REVIEW OF LITERATURE

2.1 General description

Adenovirus was discovered by Rowe and his colleagues in 1953, when they noted that adenoidal tissue, maintained in culture for two to three weeks, underwent a unique form of degeneration or showed cytopathic effect (CPE). This CPE could be duplicated when supernatant material from one culture was used to inoculate fresh epithelial cells. The agents isolated from these cultures were designated the adenoid-degenerating agent and hence named adenovirus to denote its origin (Rowe, 1954). Adenovirus was soon established as the etiological agent of acute respiratory disease by Hillemann and Werner (1954). Thus two of the fundamental properties of human adenoviruses, the acute respiratory infection and the persistent infection of lymphatic tissue, were recognized at an early stage.

So far adenoviruses have been isolated from every species of placental mammal, bird and amphibian. Thirty-one serotypes were identified as being involved in human infection by 1965 and presently their number has reached to 47 (Wadell, 1990). An isolate is identified as serotype on the basis of distinct antigenic epitopes capable of inducing neutralizing antibodies.

Recent methods for the identification of DNA viruses by the restriction enzyme analysis and DNA hybridization techniques have also been applied for adenoviruses as well (Allard et al., 1958). These methods are straightforward and can be of help when ambiguous results are obtained from serotyping attempts. In addition, application of
such techniques to clinical isolates opens up the new field of molecular epidemiology and allows probing of the genetic variability expressed with each adenovirus serotype (Fife et al., 1985).

2.2 Classification

Adenoviruses are the members of family Adenoviridae (Norrby et al., 1976). The virion has a naked icosahedral capsid, 70-90 nm in diameter and is composed of 252 capsomeres with projection at the vertices. The particle contains double stranded DNA with a molecular weight of 20-30 x 10^6 and matures in the nucleus. The family is subdivided into two genera which have distinct group specific antigens; mastadenoviruses comprising mammalian adenoviruses and aviadenoviruses comprising the avian adenoviruses. Further subdivision of the mammalian adenoviruses may be necessary if it is confirmed that certain bovine adenoviruses do not contain the group specific antigen found in all other mastadenoviruses.

Human adenoviruses were originally classified in 1960 into four sub-groups, I-IV, based on different HA properties with rat and rhesus monkey erythrocytes. This sub-division also loosely relates to the length of fibres on the virion (Rosen, 1958). By 1967, 31 serotypes were identified. They were classified into sub-genera, A-D, on the basis of their oncogenicity in new born hamsters. Sub-genus A comprises adenovirus serotypes which are highly oncogenic, i.e., causing tumors in every animal within two months. Sub-genus B adenoviruses are weakly oncogenic, meaning that few of the animals develop tumors after an observation period of one year. The non-oncogenic adenoviruses can transform rodent cells in-vitro and are divided into sub-genera C and
D on the basis of differences in the antigenicity of early T antigen (Freeman et al., 1967; Mathews, 1981). Till 1980, 47 human adenovirus serotypes were identified. They were classified into six subgenera, A-F, on the basis of the pattern of virion polypeptides obtained on SDS polyacrylamide gel electrophoresis (PAGE) (Wadell, 1980), DNA homology in terms of G+C contents of specific adenovirus genome (Green et al., 1967) and DNA restriction patterns (Adrian et al., 1986).

2.3 Viral characteristics

The adenovirion consists of a proteinaceous capsid surrounding a dense core. The core is 60 - 65 nm in diameter and contains all the DNA and about 20 per cent of the total protein of the virion. The 252 capsomers which make up the capsid are arranged into an icosahedron having 20 triangular faces and 12 vertices. The 12 capsomeres at the vertices have a fibre which consists of a rod like projection with a knob attached at the distal end. The 240 non-vertex capsomeres have six neighboring capsomeres and are called hexons. Each capsomere is seven to nine nm in diameter with a hollow center of 2.5 nm.

Proteins comprise 87 per cent by weight of mastadenoviruses and 83 per cent of aviadenoviruses. There are eight to ten polypeptides in the virions. Two are core proteins of 48,500 mol wt. and 18,500 mol wt., respectively; the latter is rich in arginine. The hexon consists of three identical polypeptides of 120,000 mol wt. Five polypeptides of 85,000 mol wt make up the penton base and three of 62,000 mol wt polypeptides comprise of fibre. There are also three hexon associated polypeptides.
The antigenic composition of adenoviruses is complex. The hexon carries four antigenic specificities; a group specific activity demonstratable by complement fixation and shared by all mastadenoviruses; a type specific activity shown in neutralization and, probably, intra-subgroup and inter-subgroup activities. The penton base carries toxic activity and weak group specific antigenicity as well as inter- and intra-subgroup activities as demonstrated by haemagglutination enhancement. The fibre has type specific antigenicity carried in the knob region and demonstrated by virion haemagglutination inhibition. Fibres longer than 12 nm also have intra-subgroup activity in their proximal region. The major core protein contains 23 per cent arginine and like other basic proteins is only weakly antigenic. Replication of DNA, transcription and maturation of adenoviruses takes place in the cell nucleus. The spliced mRNA is translated in the cytoplasm.

Infection is initiated by the attachment of the fibre, the virus attachment protein (VAP), to a receptor on the surface of permissive cells. The virus then penetrates the cell membranes, still more or less intact. The capsid is then partially removed by a process called uncoating. At this stage, the DNA is exposed sufficiently to allow some transcription of virus specific mRNA to occur. This mRNA is called 'early' to signify that it is synthesized before DNA replication has occurred (Ginsberg, 1980). Early mRNA, in turn, is translated into early virus specific protein, among which is a group of non-structural proteins called 'T' antigens. Their name derives from the fact that they were first described in adenovirus induced tumours in hamsters (Pope and Rowe, 1964). They are detected in typically infected, latently infected or oncogenically transformed cells using serum from tumour bearing animals in immunoassays (Pope and Rowe, 1964;
Baum, 1990). The exact role of these antigens is not known (Horwitz, 1990). After early events have occurred, the DNA migrates to the cell's nucleus, where DNA replication proceeds. The newly replicated DNA then forms the template for transcription of 'late' mRNA and translation of 'late' proteins, including the structural proteins. These newly synthesized proteins and DNA are assembled in the cytoplasm into new adenovirions, at which point the infected cell is lysed, releasing up to a million progeny for each input virus. Only about 1 in 100 progeny virion is infectious (Ginsberg, 1980). In oncogenically transformed cells, in vivo or in vitro, the replication cycle stops after early phase and adenoviral DNA is integrated in the cell's genome without replication of late mRNA, protein, or infectious agent. Adenoviruses can establish latent, non-lytic infection in human lymphoid cells (Huebner et al., 1954; Schlesinger, 1969) and in epithelial cells of non-human primates (Baum, 1977). The symptoms of acute adenovirus infections are due to the lysis of infected cells and the resulting immune response. No known systemic toxin is produced as a result of adenovirus infections, but isolated penton antigen is locally cytotoxic (Horwitz, 1990). The virus predilection for epithelial cells explains involvement of the eye and mucosal surfaces of the respiratory, genito-urinary and intestinal tract. Latency may represent a reservoir for infection in asymptomatic individuals, and it is probably that infection in immunocompromised patients and is due to reactivation of the latent infection rather than the person to person spread. Adenoviruses can infect and replicate at various sites of the respiratory tract as well as in the eye, gastro-intestinal tract and urinary bladder. On occasions, these viruses may cause disease in other organs, such as the central nervous system, which may be involved in meningoencephalitis complicating an adenovirus infection of the
respiratory tract (Kelsey, 1978). Although there are 47 distinct serotypes reported, most human disease is associated with only one third of these types. Many adenovirus infections are subclinical and result in antibody formation that is probably protective against exogenous reintroduction of the same adenovirus serotype. However, the virus itself may be grown, especially from gastro-intestinal tract (Fox et al., 1969) and respiratory tract (Evans, 1958), for months after initial infection and immune response.

2.4 Adenoviral diseases

The more common illnesses associated with various adenoviruses are described as follows.

