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Viral meningitis in Sudanese children

Differentiation, etiology and review of literature

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Abstract

Diagnosis of viral meningitis (VM) is uncommon practice in Sudan and there is no local viral etiological map. We therefore intended to differentiate VM using standardized clinical codes and determine the involvement of herpes simplex virus types-1 and 2 (HSV-1/2), varicella zoster virus, non-polio human enteroviruses (HEVs), and human parechoviruses in meningeal infections in children in Sudan. This is a cross-sectional hospital-based study. Viral meningitis was differentiated in 503 suspected febrile attendee of Omdurman Hospital for Children following the criteria listed in the Clinical Case Definition for Aseptic/Viral Meningitis. Patients were children age 0 to 15 years. Viral nucleic acids (DNA/RNA) were extracted from cerebrospinal fluid (CSF) specimens using QIAamp® UltraSens Virus Technology. Complementary DNA was prepared from viral RNA using GoScript™ Reverse Transcription System. Viral nucleic acids were amplified and detected using quantitative TaqMan® Real-Time and conventional polymerase chain reactions (PCRs). Hospital diagnosis of VM was assigned to 0%, when clinical codes were applied; we considered 3.2% as having VM among the total study population and as 40% among those with proven infectious meningitis. Two (0.4%) out of total 503 CSF specimens were positive for HSV-1; Ct values were 37.05 and 39.10 and virus copies were 652/PCR run (261 × 10^3/mL CSF) and 123/PCR run (49.3 × 10^3/mL CSF), respectively. Other 2 (0.4%) CSF specimens were positive for non-polio HEVs; Ct values were 37.70 and 38.30, and the approximate virus copies were 5E2/PCR run (~2E5/mL CSF) and 2E2/PCR run (~8E4/mL CSF), respectively. No genetic materials were detected for HSV-2, varicella zoster virus, and human parechoviruses. The diagnosis of VM was never assigned by the hospital despite fulfilling the clinical case definition. Virus detection rate was 10% among cases with proven infectious meningitis. Detected viruses were HSV-1 and non-polio HEVs. Positive virus PCRs in CSFs with normal cellular counts were seen.

Abbreviations: cDNA = complementary DNA, CNS = central nervous system, CSF = cerebrospinal fluid, DNA = deoxyribo nucleic acid, gDNA = genomic DNA, HEVs = non-polio human enteroviruses, HPeVs = human parechoviruses, HSV-1/2 = herpes simplex virus type-1/2, PCR = polymerase chain reaction, RNA = ribo nucleic acid, TBE = Tris Borate EDTA, VM = viral meningitis, VZV = Varicella Zoster Virus.

Keywords: HSV-1, non-polio HEV, PCR, viral neuroinfections

1. Introduction

Viral meningitis occurs at any age with young children being the most commonly affected. Unfortunately, little is known regarding the incidence of viral meningitis (VM) in Sudan and other African countries unlike that of Europe and US. In a large cohort (n = 12,000) of children in Finland, the annual incidence of presumed VM was 219 per 100,000 in infants under 1 year and 27.8 per 100,000 overall in children under 14 years.[1] The incidence of aseptic meningitis in people aged 16 years and above was lower at 7.6 per 100,000 in another smaller study from the same country.[2] Viral meningitis is a notifiable disease in England and Wales, but many cases go unreported[1,3]; in 2005 to 2006, a total of 2898 people were admitted with a diagnosis of VM, 10 times the number of cases notified over the same period.[3] In the United States, VM accounts for approximately 26,000 to 42,000 hospitalizations each year, affecting mainly infants under 1 year of age and children 5 to 10 years.[4]

In Africa, some studies reported the involvement of potentially curable causes of central nervous system (CNS) infections; 2 (0.99%) children in Burkina Faso were found positive for HSV-1 among aseptic meningitis suspected patients whereas none for HSV-2.[5] In Uganda, cerebrospinal fluid (CSF) polymerase chain reaction (PCR) testing for herpes simplex virus type-1/2 (HSV-1/2), varicella zoster virus (VZV), human

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parechoviruses (HPeV), human enteroviruses (HEV), mumps and rubella viruses were all found negative in a suspected young child.[6] Herpes encephalitis was confirmed in 1 case (0.96%) among children presented with fever and coma in 3 main hospitals in Khartoum, Sudan, during April to November of 2011.[7] Some other studies reported more aggressive and potentially fatal neuroinvasive newly discovered viruses (i.e., Ntwetwe virus)[6] or previously known viruses (i.e., West Nile, Zika, Chikungunya and Ebola viruses)[8] in sub-Saharan Africa as major causes of CNS infections in children.

HSV-1 is responsible for more than 85% of all cases with sporadic necrotizing encephalitis and is infrequently associated with aseptic meningitis while neonatal infections account for 25% of all cases of neonatal HSV infection, with an overall incidence ranging from 1 case in 2000 to 5000 deliveries per year.[9,10] Aseptic meningitis is usually caused by HSV-2 primary infection; it occurs mainly in adults, since infections are mainly acquired by sexual contact, but can also occur in young children. HSV-2 accounts for 75% of neonatal infections by HSV.[10–12] In temperate climates, most non-polio HEVs infections occur in outbreaks during the warmest months, however, sporadic cases can develop throughout the year. In the United States, infections are seen more frequently from June to October. In tropical and subtropical regions, the incidence remains stable throughout the year.[4,13] HPeVs have been identified in Japan, Netherlands and United States. In a retrospective study from the Netherlands, CSF PCR testing from 716 children less than 5 years of age identified HPeVs in 4.6%. The yearly prevalence varied from 0.4% to 8.2%.[14,15] Since the introduction of varicella vaccine, the incidence of VZV infection (i.e., chickenpox, herpes zoster) in children has declined substantially and the overall incidence of the neurologic complications in healthy children, including aseptic meningitis, is reduced to less than 1%.[16] Excluding the single case report mentioned above,[7] we couldn't find other studies on HSV1/2, VZV, non-polio HEVs and HPeVs infections in children in Sudan.

