5. Discussion
DISCUSSION

India contributes to about 42.8% of the global burden of lymphatic filariasis (WHO, 1998) and recently it has become a signatory to the filariasis elimination campaign. The fact that the elimination programme has already been launched in the country calls for rapid assessment of filariasis and delimitation of endemic areas. In filariasis an accurate and early diagnosis is important for effective cure and control of the disease. Currently, detection of infection in human is being done through the conventional technique of microscopy for the detection of microfilaria in thick blood smear made from finger prick blood collected at night as the microfilariae are absent from the peripheral blood during the day time and appear in the peripheral blood at night to reach the maximum numbers between 22.00 and 02.00 hours (Manson, 1987). This is the routine method employed for diagnosis of filarial infection, as confirmed diagnosis of filarial infection can be made only by demonstrating the mf in the night blood smear. However this method is subjective and inconvenient, inconsistent, lacks sensitivity in detecting low level parasitaemia (Harinath et al., 1996) apart from being laborious. Also, collection of thick blood smear from the people at night has got a poor community acceptance (Das et al., 1995; Weil et al., 1997).
Certain improved versions of slide smear method like use of counting chamber, filtration of blood through membrane filters for concentrating microfilariae etc have been tried to increase sensitivity of the conventional test system. But, filtration technique requires 1 to 5ml of blood and is rarely acceptable to community (Chandra et al., 1986). Though detection of microfilariae is now routinely practiced method another problem with it is early stages of infection can not be detected and majority of chronic cases are amicrofilaraemic. Because of several draw-backs inherent with these techniques the necessity of detection of infection by alternative techniques such as immunodiagnostic tests or by molecular means are therefore obvious. This situation has warranted the application of more specific and sensitive tools for the detection of infected individuals in order to treat them. There is also a need for the detection of early stages of filarial parasite developing in humans for early diagnosis of infection. The treatment of individuals harbouring developing stages of parasite would prevent pathology and appearance of clinical manifestations (Pani et al., 2000).

Thus, highly sensitive and rapid diagnostic techniques that can utilize day blood sample and yet less expensive are central towards achieving the task of filariasis elimination. Immunological tests
currently available commercially (Weil et al., 1997; More and Copeman, 1990) are expensive and need to be imported. Also, these techniques have certain technical problems. Rajgor et al. (2002) have reported that the ICT ‘Now’ card has readability problem giving variable results when read at different time points after applying the blood sample.

In the present study attempts were made to address these needs in a stepwise manner. The most important attributes of these assays would be their application to detect infection in day blood samples.

In the past, attempts were made to develop diagnostics that detected circulating filarial specific antibodies. But, now it is well known that antibody detection techniques can only be used to detect exposure which may be current or past. Parasite specific antigen detection is the appropriate way to diagnose the current infection status of individuals. Therefore, in the present investigation attempts were made to develop immuno-assays, that could detect filarial antigens circulating in host.

**Polyclonal antibodies based assays**

Filarial specific polyclonal antibodies were raised against mf antigen of *B. malayi* in animals and evaluated for their potential to detect filarial specific antigens in sera from individuals residing in an
area endemic for bancroftian filariasis. Non-endemic normal sera were included for working out the specificity of the assay and the ‘Gold Standard’ in this case was microscopic examination of thick smear (60µl) of blood sample for mf. The results of this preliminary assay showed that the specificity of the assay was 100% (with regard to NEN) and sensitivity 82% when compared to thick blood smear examination. The test also detected antigens in 32% of EN individuals. Hence, this assay system is sensitive in detecting the infection in the community than the conventional technique, although had very high specificity. In order to improve the sensitivity of the assay and also to simplify it the antibodies used in the assay were conjugated with an enzyme label and then the assay was evaluated. However, the sensitivity decreased further and hence conjugation of the diagnostic polyclonal antibodies was not helpful.

For community diagnosis and mapping of filariasis endemic areas a technique which is less specific and sensitive, unlike individual case diagnosis, would also be useful. Such tests can generally be developed using polyclonal antibodies. Also, immuno-assays based on polyclonal antibodies are generally less expensive and hence will have feasible application in large scale surveillance. In summary the need for diagnosis are:
1. A highly specific and sensitive technique for individual diagnosis and treatment of infection.

2. A stage specific immuno-assay that can detect early stages of filarial parasites developing in humans, for preventing disease resolution.

