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Non-Polio Enteroviruses Aseptic Meningitis:Embaba Fever Hospital Admissions 2010-2011

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Abstract:

Human enteroviruses (EV) cause a wide spectrum of both common and uncommon illnesses among all age groups. Enterically transmitted. The objective of this study was to identify non-poliovirus EV as a cause of viral aseptic meningitis (VAM) by two methods (cell culture and Real time PCR). From October 2010 to August 2011 cerebrospinal fluid (CSF) samples were collected from 85 patients Embaba fever hospital admitted with symptoms of aseptic meningitis of any age and both sexes. The 85 CSF samples were inoculated into RD (human rhabdomyosarcoma) cell line in three blind passages to amplify isolates producing EV-like CPE. A total of 14 (16.5%) out of 85 CSF samples showed EV-like CPE. By Real time PCR 11 out of the 14 culture positive samples and 5 out of the 14 source of virus isolation original CSF were non polio EV positive. The frequency of non-polio EV meningitis hospital admissions was in the summer season (50%), spring (25%), late autumn (16.6%) and least frequency in winter (8.4%). non-polio EV meningitis was detected in 6 out of 41 male patients (14.5%) and in 6 out of 44 female patients (13.5%). Also non-polio EV meningitis was detected in all ages with marked increase of incidence in young children (41.6%) and old age (50%) and less in adult (8.4%).

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In conclusion

Our data showed that the non-polioviruses EV was associated with the majority of VAM during 2010 – 2011 at the Embaba fever hospital which serves Embaba, Shoubra Elkheema, Qualyba and neighbors localities in Egypt. Rapid detection of non-polio EV meningitis is essential for making decisions about patient management and treatment.

Key words: Aseptic meningitis, cerebrospinal fluid, cell culture, Real time PCR, non-polio EV.

INTRODUCTION

The EVs are small RNA viruses belonging to the Picornaviridae family. Currently, more than 111 serotypes of EV have been identified divided into 4 species, including enterovirus A (EV-A) (23 serotypes including some coxsackie A viruses and enteroviruses), EV-B (60 serotypes including enteroviruses, coxsackie B viruses, echoviruses, and swine vesicular disease virus), EV-C (23 serotypes including poliovirus (PV) 1-3, some coxsackie A viruses and enteroviruses), EV-D (5 serotypes: EV-68, EV-70, EV-94, EV-D111and EV-D120) **(1).**

Enteroviruses are small, icosahedral RNA non-enveloped particles of about 30 nm diameter, composed of sixty copies of each of the four structural proteins VP 1 to VP4, that surround a positive-stranded RNA genome of approximately 7500 nucleotides **(White and Fenner, 1994)[2].** The lack of a lipid envelope contributes to the in vitro stability of the enteroviruses, permitting EVs to survive the gastric pH as well as environmental stresses. The EVs remain viable for prolonged periods in sewage, water, fomites and on hands, thereby enhancing their transmissibility. When frozen, these viruses are stable for years and even decades **(Racaniello, 2007)[3].** The non-polio EV can cause a broad spectrum of illnesses such as febrile disease, hand-foot-mouth disease, herpangina, viral aseptic meningitis and encephalitis **(Michos et al., 2007 and Irani 2008)**[4,5]. Myocarditis and neonatal sepsis **(Afifi, et al., 2009)[6].** Aseptic

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meningitis (AM) referes to a clinical syndrome of meningeal inflammation in which common bacterial agents cannot be identified in CSF **(Rotbart, 1995)[7].** Non-polio EV accounted for approximately 50% of cases of AM **(Michos et al., 2007)[4].** The diagnosis of VAM infection is documented by viral cultures from CSF or stools and blood samples. Among the limitations of viral culture for the diagnosis of VAM infection are a sensitivity of 65% to 75%, a turnaround time of 3 to 10 days, and the high degree of technical expertise required **(Rotbart, 1995)[7].** PCR with a sensitivity and specificity approaching 100% **(Ramers et al., 2000)**[8] has been shown to be an effective alternative to viral culture for rapid diagnosis. Its use improve patient management, reduce hospital related costs and enhances earlier discharge from the hospital **(Lee et al., 2006)[9].** .