A. Respiratory Diseases

In infants and young children, ten per cent of all respiratory infections are caused by these organisms (Brandt et al., 1969; Knight and Kasel, 1973). The usual symptoms include nasal congestion, coryza and cough. Other patients may have an exudative tonsillitis that is clinically indistinguishable from disease caused by the group A Streptococcus (Ginsberg et al., 1955; Horris et al., 1971). The respiratory symptoms are often accompanied by systemic manifestation such as generalized malaise, fever, chills, myalgia and headaches. The common serotypes are types 1, 2, 5 and 6 and occasionally type 3 which is usually endemic in most population. Sporadic cases may be indistinguishable from other viral respiratory infections such as influenza, para-influenza, respiratory syncytial, rhino and enteroviruses. If conjunctivitis accompanies the signs and the symptoms already described, the disease is designated pharyngo-conjunctival fever (Gold and Ginsberg, 1962). The conjunctivitis is usually follicular, unilateral or bilateral, and characteristically mild. The adenovirus serotype most commonly involved
is type 3 but types 7 and 14 within the same haemagglutination group have been isolated from such patients (Huebner et al., 1954; Parrott et al., 1954; VanDeer, 1963). In more severe cases, pneumonia may occur. Adenoviruses are probably responsible for approximately ten per cent of the pneumonia in childhood (Mallet et al., 1966). Most patients recover from these lower respiratory infections, but there have been epidemics of adenovirus type 7 that have resulted in considerable mortality. Sequelae in those who recover may include bronchitis that can clinically manifest itself years after the primary infection (Mallet et al., 1966; Simila et al., 1971).

In India, a few earlier reports from West Bengal have shown the association of adenoviruses with respiratory infections. Adenoviruses accounted for almost half of the heavy virus burden found in throats of children aged two to five years in rural West Bengal and the types commonly found were 1, 2 and 5. Many serotypes recurred periodically in given individuals despite presence of specific serum antibodies (Kloene et al., 1970). In a longitudinal study in 30 children from birth to 30 months, throat and rectal specimens were processed for virus isolation in HEp2 cells. Identification and serotyping of the isolates was done by specific NT and HAI tests. Common adenovirus types 1, 2, 5 and 6 accounted for approximately 76 per cent of isolations, all of which tended to infect persistently over periods of several weeks or months, as did less common types 3 and 8. Forty per cent of the children developed persistent infections with one common serotype; 27 per cent had persistent infections with two or more serotypes; and the remaining 33 per cent failed to develop persistent infections (Hills et al., 1973).
B. **Acute Respiratory Disease (ARD)**

In many respects, ARD is similar to the description furnished earlier of the respiratory infection of the children. The syndrome is caused by types 4, 7 and occasionally by type 3. ARD is a syndrome that tends to occur under the special conditions of fatigue and crowding created soon after the induction of young military recruits (Huebner et al., 1954; Miller et al., 1963; Mogabgab, 1968). It is less common among healthy adults. Some of the cases have had a fatal outcome from the pneumonitis that may accompany and complicate the other milder respiratory symptoms.

C. **Pertussis like syndrome**

The association of adenovirus infection with a pertussis-like syndrome has been noted and it led to some speculation that adenoviruses rather than *Bordetella pertussis* caused many of the cases of clinical whooping cough (Suzuki et al., 1981). Claims were made that even the lymphocytosis of pertussis might be related to adenovirus infection (Collier et al., 1966). This point of view was given some credence by the isolation of an adenovirus type 5 from multiple organs of a patient with severe whooping cough with lymphocytosis that ended fatally (Collier et al., 1966). Adenovirus has been isolated from many patients with this syndrome, either in conjunction with *B. pertussis* or alone (Olson, 1975; Severien et al., 1995). The large number of adenovirus isolations may be due to the conditions favorable for reactivation of latent virus from tonsillar tissue during concurrent *B. pertussis* infection (Neumann et al., 1987). A controlled study of 134 children with pertussis like illness and 101 healthy controls reported the common association of adenoviruses with whooping cough syndrome (Nelson et al., 1975).
D.  *Pharygoconjunctival Fever*

The syndrome of pharygoconjunctival fever in children is sporadically epidemic in the setting of summer camps and schools (Sobel *et al.*, 1956). The route of transmission is probably contaminated swimming water; type 3 adenovirus is the most commonly isolated agent. The onset is acute, and signs and symptoms include conjunctivitis, pharyngitis, rhinitis, cervical adenitis and fever. Both the bulbar and palpebral conjunctivae are involved and are painful and pruritic. Spread to the contralateral eye is very common. Meningismus has been reported, and poliomyelitis may be suspected. Similar syndromes can be caused by Epstein Barr virus and by enteroviruses. The lower respiratory tract is generally spared, and there is no permanent damage to the eye.

E.  *Keratoconjunctivitis*

Adenovirus was first described as the agent of outbreaks of keratoconjunctivitis in adults (Jawetz *et al.*, 1955). The syndrome occurred in shipyard workers whose eyes had sustained irritation and minor trauma from chips of paints and rust. The serotype involved was adenovirus 8. Adenoviruses are now recognized as a common cause of sporadic conjunctivitis in adults, and serotypes 19 and 37 also cause epidemic eye infections (Guyer *et al.*, 1975; Keenlyside, 1983). Vehicles for transmission of virus have included towels used in communal washrooms (Sprague *et al.*, 1973) and contaminated ophthalmic solutions (Keenlyside, 1983).

The incubation period has a wide variation, from four to 24 days, after which symptoms begin insidiously. Virus has been isolated from eye secretions more than one
week after onset of symptoms (Koc et al., 1987) and may be present even longer, since secondary household spread, which occurs in about ten per cent of cases, increases with the duration of illness in the index case (Guyer et al., 1975).

F. **Acute Haemorrhagic Cystitis**

An illness occurring almost exclusively in male children and characterized by gross haematuria and dysuria has been associated with adenovirus type 11 (Numazaki et al., 1973). Its significance lies in the potential confusion with other more serious diseases of the kidney such as glomerulonephritis. This self limited disease is usually not accompanied by fever or hypertension, and tests for renal excretory and concentrating functions have been especially normal. Type 21, like adenovirus type 11 can also cause haemorrhagic cystitis. In Japan, acute haemorrhagic cystitis in males between the ages of six and 15 years has been reported with adenovirus isolation rates from urine or with rise in neutralizing antibodies in nearly 70 per cent cases (Numazaki et al., 1973). In a report from United States, only 20 per cent cases were linked to an acute adenoviral haemorrhagic cystitis and for another 60 per cent cases the etiology remained unexplained (Mufson, 1973).

G. **Meningoencephalitis**

Several reports have directly demonstrated a total of eight adenoviruses isolated in CSF (types 3, 5, 6, 7, 7a and 12); one patient with malignant lymphoma had an adenovirus type 32 isolated from brain at autopsy (Chou et al., 1973; Kelsey, 1978). There are other cases of meningoencephalitis in which viral isolation from extraneural sites or increase in antibody titre have been used to make a diagnosis, especially those
associated with adenovirus type 7 pneumonia in children (Simila et al., 1970). A case of sudden unilateral deafness was reported to occur due to adenovirus 3 infection of nasopharynx (Jaffe and Maasaab, 1967).

H. **Gastrointestinal infections**

Adenoviruses have been associated with four to 15 per cent of all hospitalized children with viral gastroenteritis. Gastroenteritis may be a sign of systemic infection such as those caused by types 3 or 7. They may cause both respiratory symptoms and diarrhoea in a child with high fever. Type 31 is frequently isolated from stools of children with diarrhoea and may be of aetiological importance. The enteric adenoviruses type 40 and 41 which are fastidious, have been demonstrated to account for two-thirds of diarrhoea cases (Wadell, 1990). Epidemiological studies to assess the importance of these agents have been undertaken for several population groups. A report of 14 enteric adenovirus related cases of diarrhoea in 27 hospitalized patients studied during a 12-week period suggested that these viruses may be an important cause of acute gastrointestinal diseases in hospitalized young children and may be nosocomially transmitted; this report also suggested that respiratory symptoms may be prominent part of clinical manifestation (Yolken et al., 1982). The incidence of adenoviral related gastroenteritis is probably between seven per cent and 17 per cent of all of the intestinal infections in children (Cevenini et al., 1987; Wadell et al., 1987). In some population groups, 50 per cent of the children at the age of four have antibodies to the enteric adenoviruses (Shinozaki et al., 1987).
Another intestinal syndrome, intussusception, has been linked in some patients to an adenovirus infection (Bell and Steyn, 1962). The telescoping bowel characteristic of intussusception may be caused by mesenteric adenitis, acting as a lead point to the mechanical obstruction. Adenoviruses types 1, 2, 5 and 6 have been isolated both from stool cultures and from the involved lymph nodes removed at surgery (Potter, 1964; Clarke et al., 1969). Adenovirus inclusions have been seen in some appendixes removed at surgery (Yunis et al., 1975).

