CHAPTER ONE

1. INTRODUCTION

1.1. Introduction

Viral meningitis is inflammation of meninges (the membrane covering the brain and spinal cord) caused by viral infection. It is also called aseptic meningitis. Viral meningitis is the most common type than bacterial meningitis (Logan & Macmahon, 2008; Kumar, 2005). The annual incidence of viral meningitis exceeds the total number of cases of meningitis of other etiologies (Logan & Macmahon, 2008).

Enterovirus 71 (EV71) is positive sense single-stranded RNA viruses, it belongs to the Picornaviridae family, Enterovirus genus and Enterovirus A species .It is the common cause of hand, foot and mouth disease (HFMD) in children and the epidemic of severe neurological diseases (Oberste *et al.*, 1999; Bessaud *et al.*, 2014). It was first isolated and characterized from case of neurological disease in California in 1969 (Bessaud *et al.*, 2014).

Bacterial and viral meningitis appear as headache, fever, and neck stiffness (Logan & Macmahon, 2008). Other symptoms can include nausea, vomiting, photophobia, sleepiness, confusion, irritability, delirium, and coma. Infants may have Bulging fontanelle, Paradoxic irritability, High-pitched cry and Hypotonia (Kumar, 2005).

Viral meningitis has no specific treatment for it just relieving the patient's symptoms. But in some instance viral meningitis may lead to serious outcome, including death (Logan & Macmahon, 2008).

Aseptic meningitis tends to occur 3 times more commonly in males than females (Kumar, 2005).

Viral meningitis is caused by many viruses like Enteroviruses (the most common one), West Nile virus, Measles virus, Mumps virus and Herpes simplex virus type I and II (Logan & Macmahon, 2008).

EV71 is associated with many outbreaks in different areas especially the Eastern Mediterranean Region (Linsuwanon *et al.*, 2014). The prevalence rate of aseptic meningitis which caused by EV71 is differs in countries, for example the prevalence rate of EV 71meningitis in Brunei, South Korea and Thailand was 34, 19 and 9%, respectively (AbuBakar *et al.*, 2009; Ryu *et al.*, 2010; Linsuwanon *et al.*, 2014).

It is difficult to differentiate between EV71 viral meningitis from other aseptic meningitis based on clinical symptoms. Thus, EV71 diagnosis is based on laboratory diagnosis like isolation of EV71 and serologically by Enzyme Linked Immunosorbent Assay (ELISA) and molecularly by Reverse transcriptase-PCR (RT-PCR). In previous outbreaks the maim technique used for detection of EV71

was molecular technique (AbuBakar *et al.*, 2009; Ryu *et al.*, 2010; Linsuwanon *et al*., 2014).

The present study aimed to identify Enteroviruses infection in cases of aseptic meningitis with assurance on EV 71 in Khartoum State, Sudan during February to May 2015 periods. This was accomplished by subjecting cerebrospinal fluid samples (CSF) taken from aseptic meningitis patients to reverse transcription-polymerase chain reaction (RT-PCR). To the best of my knowledge no information is available about the disease status in Sudan.

1.2. Objectives

1.2.1. General objective

To generate preliminary information about Enteroviruses infection in aseptic meningitis patients in Khartoum State, Sudan.

1.2.1. Specific objectives

1. To determine the incidence of the EV71 in meningitis patients in Khartoum State, Sudan.

2. To determine the risk factors including age and gender of EV71 infection.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Definition of Enteroviruses

Enteroviruses belong to the genus Enterovirus, family Picornaviridae and are associated with different human diseases. Enteroviruses are initially classified based on neutralization by antisera pools (Melnick, 1996). Eighty nine serotypes are identified and 64 serotypes are found to be infectious to humans (King *et al.*, 2000; Lindberg and Johansson, 2002).

The human Enteroviruses are originally grouped on the basis of human disease manifestations (poliovirus), replication and pathogenesis in newborn mice (coxsackieviruses A and B), as well as growth in cell culture without causing disease in mice (echoviruses) (Melnick, 1996). Based on their molecular properties, Enteroviruses are reclassified into A, B, C and D species (King *et al.*, 2000).

In 2009, the Enterovirus genus was newly classified into 10 species, including Bovine Enterovirus, Human Enterovirus A, B, C and D, Human rhinovirus A, B and C, Porcine Enterovirus B and Simian Enterovirus A (Internatioanl Committee of taxonomy of viruses, 2010). All Enteroviruses have a positive single-stranded RNA linear genome (Schnurr, 1999; Li *et al*., 2005).

2.2. Definition of EV 71

EV 71 is very small RNA non enveloped icosehedral virus belonging to Enterovirus genus in Picornaviridae family (Schnurr, 1999; Greenwood *et al.*, 2012; Bessaud *et al.*, 2014).

2.3. History of EV 71

EV 71 was first isolated and characterized from case of neurological disease from infant stool in California in 1969 (Bessaud *et al.*, 2014). Retrospective studies in the Netherlands using the clinical specimens collected in 1963 combined with evolutionary analysis suggest that EV71 could have emerged as early as 1941(Linsuwanon *et al*., 2014).

2.4. Virus structure and genome organization

2.4.1. EV 71 Taxonomy

EV 71 is a member of Enterovirus genus, which belongs to Picornaviridae family (Greenwood *et al.*, 2012). Enteroviruses are distinguished from other Picornaviruses on the basis of physical properties (Lee *et al.*, 2009).