Patients and Specimens:

Eighty five patients, of both sexes and different ages presenting with symptoms of AM who were admitted to the Embaba fever hospital between October 2010 and August 2011 were the subjects of this study. One ml of CSF obtained by lumber puncture was sent to the laboratory department of Embaba fever hospital.

Material and Methods:

RD Cell culture:

All cell culture work was carried out in a vertical laminar air flow hood under complete sterile conditions. Growth medium was discarded from RD cell culture flask, when the cell sheet was confluent. Cell dispersing solution was added to the cell sheet for one min at room temperature then discarded .The flask was incubated at 37ºC until the cells became detached from the flask surface. The cells were resuspended in growth medium and any aggregated cells were dispersed by pipetting the cell suspension back and forth several times. Viable cell count by trypan blue vital dye exclusion was done. The cell suspension was diluted to contain 100.000 cells /ml, and distributed into a glass tissue culture tube. In order to maintain the cell line, 7ml of the cell suspension was distributed per 75cm2 tissue culture flasks, which was incubated to form a complete cell sheet before subculture. Serial subculture was carried out twice weekly.

Virus isolation in RD cell cultures:

The CSF sample was inoculated into 2 tissue culture tubes with confluent monolayer . The growth medium was aspirated and 0.2 ml of the CSF sample was inoculated into each tube and then incubated at 37ºC for 2-3 hours to allow virus adsorption, then 1ml of maintenance medium was added. The tubes were incubated at 37ºC for 15 days. Inoculated cells and control cells were checked by microscopic examination for cytopathic effect (CPE). Four uninoculated cell culture tubes were included as cell culture controls. These controls must remain healthy without any CPE if the test is considered to be valid. The virus isolation was considered positive when the characteristic EV CPE was observed in the form of visible rounding, shrinking, nuclear pyknosis with cell detachment from the glass surface and cell degeneration **(Jaianand et al., 2011)[10].**

When CPE reached third degree (+ 3) the cell culture tubes were removed from the incubator and the cell associated virus (CAV) was released by repeated freezing and thawing for three times. Then the harvested suspension was clarified by centrifugation at 1500 rpm for 10 minutes.

The supernatant containing the putative replicating virus was removed was amplified by another successive blind passages using as inoculum 0.2ml of 1: 1000 dilution of the CAV harvested suspension. This step was taken to ensure that a replicating virus in CAV suspension and to dilute out any toxic substance. The harvest of the third passage representing 1: 1000000000 (10) final dilution factor of original inoculum was divided into 0.2ml aliquots and stored at -80 C. The CSF inoculated cell cultures without CPE were

examined for 2 weeks before they were considered negative for EV like isolation.

Real time- PCR:

Tissue culture associated virus (TAV) harvests that induced CPE were tested by PCR. The reagents (CAT # KT0099; Lot # 10-0124) for Real time PCR were working provided by CDC; USA through WHO program for the global eradication of wild polioviruses.

The procedure provided by CDC was followed:

1- TAV, PCR reagents were held at room temperature.
2- Buffer B + enzyme mix: The first time a vial of Buffer B lmL is used, 2.8µl 1M DTT, 27.6 µl 40 U/ µl RNase inhibitor, 18.0 µl 20 U/ µl RT enzyme and 54.8 µl 5 U/ µl Taq polymerase and mix, this mixture is called Buffer B + E.
3- Reaction solution was done: for each sample set, 19 µl Buffer A (vortex to resuspend probe before use) and 5 µl Buffer B + E, dispense 24 µl reaction solutions into each well. So for testing all T.C samples (14 T.C samples + one sample positive and one negative) master mix of Buffer A+B was done; 16 samples × 19 µl Buffer A = 304 µl; 16 samples × 5 µl Buffer B = 80 µl and dispense 24 µl of the A + B master mix per reaction well**.**
4- 0.5µl of TAV supernatant for each sample was added into appropriate reaction strip well. One positive control: non-infectious control RNA supplied with Entero PCR kit. One negative control: Buffer A + B with no template.
5- The strips were placed in Real-time thermocycler and the amplification was carried out for 40 cycles as follows; a) RT reaction, 42°C, 45 min. b) Inactivate RT, 95°C, 3 min. c) PCR cycle. 95°C for 24 sec, 44°C for 30 sec, and then a 25% ramp speed to 60°C for 24 sec, for 40 cycles annealing step **(Bustin SA and Nalon T 2004)[11].**

The end point fluorescent data record sheet was collected at the end of the anneal step.

