from these facilities was not up to the mark due to lack of reporting from private health care providers. Moreover, Government authorities were also not taking lead actively regarding their roles and responsibilities in surveillance system. This system is no more functional in Pakistan and World Health Organization had built the technical capacity of the Government staff and had advised health department to carry out their own efforts to monitor the trends of the various diseases including Diarrhea. The objective of this study is to help in better understanding of the clinical and molecular epidemiology of enteric adenoviruses related with acute gastroenteritis in hospitalized children in Pakistan. The present study used nested PCR for identification of viruses responsible for diarrhea in children.

In order to evaluate the association of adenovirus infections with the acute gastroenteritis cases, this study was carried out during 2017-2018 at five main hospitals of the country; Benazir Bhutto Hospital, Rawalpindi (BBH), Mayo Hospital, Lahore (MHL), The Children's Hospital, Lahore, Kharadar General Hospital (KGH), Karachi and National Institute of Child Health (NICH), Karachi. In these areas to our information, no research study highlighting the prevalence of adenovirus in causing gastroenteritis had been conducted. Diarrheal ailments aided to a substantial source of illness and death particularly in the developing countries like Pakistan however, the standard data regarding the presence of adenoviruses related with gastro-enteric infections is not presently obtainable for Pakistan.

It is expected that about; 2.5 million mortalities arise because of diarrhea in children <5 years of age in poor countries. In developing countries, the total disease load is higher with an expected 1.4 billion episodes of diarrhea arising per annum and 9 million of these episodes demanding hospitalization (Finkbeiner, 2009). The increased yearly cases of diarrhea are due to the viruses, whereas bacteria are accountable for a substantial part of the cases; however, their number is yet below then those produced by viruses. Human adenovirus are responsible for causing diarrheal ailments either periodically, and in epidemics. In addition to producing diarrheal illnesses, adenovirus is also accountable for causing respiratory illnesses, conjunctivitis and haemorrhagic cystitis. In immuno-competent as well as in immunocompromised persons, adenoviruses have also been related with constant infections. In early age children, enteric adenoviruses are described with variable occurrence of infection. In developed countries, the prevalence differs from 1%-8%, while in developing countries, prevalence's from 2– 31% has been reported (Sanaei Dashti *et al.*, 2016). Among all of the mortalities occurred globally, 50% are associated with diarrhea in five countries including Pakistan, India, Nigeria, the Democratic Republic of the Congo, and China (Bhutta *et al.*, 2013). According to survey conducted in 2011, an expected about 352,000 < 5 mortalities in Pakistan, 30% are attributed to diarrheal and pneumonia infections (Gill et al., 2013). In Pakistan, the child death rate is estimated to be more than 100 deaths/ 100,000 live births with around 74,000 yearly deaths, of which 23,000 are accredited to rotavirus disease (Kawai, O'Brien, Goveia, Mast, & El Khoury, 2012). In spite of these miserable figures of mortalities, still there is

no nationwide surveillance system for monitoring the diarrheal disease load and related risks in Pakistan.

In various countries in the past decade, *rotavirus* and *adenovirus* are significant sources of viral diarrhea, which have directed to noticeable mortalities in children. Nevertheless, in few developed countries in the past, other viruses for example *noroviruses* and *astroviruses* were described for the several stated gastroenteritidis viral infections. Anyhow, diarrhea is a costly illness that effect the government budget greatly so reviewing the contributory agents comprising viruses are quiet essential for the health policy decision creators. For surveillance goals, precise determination of gastroenteritis pathogens is vital with the purpose of understanding which organisms are most predominant in particular regions and to propose precise prevention actions, vaccination policies and empiric management schedules. In the framework of epidemic studies, quick pathogen detection is also regularly essential for rapidly implementation of effective preventive strategies. For guiding the treatment of single patients principally for those having serious or continued disease or in immunocompromised hosts, laboratory detection can also be supportive. From this perspective, viral screening particularly for children below 5 years turns out to be essential (Mokomane *et al.,* 2018).