Viral meningitis is difficult to be differentiated clinically from bacterial meningitis or from other non-CNS related febrile infections that are more common in Sudan. In fact, the diagnosis of VM is uncommonly considered or reported in pediatric hospital practice in Sudan; unlike the highly inflated over-diagnosis of bacterial meningitis that was discussed and reported in our previous publication (Abdelrahim et al).[17] Besides, there is no well-established local viral etiological map. We, therefore, intended in this study to differentiate VM among suspected febrile attendees of a major referral children's hospital in Khartoum, Sudan, and to explore viruses causing neurological infections amongst those that are mostly reported in children worldwide, namely; HSV-1, HSV-2, VZV, non-polio HEVs and HPeVs.

2. Methods

2.1. Materials

This is a cross-sectional hospital-based study. All children age 0 to 15 years attending Omdurman Hospital for Children in Khartoum, Sudan during December to August of 2010 and presented with fever (>37°C), seizures and a suspicion of neuroinfections were included (i.e., complete coverage) (n = 503). Demographic (i.e., age, sex), clinical (i.e., symptoms of meningeal irritation) and conventional laboratory data (i.e., total white blood cell count and type, glucose and protein concentrations, evidence of bacterial etiology) were obtained from hospital records. An aliquot of CSF was frozen in −80°C for nucleic acid analysis at the department of Clinical Microbiology, Umeå University, Umeå, Sweden. These included; viral deoxyribo nucleic acid (DNA) and RNA extraction using QIAamp® UltraSens Virus Technology, estimation of nucleic acids concentrations using NanoDrop™ spectrophotometery, preparation of complementary DNA (cDNA) from viral RNA using GoScriptTM reverse transcription system (for HEVs and HPeVs), quantitative real time PCR using TaqMan® method (for HSV-1/2, VZV, and HEVs) and conventional PCR followed by agarose gel electrophoresis (for HPeVs).

2.2. Secondary analysis using published clinical decision rules

Data collected throughout this project were used to identify infectious meningitis and differentiate aseptic/VM according to the criteria listed in the Clinical Case Definition for Aseptic Meningitis (2004) developed by the Brighton Collaboration Aseptic Meningitis Working Group,[18] these are; presentation with meningeal irritation, CSF pleocytosis and negative CSF bacterial culture with or without identified microbial etiology by nucleic acids test, or normal CSF cellular count but with evidence of microbial origin. Following the criteria (Table 1), cases were classified as aseptic meningitis based on clinical evidence of acute meningitis, CSF pleocytosis and negative CSF Gram stain and routine bacterial culture. Cases showing CSF pleocytosis (≥5 cells/mm^3) were considered as *Infectious Meningitis*, those fulfilling all criteria of the Clinical Case Definition for Aseptic Meningitis were considered as *Aseptic Meningitis*, and cases with confirmed viral etiology were considered as *Proven Viral Meningitis* (Table 2).

2.3. Viral DNA and RNA extraction

Viral DNA and RNA were extracted using QIAamp UltraSens virus kit (Qiagen®, Hilden/Germany) following their recommended standard protocol. One mL specimen was used to

Table 1

Clinical case definition for aseptic meningitis and number of cases fulfilling the criterias.

#	Criteria for aseptic meningitis Level (1) of diagnostic certainty	Number of cases fulfilling only one criterion	Number of cases fulfilling all 4 criteria
1	Clinical evidence of acute meningitis: fever, headache, vomiting, bulging fontanel, nuchal rigidity or other signs of meningeal irritation	361 out of 361 (142 MD)	18 (4.5%; 18/404)
	AND		
2	CSF pleocytosis: >5 leukocytes/μL if patient is ≥ 2 mo, or > 15 leukocytes/μL in infants < 2 mo	23 out of 404 (97 MD)	
	AND		
3	Absence of any microorganism on Gram stain of CSF	497 out of 503	
	AND		
4	Negative routine bacterial culture of CSF in the absence of antibiotic treatment before obtaining the first CSF sample	497 out of 503	

Case definition developed by the Brighton Collaboration Aseptic Meningitis Working Group.[18]
MD = missing data 1.

Table 2

Classification of Sudanese pediatric cases based on clinical and nucleic acids findings.

♯	Defined groups	Cases out of 503*		% Out of 40†
		%	Frequency	
1	Proven infectious meningitis‡	8%	40†	100%
2	Proven aseptic meningitis	6.8%	34§	85%
3	Proven viral meningitis	3.2%	16‖	40%
4	HSV-1 meningitis	0.4%	2	5%
5	Non-polio HEV meningitis	0.4%	2	5%

*Total number of suspected febrile patients who attended the hospital during the study period and were subjected to lumber puncture.

†This number is the sum of 17 cases with positive microbial origin -but with normal cellular count-along with 23 cases with CSF pleocytosis with or without positive microbial etiology (this is the overall project findings described in 4 articles -including this one[20,21,22]).

‡This information is presented in detail in our previous publication.[20]

§This number is the sum of 18 cases with level-1 of diagnostic certainty for aseptic meningitis along with 16 cases with laboratory evidence of non-cultivable microbial etiology (further information is presented in our previous publications.[21,22]

‖Four cases with confirmed HSV-1 and HEV DNA/RNA and 12 cases with other confirmed viral nucleic acids (this information is presented in our previous publication.[21]

concentrate viral DNA/RNA. Two aliquots were prepared (30 µL each), one was preserved at –80°C and was used for detecting viral DNA, the second was preserved at –20°C and was used for the preparation of cDNA for viral RNA testing.