I. Infections in Immunocompromised Hosts

Adenoviruses are receiving increasing attention as opportunistic agents in patients with acquired immunodeficiency syndrome (AIDS) and other immunodeficiency states. These viruses have been isolated from immunocompromised hosts and have contributed to their morbidity and mortality. Children with severe combined immunodeficiency (SCID) are prone to develop severe infections with most frequently occurring persistent adenoviruses: types 1, 2 and 5 of subgenus C, and type 12, 18 and 31 of subgenus A. Furthermore, epidemics of type 3, 4 or 7 may also cause life threatening infections. In an analysis of 15 immunocompromised patients, both adults and children, of whom 12 had pneumonia with nine deaths occurring, adenovirus types 1, 2 and 6 of subgenus C occurred among children, whereas types 4 and 5 were recovered from adults. Among six adult cases four deaths occurred. There is also a report of fatal outcome of type 5 infection in a patient with X-linked lymphoproliferative disease (Zahradnik et al., 1980).
In contrast to sporadic isolations of various adenoviruses from immunocompromised patients, it has been shown that 12 per cent of AIDS patients have an adenovirus with specific genotype in their urine (DeJong et al., 1983; Horwitz et al., 1985). Restriction endonuclease analysis of viral DNA and serologic classification of 24 urinary isolates have shown that all contain more than 85 per cent of type 35 DNA. However, some of the AIDS urine isolates have the type 7 haemagglutinin, suggesting the recombination between the type 35 and type 7 group B agents. In addition to the haemagglutinins of type 7 and type 35, haemagglutinins from types 3 and 11 have been found in other cases (Horwitz et al., 1985). Many group D adenoviruses have been isolated similarly from stool and rectal swabs from AIDS patients (Hierholzer et al., 1988).

The activation of all latent DNA viruses takes place in the patients of bone marrow transplant recipient. Cytomegaloviruses are the best known and most important threat to these patients. However, there are reports which draw attention towards the risk caused by adenoviruses. These infections have been demonstrated in 8 per cent of the bone marrow transplant recipient. It is also of interest that infection with type 5 and closely related types 11, 34 and 35 is relatively frequent among these patients (Shields et al., 1985).

A prospective study of the etiology of the severe enteric infections in 78 bone marrow recipients (mean age 21 yrs) was performed in Baltimore. Adenoviruses were found in 12 of the 31 cases in which human enteric pathogens could be identified. The mortality among infected and uninfected patients was 55 and 13 per cent, respectively.
Six of the patients died within 11 days of their first positive stool culture for enteric viruses. Five of these six patients were infected with adenoviruses (Yolken et al., 1982).

The potential role of B-lymphocytes has been suggested in the maintenance of latency by several groups of investigators who have found low levels of adenovirus DNA persistent in peripheral blood lymphocytes (Abken et al., 1987). Confirmation of true latency in tonsillar tissue has been achieved by demonstrating adenovirus nucleic acid sequences in this tissue but no infectious virus, even after repeated passages (Neumann et al., 1987). Persistent adenoviruses might explain the occasional clinical illness in immunocompromised hosts who might be reinfected from an endogenous source at the time of immunosuppression. However, in some renal transplant patients, the infection seems to be exogenously acquired in the donor organ (Harnett et al., 1982). But most attempts to associate adenovirus infection with the etiology of human tumours have been unsuccessful. No viral macromolecules suggesting an adenoviral infection have been found in most studies of the etiology of human malignancies (Green et al., 1979; Wold et al., 1979; Brachmann et al., 1980).

The human adenoviruses presently comprise 47 serotypes grouped into six subgenera which roughly parallel the haemagglutination and oncogenic subgroups. Within these serotypes are multitude of atypical and intermediate strains and DNA variants that complicate the precise identification of newly isolated strains (Hierholzer, 1992). Adenoviruses are among the many pathogens that contribute to debilitating and often fatal illness in immunosuppressed patients. These viruses are not
unexpected in such patients, because they have biological properties that facilitate their spread under conditions of immunosuppression. Specific properties are as follows.

Adenoviruses are pathogenic for diverse tissues and normally cause a wide variety of clinical syndromes in humans. New serotypes and intermediate strains are frequently described (Hierholzer et al. 1975; Stalder et al. 1977). Some serotypes can produce tumours in laboratory animals. Many are known to readily become latent in lymphoid tissue and kidneys and are assumed to reactivate in immunosuppressed patients. Adenoviruses can be excreted in the stools for weeks or months after initial infection (Wadell, 1988; Horwitz, 1990). All these features are compatible with the apparent long-term presence of adenoviruses in the intestinal and urinary tracts and with their possible contribution to disease in immunocompromised hosts. At the same time, adenovirus infections in immunocompromised patients are often different from those in the normal host in terms of persistence and severity of disease and the association with a different spectrum of serotypes (Schmitz et al. 1983; Wigand and Adrian, 1986; Hierholzer et al. 1988).

Immunosuppression also probably contributes to the diversity of serotypes. The immunocompromised patients can develop chronic adenovirus disease as a result of persistent infection and an altered or ineffective immune system. This combination increases the likelihood that the patient will become infected with a second serotype while still infected with the first, thus making it possible for the two serotypes to recombine to form an intermediate type. Recombination may explain the unusual frequency and variety of intermediate strains in the gastro-intestinal tracts of AIDS
patients. An alternative explanation for the number of different adenovirus serotypes and intermediate strains is that the immunosuppressed patients may be more likely to manifest symptoms with a severe virus infection and therefore have the infection detected in standard laboratory workups (Hierholzer, 1992).

The large number of subgenus B and D antigenically intermediate strains found in the urine and stool, respectively, of AIDS patients has greatly complicated the identification of adenoviruses from these patients. In one study, 67 adenovirus isolates were identified from 48 AIDS patients. The variety of types recovered included type 31 of subgenus A, types 11,16 and 35 of subgenus B, type 5 of subgenus C and types 8,22,26,28,29 and 30 of subgenus D. Many new intermediate strains were found among these isolates (Hierholzer et al. 1988).

The most unusual aspects of the adenoviruses isolated from AIDS patients are this diversity of serotypes and the frequency of antigenically intermediate strains. The antigenically intermediate strains are particularly interesting because they may derive from the length of time and adenoviruses are in an infectious state in AIDS patients, which presumably extends from the time of onset of adenovirus infection to death of the patient. Long term infection may provide the opportunity for mutations to occur within a strain or for recombinational events between coinfecting serotypes to take place, resulting in the generation of the viruses with new antigenic makeups (Hierholzer, 1992).

2.5 Epidemiology

Adenovirus infections occur worldwide in humans as well as a variety of animals. With few exceptions, the human adenovirus serotypes are generally not pathogenic to
animals, and the animal adenoviruses are only pathogenic within the species of origin (Taylor, 1977). However, asymptomatic infections across these species barriers have been documented by antibody determinants. Simians occasionally have been shown to have antibodies to human adenovirus type 12 (Katler, 1971). Antibodies to bovine, simian and canine adenoviruses have been detected in human sera evaluated by neutralization test. The transmission of adenovirus infection and diseases varies from sporadic to epidemic. The pattern often correlates very well with viral serotype and the age of susceptible population. Since many of the adenovirus-related diseases are not clinically pathognomonic of etiologic agent, the epidemiology of adenovirus infection is crucially dependent on the techniques used to prove the identity of infectious agent. For example, measles can be diagnosed with great accuracy by most clinical observers, but adenovirus-induced upper respiratory infections or even pneumonia can be confused with a large number of other etiologies. Therefore, the incidence and clinical spectrum of sporadic adenovirus diseases were only elucidated by prospective monitoring of families, orphanages and children's homes (Vihma, 1969). Adenoviruses have accounted for 13 per cent of respiratory infections and were second only to influenza A which represented 28 per cent (Schmitz et al., 1983).

A stratification of the reported adenovirus isolations by age revealed the following: 22 per cent (<1 yr); 42 per cent (1-4 yr); 18 per cent (5-14 yr); ten per cent (15-24 yr); seven per cent (25-29 yr) and one per cent (>60 yr) (Wadell, 1990).

Serological surveys have furnished some estimates of the prevalence of adenovirus infections in various populations suffering with respiratory infections.
Antibodies to types 1, 2 and 5 are most common and are present in 40-60 per cent children (Huebner et al., 1954; Jordan et al., 1956; Brandt et al., 1972). The incidence of antibodies to types 3, 4 and 7 is low at the same ages. Adults uncommonly are infected with types 1, 2 and 5 but are more susceptible to infections with types 3, 4 and 7. During the surveillance for the virus watch studies it was documented that only about 75 per cent of the adenovirus isolates were accompanied by an antibody response, as measured by complement fixation test.

The sero-epidemiology of enteric adenovirus has been reported by Kidd and coworkers (1983). Among the sera samples tested, 86.4 per cent of specimens were seropositive for type 40 and 41 when tested by neutralization test. Sera samples were also tested for antibodies to common antigens of adenoviruses by ELISA. The incidence of antibodies rose gradually through childhood to adulthood. Antibodies were found in 20 per cent children between one and six months of age and in 50 per cent of those with 37 to 48 months. Of sera samples from young adults, 48 per cent had antibodies. Antibodies were found in ten per cent of sera samples from the aged persons (Tatsuhika et al., 1987).