Enteroviruses named by simple numerical design. The new strains are called Enterovirus and are numbered, for example EV 71, instead of subdivided into different groups (Greenwood *et al.*, 2012). This simple numerical designation started from 1969 (Levinson, 2012).

2.4.2. Genome

EV 71 genome composes of positive sense non segmented linear single strand RNA with molecular weight 2-2.6 x 10^6 and approximately 7.4 kb in length (Li *et al*., 2005; Greenwood *et al.*, 2012; Levinson, 2012; Chakraborty, 2013).

Some strains are completely sequenced (Greenwood *et al.*, 2012).

The linear genome is organized in a single long open reading frame (ORF) flanked by untranslated regions (UTR) at 5′and 3′. The 3'UTR is followed by a variable length of poly-A tract. The ORF encodes a polyprotein which can be divided into three different genomic regions from P1 to P3 and encodes a single polyprotein of 2194 amino acids. The polyprotein is processed by proteases to produce structural and non-structural proteins (Crowell and Landau, 1997; Ooi *et al*., 2010; Linsuwanon *et al.*, 2015). The P1 genomic region encodes the structural proteins VP1–VP4 which processed by the 3CD proteinase. Sixty identical units, each consisting of 4capsid proteins, form an icosahedral structure of 28 nm (Crowell and Landau, 1997).

The P2 and P3 genomic regions encode the non-structural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D which responsible for viral replication and protein processing (Ooi *et al*., 2010).

Products of the P2 region include protein 2A, 2B and 2C. 2A mediates in proteolytic cleavage of polyprotein to release P1 and in the meantime, it cleaves

3CD into 3C and 3D. Cleavage of 3CD was found to be non-essential (Lee and Wimmer, 1988). The multifunctional 2A protease also inhibits host protein synthesis and initiation of RNA synthesis. 2C is the most conserved among all enteroviral proteins (Miyashita *et al.*, 1996; Kemp *et* al., 1992).

The association between 2C and replication complex-associated vesicles suggests that it is also involved in viral replication Virus-encoded proteins 3A, 3B, 3C and 3D are in the P3 region. P3 region is cleaved into 3AB (precursor of 3A and 3B) and 3CD (precursor of 3C and 3D) (Shih *et al*., 2004). 3A is found to be closely associated with replication complex in infected cell. 3CD is a protease participating in cleavage of P1 region and after cleavage by 2C, its products are 3C and 3D. Protease 3C is the main executor for cleavage of P2 and P3 regions and this is essential for viral replication. 3D polymerase is RNA-dependent RNA polymerase which functions in RNA synthesis (Miyashita *et al.*, 1996; Kemp *et al*., 1992).

2.4.3. Nucleocapsid

EV 71 is non-enveloped, 28-30 nm in diameter. Capsid made of 60 capsomers, each of four proteins (VP1–VP4) arranged in cubic (icosahedral) symmetry (Chakraborty, 2013). VP1, VP2 and VP3 are structural proteins form the outer surface of the capsid (Hendry *et al.*, 1999).

VP1 is the immune-dominant capsid protein and contains the most important neutralization epitopes. It shows a sequence divergence and has been used for molecular type assignment and evolutionary study (Greenwood *et al.*, 2012; Bessaud *et al.*, 2014). VP4 it lies in the inner surface of the capsid and is barely exposed (Chow *et al.*, 1987).

EV 71 has six genotypes (from A to F). Genotypes B and C are divided into sub-genotypes B0-B5 and C1-C5, respectively (Greenwood *et al.*, 2012; Bessaud *et al.*, 2014).

Genotypes B and C are the most distributed genotypes in worldwide (Greenwood *et al.*, 2012; Bessaud *et al.*, 2014).

Although there is genetic relatedness between Human Enterovirus species, human Enterovirus A (HEV-A), HEV-B, HEV-C, and HEV-D, Antigenic variants exist for several Enteroviruses. The origin of prime and intratypic variant strains is speculative, but they may result from either antigenic drift or recombination (Chang *et al.*, 2007).

It is not understood why some serotypes have numerous prime strains, others have antigenic variants, and still others are antigenically homogeneous. It is possible that different serotypes have different degrees of structural constraints of their capsid proteins and thereby have different capacities for antigenic variation (Lee *et al.*, 2009).

