Isolation and Identification of Non-Polio Enteroviruses from Children in Different Egyptian Governorates

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Abstract: Enteroviruses are among the most common viruses infecting humans worldwide. Human enteroviruses are enterically transmitted and cause a wide spectrum of both common and uncommon illnesses among infants and children. The objectives of this study is to isolate Non-Polio Enteroviruses from stool specimens obtained from children aged less than 15 years from different Egyptian governorates and characterize the different serotypes of it using both immunological and molecular methods according to World Health Organization recommended protocols. Non-Polio Enteroviruses were isolated from 1000 stool samples on RD-A (human rhabdomyosarcoma) cell line and was characterized to the different serotypes using neutralization by antiserum pools and RT-PCR methods. A total of 176 (17.6%) Non-Polio enteroviruses (NPEV) strains were isolated from different Egyptian governorates. The highest percentage of positive cases was found in El Menya Governorate (49%) followed by El Fayoum (47%) then Giza (42%) while Port Said, Matrouh, South Sinai and Alwadi Algardid had no positive cases (0%). Seasonal variation of NPEV isolated from different Egyptian governorates showed the highest percentage of NPEV was recorded in the month of May (46%) followed by April (42%) then June (36%). The least percentage was found in the month of January (12%) followed by December (16%) then November (19%). There is no significant difference on the number of isolates between summer and winter months. Echoviruses (EV) had the highest prevalence in Egypt (37%). EV-14 was the most prevalent EV in Egypt (14%). The isolates (n =58) found untypable by the antiserum pools were confirmed as NPEV by PCR using Pan-Enterovirus primers. In conclusion: Study on NPEV serotypes circulating in Egypt help to formulate more effective strategies. A better knowledge of the transmission and the implications of NPEV in diseases may also justify the future studies on their molecular epidemiology. High prevalence of non-typable enteroviruses (NTEV) isolates in Egypt reflects the needs for further typing and molecular analysis.

Key words: Non-Polio Enteroviruses, NPEV, echoviruses, RT-PCR, Egyptian governorates.

INTRODUCTION

Human enteroviruses belong to the Picornaviridae family and are divided among 5 groups (poliovirus, human enterovirus A, human enterovirus B, human enterovirus C, and human enterovirus D) by molecular characteristics. These species comprise altogether 62 antigenically distinct serotypes and at least 30 additional recently described genetically distinguishable types (Blomqvist et al., 2008 and Hymas et al., 2008). Enteroviruses are non-enveloped small viruses (20-30 nm) of icosahedral symmetry. Their RNA genomes have 7.5 kb, positive sense and single strand. They are responsible for an extensive variety of diseases, although 50 to 80% of the infections are asymptomatic. They cause hepatitis, pleuridinia, stomatitis and neonatal sepsis in a significant number of patients every year. In developing countries, the poliovirus is clinically the most significant member of the genus Enteroviruses (EV) causing paralysis diseases in every 4 out of 1,000 children in school age. The non-polio enteroviruses (NPEV) are the main responsible for aseptic meningitis, myocarditis and nonspecific febrile exanthematous illnesses. Other illnesses with chronic course, such as type 1 diabetes mellitus, have also been associated with enterovirus infections Approximately 75% of infections by EV occur...
in children under 15 years of age and the attack rates are highest in children under 1 year of age (Wiedbrank & Johnston, 1993 and Gomes et al., 2002).

The determination of the relationships between EVs and clinical syndromes and the investigation of their molecular epidemiology usually requires the typing of the viruses (Manzara et al., 2002). The traditional “gold standard” for the diagnosis of EV infections is virus isolation from clinical specimens in cell culture, followed by analysis with neutralizing antisera (Grandien et al., 1995). Although the majority of cultures yield a positive result within the first week, EV typing by neutralization assay is time-consuming, expensive, and often futile (Rigonan et al., 1998 and Muir et al., 1998). Enteroviruses grow well on HeLa cells, Hep-2 cells, human rhabdomyosarcoma cell line RD cells, MRC-5 cells, human embryonic kidney cells and buffalo green monkey kidney cells (Saeed et al., 2007) but the RD cell line was found to be the most sensitive cell line for the isolation of enteroviruses (Johnston and Siegel, 1990). Enteroviruses can be isolated from feces, pharyngeal washings, cerebrospinal fluid (CSF), spinal cord, brain, heart, blood, conjunctivae and lesions of skin or mucous membrane (Bahri et al., 2005).

