5.0 Discussion
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Preparation and characterization of MAbs</td>
<td>109</td>
</tr>
<tr>
<td>5.2 Standardization of immunodiagnostic tests</td>
<td>113</td>
</tr>
<tr>
<td>5.3 Collection of NPA specimens</td>
<td>122</td>
</tr>
<tr>
<td>5.4 Detection of HRSV from NPA specimen in NIV IF test</td>
<td>123</td>
</tr>
<tr>
<td>5.5 Detection of HRSV from NPA specimens in NIV ELISA</td>
<td>126</td>
</tr>
<tr>
<td>5.6 Data analysis of HRSV positive samples in OPD and IPD</td>
<td>129</td>
</tr>
</tbody>
</table>
5.0 Discussion

Due to significant disease burden caused by HRSV and its epidemic behavior the WHO global programme for vaccine and immunization has stressed the need for extensive surveillance of HRSV in developing countries to understand its epidemiology. This involves rapid diagnosis of HRSV directly from respiratory specimens and its laboratory confirmation. The significant morbidity and mortality associated with HRSV antigen, particularly in high-risk individuals, make specific and rapid diagnosis imperative. The availability to detect viral antigens directly in clinical specimens has improved greatly with the development of immunoassays, using antibodies labeled with fluorescein, radioisotope or enzymes.

Over the past few years several commercial vendors have addressed this need by IF test and EIA assay, which provide results in hours (Bromberg et al., 1987; Krilov et al., 1988; Ahluwalia et al., 1988; Halstead et al., 1990; Kellogg, 1991; Rothbarth et al., 1991; Ramirez et al., 1992; Olsen et al., 1993b). Along with these, molecular techniques are also widely used. Nucleic acid detection methods like RT-PCR are playing an increasingly important role in the detection and typing of HRSV. Additionally, PCR products can be further characterized to study HRSV molecular epidemiology (Paton et al., 1992; Sullender et al., 1993; Gottschalk et al., 1996; Ghildyal et al., 1997; Maitreyi et al., 1999; Rajala et al., 2003). These techniques have allowed clinical laboratories to detect uncultivable, fastidious and slow growing viruses and provide diagnostic information within a clinically relevant time frame. However, there are several disadvantages in using these technologies, especially when these are applied to clinical specimens. These specimens include presence of many potential inhibitors e.g. heme, acidic polysaccharide, nuclease, EDTA, SDS and guanidium HCL have all shown to inhibit PCR (Erm, 1994). Swabs and some transport mechanism may also affect or inhibit PCR. PCR also requires a prerequisite clinical suspicion for all specific organisms in question. Sometimes interpretation of results may be difficult, since the DNA of some organisms persists even when they are nonviable.

Molecular techniques also include direct hybridization based assays. They rely on specific hybridization of a nucleic acid probe to the viral target sequences. However, the analytical sensitivity of these methods is limited; at least 10,000 copies of the target sequence are required. Hence, the clinical utility of this approach is restricted to the applications in which high concentrations of the virus are present; examples

107
include cultured virus or in situ hybridization in infected tissue. Van Dyke and Murphy Corb, (1989) developed RNA-cDNA assay for the detection of HRSV RNA in NPA. The DNA probe corresponding to N gene detected about $8.2 \times 10^5$ PFU of cell free virus from infected cultures of both the HRSV groups but the test when applied to clinical specimens was disappointing when compared to IF, EIA and culture. Sensitivity of the test was 49%, 60% and 73% and specificity was 66%, 81% and 92%.

The final and main disadvantage of this new technology is currently, relatively high cost and availability of sophisticated laboratory for the detection of pathogen. The developing country like India cannot afford these high tech facilities at primary health centers and in small-scale laboratories. Hence, while selecting a system for virus detection, technical simplicity, rapid turnaround time and cost must be considered. IF and EIA tests fulfill these criteria and offer an attractive alternative to the culture and molecular methods. IF remains the most accurate method for the detection of HRSV infection and probably has greater sensitivity than cell culture isolates. EIA, which are nearly as accurate, but require no expert microscopist or special equipments, have definite advantage for diagnostic laboratories in small hospitals.

Earlier, for the detection of HRSV in IF test; use of polyclonal antibody (PAb) was done (McQuillin and Gardener, 1968; Cradock-Watson, 1971; Chao et al., 1979). Similarly, Sarkkinen et al., (1981); Grandien et al., (1985); Hornsleth et al., (1986); Popow-Kraup et al., (1986); Hierholzer et al., (1989) employed PAb in the Ag C ELISA for the detection of HRSV from clinical specimens. However, owing to difficulties in obtaining specific and sensitive anti-sera, the use of this method of diagnosis was limited as variation among the batches of sera makes it difficult to obtain reproducible results.

After the advent of monoclonal antibody technology many groups of workers used HRSV MAbs for the detection of HRSV antigen from clinical specimens in IF test (Bell et al., 1983; Kim et al., 1983; Kao et al., 1984; Pothier et al., 1985; Freymuth et al., 1986; Kadi et al., 1986; Freke et al., 1986; Grandian, 1986; Ray and Minnich, 1987) and in ELISA (Ahaluwalia, 1987; Obert and Beyer, 1988; Hierholzer et al., 1989). Hendry et al., (1985) quantitated specific polypeptides of HRSV in NPA from HRSV-positive infants in MAb based ELISA whereas, Erdman et al., (1990) used MAb based ELISA to detect specific serum IgG, IgM and IgA antibodies to HRSV.

Though the production of MAb consumes much more time and money than the production of conventional immune serum, it has advantages of defined specificity,
homogeneity and reproducibility. Ability to immortalize the monospecific reagents by cryopreservation ensures continuous availability of MAbs that can be used in different test systems. Considering these facts, in this study we developed MAb based in-house IF (NIV IF) and ELISA (NIV ELISA) for the detection of HRSV infection from clinical specimens.

5.1 Preparation and Characterization of MAbs

MAbs (NIV MAbs) were raised against an Indian strain 955879 of group A of HRSV. A total of 29 MAbs were obtained from 9 positive hybridomas. MAbs were then characterized with respect to their reactivity in isotyping ELISA, WB, NT and IF test and used them for the standardization of immunodiagnostic tests i.e. IF and ELISA.

In the preliminary screening, all the MAbs reacted with the homologous strain of HRSV in IF test. In indirect ELISA, titers of MAb PFs were in the range of $10^3$ to $10^4$. Ig subclass determination of these MAbs in isotyping ELISA indicated that except for two IgM antibodies, all the 27 MAbs were of IgG type. In mice four subclasses of IgG i.e. IgG1, IgG2a, IgG2b and IgG3 were observed and in the preparation of MAbs, IgG has been observed to be the predominantly secreted subclass of Ig. Along with the neutralization of virus, IgG plays an important role in clearance of virus in late acute to convalescent phase of disease.

In our study, all the MAbs were directed against proteins of nucleocapsid and phosphoprotein distributed mainly in two IgG subclasses viz, IgG2a and IgG2b. Fourteen MAbs were IgG2a and 13 were IgG2b. Variation in subclass of MAbs with respect to protein specificity was not observed. Though all the N MAbs were of IgG2a type, P MAbs belonged to isotype IgG2a, IgG2b and IgM. Earlier, Perlmutter et al., (1978) reported that murine antibody response to stable proteins and carbohydrates are generally restricted to IgG1 and IgG3 subclass respectively. Similarly, enhanced IgG1 and IgG3 response and lack of IgG2 response to glycoproteins G and F of HRSV was also noted earlier. However, Stott et al., (1987) observed that the antibodies induced by VAC expressing glycoproteins G and F of HRSV in mice were mostly IgG2a and IgG2b and no antibodies of IgA, IgM, IgG1 and IgG3 were detected. They also observed that the titers of antibodies in IP inoculated animals and 28 days post inoculation were significantly higher for IgG2a and IgG2b.
In short, there is no set pattern of isotype profile of antibodies and quality of antibody response. They mainly depend upon many factors such as phase of infection, route of inoculation, nature of protein antigens etc. We used, Mv-1-Lu grown and sucrose cushion purified HRSV at our laboratory as an immunogen and the route of inoculation was IP followed by booster dose given by SC route. This combination yielded most of the MAbs of IgG2a and IgG2b type.