Faecal oral transmission accounts for most infections in young children. Initial spread may occur from the respiratory route, but the prolonged carriage in intestine makes the faeces a more common source during both the acute illness and intermittent recurrences of shedding (Fox et al., 1969). Adenoviruses have an important role to play during latency and malignancy. These were first discovered because of their propensity for latency in adenoidal tissues (Abken et al., 1987). The epidemiologic importance of
long latency in tonsil tissue is not known. The persistence of adenovirus in normal human hosts has been documented for 24 months after initial infection (Fox et al., 1969; Hillis et al., 1973).

The epidemic forms of adenovirus disease were studied in different ways from the sporadic endemic occurrence. The epidemics of acute respiratory diseases (ARD) were well known during World War II, and this awareness preceded the isolation and characterization of the first adenovirus. This ARD, which occurred almost exclusively in recently assembled military recruits, was most common in winter. It did not occur in seasoned personnel in close contact with the recruits and was later identified as a type 4 or type 7 infection in most outbreaks (Hilleman et al., 1955; Rowe et al., 1956; Hilleman, 1957). This disease did not occur in similarly congregated college students, suggesting that additional factors such as more crowded sleeping conditions or the fatigue associated with 'basic training' were contributing factors. In support of these co-factors has been the observation that ARD causing adenoviruses did not spread to civilian personnel in contact with military. In recruits congregated during the summer months, ARD often did not occur until the onset of colder weather in the fall. Influenza A could be distinguished because it affected seasonal as well as new recruits. Adenovirus induced ARD often affected 80 per cent of the recruits, with 20 to 40 per cent of them requiring hospitalization. The duration of infectivity was rather short, since virus was not demonstrable after 4 days of illness (Rowe et al., 1956). Controlled studies of route of infectivity for the ARD causing adenoviruses have demonstrated that aerosolized virus inhaled into the lungs of volunteers produced the disease, whereas, application to the mouth, the nasal mucosa, or the intestine in enteric coated capsule
failed to produce the lower respiratory disease (Couch et al., 1966). Several of the adenoviruses syndromes can probably be spread as nosocomial infections (Yolken et al., 1982).

2.6 Diagnosis

The diagnosis of adenovirus infection is based upon the isolation of respective virus or demonstrating atleast four fold rise in antibody titres to a specific adenovirus serotype. Collection of specimens from affected sites early in the illness is necessary to achieve virus isolation and to detect adenovirus antigens or nucleic acids directly in clinical samples (Kasel, 1980). Throat swabs, nasal swabs, conjunctival swabs or scrapings, and anal swabs are placed in a 'transport medium' both to stabilize the adenovirus and to inhibit the bacteria and fungi that would otherwise overgrow in the tissue culture (Gold and Ginsberg, 1962). Growth of adenoviruses from patient material is best achieved in the cells of human origin (Bell et al., 1961). Primary HEK cells are probably the best host for the replication of the entire range of adenoviruses (Rowe et al., 1956). However, HEK cells are expensive and may be contaminated with adenoassociated virus. Hence the continuous epithelial lines such as HEP-2, HeLa and KB are used for cultivation of adenoviruses (Bell et al., 1955). Human fibroblast lines are easier to maintain for a long period of time without media changes, but are less sensitive than HEK cells or continuous epithelial lines.

The cell line 293 appears to be a good host for some, but not all of the non-cultivable enteric adenoviruses like types 40 and 41 (Takiff et al., 1981). Primary monkey cell lines have been used to isolate human adenoviruses. However, there is a
large amount of accumulated evidence that most adenoviruses grow poorly in these cells unless coinfected with papova virus or simian virus 40 (Baum et al., 1972).

Adenovirus CPE consists of rounding and clustering of swollen infected cells (Kasel, 1980). Initial CPE may start at the periphery of the monolayer. However, some of the group B adenoviruses do not cause cell enlargement or clustering. Adenoviruses increase glycolysis in continuous cell lines and thereby induce the cells to produce large quantities of acid (Fisher and Ginsberg, 1957). In some laboratories, the color change of indicators caused by the lowered pH in the medium have replaced microscopic examination for CPE. Rapid cytopathology can be induced within several hours of inoculation of concentrated virus preparation and is not related to viral replication. The rounding and detaching of cells in such cases is caused by the toxicity of free viral penton capsomere, which is made in a far greater quantity than needed for viral assembly (Valentine and Periera, 1965).

Adenoviruses assemble in the nucleus of the infected cells and nuclear morphologic changes can be used for the diagnostic purposes. However, the viral proteins are synthesized in the cytoplasm, where some of these accumulate and can also be recognized by immunological techniques. The nuclear changes of adenovirus types 1,2 and 5 of group C infection include overall enlargement and intranuclear inclusions that initially are Feulgen-negative and eosinophilic but become Feulgen-positive and basophilic as infection progresses (Boyer et al., 1959). In addition, there are paracrystalline aggregates that contain viral proteins without nucleic acid (Morgan et al., 1957). Although these light microscopic cytologic changes are not absolutely
pathognomonic of adenovirus infection, they are useful for the histopathologic diagnosis of biopsies, especially of lung tissue, and as an aid to tissue culture differentiation of adenoviruses from other agents. Suggestive adenovirus isolates based on the pattern of cellular degeneration must be further characterized by adenovirus specific serological tests for definitive identification.

Monoclonal antibodies have been utilized for the epidemiological studies on the majority of serotypes involved in adenovirus infections. Monoclonal antibody based immunoassays have been described for detection of adenovirus antigen from nasopharyngeal aspirate specimens of respiratory illness, from tissue homogenates from patients with systemic infection and from stool specimens of gastro-intestinal illnesses (Hierholzer, 1987). The development of monoclonal antibody based radio-immune dot-blot (IDBT) for rapid diagnosis of adenoviral acute keratoconjunctivitis has been reported. Of the 718 conjunctival specimens tested, the sensitivity and specificity of IDBT were found to be 85.3 per cent and 92.2 per cent, respectively, when compared with the conventional cell culture isolation technique (Killough et al, 1990).

A worldwide distribution of adenoviruses can be evaluated by analysis of prevalence of antibodies and/or frequency of isolation of adenovirus strains. Screening of adenovirus specific antibodies gives reliable information on the prevalence of adenovirus infection in various populations. Mammalian adenoviruses share group specific antigen epitopes which can be detected by CFT or ELISA. Since the CF antibody response is a group reactivity, single CF antigens will detect responses to many adenovirus serotypes. CFT for adenovirus specific antibodies is ideally performed with
a pool of antigen representing a serotype of each subgenus. If this is difficult to accomplish, representative serotypes of subgenus B [eg type 7] and subgenus C [eg type 2] should be used. Sonicated, clarified, infected cells provide a good source of antigen since a ten-fold excess of structural proteins (predominantly hexons, pentons and fibres) carrying cross-reactive epitopes is produced. A four-fold or greater rise in CF antibody titre is a sign of current adenovirus infection (Wadell, 1990). However, adenovirus CF serologic responses may vary with the virus used as a source of antigen, and some adenovirus infections may not be detected by this technique (Schmidt and Lennette, 1971). The ELISA is therefore, preferred. Although HAI and SN antibody responses are more specific for a single infecting serotype, these tests may detect heterologous responses within a sub-group, especially when studying adults. Children have more specific serotype responses perhaps related to their limited exposure to adenovirus family.

There are other serological tests such as Ouchterlony agar gel diffusion test, radial gel diffusion and haemagglutination enhancement which have been used by various investigators (Grandien and Norrby, 1975). Each has some advantages such as economy of reagents, ease of performance and high sensitivity for group reactive antigens, but none has been found to have practical use in clinical laboratory.

2.7 Viral identification

A. Serological Tests

Agents growing in tissue culture and demonstrating CPE typical of adenoviruses require further identification and confirmation. Serologic tests are useful in
characterization. The fluorescent antibody test (FAT) and complement fixation test (CFT) employed on tissue culture grown viruses, measure group characteristics of the adenovirus. The group characteristics are usually shared by all human isolates (Gardner and Quillin, 1974; Kasel, 1980). The haemagglutination inhibition (HAI) and serum neutralization test (SNT) measure type specific parameters that can distinguish members of each group (Kasel, 1980).