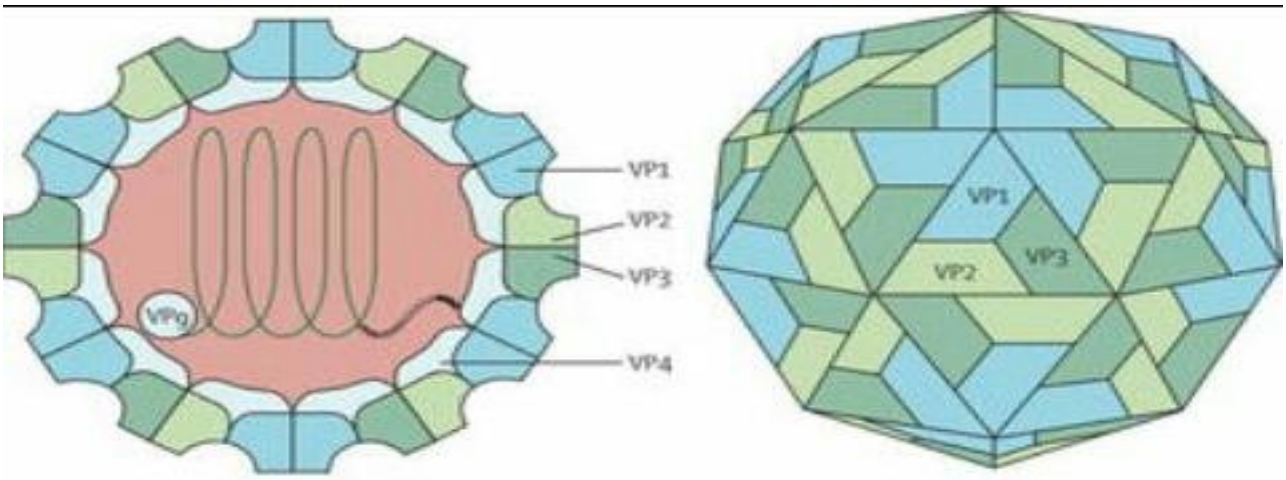

Figure1. Morphological structure of EV 71

2.5. Virus replication

Human beings are the only known natural hosts of human Enteroviruses. Viral entry into susceptible host cells is dependent on specific receptors (Solomon *et al*., 2010). Several receptors for EV71 have been identified, but a ubiquitously expressed cellular receptor, scavenger receptor B2, and a functional receptor, human P-selectin glycoprotein ligand-1, found on white blood cells, are specific for EV71. Sialic-acid-linked glycan, which is expressed in abundance in the respiratory and gastro-intestinal tracts, and dendritic-cell-specific intercellular adhesion-molecule-3-grabbing non-integrin (CD209), which is found exclusively in dendritic cells in lymphoid tissues, have also been identified (Kumar, 2005). After an EV 71 binds with a specific receptor on the cell surface, VP4 is released which leads to structural changes in the virus capsid and virion weakened (Solomon *et al*., 2010; Chakraborty, 2013).

The genome is then injected directly into the cell cytosol across the cell membrane through a channel created at one of the vertices of the virus (Solomon *et al*., 2010; Chakraborty, 2013). Virus replicate in cytoplasm (Chakraborty, 2013).

The genome is directly binds to ribosome despite the lack of 5`-cap structure. The infecting RNA translated to a large polypeptide that is promptly cleaved by the viral proteases into mature structural and non-structural proteins (Solomon *et al*., 2010; Chakraborty, 2013).

The replication of the virus genome by the error-prone RNA-dependent RNA polymerase takes place in a vesicle membrane structure (viral replication complex) (Solomon *et al*., 2010). The polymerase is estimated to misincorporate one or two bases in every genome copying event, which explains why the virus mutates and evolves rapidly (Solomon *et al*., 2010). Within the VP1gene 4.2–4.6×10 nucleotide substitutions occur per site per year (Solomon *et al*., 2010).

While the machinery of the host cellular protein synthesis is shut down by viral protease 2A, viral protein synthesis remains unaffected (Solomon *et al*., 2010; Chakraborty, 2013).

An infectious virus particle is formed after the packaging of a progeny viral RNA into a virus capsid in the cytoplasm of the infected cells (Solomon *et al*., 2010).

Mature infectious virus particles are released when an infected cell is lysed (Solomon *et al*., 2010).

2.6. Epidemiology and transmission

EV71 is an important pathogen caused large outbreaks in Asian-Pacific region (Ooi *et al*., 2010; Greenwood *et al.*, 2012).

The first recognition of this virus was in 1969 in California from faeces of 9-month old infant suffering from encephalitis and caused sporadic cases or small outbreaks of hand, foot, and mouth disease (HFMD), neurological disease, or both. In 1997, the virus caused an unexpectedly large and severe outbreak in Sarawak and Malaysia with high mortality (Ooi *et al*., 2010; Greenwood *et al.*, 2012).

It has been isolated during an epidemic of paralytic disease in Bulgaria in 1975 (Greenwood *et al.*, 2012). In Malaysia in 1997, there was a large outbreak by EV 71 (Greenwood *et al.*, 2012). In this outbreak there was 2140 children affected (Greenwood *et al.*, 2012).

In 1998 in Taiwan a large outbreak occurred by EV 71 and characterized by HFMD and caused diability and fatalities (Greenwood *et al.*, 2012). In China in 2008 outbreak caused by EV 71 has been occurred (Levinson, 2012).

EV 71 circulates at a low level in Africa, Europe, and the USA and causes sporadic cases or small outbreaks (Levinson, 2012).

Transmission of EV71 infection is a public concern. The most important risk factor of EV71 infection was contact with an infected household (Chang *et al.*, 2007).

EV 71 excreted in large number from the gut and is ingested to cause infection, this occur in the followings: fecal-oral route, eating utensils, through contaminated food and water, or through conjunctiva (rare) (Solomon *et al*., 2010; Greenwood *et al.*, 2012).

It can be also spread through contact with virus-contaminated oral secretions, vesicular fluid, surfaces or fomites (Solomon *et al*., 2010).

In acute phase the virus is present in the throat and can be spread via droplet (Greenwood *et al.*, 2012; Levinson, 2012; Forbes *et al*., 2007).

After the acute phase the only route for transmission is fecal-oral route (Collee *et al*., 2007; Solomon *et al*., 2010; Greenwood *et al.*, 2012).

Enteroviruses occur in all part of globe and infect mainly younger than adult (Brook *et al.*, 2013).

Infection occurs mainly in summer and autumn (Brook *et al.*, 2013).

Outbreaks occur in closed community (e.g. military establishment) and schools (Greenwood *et al.*, 2012). The infection rate 100% especially in children (Greenwood *et al.*, 2012).

Social factors such as poor hygiene and overcrowding are important (Greenwood *et al.*, 2012).

