Australian Journal of Basic and Applied Sciences, 3(4): 3230-3238, 2009
ISSN 1991-8178
© 2009, INSInet Publication

AJBAS
Australian Journal of Basic and Applied Sciences

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

[1]Salwa S. Afifi, [1]Samar A. Zaki, [2]Aly F. Mohamed and [2]Hend El Hosseiny

[1]Microbiology and Immunology Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt
[2]Applied Research Sector, Egyptian Company for Biological Products, Drugs and vaccines, Cairo, Egypt

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 Algadid 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, pleurodinia, 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 I diabetes mellitus, have also been associated with enterovirus infections Approximately 75% of infections by EV occur

Corresponding Author: Salwa S. Afifi, Microbiology and Immunology Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.
E-mail: afifisalwa@yahoo.com

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 140µl 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 16µl 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'-ACACGGACACCCAAAGTAGTCGGT TCC-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. 1µl diluted sample was added to 19 µl reaction mixture containing 250µM of each dNTP, 2.5ul 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 $MgCl_2$, 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.

Governorate	No. of samples	NPEV		Governorate	No. of samples	NPEV	
		No	%			No.	%
Cairo	78	17	22	Suhag	42	5	12
Giza	59	5	8	Fayoum	36	11	31
Qaliubia	62	17	27	Behaira	51	12	24
Dakahleya	46	7	15	Aswan	20	3	15
Damietta	30	2	7	Luxor	34	11	32
Kafr Al-Shaikh	38	4	11	North Sinai	17	1	6
Ismailia	25	4	16	Red sea	11	0	0
Sharkeya	45	3	7	Assiout	42	6	14
Gharbeya	40	8	20	6th of October	43	10	23
Alexandria	52	2	4	Menya	43	15	35
Menoufeya	37	7	19	Port said	10	0	0
Suez	27	1	4	Matrouh	9	0	0
Beny Sewif	51	13	25	South Sinai	8	0	0
Qena	41	12	29	Alwadi Algadid	3	0	0
		Total			1000	176	17.60%

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

Governorates		Statistic	Std. Error	Summer months	Statistic	Std. Error	Winter month	Statistic	Std. Error
Mean		7.3333	1.059		31.5000	4.68152		15.3333	1.85592
95% Confidence	Lower Bound	5.1421			19.4658			10.5625	
Interval for Mean	Upper Bound	9.5245			43.5342			20.1041	
5% Trimmed Mean	7.1944			31.2222			15.2593		
Median	6.5000			32.0000			15.0000		
Variance	26.928			131.500			20.667		
Std. Deviation	5.1891			11.46734			4.54606		
Minimum	0.00			19.00			9.00		
Maximum	17.00			49.00			23.00		
Range	17.00			30.00			14.00		
Interquartile Range	8.75			20.25			5.00		
Skewness	0.438	0.472		0.418	0.845		0.614	0.845	
Kurtosis	-0.939	0.918		-0.697	1.741		2.060	1.741	

Table 3: Independent Samples Test.

Parameters	t-test for Equality of Means						
	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						Upper	Lower
Equal variances assumed	3.210	10.000	0.009	16.16667	5.03598	4.94580	27.38753
Equal variances not assumed	3.210	6.534	0.016	16.16667	5.03598	4.08400	28.24933

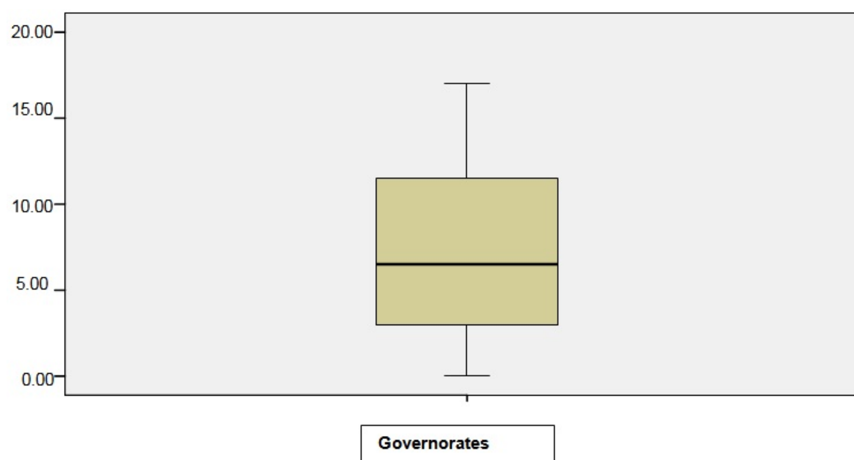

Fig. 1: Mean number of isolates.