The adenovirus CF antigen is a prepared from the hexon capsomere, which is made in at least 10-fold excess over that assembled into the virion. Although the hexon carries multiple antigenic epitopes, the alpha epitope is common to all human adenoviruses (Philipson et al., 1975). Thus, a single reference antibody can be used for initial characterization of clinical isolate as an adenovirus. The FAT using a hyperimmune sera to any of the common adenovirus serotypes will react with all the rest of the human adenoviruses and can therefore be used to confirm the group-characteristics of a clinical isolate. The type specific monoclonal antibodies that could be utilized in the FAT as well as in ELISA and latex agglutination procedures have become available for differentiating enteric adenoviruses types 40 and 41 from other adenoviruses in the faeces (Gardner and Quillin, 1974; Herrmann et al., 1987). Specific serotyping can make use of monoclonal antibodies to type specific epitopes on hexon or fibre.

In current practice, HA, HAI and SN properties of virus isolates are determined as parts of the specific adenovirus typing. Agents characterized as adenoviruses either by CFT or FAT are tested for their ability to haemagglutinate either rhesus or rat cells

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(Rosen et al., 1962). Further HAI testing using human cells as well as various other animal species can provide supplement information (Hierholzer, 1973). HA is a property exclusively of the fibre, the HAI test is only able to measure antigenicity from five percent of the genome. Neutralization properties are probably intrinsic to both the hexon and the fibre, which together represent 15 percent of the genome (Kasel, 1980).

B. **Characterization of viral DNA**

There are other properties of the virus that can be exploited both for identification of an isolate as an adenovirus and for subgroup typing. Characterization of viral DNA both by hybridization and by restriction endonuclease digestion pattern has been utilized for clinical isolates (Wadel et al., 1980; Sujuki et al., 1981; DeJong et al., 1983; Kemp, 1983). In contrast to the limited regions of genome analyzed by either HAI or SNT, restriction endonucleases recognize and cut the DNA at specific sequences found throughout the genome. More sensitive methods to identify the adenovirus is DNA-DNA hybridization which is employed after Southern Blotting of the DNA restriction digests (Suzuki et al., 1981). This procedure uses small numbers of cells and adenovirus specific radioactive DNA probes. However, this procedure is tedious for routine clinical use. The more modified form of DNA-DNA hybridization technique named dot-blot has simplified the procedure considerably. A PCR method for identification of human adenoviruses and their subtyping has recently been reported (Kidd et al., 1996).
C. **Direct microscopy**

Rapid detection by direct examination of the cytopathology of exfoliated cells has not been generally successful. It has been reported that detection of adenovirus infected cells by FAT is as sensitive as tissue culture growth (McCormick et al., 1972). However, other investigators using FAT have detected only one-third of the nasopharyngeal secretions from which adenoviruses subsequently grow in tissue culture (Collier et al., 1966). The FAT has not been useful in early diagnosis of conjunctival adenovirus lesions but may be more useful in studies of exfoliated bladder cells from patients with acute haemorrhagic cystitis (Belshe and Mufson, 1974).

D. **Electron microscopy**

The electron microscopy (EM) of clinical specimens has been utilized in special situations to rapidly and specifically identify viral agents. The discovery of the non-cultivable adenoviruses was made by EM examination of stool extracts (Flewett et al., 1975). Immuno-electron microscopy for rapid diagnosis of respiratory infection was found successful in 19 of 25 cases, throat swabs from which yielded adenoviruses in tissue culture (Edward et al., 1975). Although, less sensitive than tissue culture growth, the EM technique is rapid.

E. **Monoclonal antibodies**

Monoclonal antibodies reactive to enteric adenoviruses of sub-genus F were generated and evaluated as typing reagents in virus neutralization tests and ELISA. Two monoclonal antibodies, 40-1 and 41-1 were found specific for virus types 40 and 41,
respectively. A third monoclonal antibody reacted with all 47 human adenovirus types in ELISA (DeJong et al., 1993).

Epitope composition of human adenovirus serotype 13 (sub-genus A), 19,26 and 27 (sub-genus D) as well as 41 (sub-genus F) was studied with 23 selected monoclonal antibodies raised against different hexon types. With the help of three panels of monoclonal antibodies great differences were shown in the epitope structure of sub-genus D hexons. Comprising two adenovirus 13 strain hexons, it was shown that two epitopes recognized by monoclonal antibodies were present in different combinations even on the strains of same serotype (Adam et al., 1992). The feasibility of using monoclonal antibodies to capture specific viral polypeptides in double monoclonal antibody sandwich ELISA has been demonstrated. The monoclonal antibodies with different reactivity patterns were used for antigen capture and four of these were labeled with horse radish peroxidase enzyme and used for the detection of specifically bound hexon antigen and for the determination of the lowest reacting hexon concentration. Two combinations with monoclonal antibodies 1A3/1A3 and H12/1A3 were most sensitive for the detection of adenovirus hexons (Adam et al., 1986).

Recently, using type specific monoclonal antibodies, ELISA has been developed for direct identification of enteric adenoviruses. The test appears to be specific and rapid (Nishio et al., 1990).
MATERIALS AND METHODS

3.1 Animals

Male New Zealand White rabbits weighing 1 to 1.2 kgs, young guinea pigs and inbred Balb/C mice weighing 18-20 gms were obtained from Small Animal Facility of DRDE.

3.2 Viruses

Human adenovirus types 1-5, 7 and 8 were procured from National Institute of Virology, Pune. Polio, Coxsackie A 9 and ECHO type -4 viruses were obtained from American Type Culture Collection (ATCC), USA.

3.3 Clinical specimen

For investigation of adenovirus infection, 54 children of age group 2 months to 12 years suffering from acute respiratory disease (ARD) admitted in the local G.R. Medical College Hospital, Gwalior, were studied. The general clinical symptoms in these patients were high fever, coryza, cough, congestion and pneumonia. Throat swab samples from these patients were collected in transport media for processing in the laboratory. Collection of samples was done during winter months of September to December.

Thirty-two sera samples from same group of children admitted in the wards with ARD, 36 sera samples from acute febrile cases of respiratory illnesses in adults (20-40 yrs age group), from Military Hospital, Gwalior and 76 sera from apparently healthy controls were tested for the presence of antibodies to hexon antigen.
3.4 Cell lines

Human epithelial cell lines viz. HeLa, KB and HEp2 were obtained from National Centre for Cell Studies (NCCS), Pune for cultivation of adenoviruses from standard stocks and also for isolation of the viruses from ARD cases. Buffalo green monkey kidney cell line (BGM) maintained in our laboratory was utilized for preparing the viral antigens of Polio, Coxsackie A 9 and ECHO type 4 viruses.

Murine myeloma cell line Sp2/0 was obtained from National Institute of Immunology, New Delhi. The cell line was utilized as fusion partner in hybridoma work.

3.5 Media

The human cell lines and the BGM were maintained in the laboratory using growth media containing Eagle's minimal essential media with non-essential amino acids (MEM) supplemented with L-glutamine and foetal bovine serum in addition to antibiotic and antimycotic substances. For sub-culturing of cell lines trypsin-versene solution was used. Freezing mixture medium was prepared using growth media with high concentration of foetal bovine serum and by adding dimethyl sulphoxide as a cryo-preservative. Viruses were propagated in supplemented MEM with low concentration foetal bovine serum.

Transport media contained Hank's balanced salt solution and gelatin with antibacterial and antymycotic substances.

For checking sterility of all the media nutrient broth was used. Composition of all the media employed for virus work in given in Appendix I.
For hybridoma work, Iscove's modified Dulbecco's medium was utilized alongside hybridoma grade foetal calf serum and other supplements including HAT and HT media. For freezing of hybridoma cell lines the same basal media was used. The composition of media employed for hybridoma work is given in Appendix II.

3.6 Buffers and Solutions

Phosphate buffer saline (PBS), 0.1M and 0.5M with pH 7.3 were prepared for purification of hexon antigens. Plain PBS, 1.0M with pH 7.2, with Tween-20 and bovine serum albumin or defatted spray dried powdered milk; Carbonate-bicarbonate buffer, pH 9.6; citrate phosphate buffer, pH 5.1 were used in enzyme immunoassays. Glycine saline buffer, 0.054M, pH 8.8 was utilized for latex agglutination test. For counter-current immunoelectrophoresis, veronal buffer 0.075M, pH 8.6 and normal saline solution (NSS) were utilized. Tris buffer, 0.125M, pH 6.8 and 0375M, pH 8.8; tris glycine buffer pH 8.8 (electrophoresis buffer) and with methanol (blotting buffer) were prepared for SDS-PAGE. The composition of the buffers is presented in Appendix III.