WB analysis indicated that all the MAbs reacted with internal proteins of HRSV. Four MAbs reacted with N protein of MW 43 kd and 25 reacted with P protein of MW 33 kd of HRSV i.e. predominantly against P protein. In the study, two attempts were made to obtain MAbs against HRSV, N MAbs N1-N4 and P MAbs P1-P15 were obtained from one fusion experiment and MAbs P16-P25 from another fusion. Both the times predominantly P MAbs and a very small number of N MAbs were obtained. We could get a possible explanation for this observation in an article by Gerhard et al (1980).

The immune response to the different viral antigens may proceed asynchronously. For instance, the early immune response may involve predominantly B cells committed to the viral surface antigens, whereas the late immune response may be directed more against internal viral proteins, possibly as a result of their release from lysed, virus-infected host cell or presentation on accessory cell surface. In our study, initially immune response against HRSV in mice was very weak hence; we gave multiple doses of HRSV for better immunization of mice. Length of immunization schedule was about 35 days. This could be one of the reasons for obtaining MAbs to an internal protein P. A variety of different factors such as number of cells seeded per initial hybrid culture, immunization and fusion protocol, variation of the time interval between last immunization and sacrifice of mice used as lymphocyte donor and organ source of donated lymphocyte also have effect on protein specificity of hybridomas.

Earlier reports indicate the use of various routes of immunization, different tests for selection and different forms of HRSV antigen for immunization in the preparation of MAbs. However, analysis of these studies does not indicate any correlations of these factors with protein specificities of the MAbs obtained (Cote et al, 1981; Walsh and Hruska 1983; Gimenez et al, 1984; Muñoz et al, 1985; Orvell et al., 1987; Garcia-Barreno et al., 1989).

Gimenez et al (1984) also obtained five MAbs against P protein and only one against M protein of HRSV. They used UV irradiated sucrose cushion-purified strain RSN-2 and also RSN-2 treated with 0.1% SDS and inoculated through IP route in two
different groups of mice. Length of immunization schedule was about 30 days, selection test was cell ELISA using ethanol-acetic acid fixed HRSV infected and uninfected control cells along with indirect ELISA using sucrose cushion-purified RSN-2 strain of HRSV. Hybridomas positive in both these tests were selected. Fixing of infected cells in IF test allows reaction of MAbs to internal proteins hence, they might have selected most of the MAbs against internal proteins P. Similar to Gimenez et al., (1984) we used IP route of immunization using sucrose-cushion purified HRSV and use of indirect ELISA using the same purified HRSV for selection. One report also indicates that P protein is a major phosphorylated species in purified virus and infected cells (Lambert et al., 1988). Hence, the use of purified virus could also be the factor influencing the protein specificity of our MAbs.

The immunization of mice resulted in the development of MAbs mainly against the nucleocapsid complex of HRSV, which consists of close association of N and P protein in the HRSV. Earlier also association of both these proteins have been observed in the preparation of monoclonal antibodies. Gimenez et al (1984) obtained evidence to support the view that VP P (33 kd) was associated with nucleoprotein VP N (41 kd) using a set of MAbs against VPP of HRSV. They observed that some of the P MAbs (MAb 3 and 4) reacted with both VP N41 and VP P32. On densitometer VP P32: VP N41 ratio was 0.36 and 2.99, which concluded that MAbs were specific for VP P32 and immunoprecipitated a macromolecular complex with which VP N41 was associated. Similar findings were reported by Routledge et al., (1985) indicating a group of MAbs immunoprecipitated a 42 kd NP band, together with a heavy band of P protein of 34 kd. Garcia-Bareno and his co-workers (1996) also supported this, they studied association of N and P proteins of HRSV in the nucleocapsid complex and observed that co-expression of N and P was sufficient to induce the formation of N-P complex. They identified a protein region on P protein for the interaction of N and P protein doing deletion mutant analysis of P protein gene. It was observed that C-terminal end of this protein was essential for interacting with N. This was confirmed by finding that anti-P MAb (021/12P) reacting with a 21 residue P protein C-terminal peptide apparently displaced N from N-P complex.

Among the proteins of HRSV, glycoproteins G and F are known to play an important role in the immunization against HRSV. Antibodies produced to G protein neutralized HRSV whereas antibodies to F protein neutralized virus and also inhibited fusion of infected cells. This was observed in the study of Walsh and Hruska, (1983).
They observed complement dependent neutralization by anti-GP 90 MAb and ability to neutralize without complement of anti-F MAbs, however; MAbs to VP 37 and NP 44 were non-neutralizing.

None of N and P MAbs in our study neutralized HRSV. This was consistent with the earlier reports by Walsh and Hruska, (1983), Taylor et al., (1984) and Orvell et al., (1987). They also reported non-neutralizing anti-N and anti-P MAbs and neutralizing activity of only F and G MAbs. In contrast to our findings, Routledge et al., (1985) reported a group of MAbs reacting with internally expressed protein i.e. 34 kd P protein, neutralized virus infectivity with the titer of 256. However, Conners et al., (1991) observed transient neutralizing effect conferred by VAC-N Abs in Balb/C mice. They observed that mice immunized with VAC-N or VAC-P developed RIPA antibodies but these recombinants along with VAC-M2, SH, and M did not develop a significant neutralizing antibody response. Animals receiving VAC-P, SH and M were not resistant to replication of HRSV on day 9 and 28 after challenge. However, animals infected with VAC-N showed low but significant levels of resistance only on day 9. This effect was transient because these mice did not exhibit resistance on day 28. Only mice immunized with VAC-G and VAC-F developed neutralizing antibodies on day 9 and day 28 after challenge, this was comparable with HRSV infected animals.

Since earlier, it has been observed that depending upon specificity to HRSV proteins, MAbs exhibit different patterns of immunofluorescence in HRSV infected cells. Based on this criterion, Cote et al., (1981) grouped HRSV MAbs into two groups depending upon their style of IF staining. 1. MAbs stained cell surface associated antigen. 2. MAbs stained inclusions in cytoplasm. Kim et al., (1983) and Bell et al., (1983) also reported similar findings. Further, Walsh and Hruska, (1983) observed IF pattern of HEp-2 infected cells stained with GP 90 and VP 70 MAbs was expressed on cell surface and MAbs to NP 44 and 37 kd proteins were detected in intracytoplasmic inclusions. Consistent with this, all of our N and P MAbs reacted with small and medium sized cytoplasmic inclusions in HRSV infected cells. None of the MAbs exhibited diffuse cytoplasmic fluorescence. The Chemicon RSV MAb exhibited diffused cytoplasmic fluorescence and also reacted with cytoplasmic inclusions in HRSV infected cells. This is because Chemicon RSV MAb is a mixture of three different MAbs to HRSV. They recognize strains of both groups A and B, and are directed against the fusion protein F, glycoprotein G and nucleoprotein N (Mr. John Hermesman, Chemicon International, USA. Personal communication)
Earlier findings by Garcia et al., (1993b) indicated that nucleoprotein (N), phosphoprotein (P) and 22 kd protein of human HRSV are components of cytoplasmic inclusion bodies observed in HEp-2 infected cells. In addition, co-expression of N and P are sufficient to induce the formation of N and P complexes and generation of cytoplasmic inclusions. Later on, location of the epitopes on P protein sequence recognized by MAbs was co-related with other properties such as antigen binding competition and immunofluorescence staining of HRSV infected cells. In 1993a, Garcia and co-workers mapped 10 epitopes on the P protein of HRSV using MAbs. The antibodies that recognized the epitope on the N-terminal end revealed the presence of cytoplasmic inclusions while others gave mainly a diffuse cytoplasmic staining. Our P MAbs also stained cytoplasmic inclusions in HRSV infected cells suggesting that they recognize N-terminal end of the P protein of HRSV.

5.2 Standardization of immunodiagnostic tests

We developed immunodiagnostic tests i.e. IF (NIV IF) and Ag C ELISA (NIV ELISA) employing these N and P MAbs for the diagnosis of HRSV infection.