Molecular diagnostic tests for the detection of EVs in clinical specimens usually target highly conserved sites in the 5’ non-translated region (5’-NTR), allowing detection of all members of the genus (Nix et al., 2006). Many enteroviruses do not grow readily in cell culture; hence, these diagnostic tests based on PCR are frequently more sensitive than traditional methods. Because it is more sensitive and much faster than culture, providing reliable results in a clinically relevant time frame, PCR is rapidly becoming the new “gold standard” for enteroviruses detection (Robinson et al., 2002 and Vuorinen et al., 2003).

The aim of the present study is to isolate Non-Polio Enteroviruses obtained from different Egyptian governorates in tissue culture and to characterize the different serotypes of NPEV using both immunological and molecular methods.

**MATERIALS AND METHODS**

**Specimen:**
A total of 1000 stool specimens were included in this study. Clinical specimens were collected from VACSERA, Giza, Egypt as well as governmental hospitals from different Egyptian governorates in the period from January to December 2008.

**Testing of Fecal Specimens and Virus Isolation:**
Specimens require pre-treatment with 10% v/v chloroform, to which enteroviruses are resistant. In addition to removing bacteria and fungi, this method removes potentially cytotoxic lipids and dissociates virus aggregates (WHO, 2004). The human cell lines, including RD-A (human rhabdomyosarcoma), were used for isolation of all enteroviruses, including poliovirus (Kelly et al., 2006). The isolation of enteroviruses was determined by observation of cytopathic effect and confirmed by antisera neutralization and/or sequencing a portion of the virus genome (Oberste et al., 2000).

**Neutralization assay:**
Each virus preparation was tested in duplicate against a Coxsackievirus B1-6 pool (CP) and 20 echoviruses (A–G). 50 μl of antisera was added to the appropriate wells of microtiter plate. Each isolate was tested in duplicate against all the antiserum pools using serial dilutions of virus prepared from 10^2 to 10^−6. 50μl of all the dilutions were dropped in respective wells and incubated at 37°C for one hour. After incubation, 100μl of RD cell suspension was distributed into these wells and the plates were incubated at 37°C after covering with non-toxic sealer. Virus controls and cell controls were run along for comparison. The plates were examined daily, till the virus control showed 4+ CPE. The identification was made by analyzing the pattern of inhibition of CPE by the antiserum pools (WHO, 2004 and Saeed et al., 2007).

**Viral RNA extraction:**
Stool suspensions were prepared by adding 5 ml of phosphate-buffered saline, 1g of glass beads (Corning Inc., Corning, NY) and 0.5 ml of chloroform to 1g of stool sample, shaking the mixture vigorously for 20 min in a mechanical shaker, and centrifuging at 1,500X g for 20 min. at 4°C. For fecal specimens (10% stool suspensions), 140μl of the specimen extract was combined with an equal volume of Vertrel XF (Miller-Stephenson Chemical Co., Danbury, CT), shaken vigorously, and then centrifuged at 13,000X g for 1 min. at room temperature. The aqueous phase was transferred to a fresh tube (WHO, 2001).
Twenty micrograms of proteinase K (Roche Applied Science, Indianapolis, IN) was added to 140 μl of each liquid specimen or fecal extract, and the mixture was then incubated for 30 min. at 37°C. Nucleic acid was extracted from the digested specimen with a QIAamp Viral RNA mini kit (QIAGEN, Inc., Valencia, CA) which was used according to the manufacturer’s instructions. The eluted RNAs were passively dried in bench top desiccator under vacuum. The dried RNA was re-suspended in 16 μl of sterile nuclease-free water and stored at -20°C until use (Nix et al., 2006).