2.4. Synthesis of cDNA

The aliquot stored at –20°C was used for converting viral RNA to cDNA using RNasin® Plus RNase Inhibitor product (Promega®, Madison, WI). The enzyme is supplied in a buffer composed of 20 mM HEPES-KOH (pH 7.6), 50 mM KCL, 8 mM DTT and 50% v/v glycerol. The procedure is used to convert up to 5 µg of total RNA or up to 500 ng of poly-(A) RNA into the first strand of cDNA. In brief, per each reaction, 1 µL of random hexameric primer (0.5 µg/reaction) was added into a sterile thin-walled reaction tube containing 4 µL experimental RNA (the template) to reach a volume of 5 µL. The mixture was vortexed using Eppendorf ThermoMixer Compact® (Hamburg, Germany) adjusted at 70°C and 400 rpm for 5 minutes. Immediately, the tube was chilled in ice for at least 5 minutes, centrifuged for 10 seconds in a microcentrifuge using Eppendorf Centrifuge 5415D® (Hamburg, Germany) and 15 µL reverse transcription reaction mix was added to reach total volume of 20 µL per each cDNA reaction. The reverse transcription reaction mix was prepared, freshly for each group of cDNA reactions, by adding the following components into a clean, sterile and RNase-free 1.5 mL Eppendorf tube; 4 µL nuclease-free water, 4 µL GoScript™ (Promega®, Madison, WI) 5x reaction buffer, 4 µL $MgCl_2$ (final concentration 4 mM), 1 µL PCR nucleotide mix (final concentration 0.5 mM each dNTP), 1 µL Recombinant RNasin® (Promega®, Madison, WI) Ribonuclease Inhibitor (20 units), and 1 µL GoScript™ Reverse Transcriptase (Promega®, Madison, WI). The 20 µL RNA/RT reaction mix was then placed in the thermo-cycler machine PTC-200 Peltier DNA Engine (MJ Research®, Deltona, FL) and programmed as follows; annealing at 25°C for 5 minutes and extension at 42°C for up to 1 hour. The newly synthesized cDNA was then stored at –20°C. Before proceeding with qPCR, the reverse transcriptase was inactivated by incubating at 70°C for 15 minutes.

2.5. Quantitative real time PCR

Viral genomic DNA (gDNA) and cDNA amplification and detection was performed by real-time analysis using Applied Biosystems® 7900HT Fast Real-Time PCR (Foster City, CA). TaqMan®, (Eurogentec®, Seraing, Liège, Belgium) probe (reporter dye FAM™ on 5′ end and quencher dye TAMRA™ on 3′ end) and 2 sets of primers, forward and reverse, were used for each of the 4 target DNA templates (3 gDNAs and 1 cDNA). Primers and probes (DNA Technology®, Aarhus, Denmark) were diluted to reach final working concentration of 25 µM, which is the validated and standardized oligonucleotide concentration used by the department of Clinical Microbiology at Umeå University. Commercially provided oligonucleotide products were diluted to the suitable working solutions, as shown on Tables 3 and 4. Recommendations of (QuantiTect®, Hilden, Germany) QPCR protocols were followed: 3 out of 4 different PCR master mix solutions were prepared similarly for *Rune* (HSV-1), *Sune* (HSV-2), and *Valle* (VZV) primers (Table 3). Per 1 PCR reaction, the following components were added in clean, sterile and RNase-free Eppendorf tubes: 12.5 µL of 2x QuantiTect™ Probe PCR Master Mix (Qiagen®, Hilden, Germany), 1.0 µL of each of forward and reverse primers (final concentration of 25 µM), 0.5 µL probe (25 µM final concentration) and 7.5 µL RNase-free water (Ambion©, Waltham, MA). The mixture was pulse-vortexed and centrifuged briefly (ie, 3000–5000xg). Then the 22.5 µL single reaction mix was transferred into a well of a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems®, Foster City, CA) to which 2.5 µL template gDNA was added to reach total volume of 25 µL per 1 reaction. Experiments were conducted in groups of 10 to 96 reactions; volumes were multiplied by the total number of reactions per PCR run.

PCR master mix solution for *NM* (HEVs) primers (Table 4A) was prepared as follows: 12.5 µL QuantiTect™, (Qiagen®, Hilden, Germany) Probe PCR Master Mix (2x), 1.0 µL forward primer (25 µM), 1.0 µL reverse primer (25 µM), 0.8 µL probe (20 µM) and 7.2 µL RNase-free water were added into 2.5 µL template cDNA to reach a final working solution of 25 µL per single PCR reaction. PCR reaction plates were covered by MicroAmp™ Optical Adhesive Film (Applied Biosystems®, Foster City, CA) and concentrated at the bottom of the plate wells by spinning at low speed in a cold centrifuge (ie, 1200xg) using Allegra™ X-12R Centrifuge (Beckman Coulter©, Brea, CA). Each PCR reaction plate was designed for carrying 8 standard dilutions, 1 negative reverse transcriptase preparation, 1 non-template control containing ddH_2O and duplicates or triplicates of each experimental DNA template.

The standard (obtained from the department of Clinical Microbiology, Umeå University) was prepared with the concentrations; 5E6, 5E5, 5E4, 5E3, 5E2, 5E1, 5 and 1 virus copies. Negative reverse transcriptase preparation is a mixture containing cDNA PCR component with the exception of RNA template (replaced by RNase-free ddH_2O) and was subjected to the same cDNA PCR conditions. Non-template control is a qPCR preparation in which template DNA is replaced by RNase-free ddH_2O. Real-time cycler thermal conditions were similar for all 4 PCRs; heating at 50°C for 2 minutes (for carryover prevention since UNG will eliminate any dUMP-containing PCR products resulting from carryover contamination), followed

Table 3

Primers and probes specifications for herpesviruses.