5.2.1 Standardization of NIV MAb based IF test: NIV IF

Immunofluorescence assay provides the advantage of being able to determine specimen quality based on the number of exfoliated cells to detect virus, late in the course of illness, to report more rapidly and to reduce laboratory time and expense. The need for viable virus is not applicable to IF. But one limitation of IF is the absolute need for correctly taken NPA, that contains ciliated epithelial cells, the target cells for HRSV replication. IF test remains the most accurate method for the detections of HRSV infection in properly prepared specimens and probably has greater sensitivity than cell culture isolation.

Direct method and indirect method are the two basic methods used for the fluorescent antibody technique. Direct test requires individually conjugated MAbs for the successful diagnosis to be made. We opted for indirect method as panel of MAbs was to be screened using only one anti-species conjugate. One more advantage of indirect test lies in the increase in sensitivity as compared to direct test. The number of fluorescent antibodies combined with antigen in indirect technique can be seen to be several times greater than that combined with antigen in direct techniques. Coons, (1956) assessed the
sensitivity to be 10 times greater in the indirect techniques. Earlier, indirect IF test was used for the staining of cells in nasopharyngeal secretions and proved to be reliable for the identification and the rapid diagnosis of HRSV from NP secretions (Gardner and McQuillin, 1968; Cradock-Watson, 1971).

Initially 1:50, 1:100 and 1:150 dilutions of MAb PF were used as source of detector MAb. At the dilution of 1:50 all the N and P MAbs gave non-specific haze with HRSV infected cells and some with uninfected control cells. At the dilution of 1:100, haze with normal cells was reduced but it was still there with infected cells. However, at the dilution of 1:150 these MAbs gave bright apple green immunofluorescence with HRSV infected cells and uninfected control cells stained red in colour. The reaction was very clear, and nonspecific reaction with uninfected cells was not observed. Hence, 1:150 was considered as an optimal dilution for the standardization of the test. In the same test, Chemicon RSV MAb exhibited bright apple green diffused cytoplasmic fluorescence all over the cells and also stained cytoplasmic inclusions in HRSV infected cells.

During standardization of NIV IF test, an IF test employing strains of both the groups of HRSV were performed. Variation in the IF activity of the MAbs was observed. As compared to reaction of these MAbs with group A strains (+++ to ++++), reaction with group B strains was weak (+ to ++). In the test, N MAb N4 and P MAbs P2, P3, P5, P15, and P17 recognized most of the Indian strains along with homologous strain 955879 and the prototype strains (Long and A2) of group A giving very strong reaction (+++ to ++++), but the reaction of these MAbs with the strains 956055 and 9114497 was very weak (+ to ++). These MAbs also reacted with all the three B strains, B 8/60, B1 and 18537 of HRSV giving slightly weak reaction (+ to ++). However, in the same test these B strains and strain 956055 with Chemicon RSV MAb exhibited satisfactory reaction (+++ to ++++) indicating sufficient growth of these strains in the cells. Earlier, we observed that strain 9114497 never gave clear CPE and never grew in high titers. After completion of 14 days incubation period when the cells were harvested and tested in IF test with Chemicon RSV MAb exhibited very weak (+) fluorescence, which indicated poor growth of this strain in the cells. This could be the reason for none of our MAbs reacting strongly with strain 9114497 (Tables 7 & 8).

The remaining MAbs, i.e. N3, P4, P8, P9, P10, P13 and P20, either exhibited weak reaction or failed to detect some of the strains of both the groups. Some P MAbs P14, P15, P17, P22, and P23 occasionally gave nonspecific haze with uninfected cells. It
has been observed earlier that slides if inadequately rinsed, washed between incubation steps or drying of slides after rinse and wash steps tend to give haze. One more reason is hemolyzed; lipemic or chylous serum may bind nonspecifically to antigen masking specific antigen/antibody reaction. Hence, in order to remove this effect, we used 0.01 M PBS with 0.2% T20 instead of 0.1% T20 as wash buffer in the test. This buffer reduced haze to some extent but did not removed completely. Hence, we short-listed only four MAbs N4, P2, P3 and P5 for further diagnostic work. These MAbs at the dilution of 1:150 of MAb PF detected most of the Indian strains of groups A along with prototype strain A2 and Long and also B strains giving bright apple green immunofluoresence in a range of + to ++++ with no reaction with uninfected control cells.

In further studies we observed that MAbs P2, P3 and P5 did not give reproducible results and sometimes gave nonspecific haze with the infected and uninfected normal cells, hence not included in further diagnostic work. In contrast, MAb N4 specifically detected strains of both the groups A and B of HRSV and did not react with any of the other respiratory viruses. MAb N4 produced bright apple green immunofluoresence in the range of +++ to +++++, punctated with small and medium sized cytoplasmic inclusions with HRSV infected cells. The reaction was reproducible, and easy to interprete. Hence, we decided to use MAb N4 in further diagnostic work employing clinical specimens in IF test.

In addition to testing these MAbs discretely we tested a pool of MAbs, which included MAbs (N4, P2, and P5) short-listed during the standardization of NIV IF test. However, it was observed that reaction of MAb pool at their optimum concentration was equivalent to single MAb N4 or sometimes weak even when tissue culture stocks of HRSV was used. This mixture sometimes also gave haze with some of the cultures in the test. Hence, were not considered further.

5.2.2 Standardization of NIV MAb based Ag C ELISA: NIV ELISA

Over the past few years, ELISA has become the most widely used method for viral antigen detection. Enzyme immunoassays offer an ideal combination of sensitivity, specificity and practicality for detection of viral antigens from clinical samples (Engvall and Perlmann, 1971). In addition to live virus, these assays are able to detect inactivated virus, therefore specimen handling requirements are not as strict as those for culture or IF. Advantages of ELISA include sensitivity generated from amplification effect of enzyme, the potential of quantitative and qualitative immunoassays and choice of
enzymes with diverse physico-chemical properties. In addition, ELISA offers technical simplicity, versatility of batch testing; potential for automation, and short assay time.

In this study we developed Ag C ELISA using indigenously developed MAbs for the detection of HRSV from clinical specimens incorporating biotin-avidin system. Simple nature of biotin labeling allows the labeling of large number of different immunoglobulin molecules in a reproducible fashion. This fact permits evaluation of large number of different antibodies to the target antigen (Yolken et al., 1983). Incorporation of biotin/avidin system into ELISA affords more sensitivity than observed in standard ELISA (Kendall et al., 1983).

Standardization of ELISA was performed in two steps, viz selection of detector antibody (DAb) and selection of capture antibody (CAb).

**Determination of detector antibody (DAb):** For the selection of DAb for NIV ELISA, some high titred MAbs representing different hybrids were screened. IgG fraction of the MAbs was purified and the MAbs yielding higher concentration of IgG were used for biotinylation. This biotinylated MAb (B-MAb) was used as DAb in the ELISA. On this basis, N MAb N4, which was excellent in IF test and three P MAbs P9, P20, P21 were selected for biotinylation. Titration of B-MAbs in indirect ELISA was performed and those giving OD values above 1.0 with high P/N ratios for 1 µg of purified HR5V were considered as suitable for DAb.

As indicated in Table 10, MAb N4 and MAb P21 could not fulfill this criterion, giving OD 0.389 and 0.963 respectively. The latter was near 1.0 but gave a low P/N ratio hence was not considered for further use. Similarly, OD given by MAb P20 was also 1.008 but P/N ratio given by this MAb was low, hence not used further. MAb B-P9 observed to be satisfactory DAb as this MAb gave highest OD 1.2, hence was also considered further in the determination of CAb. Though MAb P4 later proved to efficiently capture HRSV Ag, IgG concentration of this MAb was very low, hence for all practical purposes this MAb was not labeled with biotin and could not be tested as DAb.

**Determination of capture antibody (CAb):** The capacity of each MAb to capture HRSV antigen when they are linked to solid phase was investigated. MAbs were adsorbed to wells of ELISA plate, 1 µg of purified HRSV was then added and the quantity of bound antigen was then estimated by using detector MAb (B-P9), which was reflected in P/N ratio.
Eight MAbs from 6 different hybridomas were coated in ELISA wells, MAb P4 (1:200) and MAb P9 (1:500) could capture 1 µg of purified HRSV efficiently. P/N ratio was 10.40 when MAb P4 was used as CAB and for MAb P9 it was 11.2. Rest of the MAbs could capture the virus but the reaction was very weak giving P/N value 2.0 or slightly higher, hence not used as CAB in NIV ELISA (Table 11).