Several attempts have been made in the past elsewhere to develop polyclonal antibody based assays to detect filarial specific antigens and thereby to detect the infection in its early stage. Cheirmaraj et al. (1991) developed polyclonal antibodies against the fractions Bm A-6 and the anti - Bm A SDS-soluble antigen and were evaluated for their diagnostic use in detecting filarial antigens in serum by inhibition ELISA. In their study filarial antigen could be detected in about 85% of mf, 35% clinical filariasis and up to 26% of endemic normal sera samples. (Chenthamarakshan et al., 1995). However the specificity of the assay was found to be low compared to its sensitivity.

Detecting filarial anigen in urine and in hydrocele fluid collected from infected patients also was carried out by many scientists using antibodies raised against B. malayi adult antigen. Reddy et al. (1984) of detected the presence of filarial antigen in 6 out of 10 urine samples collected from microfilaraemic patients and in 1 out of 5 urine samples
obtained from clinical cases who reside in *W. bancrofti* endemic places. The assay had the sensitivity of 60%. Malhotra *et al.* (1985) could detect *W. bancrofti* antigen by sandwich ELISA in urine samples obtained from 3 out of 21 endemic normals, 36 of 42 patients with mf, 17 of 21 with clinical filariasis and in 19 out of 25 hydrocoele fluid samples. In this assay the sensitivity in detecting urinary filarial antigen was found to be 85% and in clinical filariasis and in hydrocoele fluid the sensitivity was found to be 80% and 76% respectively. The specificity of the assay was found to be low i.e., 85.7%. Also, urine from people of Sevagram, Maharashtra and surrounding villages which are endemic for nocturnally periodic form of *W. bancrofti* were collected and by using anti-UFAC2-DE1 antibodies filarial antigen was detected in urine of 90% of microilaraemics, 30% clinical and 10% of endemic normal individuals (Padigel *et al.*, 1995). Few other assays developed include the ELISA system developed by Lunde *et al.* (1988) to detect antigen in the circulating immune complexes from 10 of 28 patients with bancroftian filariasis residing either the Cook Islands (sub-periodic form of *W. bancrofti*) or in India (periodic form of *W. bancrofti*). This test system showed only 42.8% specificity which may be due to the strain variation in different geographical variation. Polyclonal antibodies raised in mouse ascitic fluid against *W. bancrofti* microfilarial antigens were used in detecting the *W. bancrofti* antigen in
sera by sandwich and dipstick ELISA which showed 93% sensitivity (Cheirimaraj et al., 1992). However, the above test systems have their own merits and demerits in terms of their specificity and sensitivity. However, present investigation on the usefulness of the polyclonal antibody (without conjugation) based antigen detection assay showed that it may be useful in community diagnosis, as it misses only about 12% of the infected individuals. However, such technique with low sensitivity will not be useful for individual case detection, as stated earlier.

**Anti-mf MAbs for detection of microfilarial antigens**

The polyclonal antibody based assays developed in this study were found to be less sensitive than the conventional technique of examining thick blood smear collected by finger prick, during nighttime. Although this technique may be useful for use in community diagnosis in an endemic area, it may not be useful for individual case diagnosis and in areas where there is a low level of microfilaraemia, such as areas under filariasis control/elimination. Such a situation calls for, as stated earlier, a less expensive, indigenous and yet highly specific and sensitive diagnostic test that can utilize blood samples collected during the daytime. Monoclonal antibodies hold promise in this respect and already two tests based on such immunoreagents have been developed for bancroftian filariasis and are available
commercially. One is an ELISA based on a MAb (Og4C3) reactive against *W. bancrofti* antigen (More & Copeman, 1990) and another one an immunochromatographic card test based on AD1 MAb reacting with *W. bancrofti* antigen (Weil *et al.*, 1997). But, both these diagnostics have certain drawbacks. The former test is an ELISA and therefore needs to be performed in a well-equipped laboratory. The latter test is simple to perform, even in peripheral areas, but recently variation in its performance has been reported (Rajgor *et al.*, 2002). Major problems with these tests are higher costs (around Rs. 80.00, Rs.150.00 per sample, respectively) and need to be imported from outside countries. Keeping this in mind attempts were made to develop filarial specific MAbs with potential application in the diagnosis of lymphatic filariasis.

Several hybridomas were generated using *W. bancrofti* antigens and 7 high reactive clones were identified and cross-reactive studies showed that 2 of them (B5 and E2) had very high specific reactivity against filarial antigens and negligible reactivity against animal filarial and human intestinal helminth antigens. Thus, these two MAb proved to be filarial antigen specific, reacting specifically with antigens of several stages of lymphatic filarial parasites, ranging from mf till adult. MAb B5 was found to belong to isotype IgG2b while MAb E2 to
isotype IgG1. Western blot analysis showed that MAb B5 recognized two mf antigens with mol. wt. 15 and 28 kDa.

