CHAPTER-IV
DISCUSSION

Adenoviruses are responsible for a wide variety of clinical syndromes in human beings particularly in pediatric group and in immunocompromised hosts. The most common syndrome associated with adenoviruses involves respiratory tract. In infants and young children, ten per cent of all respiratory infections are caused by these organisms with considerable morbidity and mortality. These are among the many agents that take advantage of impaired or destroyed immune system to set up persistent and generalized infections in the immunocompromised hosts. In such conditions adenoviruses have been found associated with case fatality rates as high as 60 per cent in those with pneumonia and 50 per cent in those with hepatitis (Hierholzer, 1992).

Diagnosis of adenovirus infection, conventionally relies on the clinical criteria relevant to the existing epidemiological situation. Confirmation requires isolation and identification of the virus or rise in antibody titre during patient’s convalescent period. Immuno-electron microscopy is helpful in achieving disease diagnosis, but only in diarrhoeal infections. Isolation of virus is relatively easy from the clinical samples except for enteric adenoviruses. However, basic tissue culture facilities are essential. Pathognomonic CPE is observed within three to seven days, the appearance being related to the concentration of the virus in the inoculum. Confirmation of adenovirus is then possible by immunological tests like haemagglutination, haemagglutination inhibition, complement fixation, neutralization, enzyme immunoassays, immuno-fluorescence, DNA hybridization, PCR or electron microscopy. A reliable immunological test for identification is largely dependent on the availability of specific
and sensitive antibody reagent. The disease may be difficult to distinguish from an invasive bacterial infection (Sahler and Wilfert, 1974). The whooping cough syndrome has been associated with adenovirus infection in the absence of *Bordetella pertussis* (Suzuki, 1981). Patients with exudative tonsillitis are clinically indistinguishable from disease caused by group A *Streptococcus* (Ginsberg et al., 1955; Harris et al., 1971). In addition, a considerable number of patients with high WBC count and ESR have no identifiable focus of infection, making it difficult to distinguish the viral infection from the bacterial disease (Ruuskanen et al., 1985). Rapid detection of adenovirus antigen in body fluids is especially valuable in these cases. The present work was initiated with the objectives of developing suitable polyclonal and monoclonal antibodies against adenoviruses with necessary sensitivity and the specificity. Furthermore immunoassays with these reagents were to be evaluated for virus confirmation from the cases of respiratory infections in human beings.

Adenovirus types 1-5,7 and 8 were grown in three cell lines of human epithelial origin (HeLa, KB and HEp2). Among these, HEp2 provided the best growth of the adenoviruses. Log titres with adenovirus types 3,5 and 8 were comparatively higher than the rest of four types (Table 1), but the growth in all the types was sufficient enough to yield viruses for the preparation of antigens. The different adenoviral antigens, namely, the crude preparation, adsorbed viral preparation and the purified hexon antigen were resolved onto discontinuous SDS-PAGE and also tested by CIE using polyclonal antibodies to purified hexon antigen. The hexon antigen could be successfully purified as SDS-PAGE revealed a single band at mol wt of 116 kDa in the purified preparation from among the five structural proteins obtained in the adsorbed
viral antigen preparation (Fig 1). There was no contamination of penton or fibre antigen. Thus, as expected, the polyclonal antisera raised against this purified antigen preparation produced a single line of precipitin band in CIE when tested against purified, adsorbed or crude antigen preparations from the tested adenoviruses (Figs 2a and 2b). The group specificity of the hexon antigen appeared retained when tested by rabbit hyperimmune sera. The growth of three different enteroviruses in BGM was not neutralized by the rabbit hyperimmune sera, whereas adenovirus type 5 was effectively neutralized by this sera (Table 3). Therefore, this polyclonal antibody raised against the purified hexon preparation had its specificity restricted only to adenoviruses and this reagent could be utilized for identification of adenoviruses from CPE positive culture supernatants of samples from ARD cases. This hyperimmune sera had a high titre, 1:1000 in NT and 1:64,000 in indirect ELISA (Table 3 and Fig 3). Similar to NT, this polyclonal antibody reacted in ELISA to purified hexon antigen and adsorbed viral antigen preparations but not to Polio, Coxsackie A 9 and ECHO type 4 viruses, thereby again reconfirming its specificity to adenoviruses (Fig 4).