Infection is more five times prevalent in children of lower income families than in those living in favorable circumstances (Brook *et al.*, 2013).

2.7. Pathogenesis

There are conflicting theories about pathogenesis of EV71 infection and it remains somewhat unclear. However, Initial viral replication is presumed to occur in the lymphoid tissues of the oropharyngeal cavity (tonsils) and small bowel (Peyer's patches), with further multiplication in the regional lymph nodes (deep cervical and mesenteric nodes), giving rise to a mild viremia. Most infections are controlled at this point and remain asymptomatic (David, 2008; Solomon *et al*., 2010).

Further dissemination of Enteroviruses to the reticuloendothelial system (liver, spleen, bone marrow, and lymph nodes), heart, lung, pancreas, skin, mucous membranes, and central nervous system (CNS) coincides with the onset of clinical features (David, 2008; Solomon *et al*., 2010).

For EV71, viral shedding from the throat can occur up to 2 weeks after an acute EV71 infection, and virus can be isolated from stool for up to 11 weeks. EV71 is a highly neurotropic virus. The brain stem is most likely the major target of EV71 infection. Two possible routes by which the virus reaches the CNS have been suggested: the virus either enters the CNS from the blood across the blood-brain barrier (BBB), or is transmitted to the CNS through peripheral nerves via retrograde axonal spread along cranial or peripheral nerves (Chang *et al.*, 2007; Solomon *et al*., 2010).

2.8. Clinical features

Infection by EV71 is often asymptomatic, but may manifest as self-limiting disease with mild flu-like symptoms but in some cases it can death resulting from complications over days or even hours (Ooi *et al.*, 2010; Linsuwanon *et al.*, 2014).

EV71 is one of the leading causes of viral central nervous system diseases (including polio-like myelitis, brainstem encephalitis, aseptic meningitis, acute flaccid paralysis, Myoclonic jerks) and hand-foot and mouth Disease (Ooi *et al.*, 2010; Levinson, 2012; Linsuwanon *et al*., 2014).

It also causes diarrheal disease, herpangina (HA) and other severe systemic disorders, including especially Autonomic nervous system, pulmonary oedema, pulmonary hemorrhage and cardiorespiratory collapse (Greenwood *et al.*, 2012; Linsuwanon *et al.*, 2014).

2.9. Laboratory diagnosis of EV 71

Isolation of virus was the most useful method for establishing a diagnosis, but this method has been suppressed by RT-PCR (Collee *et al.*, 2007; Greenwood *et al.*, 2012; Brook *et al.*, 2013; Bessaud *et al.*, 2014).

Examination of CSF is very important for diagnosis of viral meningitis (Collee *et al.*, 2007; Greenwood *et al.*, 2012).

2.9.1. Virus isolation

The virus can be isolated from throat, rectal, and ulcer swabs, and samples of serum, urine, CSF, and fluid from vesicles (Ooi *et al*., 2010; Brook *et al.*, 2013).

The sensitivity, specificity, and usefulness of findings vary according to the sample. Virus detection in samples from sterile sites, such as vesicular fluid, CSF, serum, urine, or those gathered at autopsy, is more reliable than that in samples from non-sterile sites (Ooi *et al*., 2010).

Cell cultures which have been used for EV 71 isolation are Primary rhesus macaque kidney cells (PMK) and human dermal fibroblast (HDF) (Bessaud *et al*., 2014). Several human and non-human primate cell lines can be used, including rhabdomyosarcoma cells (RD), which is most efficient, human lung fibroblast cells (HLF) and African green monkey kidney cells (vero cells) (Ooi *et al*., 2010).

In RD cells, a characteristic Cytopathic effect (CPE) is observed typically 7–10 days after inoculation (Ooi *et al*., 2010).

Infected cells round up and show shrinkage and marked nuclear pyknosis, become refractile and eventually degenerate and fall off the glass surface. Plaque of degenerating cells is formed by EV 71 (Evans, 1984).

Blind passage might be necessary before CPE become apparent (Ooi *et al*., 2010).

Once a CPE is observed, the virus is identified by several antigen antibody

reactions like neutralization tests and indirect immunofluorescence assay (Evans. 1984; Ooi *et al*., 2010).

Cell culture isolation and serotyping using the neutralization test are accurate but have several disadvantages. Firstly, it is expensive to carry out in the general diagnostic laboratory (Rigonan *et al.*, 1998). Secondly, it is very time-consuming since virus isolation may take up to 3 weeks. Thirdly, cell culture isolation has a relatively low sensitivity which renders difficult detection of EV71 in clinical samples with low viral titer (Singh *et al.*, 2002).

Fourthly, the neutralization test itself has disadvantages. Neutralization could be hindered due to antigenic drifts or presence of multiple viruses in the clinical specimens (Schmidt *et al.*, 1974).

2.9.2. Serological approaches

2.9.2.1. Enzyme Linked Immunosorbent Assay (ELISA)

A few studies on detection of EV71 using serological assays have been carried out. ELISA has been used for various serotyping of Enteroviruses and it is sensitive and specific for laboratory diagnosis (Bendig and Molyneaux, 1996). The detection of IgM antibody in serum samples from EV71-infected patients by ELISA was shown to be effective in diagnosing acute EV71 infections (Tano *et al.*, 2002).