♯	Title	Specification
(A) Herpes simplex virus type-1		
F1	Forward primer name	Sune-1 Fw
2	Oligo scale	40 nmol
3	No. of couplings	26 bases
4	Sequence	5′ AGA TAT CCT CTT TAT CAT CAG CAC CA 3′
R1	Reverse primer name	Sune-2 Rw
2	Oligo scale	40 nmol
3	No. of couplings	17 bases
4	Sequence	5′ TTG TGC TGC CAA GGC GA 3′
Pr1	Probe name	Sune-3 Prb
2	Conc. per amount	100 pmol/µL
3	Molecular weight	7471
4	Reporter dye	VIC
5	Quencher dye	TAMRA
6	No. of couplings	20 bases
7	Sequence	5′ CGG CGG CGT TCG TTT GTC TG 3′
(B) Herpes simplex virus type-2		
F1	Forward primer name	Rune-1 Fw
2	Oligo scale	40 nmol
3	No. of couplings	18 bases
4	Sequence	5′ GGC CTG GCT ATC CGC AGA 3′
R1	Reverse primer name	Rune-2 Rv
2	Oligo scale	40 nmol
3	No. of couplings	17 bases
4	Sequence	5′ GCG CAG AGA CAT CGC GA 3′
Pr1	Probe name	Rune-3 Prb
2	Conc. per amount	100 pmol/µL
3	Molecular weight	8794
4	Reporter dye	FAM
5	Quencher dye	TAMRA
6	No. of couplings	25 bases
7	Sequence	5′ CAG CAC ACG ACT TGG CGT TCT GTG T 3′
(C) Varicella-zoster virus		
F1	Forward primer name	Valle-1 Fw
2	Oligo scale	40 nmol
3	No. of couplings	22 bases
4	Sequence	5′ TCG TGG AAT ATT GAA AGG ATC C 3′
R1	Reverse primer name	Valle-2 Rv
2	Oligo scale	40 nmol
3	No. of couplings	21 bases
4	Sequence	5′ CCG TAG GTT CGG CAA ATC TAA 3′
Pr1	Probe name	Valle-3 Prb
2	Conc. per amount	100 pmol/µL
3	Molecular weight	7801
4	Reporter dye	FAM
5	Quencher dye	TAMRA
6	No. of couplings	22 bases
7	Sequence	5′ CCC GTC AAC AGC CGG TCT GTC A 3′

by PCR initial activation step necessary for HotStarTaq® DNA Polymerase (Qiagen®, Hilden, Germany) at 95°C for 10 minutes, then denaturation at 94°C for 15 seconds and combined annealing and extension at 60°C for 60 seconds. This cycle was repeated 45 times. The duration of final PCR cycles was approximately 90 minutes including generation of the amplification curve. Resulting data were analyzed using software provided by Applied Biosystems® (Foster City, CA) 7900HT Fast Real-Time PCR system (ABI 7900HT SDS Plate Utility Version 2.4).

2.6. Conventional HPeVs PCR

HPeV nucleic acids were investigated using a validated and optimized method reported by W. Allan Nix et al.[19] In brief, 5 µL template cDNA was added to 20 µL reaction mix (12.5 µL of 2x QuantiTect™ Probe PCR Master Mix [Qiagen®, Hilden, Germany], 1 µL of 50 pmol *AN353* forward primer, 1 µL of 50 pmol *AN355* [DNA Technology®, Aarhus, Denmark] reverse primers [Table 4B], 5.5 µL nuclease-free ddH_2O [Ambion©, Waltham, MA]) to reach total volume of 25 µL per 1 reaction. The PCR amplification protocol included 35 cycles; starting at 95°C for 30 seconds, followed by 42°C for 40 seconds and 72°C for 60 seconds, with 0.4°C/s ramp from 42°C to 72°C. A representative clinical isolate for HPeVs (i.e., *Echovirus* 23) was used as positive control in the PCR procedures. The same recommended PCR amplification protocol was followed using DNA Engine PTC-200 Peltier Thermal Cycler (MJ Research©, Deltona, FL). PCR amplicons were kept at –20°C until processed by agarose gel electrophoresis.

2.7. Agarose gel electrophoresis

1% normal grade melting temperature agarose gel adequate for separating 0.1 to 10 kb DNA fragments was prepared as follows;

Table 4

Primers and probes specifications for enteroviruses.

♯	Title	Specification
(A) Non-polio human enteroviruses		
F1	Forward primer name	NMF1
2	Oligo scale	0.2 µmol
3	No. of couplings	15 bases
4	Sequence	5′ GCC CCT GAA TGC GGC 3′
R1	Reverse primer name	NMR1
2	Oligo scale	0.2 µmol
3	No. of couplings	19 bases
4	Sequence	5′ AAT TGT CAC CAT AAG CAG C 3′
Pr1	Probe name	MP
2	Conc. per amount	100 pmol/µL
3	Molecular weight	9123
4	Reporter dye	FAM
5	Quencher dye	TAMRA
6	No. of couplings	26 bases
7	Sequence	5′ CGG AAC CGA CTA CTT TGG GTG TCC GT 3′
(B) Human parechovirus		
F1	Forward primer name	AN353
2	Oligo scale	40 nmol
3	No. of couplings	29 bases
4	Sequence	5′ GAC AAT AGT TTT GAA ATN ACN ATH CCN TA 3′
R1	Reverse primer name	AN355
2	Oligo scale	0.2 µmol
3	No. of couplings	37 bases
4	Sequence	5′ CTC CAA CTA TAA TGC CAT ART GYT TRT ARA ANC CGY K 3′