One important observation was that in indirect ELISA, all these MAbs had very high titers in the range of $10^3$ to $10^4$. However, these MAbs when coated to solid phase could not capture HRSV efficiently that was reflected in lower P/N ratios. These results indicated that reactivity of MAbs with a given antigen differs greatly with the type of ELISA procedure used and also it varies depending on the form in which the antigen is presented. In this study we observed different behavior of the same MAb B-P20 in two different ELISA i.e. indirect and capture ELISA. This MAb during determination of DAb using indirect ELISA gave high OD; the same MAb could not detect HRSV captured by CAB and also this MAb was observed to be weak when used as CAB. In contrast to our results, earlier Dekkar et al., (1989) observed that neotype specific MAbs were incapable of reacting with tobacco mosaic virus (TMVP) when it was immobilized by capturing PAb or MAb. These MAbs were able to bind to TMVP when they themselves were adsorbed on to plastic and used as capturing antibody.

In our study though some antibodies worked better as CAB and some as DAb, MAb P9 efficiently captured HRSV Ag and showed high reactivity with HRSV when used as detector antibody. Similar observation was reported by Obert and Beyer (1988), in their study, of the three MAbs that reacted with N protein, one MAb RSV 22 was able to capture significant amount of purified nucleocapsid antigen when the MAb was immunobilized on the polystyrene microplate, the same MAb when biotin labeled and used as detector antibody bound strongly to nucleocapsid antigen.

Different behavior of our N MAb N4 and P MAbs P4 and P9 in two different tests was observed. Though MAb N4 was excellent in IF test this MAb was not suitable for ELISA as CAB as well as DAB. This MAb may be conformation dependent hence might not have reacted in ELISA. In IF test both the P MAbs reacted with the homologous virus giving very weak reaction and failed to detect some of the strains of HRSV group A and B. In contrast MAb P4 and P9, were found to be suitable CABs in ELISA giving high P/N ratios, and of the two, biotinylated MAb P9, also proved as suitable DAB. It is known that proteins when incubated with plastics, in seeking...
Thermodynamic stability, orient their hydrophobic region towards the adsorptive surface. To achieve their energetically favorable conformation on the surface, they may hide or change the epitope conformation normally expressed and exposed on the surface of protein in the solution. The loss of epitopes or the conformational changes of capture antibodies during immobilization are important factors that affect the sensitivity of immunoassay (Tarcha, 1990). This theory, to some extent may explain the different behavior of the same antibodies in two different assay systems.

In conclusion, in NIV ELISA only P MAb s were used. Different combinations of mix MAb pool were not tested in ELISA. After the two MAb s P4 and P9 were identified as suitable CAb s, only mixture of these two MAb s at their optimum concentration was used as CAb and MAb B-P9 as DAb. However, P/N values given by this mixture were more or less similar to using these MAb s alone.

It is known that to be useful in diagnostic solid phase immunoassay, MAb s must fulfill certain conditions. These epitopes must not be submitted to antigenic variation and directed against a viral polypeptide in large amount in the specimen. Additionally the MAb s employed in ELISA should react with distinct and non-overlapping antigenic region of the polypeptide. We have not determined epitope specificity of these P MAb s. Hence, we did not know whether these MAb s react with the same or different epitopes on the P protein. Though both are anti-P MAb s, they are from two different hybrids and might be reacting with distinct antigenic sites. With this assumption we used mixture of MAb P4 along with MAb P9 for capturing the antigen. This may be helpful when direct field specimen and cell lysates of cultures inoculated with specimens will be screened for the detection of HRSV.

Coating of 3.7 μg/ml of MAb P4 and 1 μg/ml of MAb P9 to ELISA wells resulted in maximum binding of 1 μg of purified HRSV antigen when 1 μg/ml of B-P9 was used as DAb. Earlier Hendry et al., (1985) have reported, with the exception of clone 13-1 all the MAb s at total protein concentration of 2 μg/ml (Ig fraction) as capture antibody resulted in maximum binding of HRSV antigen, and increase in concentration did not further improve this capacity of capturing the Ag. Similarly, in the study by Obert and Beyer (1988) two MAb s RSV 22 and MAb RSV 4 at the concentration of 2.5 μg/ml as CAb and B-RSV 22 and B-NC4 at the concentration of 25 μg/ml and 1.25 μg/ml respectively as DAb could efficiently detect purified nucleocapsid antigen of HRSV giving OD 2.0.
Reactivity of different respiratory viruses in NIV ELISA: After the standardization of NIV ELISA, specificity of the test was assessed employing different respiratory viruses in the test (Table 14). Along with different viral antigens, TCF from various uninfected cell lines, normal egg allantoic fluid and some contaminated cultures were also tested. NIV ELISA specifically detected only the strains of groups A and B of HRSV. No reaction was observed with any of the other viruses, contaminated cultures and with normal antigen employed. In short, NIV ELISA is able to detect specifically all the strains of group A, which included prototype (Long and A2) strains and all Indian isolates of group A and strains B1, 8/60 and 18537 of group B of HRSV. Higher P/N ratios for A strains were observed as compared to P/N ratios for B strains. As the MAbs were developed against group A of HRSV, with this group the P/N ratios were high in the range of 8.05 to 11.93 as compared to group B strains, which was in the range of 4.8 to 5.9. The homologous strain 955879 and strain 9114497 as explained earlier did not grow in high titers, which was reflected in IF and also gave low P/N ratios in NIV ELISA.

Detection of purified HRSV in NIV ELISA: Sensitivity of the ELISA depends on the processing of the antigen and the MAbs used. NIV ELISA detected 0.25 μg of purified HRSV antigen, as this concentration of virus gave P/N ratio 2.28. In our further studies employing clinical specimens, we observed that sensitivity of the ELISA increased when the specimens were sonicated at low amplitude with the detergent T20. Sonication of the purified virus enhanced the detection level, detecting lower quantities of purified HRSV up to 60 ng.

Earlier, using PAb or purified IgG fraction in capture ELISA, different groups of workers detected purified HRSV or purified HRSV proteins in the range of 1 ng to 1 μg (Sarkkinen et al., 1981; Grandien et al., 1985; Hornsleth et al., 1986; Popow-Kraup et al., 1986). In 1988, Obert and Bayer, standardized Biotin-Avidin ELISA employing three MAbs directed against distinct epitopes of HRSV nucleocapsid and observed that, the amount of purified nucleocapsid detected by ELISA varied with the pair of CAb and DAb used in ELISA. It was in a range of 1.25 ng to 40 ng/ml. Minimal sensitivity was achieved when the same MAb RSV 22 was used in both the places and maximum sensitivity was reached when MAb RSV 4 was CAb and biotin conjugated NC4 was DAb.
In our study also MAb P9 has been used in both the places. Mixture of P4 and P9 for capturing the antigen and biotin conjugated MAb P9 as DAb. This combination detected 0.25 μg of purified HRSV and 60 ng of sonicated purified virus. Using pair of MAbs with different protein specificities would have improved sensitivity of the ELISA.

**Determination of HRSV PFU detected in NIV ELISA:** Tissue culture stock of HRSV was titrated simultaneously in NIV ELISA and in tissue culture to determine number of PFU. The graph indicated that, NIV ELISA is able to detect minimal of 10 PFU in a given stock. This sensitivity is comparable to the sensitivity of commercial test kits and some molecular techniques used for the detection of HRSV. Recently Saikusa et al., (2003) used rapid test kit, Directigen Lateral flow RSV (Becton Dickinson and Company). The detection limit of the kit was about 2 x 10^3 PFU/ml for both groups of HRSV. Earlier, Yashio et al., (1996) reported higher sensitivity of RT-PCR than EIA. Their nested PCR detected 0.1 TCID₅₀ as compare to 100 TCID₅₀/ml detected by EIA. Also several reports using molecular techniques such as Light cycle RT-PCR (Whiley et al., 2002), Real Time PCR (Hu et al., 2003), using colorimetric microtiter plate (MTP) PCR system (Tang et al., 1994) reported higher sensitivity of molecular techniques which detected up to 0.01 PFU to 7.00 PFU of HRSV virion.

