CHAPTER IV

SUMMARY AND CONCLUSIONS

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Amoebiasis, is an entero-invasive disease caused by protozoan parasite *E. histolytica* as a result of the dynamic interplay of both host and parasite related factors. The relative roles of the host and parasite in determining the severity of infection have been a major area of debate. The parasite exists as pathogenic and non pathogenic forms, the expression of pathogenicity is inducible. Hence the identification of pathogenic and non pathogenic of amoebic isolates from symptomatic and asymptomatic amoebic patients for appropriate treatment is of great importance. Out of various modern approaches to distinguish pathogenic and non pathogenic amoebic isolates, isoenzymic studies are found simple, reproducible, less time consuming and more reliable as compared to biological tests for the assessment of the clinical manifestations of the disease. The discovery of isoenzyme set up of group of metabolic enzymes of *E. histolytica* isolates differed in pathogenic and non pathogenic amoebic isolates termed as 'zymodemes' has been found to be worth while to explore.

Hence the present studies have been undertaken with respect to zymodeme classification, stability, virulence potential, antigenic analysis and susceptibility of anti amoebic compounds to amoebic isolates.
The salient features of the studies are as follows:

1. Thirty (30) amoebic isolates procured from asymptomatic and symptomatic amoebic patients were classified into pathogenic and non-pathogenic zymodemes on starch gel electrophoresis by using four isoenzyme patterns of ME, GPI, PGM and HK. In addition, four axenic *E. histolytica* isolates were also assessed for zymodeme classification.

2. The labelling of arbitrary positions $\alpha, \beta, \gamma, \delta$ of the bands of these isoenzymes by previous workers have been replaced by relative electrophoretic mobilities (Rf values) with reference to migration of bromophenol blue. The Rf value calculated in this way is simple and reliable than any other method for the calculation of Rf values.

3. Isoenzyme pattern of eleven (11) amoebic isolates obtained from symptomatic patients could be grouped under pathogenic zymodeme XIV. The Rf values and zymodeme classification of amoebic isolates listed in table—10 suggested their reproducibility and different from those of non-pathogenic amoebic isolates.

4. One amoebic isolate (no. 7) from symptomatic patient showed different isoenzyme pattern than the known zymodemes and it has arbitrarily designated as zymodeme XXIV. This zymodeme XXIV had the Rf values for ME as (mean ± S.D; 0.20±0.01), for GPI (mean±S.D.
0.12±0.01), two bands of PGM (mean ± S.D.; 0.08±0.01, 0.12±0.01) and two bands of HK of Rf values (mean±S.D. 0.32±0.02, 0.60±0.02).

5. Thus, it is suggested that other pathogenic zymodemes could be existing with the commonly existing pathogenic zymodeme XIV here as well as in other parts of the country and a survey has to be done for its incidence in different parts of India.

6. Considering the Rf values from asymptomatic amoebic patients, these could be exclusively grouped under zymodeme I. Rf values and Zymodeme classification was listed in Table - 10. Since zymodeme I could be identified in all amoebic isolates from asymptomatic carriers, it is presumed that this zymodeme is commonly existing in this part of the country.

7. All the axenic E.histolica isolates were found to be belonging to pathogenic zymodeme II.

8. The virulence potential of amoebic isolates of non pathogenic zymodeme I was increased by adding cholesterol in the growing medium of amoebic isolates belonging to zymodeme I and zymodeme II. The increased virulence potential was assessed in vivo. The reassessment of isoenzyme pattern of these zymodeme I and II did not show any change tested at various intervals of time. This indicates that isoenzymes are stable. The increase in virulence
by adding cholesterol might be due to change in fluidity of membrane of the parasite, which in turn might be responsible for lysis of target cell.

