Summary and Conclusion
Acute Flaccid Paralysis (AFP) is defined as acute onset of focal weakness or paralysis characterized as flaccid (reduced tone), without other obvious cause (e.g. trauma) in children <15 years old. Enterovirus infection in humans results in wide range of acute and chronic diseases. They are undoubtedly important human pathogens with diverse serotypes of polioviruses, coxsackie A viruses, coxsackie B viruses, echoviruses and newer numbered enteroviruses. A great deal of importance given to enterovirus was due to poliovirus serotypes, for their potential to cause paralytic poliomyelitis. Considering this, all enteroviruses excluding polioviruses are known as nonpolio enteroviruses.

The eradication of poliomyelitis and wild polioviruses by the year 2000 was the aim of the WHO PEI. Oral Poliovirus Vaccine has been available for use in India since 1979 and its administration would only be stopped, when the eradication of wild poliovirus has been documented convincingly. Therefore, identifying strains as poliovirus or nonpolio enteroviruses is of paramount importance. With the last epidemiological phase of poliovirus eradication i.e. post vaccination era, the nonpolio enterovirus associated or cases isolated from acute flaccid paralysis are considered to be more important. The correct identification of these nonpolio enteroviruses had not been reported from India. This indicated the need of the present study.

Correct identification of nonpolio enteroviruses carried out by neutralization assay based serotyping is generally sufficient for establishing diagnosis but for gaining more information on the endemic serotypes, molecular characterization is required. Molecular approaches were reported to be very useful in genotypic characterization of viruses, in epidemiological studies and in understanding the disease pattern of the serotypes.

Enterovirus genome is single stranded positive sense RNA that replicates using RNA dependent RNA polymerase, resulting in very high
evolution rate. Enteroviruses cause a wide spectrum of clinical illness, showing considerable overlap between the disease spectra of different serotypes of enteroviruses. Considering this, together with the fact that all enterovirus have potential to invade central nervous system, the cases of acute flaccid paralysis from which only nonpolio enterovirus was isolated are studied.

The confirmatory diagnosis of enterovirus infection relies on virus isolation which is the "golden standard" routinely followed in the clinical laboratory. The prominent drawback is a poor diagnostic sensitivity, which can be due to low virus titer, inadequate collection, handling and processing of the samples, or insusceptibility of the cell-line used. In addition, the method is time-consuming, labor-intensive and costly. Following isolation of enteroviruses, serotypic identification is generally performed by antiserum neutralization assay.

The clinical data of acute flaccid paralysis patients from WHO poliovirus and nonpolio virus cases was compared and significant difference in the presence of fever at the onset of paralysis was found. It was also found that the most frequent nonpolio enteroviruses isolated from these patients are those that were reported to be more neurovirulent in previous reports (coxsackie B viruses and echoviruses 6, 11 and 9). But as the isolation of an enterovirus from stool does not confirm it as an etiological agent and these isolated nonpolio enteroviruses may represent background virus circulation, study of cases that live in close contact of acute flaccid paralysis patients and hence belong to same socioeconomic conditions were studied. Significant difference in isolation rate of nonpolio enteroviruses from patients and control group was found (35% vs 22%). The pattern of serotype isolated from control group was also different from that of patient group. The isolation rate of nonpolio enterovirus isolates was found to be 27% echoviruses, 35.5% coxsackie B viruses, 35% untypeable isolates and 2.5% mixed serotypes. Echovirus 11 isolates are found to be most frequent among echoviruses.
The lack of sequence data from nonpolio enteroviruses circulating in India is the major limitation for designing molecular reagents with increased sensitivity and specificity. In this view 12 echovirus 11 isolates were studied for sequence variability in the 5’untranslated region using heteroduplex mobility assay followed by nucleotide sequencing. Heteroduplex mobility assay was used to determine sequence diversity between Indian echovirus 11 isolates and prototype Gregory strain and the results were confirmed by 5’untranslated region nucleotide sequencing of five Indian echovirus 11 isolates. Heteroduplex mobility assay results showed high genomic diversity between the prototype Gregory strain and Indian echovirus 11 isolates. All isolates were grouped into five different types of heteroduplex mobility pattern with respect to Gregory strain. A 440bp 5’ untranslated region fragment of five echovirus 11 isolates representing different heteroduplex patterns, were sequenced. The sequence alignment showed that 5’ untranslated region of Indian isolates were different from prototype Gregory strain (<87.5% similarity) and identical to the echovirus 11 isolates of Finland and Hungary (>95% similarity). Phylogenetic analysis including echovirus 11 isolate sequences from different parts of the world showed that Indian echovirus 11 isolates represent a different subgroup. Results suggest that heteroduplex mobility assay can be successfully used as a preliminary screening method for sequence variability determination of enterovirus field isolates. The sequence data generated will help future studies of echovirus11 epidemiology and evolution in India. Moreover, it may help in designing new or modifying existing molecular reagents used for diagnosis.