1 g agarose (*Agarose Standard* provided by Saveen Werner AB™, Malmö, Skåne län, Sweden) was dissolved in 100 mL of 0.5x Tris Borate EDTA (TBE). The 0.5x TBE was prepared as follow; stock solution of 5x TBE concentrate buffer was firstly prepared by dissolving 54 g Tris Base and 27.5 g Boric Acid in 900 mL of deionized water, 20 mL of 0.5 M (pH 8.0) EDTA was added and the overall volume was adjusted to 1 L by adding deionized water. The working 0.5x TBE buffer solution was then prepared by adding 1 part of stock 5x TBE to 9 parts of deionized water. The solution was heated until the agarose was completely dissolved in a microwave (Electrolux®, Stockholm, Sweden) and was then allowed to cool in a water bath set at 50°C for 10 minutes. Gel casting tray was adjusted with 2 combs. After the gel solution was cooled, 5 µL of 10 mg/mL Ethidium Bromide (Bio-Rad®, Hercules, CA) was added and mixed well, then poured into the gel tray and allowed to cool and solidify at room temperature for 30 minutes. Once completely solidified, combs were removed and the gel tray was placed in an electrophoresis chamber (Separation System Inc., OWL®, Gulf Breeze, FL) and covered with 0.5 x TBE buffer. For preparing samples; 2.5 µL of 6x MassRuler™ DNA Loading Dye (Fermentas Life Sciences®, Vilnius, Lithuania) was added to 25 µL PCR amplicons. The 27.5 µL mixture was transferred into the gel wells placed in the electrophoresis chamber. Equal volumes of 2 types of standard DNA Ladders (FastRuler™ Low and Middle Range DNA Ladders [Fermentas Life Sciences®, Vilnius, Lithuania]) were loaded in each run. The agarose gel was electrophoresed at 80 Volts for 75 minutes. DNA bands were visualized by ethidium bromide staining and ultra-violet light box using Ultraviolet Transilluminator device (Spectroline®, Melville, NY). Images were transferred into a monitor (Ikegami®, Tokyo, Japan) and printed in a K65HM-CE High Density Paper (Mitsubishi Electric®, Tokyo, Japan).

2.8. Statistical analysis

We used the statistical package program IBM/SPSS version 21 (Chicago, IL) to analyze all data where numerical variables were organized into categories and expressed in frequencies and percentages. Numerical variables were described using measures of central tendency and of dispersion. Cross-tabulations to compare categorical variables and tests for detecting statistically significant differences ($P \leq .05$) and associations ($P \leq .05$, $r \geq +/- 0.5$) were conducted using Pearson chi-square or Fisher's Exact along with Phi and Cramer's V Correlation and its 95% confidence intervals.

2.9. Ethics approval and consent to participate

The ethical clearance for conducting this study was obtained from the Ethical Review Board of the National Center for Neurological Sciences on September of 2009. Patients were not contacted directly; data were obtained from hospital files and were kept anonymous at all stages of the study. Patient consent was considered unnecessary and was waived. Excess specimens were obtained from the hospital main laboratory after all officially requested tests were applied. Permission to collect data and specimens was granted from hospital authorities verbally (from hospital general director) and in writing (from Laboratories Administration, Federal Ministry of Health).

3. Results

3.1. Secondary analysis using standardized clinical codes

The criteria (Table 1) listed in the Clinical Case Definition for Aseptic Meningitis[18] were used to perform secondary-analysis using data collected throughout this project. When all criteria were met, level-1 of diagnostic certainty for aseptic meningitis was designated (Table 1). When viral nucleic acids were detected by PCR, confirmed VM was designated (Table 2). The overall clinical, nucleic acids and conventional microbial findings are illustrated in Figure 1 which describes findings from this study alongside findings reported in our other publications.[17,20,21]

3.2. General nucleic acids findings

PCR testing for HSV-1 DNA in CSF revealed 2 (0.4%) positive results out of total 503. Average Ct for both HSV-1 positive specimens was