Interpretation of Real time PCR for non-polio enteroviruses:

The results were interpreted by looking for the cycle threshold value (Ct) of between 10-28. The Ct is the cycle number where a PCR product is seen via fluorescence. These Ct values were calculated automatically by the ABI 7500 software. The Ct value cutoff is 30, with values less than 30 as positive and values more than 30 as negative.

Results:

Table (1): Displays the frequency of detection of EVs like CPE from samples of CSF inoculated into RD cell cultures. The isolated viruses were identified as NPHEV using real time PCR. Viral isolates were confirmed after 3 serial passages in RD cell cultures. It was found that 14 (16.5%) out of 85 CSF samples showed EV-like CPE in RD cell cultures. As regard Real time PCR it was found that 11 out of the 14 culture positive samples were positive as shown in the figure (1) and Table (2).

Table (1): Detection of enterovirus like cytopathic changes inducing isolates from CSF samples using in vitro RD cell cultures and the isolated viruses were identified as NPHEV using real time PCR.

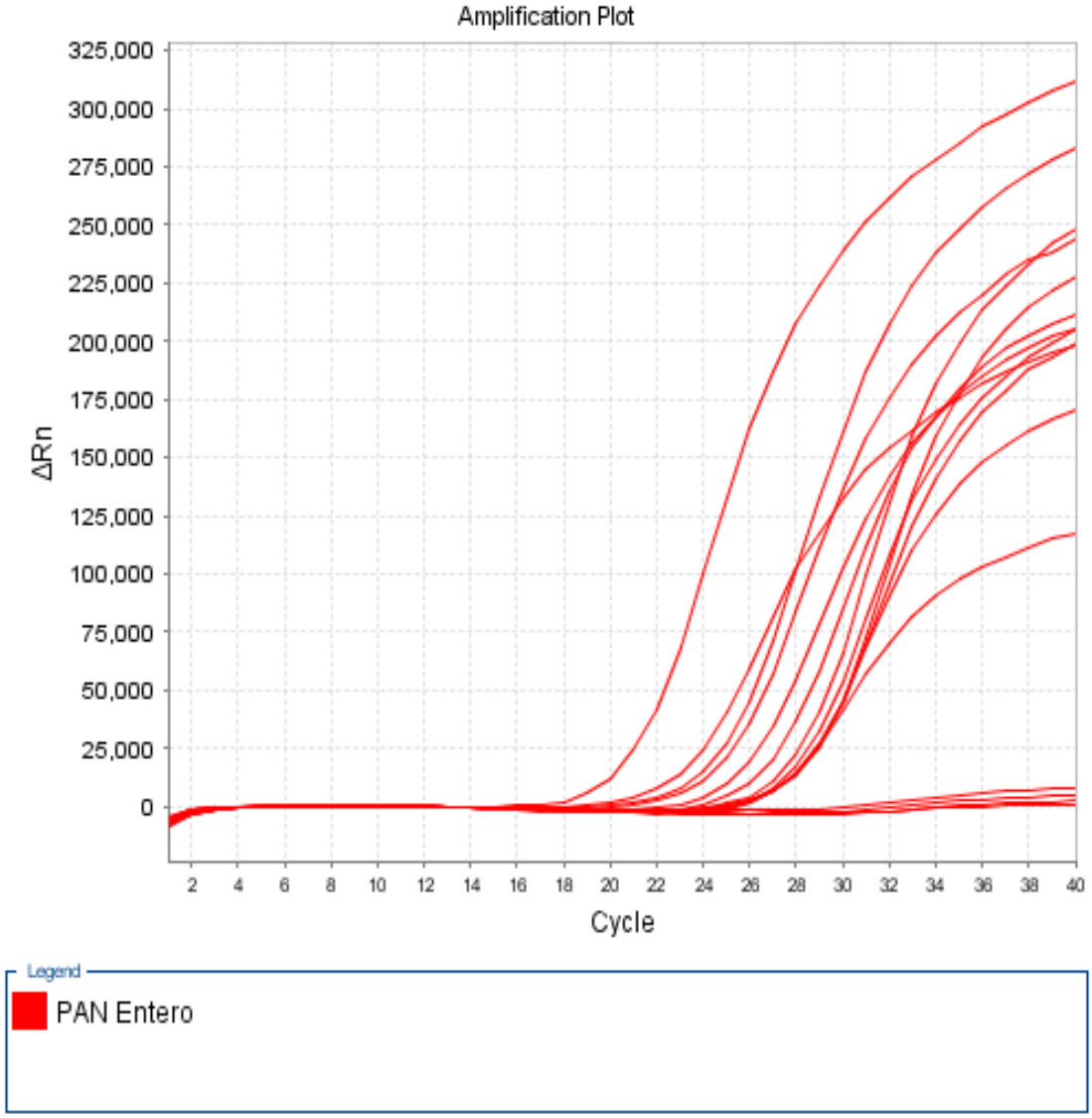
Table (1):