Compared to virus isolation and neutralization test, ELISA is rapid test in identification. It is only took 4 hours and commonly used in diagnostic

laboratories, but it has some limitations. In the IgM-capture ELISA, the whole EV71 virion was used as the coated antigen for detection of serum IgM antibody. The need to prepare large quantities of purified virions and interacting with secondary anti-human IgM in the ELISA assay made the method an expensive, laborious and lengthy process (Wang *et al.*, 2004). In addition, since the whole virus was used as the capture antigen in the ELISA assay, cross-reactions with antibodies against other Enteroviruses could result in false positives. Thus, the specificity of the IgM-based ELISA may be compromised by the presence of common epitopes of other Enteroviruses. Recently, IgM and IgG ELISA assays using recombinant purified EV71VP1 protein as a coated antigen for detection have been developed (Wang *et al.*, 2004).

Furthermore, the specifity of this test decrease with increasing age because homologous antibodies are produced when individual encounter their first Enteroviruses infection, heterologous cross-reacting IgG and IgM antibodies are produced by older children and adults following repeated infection with different Enteroviruses serotypes (Ooi *et al.*, 2010).

2.9.2.2. Indirect immunofluorescence assay

Another conventional method used by many laboratories was indirect immunoflorescence (IIF) assay (Wang *et al.*, 2004). A type-specific monoclonal antibody was raised against EV71 VP1 protein. Patient samples were subjected to

virus isolation in cell lines. Immunohistological staining with EV71 antibody was carried out then and cell culture with virus particles of EV71 could bind to the antibody. This complex was then detected by a secondary antibody labeled with FITC (fluorescent isothiocyanate-labeled) which was visualized under a fluorescence microscopy. This method does not require the time-consuming neutralization test using antisera pool but it still needs to isolate and propagate the virus. Since virus isolation involves a long time, it still delays diagnosis. In addition, this monoclonal antibody was raised against the VP1 protein of the prototype strain; it may not be able to recognize mutant viruses. It was also reported that cross reaction was observed with CA16 (Rigonan *et al.*, 1998; Tung *et al.*, 2007).

2.9.3. Nucleic acid detection

This method has become the most efficient for EV 71 detection (Collee *et al*., 2007; Greenwood *et al.*, 2012; Brook *et al.*, 2013; Bessaud *et al.*, 2014).

It depends on detection of conserved sequence within 5` non coding region of Enterovirus genome by using specific primer for it (Greenwood *et al.*, 2012).

Many studies confirmed that RT-PCR is more specific and sensitive than culture for EV 71, so it considered as the current gold standard of diagnosis of EV 71 in CSF (Greenwood *et al.*, 2012).

Molecular detection gives a lot of information which can be used for example in epidemiological studies to distinguish live attenuated vaccine from wild type virus (Greenwood *et al.*, 2012).

2.9.4. Other laboratory investigations

In mild EV 71 disease the full blood count, urea and electrolyte concentrations are generally normal, but in severe disease a raised white cell count with neutrophilia is frequently seen and hyperglycaemia might be present (Ooi *et al.*, 2010).

In CSF analysis there is mild lymphocytic pleocytosis of 10–100 cells per μL and glucose concentration is generally normal, but can be low (Ooi *et al.*, 2010).

2.10. Treatment of EV 71 infections

Generally, There is no specific treatment for viral meningitis just relieving the patient's symptoms. But in some instance EV 71 meningitis may lead to serious outcome, including death. So it need to specific treatment (Levinson, 2012).

2.10.1. Antiviral drug

Pleconaril is an antiviral drug that inhibits the entry of several Enteroviruses into cells and has been used in clinical trials of aseptic meningitis. This drug it is not, however, active against EV71 (Ooi *et al.*, 2010).

In-vitro and *in-vivo* studies show that both ribavirin and interferons might be useful, and RNA interference approaches are being explored (Ooi *et al.*, 2010).

The pimprinine family of compounds are inhibitors effective against the replication of EV71 (Wei *et al.*, 2015).

2.10.2. Intravenous Immunoglobulin

Administration of immune globulin intravenously may reduce sever disease mainly in immunocompromised patients (Ooi *et al*., 2010).

Analysis of cytokine profiles before and after immunoglobulin treatment showed substantial reductions in concentrations of some pro-inflammatory cytokines in patients with EV71 if they had encephalitis with autonomic dysfunction, but not if they had less severe disease. Intravenous immunoglobulin has, therefore, become more routinely used for the treatment of severe EV71 disease even though there are almost no data on its efficacy and this treatment is expensive (Ooi *et al*., 2010).

2.10.3. Milrinone

Milrinone is a cyclic nucleotide phosphodiesterase inhibitor currently used in the treatment of congestive heart failure. Inhibition of phosphodiesterase subtype III by this cardiotrophic agent results in an increase in intracellular concentrations of cyclic AMP, which in turn leads to increased cardiac output and decreased peripheral vascular resistance (Ooi *et al*., 2010).

Children with EV71-induced pulmonary oedema showed that those treated with milrinone had reduced tachycardia and lower mortality than those who did not receive this drug (Ooi *et al*., 2010).

2.11. Prevention and control

Standard infection control prevention (including adequate hand hygiene) is critical in preventing spread (Greenwood *et al.*, 2012). Avoidance of contact with patients exhibiting acute febrile illness is especially young children (Brook *et al.*, 2013).

After natural infection, immunity is long lasting and persists for several decades (Greenwood *et al.*, 2012).

2.12. Vaccine

EV 71 is a highly communicable and sometimes life-threatening virus but it can be prevented. Among all the measures of prevention, vaccination may be the best way (Solomon *et al*., 2010).