The two MAbs were produced in adequate quantities, purified and tested for their diagnostic potential i.e., for detecting circulating filarial antigens, in an ELISA format using sera from known antigen-positive (Microfilaraemic) and negative (Non-endemic normal) sera samples. The membrane filtration of 1 ml blood sample (collected during night) was used as ‘Gold standard’ for comparison. The MAb B5 based ELISA was found to detect *W. bancrofti* microfilaraemic individuals in a highly specific manner. However, it detected one of the non-endemic cases as positive for infection. It is possible that it may probably be detecting intestinal helminth antigens circulating in very high concentration in this individual, as in cross-reactivity studies the MAb was found to react with these antigens, although poorly. Alternatively, it is also possible that this individual might have migrated from or frequently visited a filariasis endemic area and hence harbored filarial parasites other than mf. The sensitivity of the MAb was found to be very high although slightly less than the membrane filtration technique (71.4%). It should be noted that the latter technique is highly sensitive than the conventional technique of examination of thick blood smear, nearly about three times. Thus, the MAb B5 based
ELISA is more sensitive than the conventional technique but less sensitive than the membrane filtration technique.

MAb E2 was found to be highly specific, as it did not react with any of the non-endemic individuals as infected, unlike MAb B5. However, it's sensitivity was less than that of the MAb B5 showing a sensitivity of 60% compared to the membrane filtration technique. Thus this MAb though has very high specificity it has low sensitivity. Nevertheless, 60% sensitivity of the MAb E2 based assay, compared to membrane filtration technique, means that this assay is yet more sensitive than the conventional technique. It's interesting to note that MAb B5 is slightly less specific but has good sensitivity, while MAb E2 has high specificity but has lower sensitivity. This opens up a possibility of improving the specificity and sensitivity by formatting the assay from direct to sandwich format using the two MAbs together, which needs to be explored.

Both the MAbs detected several amicrofilaraemic endemic normal individuals as having filarial infection. These observations are similar to that of MAbs used in the commercially available filarial antigen detection assays viz., Og4C3 ELISA and ICT. Using these tests it has now become clear that antigenaemia negative but amicrofilaraemic endemic normal individuals harbor single sex
infection and/or pre-adult/non-fecund worms, whose secretary/excretory antigens would be circulating in the blood of such individuals (Dreyer et al., 1996). It appears that all the MAbs developed in the present investigation also detect antigens of such parasites.

MAbs for detection of e/s antigens of early (L4) stages of filarial parasites

Any diagnostic tool which can detect the early stages of the parasite, especially the L4 stage of the parasite will be ideal for the early detection of the disease. Therefore, this study was also aimed at developing a technique for detecting the fourth stage larvae (L4) of the parasite for diagnosing the disease prior to patent stage. Filariae release a wide variety of molecules into the host environment, which are collectively called excretory/secretory (e/s) materials. It is these e/s products or the moulting fluid of parasites that contain the effective immunogens (Cheirmaraj et al., 1991). Also, the e/s products released by the living parasites are shown to be less complex and more specific in defining the infection (De Savigny & Tizard, 1977; Kaushal et al., 1982, 1984; Ottesen 1984). Hence, the present study was aimed at the development of monoclonal antibodies against the e/s antigens of L4 stage of W. bancrofti. It was also shown that the e/s antigens in the circulation of the infected host would provide an accurate and
convenient means of diagnosing active filarial infection (Ottesen, 1984). Hence, in the present study, monoclonal antibodies are developed against e/s antigens of fourth stage larvae of *W. bancrofti* and are investigated for their potential in detecting e/s antigens of developing filarial parasites of *B. malayi* (sub-periodic form) in a rodent model initially and then of *W. bancrofti* in human host.

To develop MAbs a technique for the *in vitro* maintenance of L4 stage larvae of the filarial parasite was optimized using a system developed for maintenance of L3 larvae earlier (Hoti et al., 1994). This involved collection of L4 stage larvae from the peritoneal cavities of animals and transferring them to *in vitro* culture system containing Franke’s medium supplemented with 10% normal human serum (NHS) and 15mM glutathione–reduced (GSH). Under these conditions the parasites could survive for 25 days. Using this system, e/s antigens were produced and used for the generation of hybridomas. Hybridomas generated obtained were screened for reactivity against mf and L4 e/s antigens by direct-ELISA and the positive hybridomas were cloned by limiting dilution and the high reactive clones were tested against L4 e/s antigens.