3.7 Gels

Brushite form of calcium phosphate gel was used for purification of hexon antigen. Agarose gels were used in gel precipitation tests. Ten percent polyacrylamide gels (discontinuous) both stacking and separating were used in SDS-PAGE. Composition of various gels is given in Appendix IV.
3.8 Propagation of cell lines for virus cultivation

HeLa, HEp2, KB and BGM cell lines were maintained in growth media in milk dilution bottles and incubated at 37°C with regular sub-culturing or were preserved in liquid nitrogen as per the method described by Schmidt (1979). At the growth of 2-3 million cells per bottle, subculturing was done at a split ratio of 1:3 using trypsin versene solution. The cells were suspended in growth media and distributed either in fresh milk dilution bottles or Leighton tubes (L-tubes).

3.9 Preparation of viral antigens

For the preparation of adenoviral antigens, HEp2, HeLa and KB cell lines were used and for the preparation of enteroviral antigens (Polio, Coxsackie A 9 and ECHO type 4) BGM cell line was used. Before inoculating the cell lines with the appropriate viruses, the cells were washed with serum free growth media. The adenovirus types 1-5, 7 and 8 were allowed to adsorb to the washed cells for 6 hrs at 37°C and incubated with serum free growth medium at same temperature. The enteroviruses were inoculated and adsorbed for 1 hr and further incubated in serum free growth medium as per the method of Schmidt (1979). Cell were observed daily for CPE. Forty-eight hours after the observation of 4+ CPE, the infected cells were harvested by six cycles of freezing and thawing using liquid nitrogen. This was followed by cold centrifugation at 5000 rpm in Sorvall RC 5C for 20 minutes to remove the cell debris. The supernatant fluid served as crude viral antigen preparation. Control cell line tissue antigens without virus inoculations were prepared under identical conditions.
3.10 **Titration of adenoviruses in HEp2 monolayer cultures**

The 50 per cent tissue culture infectious dose end points (TCID₅₀) of adenoviruses were determined in HEp2 cell line according to the method of Schmidt (1979). Each adenoviral type was logarithmically diluted in maintenance media and inoculated in a volume of 0.2ml to a set of four HEp2 monolayers in L-tubes. The inoculated cultures were incubated at 37°C and observed microscopically at 24 hr intervals over a period of seven days. When the cells in inoculated tubes showed viral CPE, it was considered positive and the TCID₅₀ end points were calculated by the method of Reed and Muench (1938).

3.11 **Adsorption of adenoviruses**

The 0.5 gms of bentonite particles were blended in 100 ml distilled water in a blender twice for one minute each with a gap of five minutes. This suspension was diluted to make the volume upto 500 ml with distilled water and incubated for one hr at room temperature with thorough shaking and then the particles were allowed to settle. The supernatant was decanted and centrifuged at 500 g for 15 minutes. The pellet obtained was removed. The supernatant thus obtained was again centrifuged at 750 g for 15 minutes. The pellet was collected and resuspended in 100 ml distilled water. This stock was blended for one minute and five per cent thymol in ethyl alcohol was added as preservative and the stock kept stored at 4°C.

To the sensitized particle one ml of the rabbit antisera to HEp 2 lysate was allowed to adsorb for two hrs at room temperature with in between mixing followed by overnight incubation at 4°C. Next day the unbound material was removed by
centrifugation at 750 g for ten minutes. To the pellets obtained, crude viral antigens of adenovirus types 3, 5 and 7 were added. The adsorption was carried out for two hrs at room temperature with in between mixing followed by overnight incubation at 4°C. Unadsorbed viral preparations were removed by centrifugation at 750 g for ten minutes. The pellets obtained were discarded and the supernatants containing the adsorbed viruses were collected and tested by immunoassays.

3.12 Purification of hexon antigen

Crude viral harvests of adenovirus type 5 were treated with 0.05 per cent sodium dodecyl sulphate (SDS) and dialyzed for 24 hrs against several changes of 0.1M PBS, pH 7.3. Twenty-five volumes of SDS treated virus harvests were mixed with one volume of packed gel of Brushite form of calcium phosphate and adsorbed for one hr at room temperature. The gel was then exhaustively washed in 0.1M PBS, pH 7.3. The hexon antigen was eluted with six volumes 0.5M PBS, pH 7.3. The final product was dialysed against NSS for 18 hrs at 4°C. The antigen was concentrated using membrane filter cones (Amicon) of 50,000 mol. wt. cut off.

3.13 Protein estimation of viral antigens

Protein contents of all crude viral antigens, adsorbed viral antigens and the purified hexon antigen were determined following the standard procedure of Lowry's et al. (1951).
3.14 Sodium Dodecyl sulphate polyacrylamide Gel electrophoresis

The HEP2 cell culture adsorbed viral preparation adenovirus 3,5,7 and 8, the crude viral antigen preparation of adenovirus 2 along with purified hexon antigen and all the three enteroviruses (Polio, Coxsackie A 9 and ECHO type 4) were resolved by applying the SDS-PAGE technique of Laemmli (1970). These viral antigen preparations were mixed in the SDS-PAGE sample buffer (2x) in equal volumes. Samples were boiled in water bath for five mins.

The ten per cent separating and 3.6 per cent stacking gel solutions were prepared according to the recipe given in appendix IV. The stacking gel solution was allowed to polymerize evenly over ten per cent acrylamide separating gel, 10 cm by 10 cm by 1.0 mm in dimension. An aliquot of ten μl of solubilized samples containing nearly 2mg/ml of protein were applied to wells.

Electrophoresis was performed in Hoefer's mini gel apparatus containing electrode buffer, pH 8.8. Standard molecular weight markers (Sigma) were included for measuring the molecular weights of polypeptides. The gels were run at a constant current of 15 mA per gel until the bromophenol blue marker had reached the bottom.

Gels were stained with 0.25 per cent (wt/vol) coomassie brilliant blue R (Sigma) in methanol-acetic acid-water mixture overnight. The gels were destained with same solution mixture without the dye. Destaining was carried out for 12 hrs with three to four changes and till the stained bands were clearly visible.
3.15 Preparation of hyperimmune sera

Adult rabbits and young guinea pigs were used to raise hyperimmune sera to adenovirus group specific hexon antigen. Antigen at the dose of 100 µg protein was administered in Freund's complete adjuvant (FCA) by intra-muscular route. After seven days, the animals were given another dose of the same amount of antigen in Freund's incomplete antigen (IFA) intramuscularly. A booster injection of the same amount in IFA was again given after an interval of seven days. Blood samples were collected prior to immunization and two weeks after the booster inoculation. The antibody response of the hyperimmune sera was analyzed by counter-current immunoelectrophoresis (CIE) and titrated by NT and indirect ELISA.

A. Counter current immunoelectrophoresis

Wells were punched off at a distance of five mm in agarose (0.8 per cent in veronal buffer) coated on grease free clean slides. Wells towards cathode were filled with crude and adsorbed viral antigens of adenoviral types 3,5,7 and 8, purified hexon antigen and control tissue culture antigen. Hyperimmune sera raised in rabbits or guinea pigs against hexon antigen was added in the wells towards anode. CIE was carried out in an anodic current of 20 mA for 45 minutes using veronal buffer as per the method of Graber and Williams, 1953. After this the slides were incubated at room temperature overnight and washed with NSS before staining with Coomassie brilliant blue stain.

B. Neutralization test

The hyperimmune sera of hexon antigen raised in rabbits was titrated by NT using a constant virus varying serum technique. Antibody titre was expressed as the
highest serum dilution which neutralize the test dose of the virus (Schmidt, 1979).

Serial dilutions of the serum prepared in maintenance media were mixed with adenovirus type 5 and the three enteroviruses containing 100 TCID₅₀ doses in equal volumes and incubated at 37°C for one hr.

For 'virus control', the adenovirus type 5 and the three enteroviruses at 100 TCID₅₀ were mixed with an equal volume of maintenance media and incubated under the same conditions as the serum-virus mixtures. The 100 TCID₅₀ doses of the viruses were calculated according to the method of Reed and Muench, 1938. After the incubation period, the serum-virus mixtures and virus controls were inoculated in a volume of 0.2ml onto the monolayer L-tube cultures of HEp2 cell line for adenovirus type 5 and BGM cell line for enteroviruses. A set of four tubes were employed for each serum-virus mixture and virus control. Uninoculated cells were kept as tissue culture controls. The inoculated cultures were incubated at 37°C and examined microscopically daily for the ability of the serum to inhibit the CPE of the virus. Final readings were made when the virus controls showed the presence of 100 TCID₅₀ in the test.