A clinical trial of formalin-inactivated EV71 vaccine adjuvant with aluminum hydroxide was conducted in response to the Bulgarian epidemic in 1975 but the results were not published in international journals (Solomon *et al*., 2010).

Recently, several EV71 vaccine candidates have been evaluated in animals but no clinical trial has been conducted evaluated immunogenicity and protection of three vaccine candidates (heat-inactivated whole virus, viral protein (VP1) DNA and VP1 protein expressed in *Escherichia coli*) in Mice (Solomon *et al*., 2010).

All three candidates could induce neutralizing antibodies but only the inactivated whole-virus candidate could protect mice from fatal infection. The inactivated whole-virus vaccines seem to be more immunogenic than recombinant VP1 and

DNA vaccines, but the manufacturing costs for recombinant VP1 and DNA vaccines could be cheaper than the inactivated whole-virus vaccines (Solomon *et al*., 2010).

2.13. Physiochemical Properties

In presence of organic matter Enteroviruses survive at room temperature for weak, at 4^{o}c for one month but are killed at 50-55^{o}c (Greenwood *et al.*, 2012). Enteroviruses are inactivating by 0.3% formaldehyde, chlorination and ultraviolet (UV) irradiation (Solomon *et al*., 2010; Greenwood *et al.*, 2012; Brook *et al.*, 2013). However, chemicals inactivation of Enteroviruses is ineffective when organic matter is present (Greenwood *et al.*, 2012; Levinson, 2012).

Enteroviruses are stable in acid condition (pH 3-5) (Greenwood *et al.*, 2012; Levinson, 2012).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study design

Descriptive, cross sectional study.

3.2. Data collection

Ethical approval was obtained from the Ministry of Health for collection and examination of the samples. The collected data included, gender, age, date of sample collection and place of sample collection. The obtained data were saved for statistical analysis by SPSS software program version 11.5. Personal and clinical data were collected by reviewing Hospital records.

3.3. Study period

A total of 89 CSF samples were collected from different patient groups from five Khartoum State Hospitals namely: (Mohammed Alamin Hamid Hospital, Soba Teaching Hospital, Omdurman Military Hospital, Alban Gadeed Teaching Hospital and Police Hospital) during February to May 2015.

3.4. Specimen collection

The samples were collected from patients with aseptic meningitis. Each specimen met the inclusion criteria which was a negative result for bacterial meningitis included in this study. CSF samples were collected under aseptic conditions by experience healthy workers from arachnoid space using a sterile wide born needle

inserted between fourth and fifth lumber vertebra, the CSF was allowed to drip into dry sterile container , then the samples were stored at -80^{o}C until use.

3.5. RNA extraction

Viral RNA was extracted from CSF by using (Viral Gene-SpinTM DNA/RNA Extraction Kit, Korea), according to the manufacturer's instructions. Briefly, 150 $\mu\ell$ of CSF sample was added to 250$\mu\ell$ buffer viral Gene-spinTM (lysis buffer) containing carrier RNA and then incubated at room temperature for 10 minutes, and then the resulting solution was applied to a column. A volume of 500 $\mu\ell$ of AW1 and AW2 was added for washing and the nucleic acid were eluted with 35 $\mu\ell$ of elution buffer and stored at -80^{o}C until used for reverse transcription.

3.6. Reverse transcription

Reverse transcription was performed on extracted RNA by using (Maxime RT PreMix Kit), following cDNA synthesis at a temperature 45^{o}C for 60 minutes, followed by Reverse transcriptase inactivation at 95^{o}C for 5 minutes.

3.7. Polymerase chain reaction (PCR)

The PCR was performed by processing the cDNA with primers that are specific for PAN Enteroviruses and EV71.

The primers used consisted of forwored primer

5`- CAAGCACTTCTGTTTCCCCGG -3` and reverse primer was

5`- ATTGTCACCATAAGCAGCCA -3`, which were predicted to amplify fragment of 435 bp. The primers specific for EV 71 were 5`-GTCCTTAATTCGCACAGCACAGCT-3` and 5`-CGGTCCGCACTGAGAATGTACCCAT-3`, which were predicted to amplify fragment of 507 bp.

The reaction was performed in 25 µl volume using Maximum PCR PreMix tubes (i-Taq). The volume included: 5 µl master mix, 1 µl forward primer, 1 µl reverse primer, 5 µl cDNA and 13 µl distilled water. The PCR amplification for PAN Enteroviruses and EV 71 was performed in 40 cycles consisting of denaturation for 1 minute at 94°C, primer annealing for 1 minute at 50°C and elongation for ` minute at 72°C by using Techno PCR Machine (Japan).

3.8. Gel electrophoresis

10 µl of the amplified product was subjected to direct analysis by gel electrophoresis in 2% Agarose, the gel was prepared by adding 1.5 g of Agarose to 75 ml 1X Tris Borate EDTA buffer. The product was visualized by staining with 0.15% Ethidium bromide using UV gel documentation system INGeNius. The expected size for the amplified cDNA for PAN Enteroviruses and EV71 were 435 and 507 bp, respectively.