In this study, 204/1,118 (18.24%) positive *adenovirus* cases were detected out of 1,118 children, which is related with the report from Nigeria (18%) (Babalola, Odaibo, Olaleye, & Alonge, 2015). Our finding is higher than those reported for Brazil (12.47%) (Reis *et al.,* 2016), China (4.7%) (Lu *et al.,* 2017), and India (9.30%) (Dey *et al.,* 2011). Some other reports are also showed with lower prevalence rate of HAdV then ours like New Zealand (3%) (McAuliffe *et al.,* 2013), Thailand (9.3%) (Chansaenroj *et al.,* 2017), turkey (8.6%) (Biçer *et al.,* 2011) and Venezuela (11.5%) (Alcalá *et al.,* 2018). As Pakistan is located in Asia, so in comparison with other Asian countries that revealed the prevalence rate of HAdV as Bangladesh (10.7%) (Afrad *et al.,* 2018), China (9.8%) (Liu *et al.,* 2014), India (6.1% and 7.5%) (Chitambar *et al.,* 2012), Korea (5.5%) (Lee et al., 2012) and Thailand (5.84%) (Sriwanna *et al.,* 2013) which is lower than prevalence rate of HAdV in our study. Similarly, our results are also higher from other European countries like

France (0.7%) (Cardine *et al.,* 2002), Germany (5.5%) (Mayindou *et al.,* 2016), Italy (8.73%) (Giuseppina La Rosa *et al.,* 2006), Portugal (12.4%) (Ribeiro *et al.,* 2015), Turkey (3.3%) (Öztaş *et al.,* 2016) and UK (15%) (Cunliffe *et al.,* 2010). With Middle East countries like Iran (5.18%) (Sanaei Dashti *et al.,* 2016), Iraq (3%) (Jaff, Aziz, & Smith, 2016) and Qatar (6.25%) (Al-Thani, Baris, Al-Lawati, & Al-Dhahry, 2013), the prevalence rates for HAdV are lower as compared to the present study. In case of the African countries, the comparable prevalence rate of HAdV is lower than our findings. These rates are from Sudan (16.2%) (Elhag, Saeed, El Fadhil, & Ali, 2013), Tanzania (3.5%) (Moyo *et al.,* 2014) and Nigeria (5.1%) (Arowolo, Ayolabi, Lapinski, Santos, & Raboni, 2019). There are some other reports, which showed the increased rate of detection of HAdV then our study. These reports are from different countries like Iran (20.30%) (Sharifi-Rad, Alfatemi, Sharifi-Rad, & Miri, 2015), Italy (23.1%) (Biscaro *et al.,* 2018), Gabon (19.6%) (Lekana-Douki *et al.,* 2015), China (28.94%) (Qiu et al., 2018), Brazil (43%) (Costa et al., 2017), Albania (23.2%) (G La Rosa *et al.,* 2015) and Burkina Faso (31.2%) (Ouédraogo *et al.,* 2016). These increased rates highlighted the presence of HAdV in gastroenteritis cases.

In Pakistan, adenovirus is not detected routinely and is not reported that may direct to inferiority of adenovirus disease load. Due to the absence of national laboratory based surveillance system, adenovirus infections are underdiagnosed which inferring the substantial underestimation of prevalence of community acquired and nosocomial adenovirus infection.

Presently, from developing countries like Pakistan on viral causes of acute gastroenteritis, very inadequate data is present excluding few reports that mainly focused on *rotavirus* in the latest years. There is a limited report regarding the prevalence of enteric *adenoviruses* from Pakistan. Agboatwalla (1995) reported from Karachi, Pakistan that the prevalence of enteric *adenoviruses* was 10% in 1992 in children with Gastroenteritis. During this study ELISA, technique was used for the detection of enteric *adenoviruses* and *rotaviruses* (Agboatwalla *et al.,* 1995). Another study conducted by Ahmed (2016) from Peshawar, Pakistan that showed the prevalence of HAdV as 38.94%. This study was carried out to determine the quality of water by finding the prevalence of different enteric viruses (*rotavirus, adenovirus, hepatitis A virus* and *enterovirus*) in water samples

that were taken from various regions of Peshawar (Ahmad *et al.,* 2016). No other study is conducted for screening of HAdV from diarrheal samples from young children in Pakistan. To the best of our information, this is the first comprehensive epidemiological study that concentrated on the presence of HAdV in hospitalized children having severe gastroenteritis in Pakistan.