C. Indirect ELISA

Flat bottomed polystyrene microtitre plates (Dynatech labs, USA) were coated with ten µg purified hexon antigen in 50 µls of carbonate bicarbonate buffer, pH 9.6 per well and incubated overnight at 4°C. Plates were rinsed in PBS and 200 µl of blocking solution containing one per cent BSA in PBS were added to each well and plates further incubated at 4°C overnight. Again the plates were rinsed in PBS, then serial dilutions of rabbit hyperimmune sera against hexon antigen were added in duplicate wells and
incubation carried out at 37°C for one hr in a humid chamber. Washing of the wells was carried out in PBS-Tween 20 for 15 minutes with five to six cycles. Goat anti-rabbit peroxidase conjugate (Dakopatts) in the dilution of 1:1000 was then added with 50 μls volume per well and plates incubated in moist chamber at 37°C for another one hr. Subsequent washing of the wells was done as before and the reaction was developed by adding 4mg/10ml of orthophenylene-diamine hydrochloride (Sigma) and 2ul/ml of hydrogen peroxide in citrate phosphate buffer, pH 5.1, 50 μls per well. After 15 minutes the reaction was stopped by adding 25 μl per well of 2M sulphuric acid. The absorbance of yellowish brown color was measured at 490 nm in a ELISA reader (Dynatech).

For detection of antibodies to hexon antigen in patient's sera and controls, indirect ELISA was performed in a similar way. The conjugate used for this purpose was Goat anti-human polyvalent HRP (Sigma) in 1:1000 dilution. Dot ELISA test was also performed on patient sera using the same conjugate but 3,3'-diaminobenzidine dihydrochloride as substrate for colour development.

3.16 Virus isolation

Throat swab samples were collected in transport media and stored at -20°C till used. To the samples, antibiotic and antimycotic solution were added in one ml volume and incubated at 4° for 72 hrs. Following centrifugation at 5000 rpm for 20 minutes, the treated samples were inoculated in 0.2ml volume onto the monolayers of HEp2 in L-tubes and incubated at 37°C according to the method of Schmidt (1979). A set of four tubes was used for each sample and another set of four tubes without sample inoculation was kept as controls. Inoculated cell cultures were examined
microscopically for CPE each day up to ten days post inoculation. This was designated as passage one. Samples were then blindly passaged in the similar way irrespective of CPE up to passage two level. Samples exhibiting CPE at passage two level were further subcultured for virus propagation in the same cell line. The samples not showing CPE were regarded as negative for virus isolation and discarded. Extraction of viruses from infected cell line was done by six cycles of freezing and thawing in liquid nitrogen followed by cold centrifugation at 5000 rpm for 20 minutes to remove the cell debris.

3.17 Generation of monoclonal antibodies

A. Immunization

BALB/c mice were immunized intraperitoneally with 20 μg of adsorbed adenovirus type 5 mixed with FCA. At weekly intervals two more doses of this antigen were given in IFA. Three days prior to fusion immunized mice were given two injections of the plain adsorbed antigen intraperitoneally.

B. Fusion protocol

The immunized mouse was sacrificed by cervical dislocation and spleen was removed aseptically. Procedure for the fusion was followed as reported by Kohler and Milstein (1975) with minor modifications. The spleen was placed in small sterile petridish containing ten ml DMEM and gently teased to release cells into the medium. The spleen cells suspension was centrifuged in a sterile tube at 1000 rpm for seven mins. The cell pellet was suspended in five ml ammonium chloride (0.17M in 10 mM tris, pH 7.2). The tube was placed on ice for ten mins to lyse the red cells. The suspension
was centrifuged again at 1000 rpm for seven mins and cell pellet was resuspended in ten ml DMEM. Lymphocytes were counted using haemocytometer.

Mouse myeloma cell line SP2/0 in the log phase growth was harvested and centrifuged at 1000 rpm for seven mins and suspended in ten ml of DMEM. Cells were counted using haemocytometer and mixed with spleen cell suspension in 1:2 ratio in 50 ml centrifuge tube. Cell suspension was spun at 1000 rpm for seven mins, supernatant was discarded and the pellet was dislodged. One ml of 50 per cent polyethylene glycol (1300-1600 mol wt obtained from Sigma) solution, hybridoma grade, was added drop by drop over the cells for a period of one min and the cell suspension was mixed gently for another one min. The tube was left undisturbed for two min. Then five ml of HAT medium was added slowly to the tube for a period of five mins with gentle shaking of the tube and finally 50 ml of HAT medium was added. Following this, the cell suspension was distributed, 200 µls per well into 96 well flat bottom sterile tissue culture plates (Nunc labs). The plates were kept in humidified five per cent CO₂ incubator at 37°C. After 24 hrs of fusion, medium of wells was changed with fresh HAT medium. The HAT medium in the wells was changed every three to four days. The individual wells of the plates were daily monitored using an inverted microscope (Nikon). The wells containing hybrid clones were marked. Screening of these wells was continued until growth of the clones reached appropriate size covering nearly one-third of the well surface. Supernatant of these hybrid containing wells was collected aseptically and replaced by fresh medium. The collected supernatant was tested on the same day by plate ELISA for the presence of antibodies to hexon antigen. The reactive
wells were retested after few days and the consistently positive wells were subjected to limiting dilution cloning.

C. Cloning of hybrids by limiting dilution procedure

Cloning was done by limiting dilution. The principle of this method is to adjust the cell concentration in the medium in such a way that upon distribution, statistically, every alternate well in the plate contains only one viable cell. For this the cells in the reactive wells were gently disturbed using sterile Pasteur pipette to obtain single cell suspension. Live cell count of this suspension was done by trypan blue dye exclusion test using haemocytometer. The cell count in the fresh HAT medium was calculated and adjusted to one cell per 400 μls. To each well of a new 96-well tissue culture plate, 200 μls of this cell suspension was added. Spleen cells from an unimmunised mouse were used as feeder cells at a concentration of 10^5 cells/well. Wells were monitored daily after three to four days of limiting dilution cloning and single cell containing wells were marked in each plate. Further replacement of fresh media was done only in the marked wells every three to four days. Once the sufficient growth of cells in these wells was obtained, the supernate was collected aseptically and tested for antibodies to hexon antigen by plate ELISA. Plates were always incubated in a humidified CO₂ incubator maintained at five per cent CO₂ level. Reactive wells were recloned twice similarly to get stable antibody secreting hybridomas.

D. Maintenance of hybridomas

After third subcloning, the clones were transferred to 12 well plates in HAT medium with ten per cent FCS, followed by transfer of these expanded clones to HT
medium and finally to DMEM with ten per cent FCS. For large amount of antibodies the stable clones were transferred to culture flasks with DMEM with ten per cen FCS. Cells were routinely screened for monoclonal antibody production and were also frozen.

E. **Freezing and thawing of cells**

Cells were suspended at a density of 2-5X10⁹/ml in a medium containing 20 per cent FCS and 80 per cent dimethyl sulfoxide (DMSO) and one ml aliquots dispensed in two ml cryostat ampoules were frozen at -70°C over night and then transferred to liquid nitrogen. For thawing, the ampoules were removed from liquid nitrogen and DMEM prewarmed to 37°C was added. Cells were washed twice with DMEM, suspended in culture flasks with medium containing 20 per cent FCS. Feeder cells were added and incubated at 37°C in a CO₂ incubator.

F. **Production of monoclonal antibodies in ascites**

Antibodies were produced in bulk by growing the cells in the peritoneal cavity of pristane primed mice. BALB/c inbred mice were injected intraperitoneally with 0.5 ml of pristane (2,4,10,14- tetramethyl pentadecane) (Sigma). After seven days, three to four million hybrid cells were injected intraperitoneally into these mice. Ascitic fluid was obtained after about ten days by tapping intraperitoneal cavity and fluid was collected with help of 16 gauge needle. Ascitic fluid obtained was rendered cell free by centrifugation at 1000 rpm. The supernatant was further centrifuged at 10,000 rpm to remove debris.
G.  *Delipidification of ascites*

The ascitic fluid was delipidified using organic solvents; four parts of diethyl ether were mixed with one part of butanol. The ascitic fluid was mixed with an equal amount of the mixture of organic solvents and incubated at room temperature for one hour. After centrifugation at 8000 rpm for 20 mins., lower phase containing ascitic fluid devoid of lipids, was carefully aspirated without disturbing lipid layer at the interface of organic and aqueous layers.

3.18  **Characterization of monoclonal antibodies**

*Isotyping*

Immunoglobulin class of Moabs was determined by plate ELISA using HRP labeled goat antimouse IgG, IgA, IgM conjugates (Sigma). The procedure followed was nearly the same as described for indirect ELISA.

3.19  **Specificity testing of monoclonal antibodies**

Specificity of monoclonal antibodies to hexon antigen of adenoviurs 5 was assessed using different viral preparations of adenovirus 2,3,5,7 and 8; Polio virus, Coxsackie virus A 9 and ECHO type 4 virus by performing both the Western blotting and dot ELISA tests.