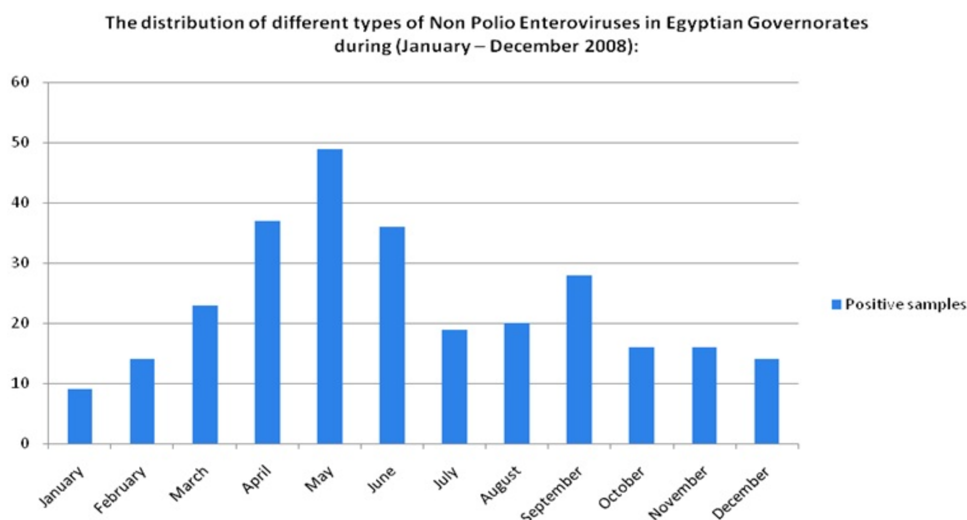

Fig. 2: The distribution of different types of non polio enteroviruses in Egyptian governorates from January to December 2008.