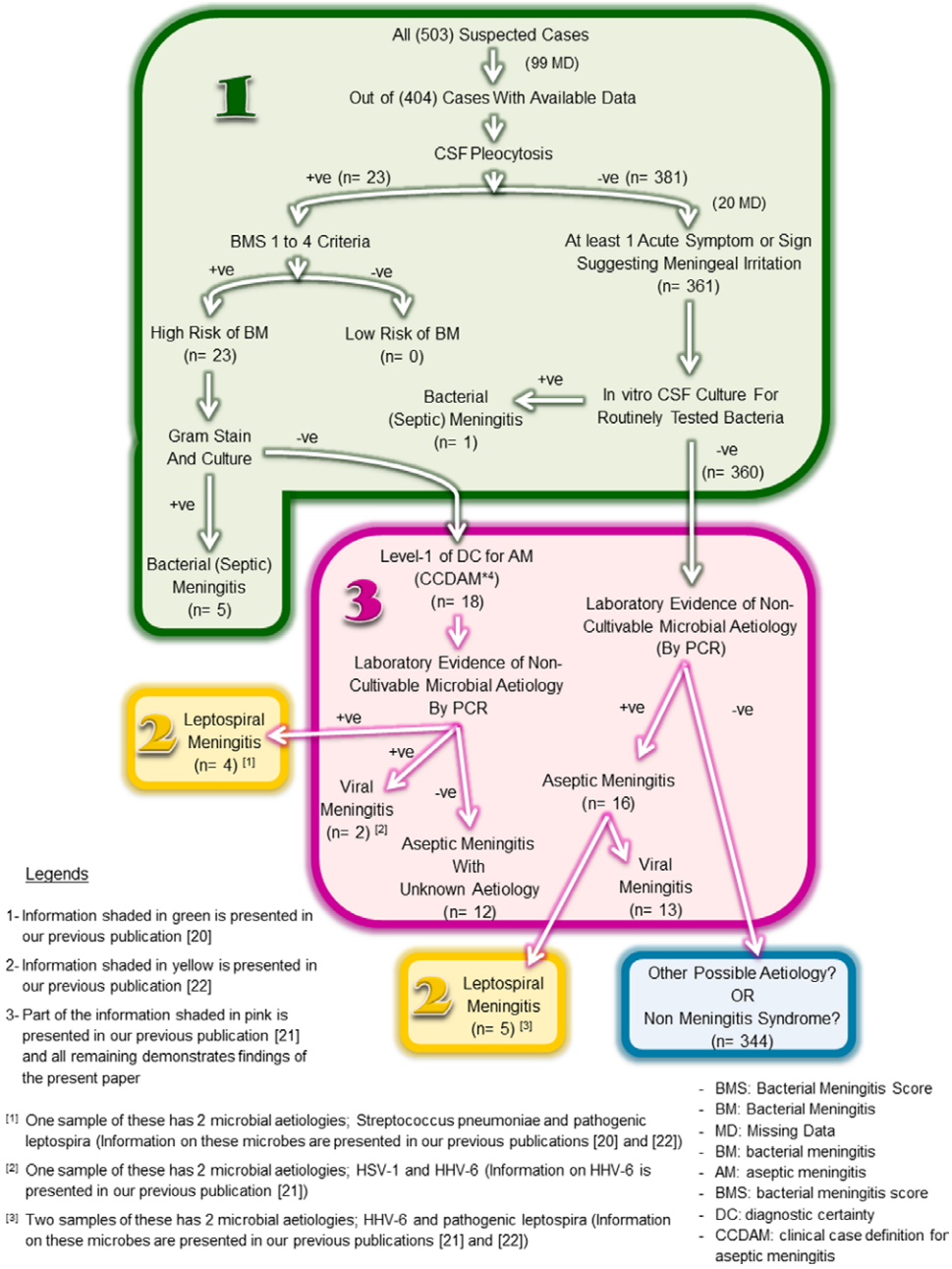

Figure 1. Categorization of all cases based on clinical case definition for aseptic meningitis, bacterial meningitis score and molecular and conventional microbial findings.

38.67 + 0.96 SD and median virus copy was 2.5×10^2/PCR run (1×10^5 virus copies/mL CSF). PCR testing for non-polio HEVs cDNA in CSF revealed another 2 (0.4%) positive results. Average Ct for both HEVs positive specimens was 38 + 0.4 SD and median virus copy was 3.5×10^2/PCR run (1.4×10^5 virus copies/mL CSF). No genetic materials were detected for HSV-2, VZV and HPeV. On TaqMan-based real-time PCR procedures, standard dilutions with viral copies of 5E6 to 5E2 produced amplification curves between Ct 24 to Ct 36. The generated standard curve plot showed perfect negative association between Ct and viral quantity. This was repeatable in all viral PCR runs discussed hereunder.

3.3. Hospital diagnosis and therapy

Based on the available final diagnosis (356/503 [147 missing]) written in hospital records and obtained during our study period; 61.8% (220/356) of admissions were diagnosed as having a CNS associated condition; 55.9% (199/356) as bacterial (septic) meningitis, 0.6% (2/356) brain abscess and 5.3% (19/356) febrile convulsions. The remaining 38.2% (136/356) were diagnosed as having other non-CNS related infections. These are; 18.5% (66/356) pneumonia, 11.2% (40/356) tonsillitis, 3.7% (13/356) malaria, 1.4% (5/356) sepsis, 1.1% (4/356) tonsillitis and severe pneumonia, 0.8% (3/356) severe malaria and pneumonia, 00.8% (3/356) fever of unknown origin, 0.3% (1/356) urinary tract infection, and 0.3% (1/356) bronchitis. All patients were treated during hospitalization by first line and/or second line antibiotic therapies (ampicillin, penicillin, cephalosporin, gentamycin, chloramphenicol, quinin) as described in detail in our previous publication (Abdelrahim et al).[17]

3.4. Findings on cases with positive viral etiology

3.4.1. Herpes simplex virus type-1. Both cases were admitted in December of 2009. One was an infant 9 months old, she was admitted after 2 days of symptoms onset; fever, seizures and vomiting. On CSF nucleic acids examination; the target product was amplified at Ct 37.05 and produced virus copies of 6.5×10^2/PCR run (2.6×10^5 virus copies/mL CSF). CSF specimen was clear, showed leukocytosis (50 cells/mm^3), normal glucose concentration (76 mg/dL) and high protein concentration (60 mg/dL). This patient was co-infected with HHV-6.[20] The second case was 14 years old and was admitted after 1 day of symptoms onset. He presented with only fever and headache. CSF nucleic acids examination revealed amplification at Ct 39.10 and virus load of 1.23×10^2/PCR run (5×10^4 virus copies/mL CSF). CSF specimen was clear as well, showed normal cellular count (<5 cells/mm^3), normal glucose concentration (53 mg/dL) and high protein concentration (127 mg/dL). CSF specimens from both cases were negative for meningitis common rapid growing bacterial pathogens. Both cases were hospitalized for 9 days, they were treated with ampicillin, and were diagnosed as having septic (bacterial) meningitis on discharge.

3.5. Non-polio human enteroviruses

Both cases were infants 8 months old; 1 of them was a male. Clinical data were not available for these cases unfortunately. The male patient was admitted in autumn (July). On CSF nucleic acids examination; the target product was amplified at Ct 38.30 and produced virus copies of 2×10^2/PCR run (8×10^4 virus copies/mL CSF). The CSF specimen was turbid, measuring 1000 cells/mm^3 with lymphocytic predominance (80%), with normal glucose concentration (51 mg/dL) and high protein concentration (151 mg/dL). The female patient was admitted in summer (March). On CSF nucleic acids examination; the target product was amplified at Ct 37.70 and virus copies of 5×10^2/PCR run (2×10^5 virus copies/mL CSF). The CSF specimen was clear, showed normal cellular count (<5 cells/mm^3), normal glucose (65 mg/dL) and normal protein (14 mg/dL) concentrations. CSF specimens from both cases did not show evidence of bacterial origin.