Following immunization with the adsorbed adenovirus type 5 and selection of clones using purified hexon antigen, eight stable reactive clones were obtained. Of these clones, ADV-1, ADV-3 and ADV-5 had high sensitivity for hexon antigen (Fig 5). There was a differential pattern in reactivity of these clones when tested with various adenovirus types. In dot ELISA as well as Western blotting, ADV-1 and ADV-3 reacted with all the adenoviruses tested, thus showing a group-specificity (Figs 9a and 9b). ADV-3 in addition, showed reactivity at 80 kDa region of the viruses, the region of penton protein (Fig 9b). ADV-5 showed a type specific activity, reacting only
to adenovirus type 5 both in dot ELISA and the Western blotting (Figs 8 and 9c). Monoclonal antibody ADV-5 can be of immense use as a typing reagent for adenoviruses. The epitope for this monoclonal antibody might be in the Epsilon portion of Hexon antigen, the established region of type specificity (Horwitz, 1990). All the three clones did not react to any of the enteroviruses tested (Figs 9a, 9b, 9c).

Epitope composition of human adenovirus serotype 13,19,26,27 and 41 was studied with 23 selected monoclonal antibodies raised against different hexon types by Adam and his associates (1986). Differences were shown in the epitope structure of hexons particularly of subgenus D. The feasibility of using monoclonal antibodies to capture specific viral polypeptides in double monoclonal antibody sandwich ELISA has also been demonstrated. The monoclonal antibodies with different reactivity patterns were used for antigen capture. Two combinations with monoclonal antibodies 1A3/1A3 and H12/1A3 were most sensitive for the detection of adenovirus hexons.

Adenoviruses are common pediatric viral pathogens. They generally produce benign respiratory illness, but can cause severe respiratory disease such as obliterative bronchiolitis and necrotizing pneumonitis that can result in death or long-term pulmonary sequelae (Abzug, 1989). Less commonly, neonates and children infected with adenoviruses have severe extrapulmonary disease as a result of a virus-induced toxin like effect of viral dissemination (Abzug, 1989). Of the 54 hospitalized children with ARD investigated in the present work, adenovirus was found associated in nearly 40 per cent of the patients. Adenoviruses, therefore, are important respiratory pathogens of pediatric group in this region. The only study undertaken earlier in West
Bengal also documented a high rate of adenovirus respiratory infections in children. Adenoviruses accounted for almost half of the heavy virus burden found in throats of children aged two to five years and the commonly found types were 1, 2 and 5 (Hillis, 1973).

The time required for laboratory diagnosis of adenovirus respiratory infections takes very long and invariably exceeds the duration of the illness. Early diagnosis of the infection therefore, becomes important for the timely clinical management. The rabbit polyclonal antisera to purified hexon antigen and the specific monoclonal antibodies to adenoviruses were utilized for the development of rapid and sensitive immunoassays for the detection of adenoviruses from clinical cases of ARD in children.

Colored *Staphylococcus aureus* Cowan I sensitized with rabbit hyperimmune sera to hexon antigen could be used as a simple coagglutination test for detection of adenoviral antigens. Results with this test are obtained within three to five minutes. This test specifically detected all adenoviral types and remained negative with the control enteroviruses. This test was applied on culture supernatant of 31 CPE positive samples from the patients of ARD. The coagglutination test revealed nine of the samples as positive. The same nine cases were also found positive by the conventional confirmatory NT. There were three more NT positive cases among the remaining 22 samples that stayed negative for coagglutination (Table 6). Therefore, it appeared that the coagglutination test had a very good specificity for adenoviral identification (100 per cent) but with a relatively low sensitivity (75 per cent). Interestingly, the latex agglutination test using latex beads sensitized with the rabbit hyperimmune sera to
hexon antigen, showed identical results to those obtained with coagglutination test. Here also the same nine samples from clinical cases showed the positive reaction with the rest remaining negative. Likewise all the three enteroviruses also failed to react in this test as well. Therefore the coagglutination test and the latex agglutination test had the same level of sensitivity and specificity in detecting adenoviruses from tissue culture grown viral isolates from ARD cases.