Two clones A6 and A7 which showed high reactivity against filarial antigens were obtained and investigated further with reference
to their filarial antigen reactivity and their potential in detecting the e/s antigens of L4 stages *vis-a-vis* other stages in mammalian host. The clones were cultured *in vitro* as well as *in vivo* (as ascitis) successfully for producing monoclonal antibodies and purified for further studies. Isotyping of the two clones showed that MAb A6 was isotype IgG2a and MAb A7 is of IgG2b isotype.

In an immunofluorescence assay MAb A6 reacted with the surface of antigens of mf of *B. malayi* and *W. bancrofti* while MAb A7 did not. In western blot analysis MAb A7 recognised 29.0 kDa antigen among e/s antigens of L4 stage of *W. bancrofti*.

Antibodies against nematodes are known to exhibit cross reactivity against other filarial nematodes (Dissanayake, 1980) as well as the intestinal helminths such as *Ascaris* sp. Earlier cross-reactive antigens/antibodies of the MAbs between the bovine filarial parasite, *S. cervi*, and human filarial parasite, *B. malayi*, have been identified in immuno-blotting analysis using hyper-immune rabbit sera (Kaushal, 1987). This becomes all the more important as in a tropical filarial endemic area, the chances of encountering antigens of zoonotic/intestinal helminths are more. Therefore, antigen cross reactive studies with these two MAbs were carried out against homologous (various stages of *B. malayi* and *W. bancrofti*) and
heterologous (adults of S. cervi) filarial antigens and also against the non-filarial antigens (adults of A. lumbricoides). MAbs A6 and A7 were identified as having high reactivity against W. bancrofti antigens and least cross-reactivity against adult antigens of S. cervi and A. lumbricoides. A7 was more specific to e/s antigens of 4th stage larvae of W. bancrofti, whereas A6 to mf antigens of W. bancrofti as well as B. malayi.

Although these two antibodies reacted with e/s antigens of L4 and crude mf antigens, the efficacy of these antibodies to detect various stage specific antigens present in the host-serum had to be studied. For this purpose, sera samples collected from an animal model, M. coucha, experimentally infected with B. malayi and having defined stages of this parasite was chosen. This system was chosen because of the reason that M. coucha inoculated with B. malayi L3 and amicrofilaraemic is known to simulate amicrofilaraemic and asymptomatic human filariasis in an endemic area (Murthy, 1983). MAb A7 showed high reactivity with sera samples collected on 20th day post-inoculation of L3. The pre-patent period of B. malayi in M. coucha is 90-120 days post-inoculation of L3 and the development of L3 to L4 takes 15 to 30 days and L4 to adult upto 90 days (Murthy et al., 1983) This indicates that MAb A7 detected the antigens of L4 stage larvae, as L3 develop to L4 stage in 20 days of inoculation in to
the animal. It also showed moderate reactivity against sera samples collected on time points corresponding to stages later than L4. MAbs from the clone A6 showed high reactivity against sera samples from animals 80th day post-L3 inoculation i.e., when the L3 inoculated would have developed into adult stage.

When the MAb A7 was tested against the sera samples from human individuals who were amicrofilaraemic and asymptomatic, it detected a significant proportion of them as antigen positives indicating that they may be harbouring developing filarial parasites (L4). These people are referred to as 'endemic normal individuals' as they are free from infection as detected by conventional blood smear examination (Ottesen, 1984) but may harbour developing parasites. As the MAb showed reactivity with crude antigens of *W. bancrofti* mf also, it could detect 84% of microfilaraemic cases as positives. Membrane filtration technique is considered as the more sensitive technique for mf detection than the conventional standard technique of examining night blood smear and the MAb A7 was sensitive enough to detect about 85% of those found positive in membrane filtration. MAb A6 also exhibited a sensitivity of 86% in the detection of microfilaraemic cases. The two MAbs had slightly lesser specificity, compared to membrane filtration technique, as they showed antigen positivity in a few sera sample from non-endemic normals. The two MAbs exhibited a slightly
lesser sensitivity compared to membrane filtration technique, mainly because they did not detect samples with very low mf count ($3\geq$). With respect to very low mf count, lack of sensitivity has been reported also for the two commercially available kits viz., ICT card test and Og4C3 (Trop-Bio) ELISA test (Pani; 2000; Rocha, 1996). Thus, the immuno-assays developed in the present investigation have shown potential in the diagnosis of filarial infection in humans. However, the diagnostic potential of these assays needs to be evaluated on a larger number of samples.