4. Discussion

Aseptic/VM is used to include all types of infectious meningitis with negative CSF bacterial cultures.[22] Interestingly, on reviewing data collected from hospital records, none of our cases was diagnosed as having aseptic or VM on discharge. In addition to the inflated bacterial meningitis diagnosis (56%) discussed in our previous publication for this same population,[19] the 0% diagnosis of aseptic/VM was another odd finding. Indeed, failing to diagnose patients as having aseptic/VM when fulfilling the required criteria is unrealistic. According to the clinical case definition for aseptic meningitis and to our nucleic acids findings, we considered 34 cases (i.e., 18 + 16, 6.8%) as having aseptic meningitis out of the total study population. Among the 40 cases with confirmed infectious meningitis, proven aseptic meningitis represents 85% with verified viral etiology in 40% (Tables 1 and 2, Fig. 1).

The diagnosis of viral infections of the CNS has been revolutionized by the advent of molecular diagnostic technologies to amplify viral nucleic acids from CSF[23,24]; even though up to 70% of cases of CNS infections remain of unknown etiology in modern surveys.[25] The virus detection rate was 46% out of 65 suspected pediatric patients in Eastern Iran[26] and 32% out of 22 patients with aseptic meningitis in China.[27] In the present study, the virus detection rate was 3.4% (n = 17) out of 503 pediatric patients. This rate is comparatively low because patients were poorly differentiated. Clinically, some inflammatory and infectious diseases may mimic infectious meningitis resulting in inclusive disease definition. The virus detection rate was 50% (17/34) among our cases with proven aseptic meningitis; this estimate is comparable with the aforementioned studies.[26,27]

Among other herpes viruses, HSV-1, HSV-2 and VZV are considered as major agents of CNS infections.[10,11] Infection with HSV-2 beyond the neonatal period in an otherwise healthy individual is almost always associated with a self-limited form of meningitis.[28] In contrast, HSV-1 typically causes rapidly progressive fatal necrotizing encephalitis[29] with a mortality rate of up to 70% in the absence of therapy.[12,30] In our study, all patients were fully recovered and discharged. We, therefore, report benign conditions of herpes meningitis caused by HSV-1. Several studies concur; they described mild or atypical meningitis caused by HSV-1.[26,31–33] HSV-2 was not detected in our population, which reflects the rare incidence of this primarily sexually transmitted pathogen in Sudan in comparison with some other countries.[34,35] Because CNS HSV can present with seizures, low grade fever, and other nonspecific symptoms in the absence of the vesicular skin rash, the clinical diagnosis of HSV meningitis or encephalitis is not possible.[36–38] Among our HSV-1 identified cases, 1 presented with fever, seizures and vomiting and the second presented with fever and headache. It is worth to mention that the febrile seizures were seen in 9 month old female, and more subtle symptoms in a 14 year old male. Typical CSF glucose and proteins findings in VM[3,18] were demonstrated in both specimens. The infant specimen showed CSF pleocytosis, whilst the other showed normal cellular count. Normal CSF cellular counts in cases with VM have been reported.[3,13]

HSV positivity rate among our 40 cases with proven infectious meningitis was 5%, among our 34 cases with proven aseptic meningitis was 6%, and among our 16 cases with proven VM was 12.5%. Our finding among highly suspected groups was in good accordance with the literature. The HSV positivity rate in CSF (4%; 11/288) in a study by Reil et al[39] was found to be significantly lower than in respiratory specimens (30%; 20/67) and skin lesions (47%; 18/38). Several other studies reported low positivity rates from CSF ranging from 2.5% to 5%.[40,41] The same finding was reported from the very few studies in Africa; out of 202 CSF specimens collected from aseptic meningitis suspected patients in Burkina Faso, 2 (0.99%) were positive for HSV-1.[5] A single recent study from Sudan confirmed 1 case with herpes encephalitis out of 104 children presenting with fever and coma.[7]

Amplification at high Ct values indicates low viral copies. In this study, HSV-1 DNA in 1 case was amplified at high Ct of 39 while the second at a lower Ct of 37, corresponding to viral loads of 49.3×10^3 and 261×10^3 per 1 mL CSF, respectively. The study from Burkina Faso detected a lower limit estimated to be 2.45 copies per µL.[5] Further, Hosseininasab et al[26] reported wide range of HSV Ct values of 16 to 44 with viral loads from less than 1E2 to more than 1E9 copies per mL. However, their reported median viral load in CSF was lower than in other types of specimens reaching Ct of 31 or higher and corresponding to a moderate virus load of less than 1E5 HSV copies per 1 mL CSF; a result that strongly supports ours. The viral load of HSV in CSF was correlated with morbidity and mortality in some studies.[42,43] Domingues and colleagues[43] reported that patients with a high HSV copy number (>10^5/mL) had a poorer prognosis than those with lower copy numbers. Barnetson and Rogers[44] supported this view, whereas others[45,46] did not see such a correlation. However, quantification of HSV DNA may also be important in monitoring the response to treatment, as HSV DNA can be detected in CSF for up to 40 days after onset of symptoms; a time period where treatment is not usually continued.[46] Additional studies are required to determine the actual value of CSF HSV load in establishing the prognosis and improving treatment results. Despite the fact that HSV meningitis treatment is mandatory,[12,26,30] our patients were not treated with antiviral drugs when hospitalized. Antiviral therapy is not frequently prescribed in Sudan, especially when viral CNS infections are not expected

and therefore not considered in the diagnosis. Both cases with herpes infections were found to be treated with ampicillin, as they were thought to have bacterial (septic) meningitis despite the lack of bacterial Gram stain and culture confirmation.