According to our results, the adenovirus was found all over the year with no seasonal peculiarity. According to data from BBH and MHL, the virus was found more in summer months as compared to winter months while in rest of the hospitals data it was more prevalent in winter then in summer. This presence showed the presence of human adenovirus throughout the year. According to the results monsoon season also increase the likelihood of adenovirus infections. We observed a peak in incidence of *adenovirus* in April and May. This peak coincided with the report from Albania where *adenovirus* peak occurs in April and May as well (G La Rosa *et al.,* 2015). Few other studies assured this pattern of seasonality for the virus, though former data described HAdV to be isolated during whole year without any seasonality or any sharp increase in incidence of *adenovirus* over the year (G La Rosa *et al.,* 2015).

HAdV-*rotavirus* co-infection rate in this study was 26.76% with repeated co-infection in January by the rate of 30.18% of all co-infections. In gastroenteritis incidences, co-presence of over one agent is not uncommon. In this study, the incidence of viral co-infection was more in male cases comparative to females without any statistical difference present among them. Here in our report there were significant relationships among co-infection and the prevalence of clinical symptoms detected in children. The co-infection rate in this study is somehow consistent with report from Albania (G La Rosa *et al.,* 2015), which showed the rate as 27.2%. Moreover, children with coinfection possessed quite serious clinical manifestation with greater possibility of becoming seriously dehydrated, irrespective of age and living style. According to our results, children with co-infection did not that much differ from those with mono-infection with regard to clinical parameters. Different co-infection rates were reported in various studies (Babalola *et al.,* 2015).

*Human adenovirus* (HAdV) is one of the pathogens accountable in industrialized and unindustrialized countries for gastroenteritis between infant and children. In our study, peak incidence of HAdV infections has been detected in children between 1 to 12 months of age. The greater ratio of HAdV infection produce frequently in ≤ 2 years of children. In our findings, the rate for ≤ 2 year children is 95% which is higher than reported data for Iran (53%) (Motamedifar, Amini, & Shirazi, 2013), Turkey (73.7%) (Biçer *et al.,* 2011), Taipei (76.6%) (Lin *et al.,* 2000) and Iraq (78 %) (Jaff, Aziz, & Smith, 2015). This elevated level in ≤ 2 years old children proposes that the younger group is quite simply infected by *adenovirus* as compared to the elder group. The reason behind this may be by the fact that young children grows more susceptible to infections then elder with improved fortified with the growing innate and adaptive immune systems (Simon, Hollander, & McMichael, 2015).

The molecular analysis of adenovirus positive cases revealed the presence of several genotypes in the diarrheal cases. The prevalence of these diverse genotypes pose the great impact of the variants to cause disease severity in pediatrics population. The detected genotypes include HAdV-B3, B7, C2, D28, F40, and F41. Among these types, HAdV-F40 was isolated with increased frequency followed by HAdV-F41. All of this strain possess increased homology with the reference strain. The presence of these strains were stated in accordance with reports from various studies (Colak *et al.,* 2017; G La Rosa *et al.,* 2015; Liu *et al.,* 2014; Lu *et al.,* 2017; Moyo *et al.,* 2014; Qiu et al., 2018).

**Study limitations:**

It is the reality that in initial attempt no scientific study is out of limitations. However, these limitations highlighted the points in the study that require re-investigation, which produces the encouragement for future analysis. This study also has certain limitations as follows:

1) For AFP study, the samples collected were over a single year i.e. 2017, which makes it difficult for identification and differentiation of adenovirus types that are circulated constantly or

intermittently. But despite this limitation this study let us to distinguish an excessive variety of genotypes flowing in the population of Pakistan.

2) Another limitation in case of the diarrheal samples is the inability of including all of the samples during the study period due to financial limitations. However, in spite of this limitation, the selected samples at the time of delivery signifies a symbolic samples counting all age groups, geographical distribution, seasonality, and different years of study.

3) Additionally, climatic variables, which play important role in notifying the local epidemiology of adenoviruses, are not collected in this study. These variables additionally provide auxiliary data, which is helpful in providing safety measures for monitoring this frequent disease in our country. Hence, this investigation may provide significant support in the employment of extensive collection of studies for the identifications of all HAdV species, in studies founded on the adenovirus etiologies of gastroenteritis and AFP cases.