**RT-PCR:**

*In vitro* amplification of the virus isolates was performed by PCR using the following primers for pan-Enterovirus (Pan-EV): 5’-ACACGGACACCCAAAGTAGTCGGTTCC-3’ 5’-TCCGGCCCCTGAATGCGGCTAATCC-3’ (114 base pairs). The primers used for PCR amplification were selected from the highly conserved 5’ non-coding region of the enterovirus and product was amplified by one step RT/PCR. Non-infectious control RNA was used as positive control, culture supernatant from uninfected cells was used as negative control. Virus samples were diluted 1:4 in RNase free water. 1 μl diluted sample was added to 19 μl reaction mixture containing 250 μM of each dNTP, 2.5 μl of 10× PCR buffer and 40 pmol of each forward and reverse primers. The reaction mixture was first incubated at 95°C for 5 minutes and then chilled on ice for 5 minutes. Once tube contacts cooled, 5 μl mixtures containing 2.0 mM MgCl₂, 0.5 mM DTT, 0.2 units Placental RNase inhibitor, 0.07 units AMV reverse transcriptase and 1.5 units Taq polymerase was added. The tubes were placed in thermocycler (GeneAmp PCR System 9700, Foster City, CA) and cycled as follows: 42°C for 20 min., 95°C for 3 min., 30 cycles at 95°C for 45 sec, 55°C for 45 sec, 60°C for 45 sec. respectively. Bands of the PCR amplified products were visualized under UV illumination on 10% Poly-acrylamide gel after ethidium bromide staining (WHO, 2004 and Saeed et al., 2007).

**Statistical Analyses:**

Statistical analyses were performed using Descriptive Statistics, Tests of Normality, t-test for Equality of Means and Variances for seasonal NPEV isolates in different months of the year and comparison between Egyptian governorates.

**RESULTS AND DISCUSSION**

This study comprises 1000 stool samples obtained from children under 15 years old from all governorates in Egypt. Non polio enteroviruses detection and characterization was performed by isolation on cell culture followed by neutralization and RT-PCR according to World Health Organization recommended protocols. Descriptive Statistics of distribution of Non Polio Enteroviruses in Egyptian Governorates during (January – December 2008) showed that maximum and minimum numbers of isolates were 17.0 and 0 respectively, with mean value ± SD = 7.33 ± 5.19 (Fig. 1 and Table 2).

A total 176 samples were positive for non polio enteroviruses (17.6%). The highest percentage of positive cases was found in EL Menya governorate (49 %) followed by El Fayoum (47%) then EL Giza (42%) while Port Said, Matrouh, South Sinai and Alwadi Algadid had no positive cases (0%) (Tables 1 and 2).

T-test for summer month's group and winter month's group show that mean value of number of isolates ± SME= 31.5± 4.68 and 15.33±1.86 respectively. There is no significant difference between summer and winter months (Table 3).

Fig. 3 (a and b) showed the expected normal and the Deviation from normal seasonal variation of non-polio enteroviruses isolated from different Egyptian Governorates. The highest percentage of enteroviruses was recorded in the month of May (46%) followed by April (42%) then June (36%). The least percentage was found in the month of January (12%) followed by December (16%) then November (19%) (Figs. 2 and 3).

Fig. (4) Showed the distribution of non-polio enteroviruses isolated from Egyptian governorates. EV had the highest prevalence (37%) followed by non-typeable enteroviruses (NTEV) which had a prevalence of 33% and finally COX. B viruses with a prevalence of 30%.

The most prevalent echovirus in Egypt was EV-14 (14%) followed by EV-27 and EV-29 (12%) while the least prevalent was EV-4 which had a prevalence of 5% followed by EV-3 and EV-6 with a prevalence of 6%.
Table 1: The distribution of different types of Non Polio enteroviruses in Egyptian Governorates during January – December 2008.