CHAPTER FOUR

4. RESULTS

Detection of Enteroviruses

A sum of 89 samples were tested for Enteroviruses, 46 (17 males and 29 females) were positive for Enteroviruses by RT-PCR **(Table 1).**

Based on age group, the distribution of 46 patients positive for Enteroviruses were 40 and 6 in age group 1day-15years and 16-30years, respectively **(Table 2).**

Detection of EV 71

A total of 46 samples that were Enteroviruses positive tested for EV 71 using RT-PCR. Out of these 10 samples, (3 males and 7 females) were positive for EV 71 **(Table 1).**

Based on age group, the distribution of 10 patients positive for EV 71 was 8 and 2 in age group 1day-15years and 16-30years, respectively **(Table 2).**

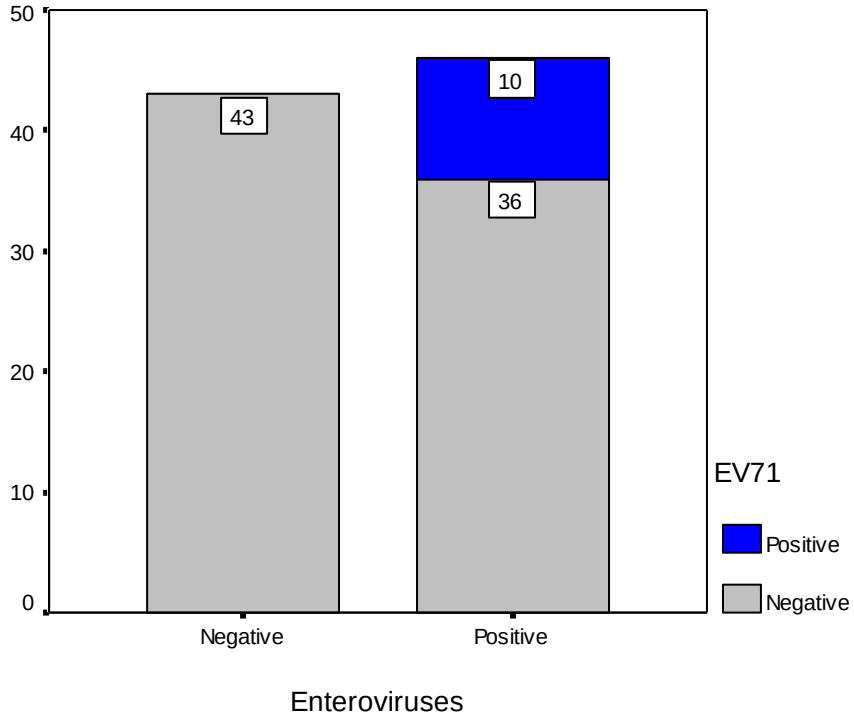

Figure (2) Frequency of Enteroviruses and EV 71 in aseptic meningitis

Khartoum State Hospitals, Sudan 2015

*Percentage of Enteroviruses 52% (46/89)

* Percentage of EV 71 22% (10/46)

Table (1) Patients grouped by gender and detected with Enteroviruses and EV71

Gender	Enteroviruses		EV 71		Total
	Negative	Positive	Negative	Positive	
Male	26 (60%)	17 (40%)	40 (93%)	3 (7%)	43
Female	17 (40%)	29 (60%)	39 (85%)	7 (15%)	46
Total	43	46	79	10	89

Table (2) Patients grouped by age and detected with Enteroviruses and EV 71

Age	Enteroviruses		EV 71		Total
	Negative	Positive	Negative	Positive	
1 day-15 years	39 (49%)	40 (51%)	71 (90%)	8 (10%)	79
16-30 years	4 (40%)	6 (60%)	8 (80%)	2 (20%)	10
Total	43	46	79	10	89

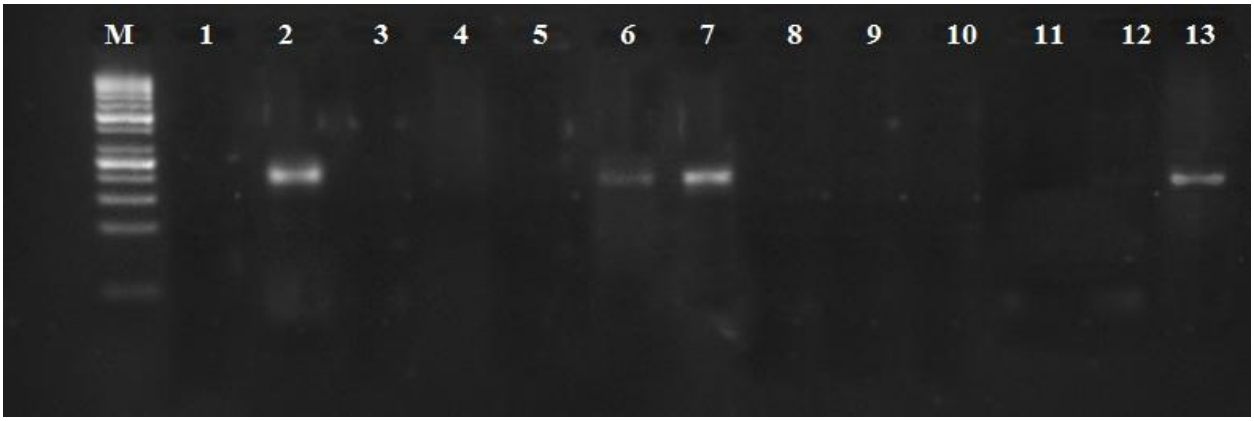

Figure3: Enteroviruses result (435 bp) on 2% agarose. Lane 1 and 2 show negative and positive controls, respectively and lane 6,7and 13 show positive results, M: 100bp DNA marker.