Knowing that aseptic meningitis and meningoencephalitis can be caused by VZV infection frequently without the zoster rash,[47–51] we examined our 503 CSF specimens for the DNA of this virus and identified 0%. Despite our failure to recover VZV, other studies suggest this virus as an important etiological agent in acute aseptic meningitis.[2,52,53]

There is worldwide circulation of enteroviruses,[4] except for poliovirus which remains endemic in only 4 countries; Pakistan, India, Nigeria and Afghanistan.[54] Although non-polio human enteroviruses are the leading recognizable cause of aseptic meningitis accounting for 80% to 92% of all cases in which a pathogen is identified, as stated by Rotbart in his review in 1995,[55] studies published later do not concur. Beld et al in 2004[56] reported low isolation rate of 12.8% in the CSF of 281 highly suspected groups and Lo et al in 2010[57] reported a lower rate of 2.7% among 9107 enteroviral infection suspected pediatric patients. Our study, which examined suspected pediatric patients in 2010, is in accordance; the detection rate was 9% among our 22 cases with identified aseptic microbial etiology; 6% among our 34 cases with proven aseptic meningitis; and 5% among our 40 cases with proven infectious meningitis (Table 2). However, the detection rate is much lower (i.e., 0.4%) among our total study population of 503. Further, studies of longer duration may succeed in identifying outbreaks at certain point in time while normal findings are observed in the remaining periods. In a French retrospective surveillance, which continued for twenty years (1985 to 2005), only 2 peaks of enteroviral meningitis were identified; the first was in 2000 and reported 191 cases, the second was in 2005 and reported 305 cases.[58] They also identified 9 years of significantly high HEV isolation frequency ($n \geq 7$ cases per year), and the remaining 11 years showed either 0% isolation rate or sporadic cases of no significant increase in incidence.

The peak incidence of enterovirus meningitis occurs in summer and early autumn in temperate regions,[4] with considerable year-to-year variability in the incidence.[59] Epidemiological data in less temperate regions (Saharan and sub-Saharan) were not found, however, the 2 identified cases in the present study occurred in summer and autumn. In general, enteroviral meningitis is more common in young patients, especially those under 1 year of age.[60,61] In the present study, both of our cases were infants aged 8 months; 1 was a male. Clinical signs and symptoms of enteroviral CNS disease in young children are mostly nonspecific[48,62,63] whereas specific ones may or may not be present, and seizures occur in <10% of patients.[64–66] In the present study, all children were febrile and had seizures in addition to some other nonspecific symptoms as described in the results above.

Laboratory findings in pediatric enteroviral meningitis vary widely.[48] CSF leukocyte count varies from none to several thousand cells per mm^3, but most often is less than 1000.[67] Lymphocytes are generally the most numerous cells in CSF, but the predominance of neutrophils is common, especially early in the disease.[48,67] However, studies suggesting CSF neutrophil predominance (>50%) during the early course of aseptic meningitis followed by a decrease over time corresponding to subsidence of symptoms[68] were opposed by the findings of more recent studies[69,70] which did not acknowledge this pattern. One of our CSF specimens was turbid and showed CSF leukocytosis of 1000 cells/mm^3 with 80% lymphocytosis. Several other studies also reported lymphocytic predominance in the early course of aseptic or VM.[3,18] Our second positive CSF specimen was clear and showed normal CSF cellular count with less than 5 cells/mm^3. Normal CSF cellular counts were reported frequently in pediatric patients with CNS infections[3,13,39] occurring in as low as 3% of patients to as high as 40%.[65,71–75] It should, therefore, be kept in mind that when clinical signs and symptoms suggest the diagnosis of enteroviral meningitis, even if the CSF contains no leukocytes, an enterovirus may still be present. Henquell et al[76] and Landry[77] reported high rates of CSF leukocytes in only 9% to 15% in children with viral CNS infections. In addition, Lee et al)[66] observed normal CSF white cell counts in 22% of young infants with proven enteroviral meningitis.

In most patients with enteroviral meningitis, CSF total protein content is within normal limits, but in a small percentage, it may be slightly or even markedly elevated.[64,65,76,77] Our results supported both possibilities; the CSF specimen with leukocytosis showed high CSF protein levels, reaching 151 mg/dL, and the one with normal cellular count showed a normal CSF protein level of 14 mg/dL. CSF glucose content in enteroviral meningitis is usually normal but can be diminished in up to 18% of cases.[48,64,65] Our findings were consistent; both positive specimens had normal glucose level of average 58 + 10 mg/dL.

Since conventional PCR is considered highly specific and sensitive in diagnosing parechoviral infections,[36,78] we adopted this method and found no parechoviral cDNA (0%) in CSF specimens from the present population.

A major limitation in this study was that the hospital's provisional diagnosis of infectious CNS disease was too inclusive, leading to distracted efforts on examining few viruses in large 503 specimens instead of focusing in only 40 specimens and testing more viruses.

5. Conclusions

We conclude that patients fulfilling the recommended clinical criteria were not diagnosed as having aseptic or VM. We differentiated 6.8% as proven aseptic meningitis among the total study population and 85% among those with confirmed infectious meningitis. Viral etiology was identified in 3.2% among the total study population and in 40% among those with proven infectious meningitis. The detected viruses were HSV-1 and non-polio HEVs with viral detection rates of 0.8% among the total study population and of 10% among those with proven infectious meningitis. HSV-2, VZV, and HPeVs were not involved in neuroinfections in children in this population. Positive PCRs in CSFs with normal cellular counts were seen.

6. Recommendations

We recommend applying the clinical case definition for the diagnosis of aseptic/VM in suspected patients, exploring further viral etiologies, and that a multiplex or broad range real-time PCR protocol for the rapid diagnosis of genetically related species in a single reaction be developed, to minimize costs.

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Author contribution

NA developed research questions and design, collected and managed all data, performed all laboratory work, statistical analysis and interpretation, wrote and edited the text. NM advised on the approach and methodology and guided and supervised the molecular laboratory work. ME and CA contributed on the approach and methodology, supervised the molecular laboratory work, edited and proofread the manuscript and were major contributors in the overall study. IF supervised the research process throughout; contributed in the development of research questions, design and methodology, managed all logistics and clinic based activities. All authors have read and approved the final manuscript.

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