<table>
<thead>
<tr>
<th>Governorate</th>
<th>No. of samples</th>
<th>NPEV No.</th>
<th>%</th>
<th>Governorate</th>
<th>No. of samples</th>
<th>NPEV No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cairo</td>
<td>78</td>
<td>17</td>
<td>22</td>
<td>Suhag</td>
<td>42</td>
<td>5</td>
</tr>
<tr>
<td>Giza</td>
<td>59</td>
<td>5</td>
<td>8</td>
<td>Fayoum</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>Qaliubia</td>
<td>62</td>
<td>17</td>
<td>27</td>
<td>Behaira</td>
<td>51</td>
<td>12</td>
</tr>
<tr>
<td>Dakahluya</td>
<td>46</td>
<td>7</td>
<td>15</td>
<td>Aswan</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Damietta</td>
<td>30</td>
<td>2</td>
<td>7</td>
<td>Luxor</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td>Kafr Al-Shaikh</td>
<td>38</td>
<td>4</td>
<td>11</td>
<td>North Sinai</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>Ismailia</td>
<td>25</td>
<td>4</td>
<td>16</td>
<td>Red sea</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Sharkeya</td>
<td>45</td>
<td>3</td>
<td>7</td>
<td>Assiout</td>
<td>42</td>
<td>6</td>
</tr>
<tr>
<td>Gharbeya</td>
<td>40</td>
<td>8</td>
<td>20</td>
<td>6th of October</td>
<td>43</td>
<td>10</td>
</tr>
<tr>
<td>Alexandria</td>
<td>52</td>
<td>2</td>
<td>4</td>
<td>Menya</td>
<td>43</td>
<td>15</td>
</tr>
<tr>
<td>Menoufeya</td>
<td>37</td>
<td>7</td>
<td>19</td>
<td>Port said</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Suez</td>
<td>27</td>
<td>1</td>
<td>4</td>
<td>Matrouh</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Beny Sewif</td>
<td>51</td>
<td>13</td>
<td>25</td>
<td>South Sinai</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Qena</td>
<td>41</td>
<td>12</td>
<td>29</td>
<td>Alwadi Algadid</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>176</td>
<td>17.60%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Statistical descriptive data of distribution of different types of Non Polio enteroviruses in Egyptian Governorates during summer and winter months of 2008.

<table>
<thead>
<tr>
<th>Governorates</th>
<th>Summer months</th>
<th>Winter month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.3333</td>
<td>5.5625</td>
</tr>
<tr>
<td>Std. Error</td>
<td>1.059</td>
<td>1.85592</td>
</tr>
<tr>
<td>95% Confidence Interval for Mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower Bound</td>
<td>5.1421</td>
<td>3.5625</td>
</tr>
<tr>
<td>Upper Bound</td>
<td>9.5245</td>
<td>7.1041</td>
</tr>
</tbody>
</table>

Table 3: Independent Samples Test.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>t-test for Equality of Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>t</td>
<td>df</td>
</tr>
<tr>
<td>Equal variances assumed</td>
<td>3.210</td>
</tr>
<tr>
<td>Equal variances not assumed</td>
<td>3.210</td>
</tr>
</tbody>
</table>

Fig. 1: Mean number of isolates.
Fig. 2: The distribution of different types of non polio enteroviruses in Egyptian governorates from January to December 2008.

Fig. 3: The expected normal and The Deviation from normal of seasonal variation of non-polio enteroviruses isolated from different Egyptian Governorates.
Fig. 4: The distribution of non-polio enteroviruses isolated from Egyptian stool samples.

Fig. 5: The distribution of echoviruses isolated from Egyptian stool samples.

Fig. 6: Polyacrylamide gel electrophoresis (10%) stained with Ethidium bromide.

Fig. (6) shows the amplification of Enteroviruses universal gene by RT-PCR. RNA extracted from Cell culture samples used as the template in RT-PCR Lane (1 to 10): PCR products from samples from 1-10. Lane(11): Negative Cell culture control. Lane (12): Negative Media control; Lane (13): DNA Negative control; Lane (14): Positive control: Lane (15): 100 bp Molecular weight Marker, Sigma. The gel was photographed using a Polaroid camera.

Discussion:

Millions of non-polio enterovirus infections occur globally exhibiting a peak in summer and autumn and are caused by more than 65 non-polio enterovirus serotypes, including coxsackie A viruses, coxsackie B viruses, echoviruses, and numbered enteroviruses. According to current virus classification, enterovirus serotypes are divided among five species within the genus Enterovirus: Poliovirus 1–3; human enterovirus A
Enteroviruses are most often associated with non-specific febrile illness, but can also be responsible for severe clinical manifestations e.g., aseptic meningitis, encephalitis, flaccid paralysis, and myocarditis (Bingjun et al., 2008). Enteroviral infections are more prevalent in children than in adults (Morens, 1978; Cherry, 1998; Carron & Geoffrey, 1999). The age distribution of enterovirus-associated illness in a 10-year surveillance report from the United States recorded that most of the cases were reported in young children (Moore, 1982). The present study was enrolled on specimens of children less than 15 years and that the isolation of NPEV decreased significantly with the increase in age. Similar pattern was observed by Morens who analyzed the reports to the CDC on isolation of NPEV for the years 1971–1975 and found that the incidence of reported isolations decreased with increasing age (Morens, 1978).