Thirty-eight out of the 54 samples from the clinical cases showed CPE in HEP2 cell line. The culture supernatant of 54 samples when tested by sandwich ELISA showed 22 being positive (Table 8). Of these 22 cases, 20 exhibited CPE. The two ELISA positive but CPE negative samples could be explained possibly due to a low level of infection in cell line before reaching a visible CPE or could be the false positive detections. NT could be performed on 13 of CPE and ELISA positive samples. Except for one case, rest all were found positive to NT. Of the 32 ELISA negative cases, 14 samples did not exhibit CPE, therefore, NT was not required to be performed on these samples. The remaining 18 ELISA negative but CPE positive cases also stayed negative by NT (Table 10). The sandwich ELISA, therefore, appeared to be a very reliable test for identification of clinical isolates of adenoviruses. The time taken to undertake the ELISA was nearly four to five hrs if the antibody coated and pre-blocked plates were used. Compared to this, NT is very laborious and time consuming test, requiring nearly four to five days to observe the final results. In an effort to further simplify the monoclonal antibody ELISA procedure, direct ELISA on a nitrocellulose matrix (dot ELISA) was standardized. Results obtained with dot ELISA were similar to that obtained with sandwich plate ELISA. Instead of two cases picked up by sandwich plate
ELISA out of the CPE negative samples, dot ELISA detected three as positive (Table 11). Interestingly, dot ELISA also detected the same two sandwich plate ELISA positive cases that were CPE negative. It becomes difficult to comment on the status of these two cases. If these were to be considered false positive detection, how could two different ELISA systems using totally different coating matrix detect the same two cases from among the CPE negative group of 16 samples? If these were adenovirus positive cases, why the CPE was not elicited? There was no scope of NT to be performed on these samples in the absence of CPE. The possibility thus remains that these were cases of low level of infection of adenoviruses in HEp2 cell line. Perhaps electron microscopy could have helped but was not applied in the present work.

Overall dot ELISA like sandwich plate ELISA showed a good reliability for adenovirus identification. The total time taken to perform the dot ELISA was three to four hrs and the test was relatively more simpler and convenient than sandwich ELISA. Detection of adenoviral antigens directly from clinical throat swab samples by monoclonal antibody based sandwich ELISA or dot ELISA was not attempted. However, dot ELISA, if found useful for the testing of clinical samples, would be a preferred test for bedside disease diagnosis in patients. Detection of adenoviruses directly from clinical samples of throat swab, was tried using coagglutination test. Of the eight samples tested, three showed a clear positive result. The same were confirmed by NT. The other two NT positive samples produced a weak coagglutination reaction and the three NT negative cases were clearly negative by coagglutination test. In general the results appeared promising. The stability of the sensitized coagglutination reagent was found to be over nine months at 4°C. Being a plate agglutination test, only
one step testing was required and the appearance of results was faster (five minutes). This could have served as the desired bedside testing system but for its limitation of lower sensitivity. Probably monoclonal antibody dot ELISA could do away with this limitation and merits to be fully explored on large number of cases to serve as the desired field based testing system.

Other workers have also attempted direct detection of adenoviruses from clinical specimens by rapid tests that measure the group specific hexon antigen. Fluorescent antibody tests are fast, convenient, and qualitative; enzyme immunoassays, radio-immunoassays, and time-resolved fluoroimmunoassays are quantitative in measuring the hexon component but are relatively less convenient (Hierholzer, 1992). All these tests can be performed within half a day provided that the laboratory has the expertise to do this testing and the tests being performed as a routine. These tests detect the presence of adenoviruses in the specimens but cannot identify the serotype. For serotyping, the virus must be grown out in cell cultures and typed by haemagglutination inhibition and neutralization tests with hyperimmune type specific and animal antisera (Wadell, 1988; Hierholzer et al., 1991). To show the genome variant groupings and relationships among strains, recent methods of restriction fragment length polymorphism using around six to ten restriction endonucleases has been reported (Hierholzer, 1992). Monoclonal antibody based immunoassays have been described for detection of adenovirus antigens from nasopharyngeal aspirate specimens from respiratory illness, with tissue homogenates from patients with systemic infection and with stool specimens from gastro-intestinal illness (Hierholzer et al., 1987). Monoclonal antibodies reactive to enteric adenoviruses of sub-genus F were generated and evaluated as typing reagents.
in virus neutralization tests and ELISA. One among them reacted with all 47 human adenovirus types in ELISA (DeJong et al., 1993).

The development of monoclonal antibody based radio-immune dot-blot (IDBT) for rapid diagnosis of adenoviral acute keratoconjunctivitis has also been reported. Of the 718 conjunctival specimen tested, the sensitivity and specificity of IDBT were found to be 85.3 percent and 92.2 percent, respectively, when compared with the conventional cell culture isolation technique (Killough et al., 1990).

Recently, using type specific monoclonal antibodies, ELISA has been developed for direct identification of enteric adenoviruses for laboratory diagnosis of acute gastro-enteritis. The test appears to be specific and rapid (Nisho et al., 1990). The type specific monoclonal antibody generated in the present work can also have merit in such conditions.