One of the possible ways of changing of zymodemes may be due to a novel way of genetic exchange. This was assessed by mixing two clones of HMI-C-131 and HMI-C-145 of different electrophoretic mobilities. Before mixing one clone HMI-C-131 had three bands of HK of Rf Values 0.20, 0.37, 0.11 and a single band of PGM Rf value 0.075. The other clone HMI-C-145 had two bands of HK of Rf values 0.21 and 0.38 and single band of PGM of Rf value 0.087. The virulence potential determined by BHK monolayers was 32±1.8 percent, 43±2.3 percent of HMI-C-131 and HMI-C-145 respectively before mixing of these clones. The mixed clonal population in ratio of 1:1 of parasites of above two clones was maintained for a period of 30 days. After this period, a new progeny of *E. histoytica* population of different electrophoretic mobilities appeared which were entirely different from both the parent clones as determined by virulence potential and electrophoretic studies. These changes in electrophoretic mobilities may be due to novel way of genetic exchange which definitely needs further investigation. Moreover, virulence potential determined by BHK monolayers at different time intervals after mixing of clones
showed a progressive increase. It was 46±1.4 percent, 50±1.2 percent and 59±1.2 percent after 10, 20 and 30 days respectively. However electrophoretic studies of HK showed alterations up to 20 days and then slowest moving band of one of the clones (HMI-C-131) disappeared with hardly any change in other two bands. After 30 days, new electrophoretic bands with completely altered Rf values i.e. 0.24, 0.40, 0.75 from those of the parent clones appeared. Similarly, PGM showed a single band of Rf value 0.087 upto 20 days. Like HK, after 30 days a slower band of Rf value 0.080 appeared different from parent clones.

To broaden zymodeme classification two enzymes G6PD and SOD were studied. These two enzymes are important for detoxification mechanism of *E.histolytica*, which is very essential for eradication of the parasite. However, in the present study, either of these enzymes did not show any difference in their electrophoretic behaviour for virulent or avirulent amoebic isolates. To best of the knowledge of the present investigator, the presence of these enzymes in *E.histolytica* have been shown electrophoretically for the first time. It is worth to probe the monitoring of these enzymes for the screening for the anti-amoebic drugs.
Antigenic analysis of the extracts of amoebic isolates belonging to pathogenic and non-pathogenic were subjected to SDS-PAGE. It showed no significant variations amongst pathogenic and non-pathogenic zymodemes. However, quantitative differences were found in the range of 50-56 Kda region amongst pathogenic and non-pathogenic zymodemes, which is again of interest with respect to identification of symptomatic and a symptomatic amoebic patients.

In vitro, the susceptibility of anti amoebic compounds i.e. of metronidazole, furazolidone separately and in combination of metronidazole and furazolidone were identical for pathogenic and non-pathogenic amoebae. The lethal concentration of metronidazole was assessed as 5µg/ml for amoebic isolates of symptomatic and asymptomatic amoebic patients. However, higher concentrations of furazolidone ranging from 125 µg/ml to 250µg/ml were required to kill the amoebae belonging to pathogenic and non-pathogenic zymodemes. The combination of M:F in the ratio of 3:1(V/V) was found to be equally sensitive to pathogenic and non-pathogenic amoebic isolates, which is lesser in concentration than both the individual compounds. The minimum range of this combination of M:F was found as to be (1.86±0.62).
μg/ml to kill the invasive and non-invasive amoebae. This was probably due to synergistic action of one of the anti-amoebic compounds. This has a great advantage in treatment of amoebiasis especially with respect to asymptomatic amoebic patients or complicated amoebiasis.

Conclusion

Isoenzymes of *E. histolytica* are stable, reproducible and can distinguish pathogenic and non pathogenic zymodemes. Still a large number of amoebic patients are to be studied, to find out more pathogenic zymodemes, which would be of great interest to monitor, the diagnosis and prognosis of the disease.

2. The changing of electrophoretic mobilities observed in the studies, may be due to novel way of genetic exchange, which needs further investigation.

3. Two new enzyme G6PD and SOD were investigated for their zymodeme classification of amoebic isolates. Although, these could not differentiate pathogenic and non pathogenic amoebae, these are very important for the detoxification mechanism of the parasite. Hence these enzymes should be exhaustively studied quantitatively and qualitatively with a large number of amoebic isolates. These studies will help in eradication of the disease. In addition, more enzyme systems
should be tried to distinguish virulent and avirulent amoebae.

4. The extracts of pathogenic and non-pathogenic amoebae did not show significant variations on SDS-PAGE in higher molecular range. However, quantitative differences were observed in low molecular range of 50-56 Kda. for pathogenic zymodemes, while non-pathogenic zymodemes had weak bands in this range. Thus, majority of antigens are on cell surface of the parasite. Hence in depth, all surface antigens should be assessed to find out marker antigens to distinguish pathogenic and non-pathogenic amoebae.

5. In view of the fact that the anti amoebic compounds i.e. metronidazole and furazolidone separately and in combination of other anti amoebic compounds should be evaluated on symptomatic and asymptomatic amoebic isolates to minimize the side effects with higher therapeutic potency. More appropriately, specific metabolic inhibitors for pathogenic and non-pathogenic amoebae has to be studied in details for the effective control of the disease.