Mean titer of HRSV present in nasal washes of children hospitalized with acute HRSV infection ranged from 1.3 x 10^5 to 1.0 x 10^6 TCID₅₀/ml (Hall et al., 1975b). Hence, detection of 10 PFU by NIV ELISA will be very useful for the rapid detection of HRSV from clinical specimens obtained not only from hospitalized patients but also from patients attending OPD with URI.

**Comparison of NIV ELISA with ELISA without CAB:** Use of MAbs as a solid phase immunosorbent to measure the viral antigens in unpurified preparation has been reported earlier. The advantage of using MAb for capturing the Ag in the assay is ability of MAb to bind and detect specific antigens in crude preparation with good sensitivity. Comparison of NIV ELISA with ELISA without CAB was done.

Purified preparation of strain 955879 along with crude preparation of 95587 and 955877 strains and uninfected normal cell antigen was used as source of Ag for ELISA. When purified 955879 was used as antigen, the detection level of both ELISAs was same. Both the ELISA detected up to 0.25 μg of purified (non-sonicated) HRSV but variation in P/N ratios with higher P/N ratio in NIV ELISA was observed. In case of unpurified preparation of both the strains, ELISA without CAB could not detect HRSV.
from neat or undiluted stock; giving P/N ratios 1.87 and 1.36 for strains 955877 and 955879 respectively. NIV ELISA detected HRSV from neat stock, giving P/N ratios 8.76 and 7.49 for strains 955877 and 955879 respectively. Higher detection limit for the strain 955877 was observed (up to 1:80 dilution of antigen) as compared to ELISA without CAb, it was up to 1:40. No nonspecific signals were obtained for normal antigen in both the ELISA. In short, the NIV ELISA using CAb was specific and more sensitive for both unpurified and purified viral antigens. Signals were low in ELISA where CAb was not used for capturing the antigen.

Consistent with our reports Hendry et al., (1985) observed low absorbance values when no MAb or heterologous MAb (2Hx-2) was added in capture ELISA for detection of HRSV. Values were in the range of 0.151 to 0.021 for 2Hx 2 and 0.173 to 0.019 when no MAb was added as compared to 1.679 to 0.553 when one of the N MAbs to HRSV was used as CAb. It was 10 times more than no MAb used.

Use of N and P MAbs for diagnosis: Detection of strains of both the groups by NIV IF and NIV ELISA with N and P MAbs respectively is consistent with the earlier reports. In the analysis of antigenic variation among the field strains isolated in Sapporo, Japan during 1980 to 1988 by IF, ELISA and RIP using anti G, F, N and P MAbs raised against Long strain, variation in the reactivity was observed with G and F MAbs of these strains and strains could be grouped into two groups A and B. But all the field strains reacted with all the anti-N and P MAbs indicating conserved nature of these proteins in both the groups (Tsutsumi et al., 1989). Similarly, Orvell et al., (1987) used nucleocapsid MAbs against WV 4843 strain of group B of HRSV for the detection and strain analysis of HRSV. MAbs directed against M, N and P proteins could not distinguish between strains of group A and B. Whereas G MAbs reacted only with the homologous group B. This indicated higher antigenic homology between the nucleocapsid proteins than between envelope components. These studies supported earlier findings of Lambden, (1985). cDNA clone of phosphoprotein gene of Edenberg strain of HRSV when sequenced and compared with sequence of P protein gene of A2 strain, revealed that homology at nucleotide level was 96.38% and at amino acid level it was 99.17%.

Similarly, Johnson and Collins (1989) indicated that N gene to be the most highly conserved between both the groups. They compared sequence of 2330 nucleotide spanning NS2, NS1, N gene and intergenic region of human HRSV strain 18537 of group B and compared with previously reported sequence of strain A2 of group A. It
was observed that NS2, NS1 and N were highly conserved at nucleotide level, 78%, 78% and 86% respectively and at amino acid level 92%, 87% and 96% identity respectively. The high level of sequence identity between N genes of both the groups indicated that N would be the gene of choice for use as hybridization probe for detection of HRSV RNA. Later, Van Dyke and Murphy Corb (1989) used HRSV N gene as a probe in RNA cDNA hybridization assay for the detection of RNA. This probe was chosen because this is a major structural protein of virus and it is abundantly transcribed in infected cells. In addition this gene is relatively conserved between both the antigenic groups of HRSV allowing single probe to detect all strains of the virus.

These reports support our observation of detection of both the groups by N and P MAbs raised against strain of group A of HRSV.


5.3 Collection of NPA specimens

Success of detection of respiratory viruses depends on variables like site of collection and the type of specimens used. TS/NS are more practical to obtain and involve less expense than NPA but they do not access the nasopharynx and therefore, unlike NPA do not provide an optimal specimen for diagnosis of respiratory viruses performing IF test. Earlier reports also indicate that samples of nasopharyngeal secretion recovered by nasal washing or direct aspiration are two to three times more likely to yield HRSV in cell culture than specimens obtained by swab. Halls and Douglas, (1975c) also documented superior virus titer and shorter time for the detection of HRSV in cell culture from NPA, they also reported significantly higher ELISA absorbance values for NPA than the swab specimens. Similarly, Sarkkinen et al., (1981), McIntosh et al., (1982), Trehauf et al., (1985), Cruz et al., (1987), Frayha et al., (1989) also found NPA to be superior specimens.

Ahluwalia et al., (1987) studied paired NPA and NS from 32 hospitalized children with X-ray confirmed pneumonia and bronchiolitis, when tested for HRSV by culture, IF, ELISA and spot hybridization with human genomic probe, sensitivity of all
the four tests was higher for NPA specimen. However, specificity for both the specimens was 100%.

5.4 Detection of HRSV from NPA specimen in NIV IF test

Standardization of IF was done using tissue culture infected with strains of HRSV. As compared to infected tissue culture, the respiratory specimens contain some additional factors such as mucous and BSA, FCS or gelatin from TM. In order to avoid any nonspecific reaction with these factors in the test, standardization of the tests employing NPA specimen was required. Hence, when NPA specimens were employed, some standardization or modification in the test protocol was done.

Initially, after bringing the specimens to the laboratory, NPAs collected in TM were centrifuged; supernatant was taken out, distributed and stored at ~70°C. Pelleted epithelial cells from NPA were used for IF test immediately i.e. all the exfoliated cells from the NPA were taken out and used for IF and only supernatant was used for ELISA. Cell fractions were washed three times with PBS and cells were spotted on to multistep slide. Use of PBS for washing NPA cells has been reported to remove mucous from the specimens (Kadi et al., 1986; Freymuth et al., 1986; Swenson and Kaplan, 1986; Ahluwalia et al., 1987; Van Dyke and Murphy Corb, 1989; Waner et al., 1990; Cubie et al., 1990).

After washing cell fraction with PBS, cells were probed with the MAb N4 at the dilution of 1:150 along with Chemicon RSV MAb and NMS. Chemicon RSV MAb detected HRSV from NPA (024645) exhibiting bright apple green immunofluorescence (++++) in plenty of epithelial cells from NPA in contrast our MAb N4, could not detect HRSV from this NPA. Being N MAb, this MAb reacted with internal proteins of HRSV nucleocapsid, hence washing of mucous from cell fraction of the NPA was very critical for this MAb as compared to Chemicon RSV MAb, which is a mixture of three different MAbs to HRSV. They are directed against two surface proteins fusion protein F, and glycoprotein G and internal protein N of HRSV. This stressed the need of stringent washing of the cells to remove the mucous. Thereafter, for the next lot of specimens, we increased the number of washes with PBS and reduced dilution of MAb N4 to 1:10, 1:20, and 1:50 to probe NPA cells. 1:10 dilution of MAb N4 detected HRSV from NPA cells but the intensity of the IF reaction was up to +++. We considered these specimens as positive for HRSV as the reaction fitted in the CDC criteria.
During the study some of the specimens showed non-specific haze with NMS also, which indicated the presence of mucous in the specimen. In order to remove all the mucous from the specimen and improve the intensity of IF staining we used a mucolytic agent, 0.5% N-acetyl L-cysteine in the washing of cell fraction. Earlier Miller et al., (1986) used this reagent in the treatment of nasopharyngeal specimens, which effectively reduced the nonspecific fluorescence encountered during direct examination of the specimen by IF. This treatment did not affect specific antigen staining or viability of several common respiratory viruses. We also observed a striking increase in the intensity of IF after the use of mucolytic agent.