**Conclusion:**

Right now, due to tremendous effort conducted by Pakistan, the country moved towards the eradication of polio disease, however, the country still faces the huge amount of non-polio AFP cases. These cases may provide the miserable picture of mortality and morbidity in whole country. Hence, these conditions produce the alarming situation for the identification of non-polio AFP cases in our country. Therefore, with the help of this study, a sufficient and consistent data is produced that helps in considering the incidence, etiology, clinical manifestation and molecular epidemiology of adenoviruses disseminated in country.

1) This investigation also aids in recognizing the alternation in the array of adenovirus infections and disease relation. Molecular characterization done in this analysis deliver evidences on antigenic disparity in evolutionary mechanism of adenoviruses. In order to save our country from this crippling infection, the results of our analysis discover the sources of AFP other than

poliovirus. Moreover, for the recognition of connotation of adenoviruses in producing AFP and its pathogenicity, animal model requests to be established.

2) Conclusively the presence of adenoviruses in gastroenteritis cases in the native population of the country is quite high. Pakistan is among one of the Asian countries with higher diarrhea related deaths. Regardless of this, screenings of the viruses are not completed in our country hospitals because of the lacking appropriate laboratory services attributable to limited assets. Besides, absence of awareness between the clinicians leads to the undiagnosed or misdiagnosed viral infections, which are commonly cured, with needless antibiotic schedule. The presence of higher disease load in early age children of our country produced additional emphasis on the prioritization of the analysis of HAdVs particularly in the neonates and early-age infants.

3) In conclusion, our results may describe the association of adenoviruses in AFP and diarrheal cases in Pakistan. The outcomes of this study provide the epidemiological picture of adenoviruses in cases of AFP and gastroenteritis. These outcomes are constant with the reports of other studies that signifies the relation of adenoviruses with AFP and gastroenteritis. However, additional molecular and clinical analysis are desirable.

**Recommendations:**

With the help of our study, suitable and consistent data is produced which help in enhancement of the technical aptitudes of national public health organizations for implementation of innovative molecular diagnostic facilities at the main country hospitals. In diarrheal infection, a precise considering of the association of HAdV is probably to add to infection prevent policies. Briefly, this study demonstrates that may be HAdV is the source of gastroenteritis and AFP and is more predominant among 1 to 24 months (gastroenteritis) and less than 15 years (AFP) old children. For better detection, epidemiology based studies must be conducted on largescale to include other areas of Pakistan.

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# Appendix 1

Diarrhoea case report form
Information extracted from patient management file

**Medical Record Number**

**Hospital/ Health Facility/City**_____

**Date of admission:** __/__/__ (day/month/year)
**Treated at:** ( ) **Emergency Room,** **Duration in hours** ( )
( ) **admitted to Ward** **Duration in days** ( )

**Patient information**
Patient ID number _____
Patient name: _____
City/village of residence: _____
Age (months): ____
Sex: M ( ) F ( )
**Vaccine Dose:** **Nill** ( ) **One** ( ) **Two** ( )
**Date of Vaccination:** __/__/__ __/__/__ (Day/Month/Year)

**Clinical information**
Duration of symptoms: ____days
History of fever: Yes ( ) No ( )
Temperature at admission: _____°C (axillary)
Vomiting: _____ (Yes/No) No. of episodes/24 hr: _____ Duration (days): _____
Diarrhoea: _____ (Yes/No) No. of episodes/24 hr: _____ Duration (days): _____
Dehydration status (as defined in the notes): ( ) severe ( ) some ( ) none
Treatment ORT: ( )
IVF: ( )
Outcome (mark one): ( ) improved ( ) Died
( ) Transferred ( ) unknown

Date of discharge, transfer or death: __ __/__ __/__ __ (day/month/year)

Laboratory information

Stool collected: Yes ( ) No ( )
Date stool specimen collected: __/__/__ (day/month/year)

Patient ID Number __/__/__/__/__/__/__/__/__/__

Date of sending the specimen to laboratory__ __/__ __/__ __

Date of receiving Lab results: __ __/__ __/__ __(day/month/year)

Rotavirus EIA results_____

**Person who completed the form:**

Name: _____ Signature: _____
Date of report completed: __ __/__ __/__ __ (day/month/year)