Coxsackie B viruses (serotype 1-6) are implicated in several acute and chronic diseases. Often from such infections like meningitis and myocarditis, their isolation and hence identification could not be achieved. The conventional serotyping assay often takes very long time, usually 10-15 days for correct identification of virus isolates. Also the isolation of virus from samples such as cerebrospinal fluid and myocardial fluid has its own
limitations. Keeping this in mind a rapid RT-PCR based RFLP assay for subtyping of coxsackie B viruses clinical isolates was designed. This assay is based on a simple and sensitive RT-PCR followed by subtyping using RFLP with a single enzyme BsaJI. The assay is able to differentiate all six prototype strains of coxsackie B viruses. Serotyping of 31 clinical isolates was attempted by RFLP assay of which, 29 were correctly identified when compared to the neutralization assay results. Two of them were typed as CBV 6 but remained untypeable by neutralization assay. This discrepancy may be due to the fact that neutralization utilizes antibodies most of which are directed towards VP1 capsid protein. In capsid region mutations are very common and that may be a reason for failure of neutralization assay for these isolates. They may be prime strains, although possibility cannot be excluded that their 5' untranslated region had undergone mutation to produce intermediate strains. The specificity of the assay is comparable to serotyping and this assay could be used in combination with antiserum based serotyping and also for the samples where isolation of virus in cell culture is not possible.

All untypeable isolates were resistant to typing by the widely employed neutralization test using WHO antiserum pools. Twelve untypeable isolates which were different from the other viruses in neutralization pattern were considered as variants viruses. Partial nucleotide sequencing by targeting the 5' untranslated region of these viruses was carried out. IndUT1, 2, 3 and IndUT5 were found to be echovirus 11. The average homologies among these isolates were 92% and were clustered near to Indian echovirus 11 isolates (AY335796, AY335798, AY335795, AY335797). IndUT4 was found to be poliovirus and its sequence was 95% homologous with type 1 wild-vaccine poliovirus recombinant of China. Other 7 virus isolates which were growing in L20B cells but poliovirus isolation was negative including IndUT7, 8,9,10 and 11 were found to be poliovirus type 1 wild and showed 95% homology with type 1 wild-vaccine poliovirus recombinant of China as in case of IndUT4.
IndUT6 and IndUT12 were distinct from this group and were found to be EV71 and echovirus 11 respectively. IndUT12 was 96% homologous with Indian echovirus 11 isolate (AY335799).

The commonest nonpolio enterovirus that was reported to cause paralytic disease like acute flaccid paralysis is enterovirus-71. Of the total nonpolio enteroviruses isolated, many remained untypeable by using WHO intersecting antiserum pools. As WHO antiserum pools did not include antiserum against enterovirus-71 together with the fact that these untypeable viruses were isolated from patients having acute flaccid paralysis they were investigated for the presence of enterovirus-71. Antiserum was raised against enterovirus-71 prototype BrCr strain and used to identify 70 untypeable enterovirus isolates. But, some of the isolates escaped neutralization against raised antiserum, due to rapid evolution rate of enterovirus-71, resulting in change of neutralizing epitopes. The 5'UTR is considered as the most conserved part of enterovirus genome, so a DIG labeled DNA probe was made for screening of untypeable isolates for presence of enterovirus-71. The hybridization assays were able to pick up the isolates reactive to raised antiserum as well as some other isolates also.

The specificity of the probes was determined by subjecting the reactive isolates to RT-PCR using two different sets of highly specific primers. The RT-PCR primers confirmed the presence of enterovirus-71 in one isolate. This is the first report on isolation and molecular characterization of enterovirus-71 isolates from India. The hybridization assay can save costly reagents required for RT-PCR for large number of isolates to be screened. These probes can replace the need of antiserum that is not sufficiently available and also needs laborious procedure of virus purification, ultra purification, two months time and animal to be sacrificed. The sensitivity of these probes could not be compared with RT-PCR as primers target only 440bp region and if that region is mutated during evolution it may result in false negativity. Furthermore, these primers were
not designed keeping in view the molecular makeup of enterovirus-71 Indian isolate.

To conclude, in the present study attempts were made to characterize the nonpolio enteroviruses isolated from acute flaccid paralysis cases by analyzing their phenotypic and genotypic characteristics. Molecular characterization was carried out using RT-PCR, RFLP, Probe hybridization, Heteroduplex mobility assay and Partial nucleotide sequencing. Heteroduplex mobility assay is a powerful approach and in this study it has been used for the first time to determine the sequence variability among nonpolio enteroviruses. RT-PCR based RFLP assay was developed for the 5' untranslated region, which is a useful approach to differentiate coxsackie B virus clinical isolates and is a valuable supplement to enterovirus identification for diagnostic and epidemiological studies. In addition, a DIG labeled DNA probe was developed for screening the untypeable isolates for the presence of enterovirus 71, the most common nonpolio enterovirus reported to cause paralytic disease like acute flaccid paralysis.