In conclusion, MAb N4 at 1:10 dilution could detect HRSV from all the OPD and IPD specimens except for one OPD (025917) and one IPD (024645) specimens. This time intensity of IF staining was in a range of +++ to ++++ with few to plenty of infected cells and the results were comparable with the ‘gold standard’ Chemicon IF test. MAb N4, being N MAb reacted with small and large cytoplasmic inclusions in contrast to Chemicon MAb exhibiting diffused cytoplasmic fluorescence and also showed staining of small and large cytoplasmic inclusions in infected epithelial cells from NPA.

One observation was that, number of fluorescent cells per specimen with MAb N4 was comparatively less than that of Chemicon MAb. Consistent with our observation earlier Kao et al., (1984) used F and N MAbs for the rapid detection of HRSV. Both the MAbs had identical sensitivities in IF test but N MAbs stained fewer cells per specimen than anti F MAb. However, in our study total positive specimens and intensity of immuno-fluorescence using both the MAbs N4 and Chemicon HRSV MAb were comparable (Table 19 & 20).

As per Ahluwalia et al., (1987) specimens were considered positive for HRSV if the smears showed greater than or equal to 2+ particulate green cytoplasmic fluorescence in one or more nasopharyngeal epithelial cells at 10X magnification and specimens were considered insufficient if less than 8 to 10 nasopharyngeal epithelial cells were present at 10X magnification.

Of the 134 NPA specimens collected by us during the year 2002, 53 from IPD and 81 from OPD, 23 specimens had insufficient cells and hence could not be tested in IF test. Initially during the standardization of the specimen collection and processing, 25 NPA specimens were tested only with Chemicon IF, and were not tested with the NIV IF (MAb N4). Subsequently 86 specimens were tested in both the Chemicon and NIV IF tests. Of the 26 IPD and 7 OPD specimens diagnosed positive for HRSV in Chemicon IF
test, NIV IF test could detect HRSV from 25 IPD and 5 OPD specimens. OPD specimen 02762 could not be tested with MAb N4, as this was very first OPD NPA specimen collected and number of exfoliated cells was very less.

The two clinical specimens (025917 and 024645) positive by Chemicon assay were negative by NIV IF. But due to scarcity of the specimens the test could not be repeated giving more number of washes with mucolytic agent. Misidentification of these two specimens could be due to difference in sensitivities of Chemicon IF test and NIV IF. As mentioned earlier, Chemicon HRSV MAb is a mixture of MAbs to surface glycoprotein G and F and also to internal protein N of HRSV. However, in NIV IF only one MAb reacting to internal protein N of HRSV was used. Due to which, removal of mucous from the clinical specimens was also very crucial for NIV IF test as compare to Chemicon IF.

We tested some of these NPA specimens with mix MAb pool (N4, P2, and P5) that was used earlier with infected cultures. Of the 41 specimens tested, 46% specimens were positive for HRSV by Chemicon IF test, 39.0% by single MAb N4 and HRSV was satisfactorily detected only in 22% of the specimens using mix MAb pool. The overall reaction exhibited by this mix MAb pool was weak (1+ to 2+) as compared to MAb N4 alone (3+ to 4+). As with the tissue culture this mixture sometimes gave haze with some of NPA specimens. Due to these results given by MAb mix we did not use mix MAb pool in further IF test.

**Evaluation of NIV IF test:** Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of NIV IF test were estimated by plotting 2X2 table. NIV IF test showed sensitivity 93.75%, specificity 100%, PPV 100% and NPV 96.36% when compared with Chemicon IF test, which was used as ‘gold standard’. The results of the evaluation test demonstrated that NIV IF test is comparable to Chemicon kit and hence will be very useful for HRSV diagnosis.

Of the 111 specimens, we could test 86 specimens in both the IF tests. A total of 32 specimens were positive and 54 were negative for HRSV in Chemicon IF test. Of the 32 specimens our MAb detected HRSV from 30 specimens giving sensitivity of the test 93.75% but these MAbs failed to detect HRSV from 2 specimens giving false negative results resulted in NPV 96.36%. Importantly, NIV IF test did not give false positive reaction with any of the specimens positive for other respiratory viruses detected by Chemicon kit, this yielded PPV and specificity of the test 100%.
Lower sensitivity of NIV IF test can be attributed to use of single MAb N4 reacting to internal protein N of HRSV in the test. We used ascites fluid of MAb N4 as source of antibody in NIV IF test instead of its purified counterpart as we observed that latter was not very satisfactory. Initially we tested purified MAb N4 in IF test, using cells infected with strains of HRSV. This MAb even at lower dilutions (1:20, 1:40) gave very weak (1+ to 2+) IF reaction with some strains and sometimes failed to detect HRSV when compared with its unpurified counterpart in the same test. We also tested 9 NPA specimens with this purified MAb, of them four specimens were positive by Chemicon IF test and by unpurified MAb N4 however, purified MAb failed to detect HRSV from any of these specimens.

We also tested reaction of purified MAbs N3, P4, P9, P16, P20, P21, and P23 with tissue culture stocks of strains of HRSV but the results obtained were more or less same as purified MAb N4.

Use of MAbs against the highly conserved proteins of nucleocapsid complex of HRSV group A and B for the detection of HRSV in IF has been documented before. Freke et al., (1986) used the pool of P and N MAbs of HRSV along with polyclonal antibody (PAb), and observed that the pool of MAbs was superior reagent for detecting HRSV. HRSV was detected from 33% of 227 NPA specimens as compared to PAb detecting HRSV from 30% of the specimens in IF test whereas HRSV was detected in only 28% of the specimens by culture. Similarly, Ray and Minich, (1987) also reported higher sensitivity of DFA using pool of MAbs as compared to PAb and culture for the detection of HRSV. Hughes et al., (1988) also reported higher sensitivity of MAb IF over PAb IF and EIA kits (Kalstead EIA kit). EIA kit was less sensitive than DFA MAb detecting HRSV in 48% of the specimens; PAb IF detected 53% as compared to 54% of the specimens in DFA MAb. Similarly, Waner et al., (1990) when evaluated Directigen HRSV EIA kit for the detection of HRSV. They compared with IF test using PAb and MAbs, the latter reagent was found to be significantly more sensitive and specific than PAb.

5.5 Detection of HRSV from NPA specimens in NIV ELISA

Standardization of NIV ELISA was done employing purified and tissue culture stock of HRSV. As mentioned earlier, as compared to purified HRSV and laboratory-grown virus, the respiratory specimens contain some additional factors. As
with immunofluorescence assay adequate preparation of clinical specimen is essential for optimum results in immunoassays. Hence when clinical specimens were employed minor modification in the test protocol was done.

Rapid detection of HRSV from the clinical specimens using NIV ELISA was done taking Chemicon IF test as ‘gold standard’. All 134 NPA specimens could be tested in this test because presence of infected intact cells in sufficient number in clinical specimens is not the limiting factor as is for immunofluorescence.

Initially, supernatant fractions after centrifugation from the fresh specimens collected in TM were employed in ELISA. Of the 8 IPD (024645-025688) and 5 OPD (02762-025685) specimens diagnosed positive for HRSV in Chemicon IF test, NIV ELISA could detect HRSV from 2 IPD specimens (024645 and 025439) and 4 OPD specimens (025437, 025510, 025684, 025685). NIV ELISA failed to detect HRSV from six IPD (025511 to 025688) specimens and one OPD specimen (02762) indicating lower sensitivity of ELISA (Table 19 & 20).