A.  **Western blotting**

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets (0.45μm pore size, Sigma) was performed as described by Towbin and Gordon (1984) using tris-glycine buffer, pH 8.3, containing 20 per cent methanol. Transfer was affected with a constant voltage of 45 volts for one hour in a transblot cell (Hoefer
transfer apparatus). The free protein sites of nitrocellulose blots were blocked by incubating in five per cent fat free milk powder in PBS, pH 7.4 overnight at 4°C. Blots were washed three times with PBST for ten mins each with shaking. Blots were incubated with three monoclonal antibodies to hexon antigen individually, for one hour at 37°C. Following washing of blots in PBST for 20-30 minutes with five to six changes, incubation of the blots was carried out either with Goat antirabbit polyvalent HRP conjugate (Dakopatt labs), 1:2000 dilution in PBS, or with goat anti-mouse polyvalent HRP conjugate (Sigma), 1:1000 at 37°C for one hr. After 30 minutes of washing in PBST, the blots were developed with diaminobenzidine dihydrochloride (Sigma) and hydrogen peroxide in PBS, pH 7.2.

B. Dot ELISA

Crude viral preparations from clinical samples, adenoviruses, purified hexon antigen and the three enteroviruses were tested by dot ELISA using one of the monoclonal antibodies (ADV-1) previously characterized by Western blotting. Two µls each of the viral antigen preparations was coated onto the centre of nitrocellulose tips on six or 12 projection combs (Sunchem labs, Gwalior). The coated combs were incubated for 30 minutes at 37°C and then blocked with five per cent defatted powder milk in PBS overnight at refrigeration temperature. Combs were rinsed in PBS and dipped in the culture supernatant of monoclonal antibody producing cell line (ADV-1). Incubation was carried out for one hr at 37°C in a humid chamber. Washing was done using PBST for 15 minutes with five to six changes. Goat anti-mouse polyvalent HRP conjugate (Sigma) in the dilution of 1:1000 was added into the wells of microtitre plate and the combs dipped in these wells for one hr at 37°C. Washing was again done in
PBST for 20-30 minutes with five to six changes and the color developed using 3,3'-diaminobenzidine dihydrochloride and hydrogen peroxide in PBS, pH 7.2. Appearance of a colored dot in the centre of nitrocellulose tip of the combs was considered positive for adenovirus hexon antigen.

3.20 Identification of viral isolates by hyperimmune sera

A. Neutralization Test

The virus isolates were identified by conventional NT. The test procedure followed for NT was nearly similar to the one described for titration of hyperimmune sera. For NT, the hyperimmune sera raised in rabbits against hexon antigen was used to neutralize the infecting capacity of the viral isolates by preventing the appearance of CPE using a constant serum-varying virus procedure according to the method described by Schmidt (1979). Dilution of hyperimmune sera used for this procedure was 1:1000 and two ten-fold dilutions (1:10 and 1:100) of each virus isolate were tested. Incubation of this virus-serum mixture was done for one hr at room temperature. Each virus-serum mixture was inoculated in a volume of 0.2 ml in a set of four L-tube HEp2 monolayer cultures. For virus isolate control, each dilution of the isolate was mixed with an equal volume of maintenance media, incubated like that of serum-virus mixture and inoculated in 0.2 ml volume onto a set of four HEp2 monolayer cultures. A set of four uninoculated HEp2 monolayer cultures in L-tubes served as tissue culture controls. The inoculated and uninoculated cultures were incubated at 37°C and examined microscopically daily for the ability of the serum to inhibit the CPE of the virus. Final observations were made till the appearance of CPE generated by the virus isolate controls.
B. **Coagglutination Test**

Procedure for coagglutination test using coloured *Staphylococcus aureus* organisms was essentially the same as described by Batra et al. (1987). Briefly, the *S. aureus* Cowan I strain obtained from Christian Medical College, Vellore was grown in trypticase soya broth supplemented with peptone and yeast extract. The culture flasks were incubated in a shaker water bath at 37°C for 12 hrs. The organisms were pelleted by centrifugation at 3000 g for 15 minutes and were given three washings in 0.01M PBS, pH 7.2. The finally washed pellet was suspended in PBS to five per cent volume/volume. Formalin was added to it at a concentration of two per cent and cells were kept at 4°C overnight. The cells were again washed with PBS twice and suspended in PBS to five per cent volume/volume. The suspension was heated at 80°C in a water bath for five minutes followed by rapid cooling. The *S. aureus* suspension thus obtained was stained with Giemsa stain at 1:20 dilution for six to eight hrs at 4°C. Stained cells were washed with PBS five times to remove unbound stain and adjusted to five per cent volume/volume in PBS. For adequate coating, the optimal volume of serum was determined by adding varied amounts of pooled rabbit hyperimmune sera against hexon antigen (NT titre 1:1000) to one ml of five per cent coloured cell suspension. The cells were incubated at 37°C for two hrs with intermittent shaking. After this the antibody coated cells were washed clean of unbound antibodies with PBS. The finally washed suspension was readjusted to five per cent. The agglutination activity of the coated cells was assessed by employing different concentrations of hexon antigen. A drop of 40 μls from crude clinical viral isolates, adenoviruses and enteroviruses and was mixed with same volume of coagglutination reagent on a clean glass slide and rotated for three to
five minutes to observe the appearance of agglutination. For reagent control, HEp2 tissue culture antigen preparation was used.

C. **Latex agglutination Test**

Latex beads with particle diameter of 0.885 μm (Sigma), ten per cent wt/vol were used. Fifty μl of these beads were suspended in 1.25 ml of glycine saline buffer, pH 8.8. To this, 20 μl of 1:20 diluted rabbit serum to hexon was added and incubated for one hr at room temperature with intermittent shaking. This was followed by washing the beads in glycine saline buffer by centrifugation at 4000 rpm for 15-20 minutes. The uncoated sites of the beads were blocked with one per cent BSA in glycine saline buffer at room temperature for one hr. A drop of this antibody sensitized latex beads reagent was mixed with a drop of viral preparation on a glass slide. The slide was rotated for five to ten min and observed for agglutination of latex beads. Parallel positive control of hexon antigen and negative control of HEp2 tissue culture antigen were also tested.

3.21 **Identification of viral isolates by monoclonal antibodies**

A. **Monoclonal antibody Sandwich ELISA**

Monoclonal antibodies of IgM class were utilized as revealing antibodies in association with a guinea pig polyclonal antihexon as capture antibodies in a sandwich ELISA system. The 96 well polystyrene ELISA plates (Dynatech Labs) were coated with 1:1000 dilution of guinea pig anti-hexon polyclonal antibodies diluted in carbonate-bicarbonate buffer, pH 9.6 with 100 μls volume per well. The plates were left in refrigerator overnight and blocked with one per cent BSA in PBS and again kept in refrigerator overnight. After blocking, plates were rinsed with PBS and 100 μls of each
viral preparation (clinical samples, standard adenov- and enteroviruses, purified hexon antigen and tissue culture antigen of HEp2 and BGM cell lines) was added in duplicate wells and incubation carried out at 37°C for one hr in a humid chamber. Plates were washed using PBST for five to ten minutes with five to six changes. Culture supernates (100 μls/well) of IgM producing monoclonal antibody (ADV-1) was added for all the viral preparations and plates incubated at 37°C for one hr. Following washing of the plates with PBST, rabbit anti-mouse IgM peroxidase conjugate (Sigma) in 1:1000 dilution was added to the wells, 100 μls/well and incubation carried out for another one hr at 37°C. After thorough washing of wells, color was developed as described earlier.

B. **Monoclonal antibody Dot ELISA**

Tissue culture inoculated all the 54 samples from ARD cases in children were dotted neat in two μl volume each on to the nitrocellulose tip of coombs. Standard adenoviruses and enteroviruses were also dotted. The dot ELISA used was as per the procedure described under specificity testing of monoclonal antibodies.

3.22 **Antibody detection in human serum samples**

A. **Dot ELISA**

Serum samples from 32 ARD cases in children were tested by dot ELISA using tissue culture grown adenoviral antigen of types 3, 4, 5, 7 and 8. Dot ELISA was performed as per the procedure described earlier. All the serum samples were tested in 1:100 dilution. Conjugate employed was rabbit anti-human whole immunoglobulins conjugated to peroxidase enzyme (Sigma) in 1:1000 dilutions.
B. \textit{Plate ELISA}

Procedure followed for plate ELISA was similar to one used for titration of hyperimmune serum. Rabbit anti-human peroxidase conjugate in plate ELISA was used at 1:2000 dilutions.