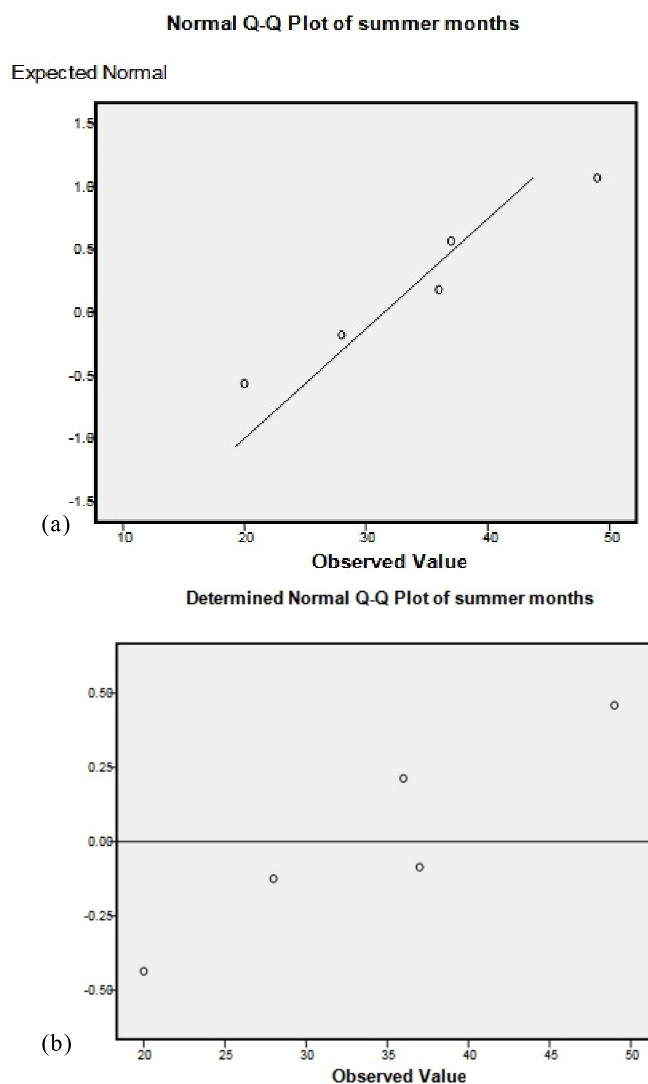

Fig. 3: The expected normal and The Deviation from normal of seasonal variation of non-polio enteroviruses isolated from different Egyptian Governorates.

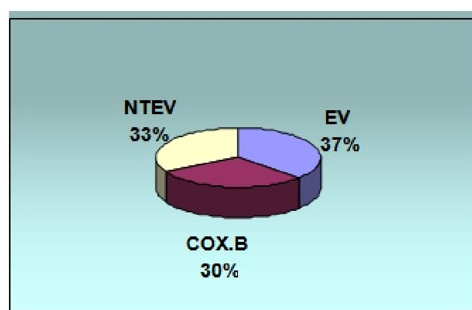

Fig. 4: The distribution of non-polio enteroviruses isolated from Egyptian stool samples.

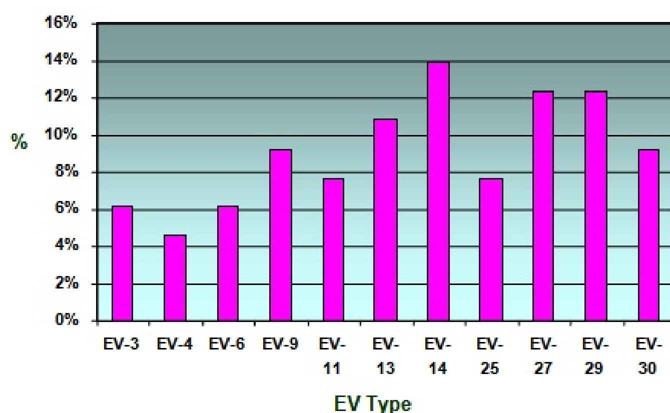

Fig. 5: The distribution of echoviruses isolated from Egyptian stool samples.

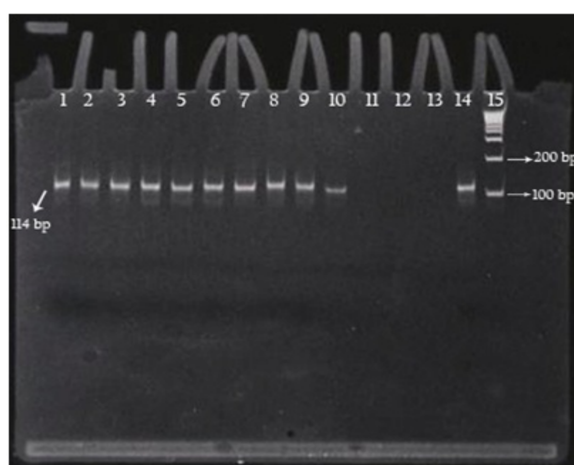

Fig. 6: Polyacrylamide gel electrophoresis (10%) stained with Ethidium bromide.

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

(coxsackievirus A2–8, 10, 12, 14, 16, and enterovirus 71); human enterovirus B (coxsackievirus A9, coxsackievirus B1–6, echoviruses 1–7, 11–21, 24–27, 29–33, enterovirus 69 and 73); human enterovirus C (coxsackievirus A1, 11, 13, 15, 17–22, and 24) and human enterovirus D (enterovirus 68 and 70) (King *et al.*, 2000). 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 antiserum 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 antiserum, 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

Bahri, O., D. Rezig, B.B. Oueslati, Ben A. Yahia, Ben J. Sassi, N. Hogga, A. Sadraoui and H. Triki, 2005. Enteroviruses in Tunisia: virological surveillance over 12 years (1992–2003). J. Med. Microbiol., 54: 63-69.