Hence, instead of using only supernatant or cell phase in ELISA the complete whole nasal secretion without centrifugation was tested, which would give higher EIA signals. Similarly, sonication and treatment with detergents are reported to increase signals in EIA, presumably by releasing antigens from cells (Sarkkinen et al., 1981). Their studies indicated that original undiluted NPA collected by suction should be used as specimen instead of supernatant fraction or swab specimen. This conclusion was based on the fact that several HRSV IF positive specimens were found negative or borderline positive when only supernatant fraction was used in immunoassay. Hendry et al., (1986) compared washed nasal epithelial cells with uncentrifuged nasal secretions for the detection of HRSV in ELISA. 96% of the specimens were positive with whole nasal secretions whereas 68% were positive by ELISA with matching washed cells fraction. In the former, absorbance was greater than in cell fractions. Similarly, Popow-Kraup et al., (1986) also observed that, of the 16 HRSV positive specimens of which cell fractions were tested in IF test, 37.5% were false negative by ELISA indicating the supernatant of an unsonicated nasal secretion is unsuitable for ELISA testing.

Thereafter, we adopted a revised protocol of sample processing (sonication at low amplitude with 0.2% T20) to improve the sensitivity of the test. Using the revised protocol, we could test 90 specimens in both the tests (Chemicon IF test and NIV ELISA). Among them, of the 18 IPD (025802-027081) and 2 OPD (025917-025919) specimens diagnosed positive for HRSV in Chemicon IF test, NIV ELISA could detect
HRSV from 16 IPD and 1 OPD specimens (Table 19 & 20). Due to this changed protocol of specimen processing a marked increase in the positivity of the NPA specimens was observed. HRSV is an enveloped virus and dissociates into individual protein or protein complexes when exposed to detergent T20, which might have led to increase in the detection of HRSV from more number of specimens using P MAb.

In ELISA, a specimen giving P/N ratio ≥ 2.0 was considered as a positive reaction for HRSV. P/N ratios for positive specimens were in the range of 2.0 to 7.0, which indicated the virus load in the NPA. However, no co-relation between P/N ratio and disease severity i.e. patient attending OPD and hospitalized patients was observed.

During standardization we also considered mean OD + 2SD and mean OD + 3SD. When the former was considered, three more NPA specimens which were negative in 'gold standard' IF and NIV IF also became positive i.e. false positive. When the latter was considered two samples, previously positive in both the IF tests, became negative i.e. false negative. However, positivity obtained, taking P/N ratio above two was matching with that obtained by 'gold standard' Chemicon IF test.

Evaluation of NIV ELISA: For the evaluation, specimens processed using revised protocol of sonication with T20 were considered. Sensitivity, specificity, PPV and NPV of the NIV ELISA was estimated plotting 2X2 table. NIV ELISA showed sensitivity 85%, specificity 100%, PPV 100% and NPV 95.89%, when compared to Chemicon IF test, which was used as gold standard. The results of the evaluation test demonstrated that NIV ELISA would be useful for the diagnosis of HRSV.

Of the 134 specimens, 90 specimens could be tested in both the Chemicon IF test and NIV ELISA employing revised protocol. Of the 90, 20 specimens were positive and 70 were negative for HRSV in Chemicon IF test, NIV ELISA detected HRSV from 17 specimens. These included one of the two IF positive NPA from OPD and 16 of 18 IF positive NPA from IPD. This yielded sensitivity of NIV ELISA 85%. However, NIV ELISA failed to detect HRSV from one OPD and 2 IPD specimens positive in both the IF tests giving false negative results, this yielded NPV 95.89%. Importantly, NIV ELISA did not give false positive reaction in any of the specimens. This yielded PPV and specificity of the test 100%.

In ELISA, the detector and capture antibody should ideally be reacting with different epitopes. In NIV ELISA, a mixture of two MAb P4 and P9 reacting to P protein of HRSV was used as CAB and one (P9) of them was DAb i.e. single MAb P9 in
both the places was used. This could be one of the factors for not detecting HRSV from three clinical specimens (025917, 026315, 026318) even after sonication.

Lower sensitivities of different commercial EIA kits are also reported earlier. Ahluwalia et al., (1988) and Halstead et al., (1990) reported sensitivity in a range of 75%-95% with specificities above 95% when compared to IF and culture. Halstead et al., (1990) compared different commercial EIA and IF kits with isolation of HRSV from 117 specimens. These kits included DFA (Bartel Immunodiagnostics Supplies Inc. Bellevue, Washington), Directigen EIA (DTG) (Becton Dikinson Micorbiology Systems, Cockey Sville Md), the Test-pack EIA (TP) (Abbott Laboratories, North Chicago III) and RSV EIA (Abbott Laboratories). DFA test included pool of MAbs to G and capsid protein, DTG had MAb to N and F proteins whereas, TP and RSV EIA had PAb in the test. In the study MAb based DFA fulfilled the criteria of rapid and cost effectiveness as compared to EIA kits. Todd et al., (1995) also supported earlier findings reporting lower sensitivity, specificity, PPV and NPV of Test-pack EIA kit than IF. They were 83%, 87%, 83% and 94% respectively for Test-pack EIA as compared to IF.

Some reports also indicate higher sensitivities of EIAs when compared to other tests. Lauer et al., (1985) when compared ELISA kit (Ortho Diagnostic Inc) with virus culture and indirect IF using nasal washing of the 337 specimens. ELISA detected HRSV from 49% of the specimens, IF detected from 32% and culture from 37% of the specimens indicating Ortho ELISA to be more sensitive. Consistent with these Johnston and Siegal, (1990) also reported higher sensitivities of EIA. In the comparison of DFA, EIA, SV and CC, EIA identified 87%, DFA 83%, SV 73% and CC 40% of the HRSV positive cases.

We observed that, sensitivity of NIV ELISA was less (85%) as compared to NIV IF test (93.75%). But both the tests have 100% specificity. One reason for these kinds of results is, even with one cell containing viral antigen, a positive result can be obtained by IF, whereas certain concentration of antigen is required for a positive ELISA result.

5.6 Data analysis of HRSV positive samples in OPD and IPD

The main objective of the present study was to develop IF and Ag C ELISA using MAbs developed at NIV for the detection of HRSV from clinical specimens. However, during evaluation of the tests, it was possible for us to investigate a local
outbreak of HRSV during the year 2002 in and around Pune City, with peak activity in monsoon season. We conducted this small preliminary study in Pune, Maharashtra during the year January through December 2002. The study population included only children who were brought to the OPD and IPD of KEM hospital for the treatment of respiratory tract disease, hence represented more severe cases of ARI.

In developing countries, the distribution of viral pathogens affecting the respiratory tract can be summarized, as HRSV 15%-20%, PIV 7%-10%, influenza A, B and adenovirus 2%-4%. In children with ALRI, incidence of HRSV infection during the epidemic season may reach 50% (Berman, 1991, John et al., 1991, Agarwal et al., 1971). Our results were consistent with the earlier reports. In our study during the year 2002, 111 specimens were tested in Chemicon IF test. Of them 29.7% specimens were positive for HRSV. This percentage was even higher during HRSV epidemic season. Of the specimens collected from the patients hospitalized for ALRI during this season, 53.1% had HRSV whereas in OPD patients the percentage was low, up to 11.3%.

Temporal trends in the appearance of HRSV epidemics across continents and within the countries and regions have been reported in earlier studies. Appearance of epidemics seems to vary with climate including changes in temperature and humidity. North America and Europe have similar temporal trends in HRSV epidemics, where HRSV activity starts in September/October and lasts till January. In Central and South America, HRSV activity is observed from December through January and then travels south from March through May. In Africa, epidemic starts at very southern coasts during January and moves northward during next six months. However, in some areas like Cape-Town, and some places in Uganda and Nigeria it is present throughout the year. In Asia, HRSV epidemic appears in equatorial region in March and appear to move away from equator during following months. In South of equator in Australia, epidemic starts in March/April and in north of equator in Hong Kong in April and spreads around Pacific Rim in clockwise direction. It appears in Seoul in October, Japan in October/November, Anchorage in November and Seattle and Vancouver in December (Stensballe et al., 2003).

In India, HRSV epidemic follows south-north progression appearing in South India in July/August coinciding with rainy months (Cherian et al., 1990; John et al., 1991; Steinhoff et al., 1985), Kolkata in August/September, post monsoon season with warm and hot season (Hillis et al., 1971; Ota and Bang, 1972), New Delhi in September,
Similar to South India, during our study in the year 2002 in Pune, increased activity of HRSV was observed in July through October, with peak activity in August/September. This corresponded with the monsoon season in this area. During the first half of the year, though there was a peak of ARI, only one case of laboratory confirmed HRSV was detected in February 2002. However, in the peak season, 32 cases were positive for HRSV disease. In July, 9.09%, in August 37%, and in September 40% of the patients were positive for HRSV. In October the number was reduced, only 28.57% of patients showed HRSV etiology (Table 24).