No of CSF samples	Virus isolation in RD cell culture on the basis of enterovirus like cytopathic changes
85	14/85 (16.5%)

Table (2):

No of virus isolated in RD cell culture on the basis of enterovirus like cytopathic changes	No of Real time PCR results using EVs common primers
14	11

Figure (1): Shows eleven RNA samples out of the 14 suspected enterovirus isolates from CSF inoculated in RD cell cultures positive samples were positive by Real time PCR using common non polio enteroviruses primers (CDC., 2008). The upper lane corresponding to positive control followed by positive samples (11samples) and the flat lanes represent the negative samples (3 samples) and one negative control



According to the results of virus isolated in RD cell culture the higher frequency of VAM was detected by virus isolation from the CSF of clinically suspected patients in the summer season 7/14 (50%) positive cases followed by spring 3/14 (21.4%) positive cases and late autumn 3⁄14 (21.4%) positive cases with less frequent in winter 1⁄14 (7.2%) positive case. VAM was detected in 8 out of 41 male patients (19.5%) and in 6 out of 44 female patients (13.5%). Also VAM was detected in all ages with 7⁄14 (50%) cases incidence in young children and 6⁄14 (42.4%) old age and less in adult 1⁄14 (7.1%) case.

Discussion:

AM is frequently caused by viral agents (VAM), particularly the human EVs **(Irani, 2008)[5].**

The present study was carried out to investigate the role of non-polio EV in suspected VAM in patients admitted to the Embaba fever hospital from a large zone of greater Cairo that stretches from the industrial Shoubra Elkheema to the rural villages around Qualyub. CSF samples from 85 patients presenting with clinical data suspected to have VAM were subjected to routine virus isolation procedure using RD cell culture. Possible non polio EV isolation were obtained from 14⁄85 (16.5%) CSF samples. Further documentation of these virus isolates by real time PCR showed that eleven out of these 14 were non polio EV.

The current study comes in agreement with results of **Verstrepen et al., (2002)[12],** found that from 186 CSF obtained from VAM patients, isolation of viruses by cell culture inoculation was positive for enterovirus in 31(16.5%) CSF samples. By contrast they detected enterovirus RNA in 45⁄186 (24%) of CSF samples by real-time PCR. They concluded that direct testing of samples by real-time PCR is more sensitive for diagnosis.