Bingjun, T., H. Yoshida, W. Yan, L. Lin and T. Tsuji, 2008. Molecular Typing and Epidemiology of Non-Polio Enteroviruses Isolated from Yunnan Province, the People's Republic of China. J. Med. Virol., 80: 670-679.

Blomqvist, S., A. Paananen, C.S. Kopra, T. Hovi and M. Roivainen, 2008. Eight Years of Experience with Molecular Identification of Human Enteroviruses. J. Clin. Microbiol., 46(7): 2410-2413.

Carron, N. and B.C. Geoffrey, 1999. A study of enterovirus isolation in Glasgow from 1977 to 1997. J. Med. Virol., 58: 304-312.

Cherry, J.D., 1998. Enteroviruses: coxsackieviruses, echoviruses, and polioviruses, p. 1787–1839. *In* R.D. Feigin and J.D. Cherry (ed.), Textbook of pediatric infection diseases, 4th Ed., The W.B. Saunders Co., Philadelphia, Pa.

Dhole, T.N., A. Ayyagari, R. Chowdhary, A.K. Shakya, N. Shrivastav, T. Datta and V. Prakash, 2009. Non-polio enteroviruses in acute flaccid paralysis children of India: vital assessment before polio eradication. J. Paediatr. Child. Health., 45(7-8): 409-13.

Diedrich, S., E. Schreier, 2001. Aseptic meningitis in Germany associated with echovirus type 13. BMC Infect. Dis. 1: U1-U5.

Gomes, M.C., De C.M. Castro, M.J.C. Olivera and Da E.E. Silva, 2002. Neutralizing Antibodies to Enterovirus 71 in Belém, Brazil. Mem Inst Oswaldo Cruz, Rio de Janeiro, 97(1): 47-49.

Grandien, M., M. Forsgren and A. Ehrnst, 1995. Enterovirus, p. 279-297. In E. H. Lennette D, Lennette A and Lennette ET (Ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections. American Public Health Association, Washington, D.C.

Heim, A., 2005. From poliovirus surveillance to enterovirus surveillance: A complete picture? J. Med. Microbiol., 54: 1-2.

Hymas, W.C., W.K. Aldous, E.W. Taggart, J.B. Stevenson and D.R. Hillyard, 2008. Description and Validation of a Novel Real-Time RT-PCR Enterovirus Assay. Clinical. Chemistry, 54: 406-413.

Johnston, S.L.G. and C.S. Siegel, 1990. Presumptive identification of enteroviruses with RD, Hep-2 and RMK cell lines. J Clin Microbiol., 28:1049-1050.

Kapoor, A., Ayyagari, A. and Dhole, T.N. 2001. Non-polio enteroviruses in acute flaccid paralysis. Indian J. Pediatr., 68: 927-929.

Kelly, H., K.A. Brussen, A. Lawrence, E. Elliot, J. Pearn and B. Thorley, 2006. Polioviruses and other enteroviruses isolated from faecal samples of patients with acute flaccid paralysis in Australia, 1996–2004. Journal of Pediatrics and Child Health, 42: 370-376.

King, A.M.Q., F. Brown, P. Christian, T. Hovi, T. Hyypia, N.J. Knowles, S.M. Lemon, P.D. Minor, A.C. Palmenberg, & other authors 2000. Family Picornaviridae. In Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses, pp: 657-678.Edited by M.H.V. Van Regenmortel, C.M. Fauquet, D.H.L. Bishop, E.B. Carsten, M.K. Estes, S.M. Lemon, J. Maniloff, M. A.Mayo, D.J. McGeoch, C.R. Pringle & R.B. Wickner. San Diego: Academic Press.

Manzara, S., M. Muscillo, G. La Rosa, C. Marianelli, P. Cattani and G. Fadda, 2002. Molecular Identification and Typing of Enteroviruses Isolated from Clinical Specimens. J. Clin. Microbiol., 40(12): 4554-4560.

Morens, D.M., 1978. Enteroviral disease in early infancy. J. Pediatr., 92: 374-377.