Peak of HRSV activity has been associated in time with a dramatic increase in the number of infants and young children who were hospitalized with bronchiolitis and pneumonia. In Washington D C it was observed that 43% of patients had bronchiolitis, 25% had pneumonia and 23% of the patients had ALRTI during peak HRSV activity (Kim et al., 1973; Brandt et al., 1973). In tropical climate also, annual outbreaks of HRSV infection are co-related with the outbreaks of bronchiolitis and pneumonia with pneumonia being the commonest type of HRSV-LRI (Berman et al., 1983; Avila et al., 1989). In our study, of the 26 hospitalized patients diagnosed positive for HRSV etiology, 8 patients had bronchopneumonia, 7 patients each with bronchiolitis and pneumonia and 4 patients had ALRI. Similar to our studies, earlier in India Hills, (1971); Agarwal et al., (1971a); John et al., (1991); Chattopadhya et al., (1992); Jain et al., (1991); Maitreyi et al., (2000) also reported etiology of HRSV in bronchiolitis, pneumonia, bronchopneumonia and ALRI patients.

It is known that the risk of severe HRSV infection and mortality increases with chronic congenital heart or lung disease, immunodeficiency or immunosuppression, but due to effective modern intensive care, mortality due to HRSV infection is less than 2% (Walsh, 1997). In our study, a male patient (025922) with bronchiolitis aged two months had a history of CHD and one (025688) with ALRI having a history of neuroblastoma and undergoing chemotherapy, were positive for HRSV and required hospitalization. In this study the mortality was very low, only one patient with HRSV etiology expired but this patient was from orphanage and undernourished. Lack of breast-feeding, lower socioeconomic status, and crowding are known to be associated with more severe HRSV disease (Washburne et al., 1992).
During the peak activity of HRSV, all the OPD patients confirmed to be positive for HRSV were resident of Pune City. However, in case of 26 IPD patients positive for HRSV, 12 patients were resident of Pune city and 14 were from peri-urban Pune and from the places about 200 km surrounding Pune city. This could be because that all the NPA specimens were collected from KEM Hospital, Pune. This hospital is a large referral hospital centrally located in Pune city. Hence the patients attending OPD for mild respiratory infection, all were resident of Pune whereas in case of patients requiring hospitalization for LRI i.e. IPD patients came from the Pune city, peri-urban Pune and also from the places surrounding Pune city. This indicated that HRSV was active in and around Pune during the monsoon, in the months July, August and September 2002. Earlier also John et al., (1991) in his study reported a local outbreak of HRSV in Vellore, South India. Among the 256 inpatients, 73 patients were from Vellore or peri-urban Vellore, and rest were from other towns and rural areas surrounding Vellore.

HRSV is known to be the most important cause of LRI in early childhood especially in early months of life all over the world. Though all age groups are susceptible to HRSV infection, majority of hospitalizations for HRSV disease occur in 2-6 month old children. Frequencies of HRSV infection did not vary widely with age between 1-18 months in those, whose respiratory illness did not require hospitalization. In contrast among those who were hospitalized, there was a distinct peak at two months of age (Parrott et al., 1973). In our study, in 26 hospitalized patients positive for HRSV etiology maximum incidence of HRSV-ALRI was observed in young infants; 1-3 months 10 patients, 4-6 months 7 patients, 7-9 months 4 patients, 10-12 months 1 patient and above 1 year of age 4 patients. In hospitalized patients, age group of 1-3 months was most affected as compared to OPD patients where only one patient in the age group of 1-3 months was identified. In OPD most affected age group was above 12 months. Our results supported findings of Subramanian and his co-workers, (1980) in Madras obtained maximum isolates in the young infants of age group 7-12 months. In Vellore, South India John et al., (1991) detected HRSV in only 12% of 450 children greater than or equal to 1 year old as compare to other respiratory viruses. Such striking association with age was not seen for any other viruses.

It has been reported that along with the other risk factors, males appear to have an increased risk for HRSV-ALRI. Earlier, Glezen et al., (1971) in their epidemiological studies have shown that males are more likely than females to have
HRSV-LRI up to 5 years of age. However, Parrott et al., (1973) reported the sex distribution among children with HRSV infection severe enough to be admitted to the hospital in industrialized countries is 2.0:1.5 (Male: Female), but in milder cases the distribution of sexes was equal. Holberg et al., (1991) observed the peak activity of HRSV in children between 3 to 6 months of life and more susceptibility of males of particular ethnic group i.e. Hispanic males to HRSV-LRI in the early months of life. There was no HRSV-LRI in Hispanic females in the age range of 1-3 months. Recently, Weber et al., (2002) reported similar findings. They reported higher incidence of HRSV-ALRI in male than female children in Gambia in the ratio of 1.26:1 (Male: Female). Consistent with the earlier findings, in our study, of the 26 HRSV positive hospitalized children, 21 were male and 5 were female (4.2:1). However, in HRSV positive OPD patients, 5 were male and 2 were female (2.5:1).

Some earlier studies explain the reason behind this kind of observations. Taussig, (1977) demonstrated that males are more likely to have smaller airways for their lung size than females. This potentially could put them at increased risk for symptomatology with viral infection, and could be of more clinical importance in the first few months after birth before considerable growth has occurred.

In conclusion, our data shows that more than 50% of the cases admitted during the year 2002 for ARI were due to HRSV. These data strongly suggest the need for vaccination against HRSV. Understanding the epidemiology of the HRSV is the foundation for the introduction of a vaccine in the community. With this intention the study was undertaken and specimens collected during the year 2002 were studied. As the seasonality of HRSV in Pune has not yet been studied, we continued testing of NPA specimens through 2003. However, no HRSV activity was noted during this year and only data of HRSV positive samples in the year 2002 could be analyzed. From the viewpoint of epidemiology present study is very small, based on only one HRSV season. It is also important to analyze activity of both the groups A and B during epidemic season using RT-PCR and genotyping studies. As we did not perform these studies during the year 2002, we were unable to differentiate A and B groups and study their circulation during the same season.

Rapid, cheap, sensitive and less labor-intensive tests are required to undertake extensive epidemiological studies. With this aim we developed the immunodiagnostic tests; NIV IF and NIV ELISA using N and P MAbs respectively, for the detection of HRSV from clinical specimens. However, MAbs developed against group A strains may
not be adequate for the detection of all the antigenic variants of HRSV. Ideally, MAbs
developed against different proteins of both the groups A and B of HRSV should be
pooled and used in immunoassays to give optimum results. Since we do not have Indian
isolate of group B, we were unable to develop MAbs against B strain. Our MAbs were
specific to conserved proteins N and P of HRSV. These MAbs in both tests reacted with
strains of the groups A and B of HRSV grown in tissue culture. Hence, considering the
possibility that these MAbs are likely to detect strains of both groups A and B, further
work was undertaken.

NIV ELISA has proved to be highly sensitive and specific and large number
of samples can be screened at a time. However, sonication of samples before testing in
ELISA to improve the ELISA sensitivity might be the limiting factor for its use in
routine laboratory diagnosis. So far our tests are at institutional level where these
sophisticated facilities are available. We are planning to investigate some more HRSV
outbreaks in Pune, employing these tests during which alternative procedures of sample
processing which can replace sonication will be tried. One of the alternatives is use of
different agents like Nonidet P40 (NP 40) or Triton X-100 for treatment of specimens before
testing in ELISA.

NIV IF test has also proved to be a very useful tool. Though labor intensive
this test gives reliable results in 2-3 hours. Detection of HRV using both the tests will
also be useful in controlling the HRV disease by cohorting of patients in the hospital,
day-care centers, orphanages etc. as nosocomial infections of HRV are known.

Thus, with these in-house diagnostic tools and the baseline data obtained on
HRV in this study, larger epidemiological studies on HRV can be undertaken.