A total of 1000 stool specimens were tested during January–December 2008 (Tables 1 and 2). In general, these samples were taken from different governorates in Egypt from children age less than 15 years. Non Polio Enteroviruses activity lowest peak was found in South Sinai and Alwadi Algadid (0 %). The highest peaks of NPEV detection were observed in El Menya governorate (49 %) followed by El Fayoum (47%) during May (46%) and April 2008 (42%) (Fig.2), the least percentage of detection of NPEV was found in the month of January followed by December then November (12%, 16%, 19%) respectively. This result was similar to a study conducted by Diedrich and Schreier (2001) they reported that two to three serotypes are usually found to be predominant during one season with approximately other 10–15 serotypes sporadically detected. The type distribution varies from season to season. Although sero- or genotyping has little influence on the clinical management of patients, it helps to identify circulating virus types with increased virulence in order to check trends in enteroviral disease and guide outbreak investigation (Heim, 2005).

The current study in Egyptian governorates shows that echoviruses (EV) had the highest prevalence (37%) followed by non-typable enteroviruses (NTEV) which had a prevalence of 33% and finally coxsackievirus B with a prevalence of 30%. The serotyping of the NPEV isolated from the stool specimens in Egypt showed that the most prevalent echovirus in Egypt was EV-14 (14%) followed by EV-27 and EV-29 (12%) while the least prevalent was EV-4 which had a prevalence of 5%.

In other study involving serotyping of the NPEV isolated from stool specimens in India, results showed that Echovirus 6, 11, 9 and coxsackievirus B were the most frequent serotypes of NPEV (Kapoor, et al., 2001). In the present study, 58 isolates (33%) could not be identified by the antisera pools. The pools contained antibodies against only 21 of the 68 known human enterovirus serotypes, so their failure to neutralize a given isolate could have been due to the absence of homologous antibodies in the pools used, virus aggregation or presence of a virus mixture which is similar to study by Sokhey et al. (1996). Similarly, many enterovirus isolates could not be typed by the microneutralization test during a study of epidemiology of different enterovirus serotypes in Netherlands (Verboon et al., 2002). In India, Dhole et al. (2009) found that the percentage of NTEV in their study was 32.6% which is very similar to that of the current study (33%). Standard methods for enterovirus detection and identification are based on virus isolation on cell culture followed by serotyping the isolated viruses by microneutralization assays using pools of serotype specific antisera (Muir, et al., 1998). This procedure is time consuming and labor intensive and the availability of specific antisera gradually becomes restricted. Several techniques for rapid detection of the enterovirus genome in clinical samples, most of them based on PCR amplification in the 5' non-coding (5' NC) of the genome have been developed. In current study similar method was used to identify the isolates (n = 58) that could not be serotype by enterovirus-specific pools available. However such methods do not allow serotype identification and genetic characterization of the detected viruses beyond the genus level (Van Loon et al., 1999). Thus when information on the serotype is needed, virus isolation on cell culture remains the most appropriate technique. To overcome the problems related to the specific antisera, attempts have been made to develop new methods for typing enteroviruses by PCR amplification and partial sequencing in the VP1 region of the genome (Norder et al., 2003; Oberste et al., 2003 and Rahimi et al., 2009).

In conclusion, serotyping of the NPEV isolated from the stool specimens from different Egyptian governorates indicate that characterization of NPEV isolates could provide better understanding of the epidemiology of NPEV. Data generated from this study will help future studies on NPEV serotypes circulating in Egypt and to formulate more effective strategies. A better knowledge of the transmission and the implications of NPEV in diseases may also justify the future studies on their molecular epidemiology.
REFERENCES