Moore, M., 1982. From the Centers for Disease Control. Enteroviral disease in the United States, 1970–1979. J. Infect. Dis., 146: 103-108.

Muir, P., U. Kammerer, K. Korn, M.N. Mulders, T. Poyry, B. Weissbrich, K. Kandolf, G.M. Cleator, and Van Loon, A.M. 1998. Molecular typing of enteroviruses: current status and future requirements. Clin. Microbiol. Rev., 11: 202-227.

Nix, W.A., M.S. Oberste and M.A. Pallansch, 2006. Sensitive, Seminested PCR Amplification of VP1 Sequences for Direct Identification of All Enterovirus Serotypes from Original Clinical Specimens. J. Clin. Microbiol., 44(8): 2698-2704.

Norder, H., L. Bjerregaard, L. Magnius, B. Lina, M. Aymard and J.J. Chomel, 2003. Sequencing of 'untypable' enteroviruses reveals two new types, EV-77 and EV-78, within human enterovirus type B and substitutions in the BC loop of the VP1 protein for known types. Gen. Virol., 84: 827-36.

Oberste, M.S., K. Maher, M.R. Flemister, G. Marchetti, D.R. Kilpatrick and M.A. Pallansch, 2000. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. J. Clin. Microbiol., 38: 1170-4.

Oberste, M.S., W.A. Nix, K. Maher and M.A. Pallansch, 2003. Improved molecular identification of enteroviruses by RT-PCR and amplicon sequencing. J. Clin. Virol., 26: 375-7.

Rahimi, P., H. Tabatabaie, M.M. Gouya, M. Mahmudi, T. Musavi, K.S. Rad, T.M. Azad and R. Nategh, 2009. Direct identification of non-polio enteroviruses in residual paralysis cases by analysis of VP1 sequences. J. Clin. Virol., 45(2): 139-41.

Rigonan, A.S., L. Mann and T. Chonmaitree, 1998. Use of monoclonal antibodies to identify serotypes of enterovirus isolates. J. Clin. Microbiol., 36: 1877-1881.

Robinson, C.C., M. Willis, A. Meagher, K.E. Gieseker, H. Rotbart and M.P. Glodé, 2002. Impact of rapid polymerase chain reaction results on management of pediatric patients with enteroviral meningitis. Pediatr. Infect. Dis. J., 21: 283-286.

Saeed, M., S.Z. Zaidi, A. Naeem, M. Masroor, S. Sharif, S. Shawkat, M. Angez and A. Khan, 2007. Epidemiology and clinical findings associated with enteroviral acute flaccid paralysis in Pakistan. BMC Infect. Dis., 7: 6.

Sokhey, J., B. Sharma, H. Singh and D. Sood, 1996. Isolation of poliovirus from cases of acute flaccid paralysis. Indian Pediatr., 33: 917-920.

Van Loon, A.M., G.C. Cleator and A. Ras, 1999. External quality assessment of enterovirus detection and typing. Bull WHO, 77: 217-223.

Verboon, M.A., T.G. Krediet, Van A.M. Loon, J. Kaan, J.M.D. Galama, L.G. Gerards and A. Fleer, 2002. Epidemiological survey of neonatal non-polio enterovirus infection in the Netherlands. J. Med. Virol., 66: 241-245.

Vuorinen, T., R. Vainionpää and T. Hyypiä, 2003. Five years' experience of reverse-transcriptase polymerase chain reaction in daily diagnosis of enterovirus and rhinovirus infections. Clin. Infect. Dis., 37: 452-455.

Wiedbrauk, D.L. and S.L.G. Johnston, 1993. Enteroviruses. In Wiedbrauk DL, Johnston SLG (Eds.), Manual of Clinical Virology, Raven Press, New York, pp: 92-97.

World Health Organization, 2001. Manual for the virological investigation of polio WHO/EPI/GEN/97.01. World Health Organization, Geneva, Switzerland.

World Health Organization, 2004. Polio laboratory manual, 4th edition, 2004. World Health Organization, Geneva, Switzerland. pp: 87-121.