Lourdes et al. (2007)[13], have studied 279 VAM patients, of both sexes; aged from 1 month to 75 years and found that 30⁄279 (10.7%) CSF were EV positive by RD cell culture. The isolated EV were checked with RT-PCR and 18/30(60%) were EV. Nearly their results are in agreement with our results where's we found that 14⁄85 (16.5%) CSF samples yielded EV like using RD cell lines. Out of these TAV isolates non polio enterovirus RNA was detected in 11⁄14 (78.5%) by Real time PCR. RD cells were also used by **Silva et al. (2002)[14],** for virus isolation from CSF obtained from VAM patients. The isolation rate was as low as 7.5% (7/94) but **dos Santos et al., (2006)[15]** reported higher virus isolation from CSF as 162/1,022 (15.8%) using RD cell culture. A higher isolation rate 36/68(53%) from CSF of VAM was reported by **Guney et al., (2003)[16]**. Thoren and Widell (1994) reported virus isolation from VAM CSF samples using green monkey kidney (GMK) cells and human embryonic fibroblasts (HEF) from 6/27(22%). The type of cell and their different origins influence EV isolation rates and RD cells seem to be the choice cell culture for EV isolation.

Pérez-Ruiz., (2003)[17] compared the ability of the RD and MRC-5 cell-lines to detect enteroviruses in 33 clinical samples including (CSF, stools and throat swabs). The samples were tested by traditional tube-culture and 100% and 85% of samples yielded enterovirus isolation in RD and in MRC-5 cells, respectively. RD cells supported growth of all enterovirus serotypes, whereas MRC-5 cells were not able to detect coxsackievirus A9 and two coxsackievirus B5 that were isolated in RD cell cultures.

Bráulio et al., (2007)[18], found that 8⁄18 (44%) EV isolates from CSF were obtained after 2 passages in RD cell line. In the present study non polio EV isolates were obtained by inoculation followed by 3 passages in RD cell culture from VAM CSF samples. The EV were isolated from 14/85 (16.1%) of the CSF samples. This different isolation rates may be due to the type of EV or the different number of passages, as the third passage differentiate toxicity from CPE, so toxic components of the sample can be diluted and the third passage will allow cell to maintain viability or alternatively for the virus to amplify and produce CPE and confirm the diagnosis (**Pallansch and Roos, 2001)[19]**.

Ceyla et al., (2009)[20], collected their VAM CSF samples (91samples) from January 2005- December 2006 and the age of their patients ranged from < one year old to > 60 years old and confirmed viral isolation from CSF after 3 passages in RD cell cultures and found EV in 6/91(6.5%) of their CSF samples. Also they found that 18

⁄91 (19.8%) isolates were EV by RT-PCR. Their infected RD results (19.8%) were less than the results in the present study (35%), this may due to the diagnosis in this work was achieved by Real time PCR which is more specific and accurate **(Sylvie et al., 2010)[21].**

This study comes in agreement with results of **Bottner et al.,(2002)[22],** who found that non polio enteroviruses were detected in 30⁄70 (42%) patients using PCR. However the percentage in their study more than the percentage in this study, this may be due to all patients in their study from children with developing immune system.

Dalwai et al., (2010)[23] found that the majority 75 ⁄ 92 (82%) of the enteroviral meningitis cases were among children < 2 yrs of age. Furthermore, the frequency of VAM cases in children < 2 yrs of age (75 of 281, 27%) was significantly higher than the frequency (3 of 38, 8%) seen in older (4-12 year) children (p = 0.011). The findings are consistent with earlier observations showing that infants and young children, due to their developing immune system, are more susceptible to enteroviral infections **(Rotbart 1995 and Siafakas 2004)**[7,24]. Enteroviral infections in older children are less common and are often associated with recreational water activities **(Berlin, 1993)[25]**. In the present study enterovirus meningitis was detected in all ages with marked increase of incidence in young children and old age. In our study 41.6% of patients were from 2 months up to 17 years old. Of patients were up to 15 years old, **Lourdes., et al., (2007)[13],** reported a rate 51.6%. A rate of 58.7% was reported by **Bedoya et al. (1998)[26]** in cases of acute meningitis, with patients aged between 1 and 15 years. They also reported that meningitis was uncommon in patients less than one month old and above 17 years old. The current study also did not find patients above 17 years up to 25 years; however, several patients above 45 years of age were identified (50%).

We conclude that the non-polioviruses human enteroviruses were associated with the majority of aseptic meningitis during Oct 2010 to August 2011 in locations served by Embaba fever hospital. Rapid detection EV meningitis is essential in making decisions for patient management and treatment.

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