Commonly used CF serology for antibody responses detects 25 per cent to 80 per cent of the cases and is not the optimal test (Welliver et al., 1983; Ruuskanen et al., 1984; Ruuskanen et al., 1985). The sensitivity of ELISA system was 100-200 fold greater than CF. The ELISA IgG antibody response when tested in paired sera samples was found to correlate with acute adenoviral infections (Roggendorf et al., 1982). In the present study, therefore, antibody detection from cases of ARD in children was undertaken by dot ELISA using tissue culture antigens of adenovirus types, 3, 4, 5, 7 and 8 (Fig 11). Simultaneous to antibody testing, the throat swab samples of the patients were also processed for adenovirus isolation. From twelve of the 32 patients
adenoviruses could be isolated and sera samples from ten of these twelve patients showed presence of antibodies to adenoviruses by dot ELISA (Table 12).

In two cases adenoviruses were recovered from the ARD patients but simultaneous antibodies were not demonstrable by dot ELISA. Probably these were the cases with early infection where antibodies were yet to reach to a level detectable by dot ELISA. In one case, sample no. 4, antibody response to adenovirus type 3 was observed, the throat swab sample of the patient did produce CPE but negative for adenovirus by NT. Interestingly, the same sample was found positive to adenovirus hexon antigen by both monoclonal antibody sandwich ELISA and dot ELISA. There is a possibility that the virus, due to some reason, could not be correctly identified by NT. Or this could be a virus showing extensive crossreactivity to the adenoviruses. Emergence of atypical, intermediate strains and DNA variant types of adenoviruses have been recently reported in literature that complicate the precise identification (Hierholzer, 1992). In 19 of the patients antibody response to adenoviruses by dot ELISA test and also the adenovirus isolation were negative. Overall a good correlation existed between virus isolation and demonstration of antibodies by dot ELISA. Since majority of the patients were from children below five yrs of age, the chances of their encountering the infections were few. These were probably the freshly exposed cases to adenoviruses. It appears that in young children the value of antibody detection test is equally significant to that of virus detection and can be an alternative to the tedious, time consuming and resource intensive tissue culture facility needed for virus isolation and identification. Studies by Ruuskanen et al (1985) on hospitalized children with adenoviral infections have also shown that 93
per cent of patients with positive adenovirus isolation from nasopharyngeal specimens developed serologic response of IgG type when measured by ELISA.

In children with ARD in the present instance, the antibody response was evident to multiple adenoviral types in most of the cases (Table 12). Mixed infection with adenoviruses has frequently been observed (Hillis, 1973). There still exists the possibility that the antibody reactivity picked up by different adenoviral types could be as a result of response to certain group specific antigens. Hexon as well as penton proteins are the known contributors to group specific activity in adenoviruses (Horwitz, 1990).

Serotypes 1,2,5 and 6 predominate in young children and may cause coryza, pharyngitis or occasional severe lower respiratory tract disease (Brandt et al., 1969; Knight and Kasel, 1973). Serotypes 3,4 and 7 are associated with pharyngoconjunctivital fever, influenza syndrome and atypical pneumonia in older children and young adults (Huebner et al., 1954; Parrott et al., 1954; VanderVeen, 1963). In the present investigation, prevalence of antibodies to adenovirus type 3 was seen in maximum number of pediatric ARD cases, followed by adenovirus types 7, 5,8 and 4 (Table 12).

Majority of hospitalized children with adenoviral infection in the present investigation were males. Reports from other parts of the world have documented a preferential occurrence of adenoviral infections in males (Schmitz et al., 1983). A predilection for males was also observed in all species of subgenera B and C, and in type
4 and 19 (Schmitz et al., 1983). In the present work, the types frequently involved were 3, 7, 5, 8 and 4. Types 3 and 7 belong to subgenera B and type 5 to subgenera C.

After obtaining encouraging results of antibody detection by ELISA in children with ARD, acute febrile cases with respiratory illnesses and apparently healthy individuals of adult population were screened for antibodies to purified hexon antigen by plate ELISA. There was a contrasting difference in O.D. values at different dilutions of sera among majority of cases in acute febrile respiratory illness group when compared to healthy subjects (Fig 12). These results are highly suggestive of high incidence of adenoviral respiratory infections even in adult population in this region. In a laboratory animal study, Lengyel et al. (1992) reported latex agglutination test for antibody detection. Latex particles coated with purified hexon, penton or fibre antigens displayed specific agglutination with 21 rabbit immune sera directed against the different antigens and the complete virus of ten human and two animal adenoviral types.