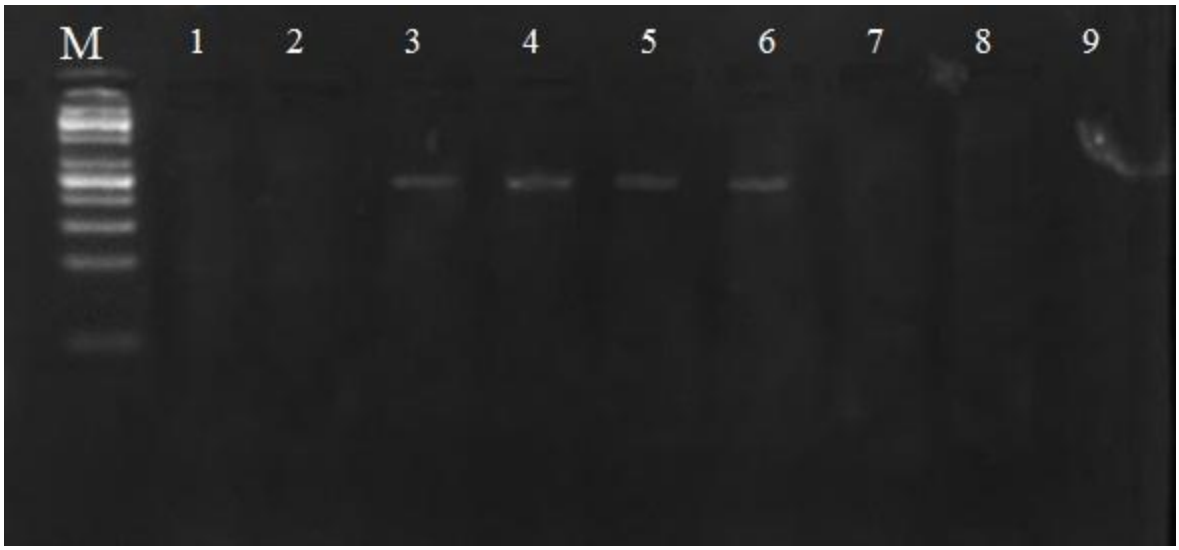

Figure4: EV 71 result (507 bp) on 2% agarose. Lane 2 and 3 show negative and positive controls, respectively and lane 4,5and 6 show positive results, M: 100bp DNA marker.

CHAPTER FIVE

5. DISCUSSION

5.1. Discussion

EV 71 is positive sense single-stranded RNA viruses, belongs to the Picornaviridae family and Enterovirus genus. It is the common cause of HEMD in children and the epidemic of sever neurological diseases. Aseptic meningitis is the most common type than bacterial meningitis. Viral meningitis has no specific treatment for it just relieving the patient's symptoms. But in some instance viral meningitis may lead to serious outcome, including death.

Little is known about the epidemiology of Enteroviruses in Sudan in particular and Africa in general. Hence, there is a need to know the Enteroviruses and EV 71 occurring in particular geographical area.

The present study focused on molecular detection of Enteroviruses infection in case of aseptic meningitis with emphasis on EV 71.

The current study in Sudan is the first one to report directly measured rate of Enteroviruses with emphasis on EV 71 associated meningitis in CSF samples.

This study had been conducted to detect RNA of EV 71 in CSF samples from patients with aseptic meningitis using RT-PCR.

A total of 89 samples of CSF had been involved in this study. 46 (52%) samples were positive for Enteroviruses. 10 (22%) samples from these positive samples

were positive for EV 71.This in turn will be considered as an alarming indicator of the disease in the population in Sudan. Taken all of the above finding into consideration, these may indicate complex epidemiology in Sudan.

The result obtained in this study using RT-PCR, were in disagreement with several published studies in different outbreaks in Brunei, South Korea and Thailand with those reported of EV 71 38%, 19% and 9%, respectively (AbuBakar *et al.,* 2009; Ryu *et al.,* 2010; Linsuwanon *et al*., 2014).

In this study the EV 71 infection occurred mainly in adult more than children and there is no significant statistical relation between EV 71 infection and patient's age. In Cyprus, Gaza, China and Taiwan outbreaks EV 71 mainly affects children more than adult which disagree to this study result, but in Romania outbreak the adult are more affected than children (Logan & Macmahon, 2008).

According to the results female are more affected than male and also there is no significant statistical relation between gender and EV 71 infection.

In India it was reported that the aseptic meningitis is common in male more than female whish disagree to this study results (Kumar, 2005).

Hence, there is a need to know the type of Enteroviruses in meningitis patients occurring in particular geographical area to serve as guide in choosing a specific type of treatment.

Laboratory diagnosis is important in managing Enteroviruses infection. Rapid and accurate Enteroviruses diagnosis improve medical management by allowing timely provision of antiviral therapy, implementation of appropriate infection control strategies for individual and public health response to meningitis, and limitation of unnecessary investigation or antibiotic therapy (Kumar, 2005; David, 2008; AbuBakar *et al.*, 2009; Ryu *et al.*, 2010; Linsuwanon *et al*., 2014).

Meningitis disease cannot be reliably diagnosed on clinical features alone. The clinical picture may be difficult to distinguish from infection with bacteria like: (*Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Treponema palladium*, *Borrelia burgdofi*, Leptospira spp and Brucella spp) (Kumar, 2005).

These findings should highlight the need for the establishment in Sudan of rapid, sensitive and specific diagnostic techniques (such as one used her) for better management of Enteroviruses infections especially in the high risk group.

To best of our knowledge this is the first attempt to identify the causative viral agents of meningitis infections in Sudan using molecular techniques.

Finally, the results obtained should call for wider surveillance at the national level in order to fully elucidate the true status of Enteroviruses, EV 71 and other viral meningitis in Sudan.

5.2. CONCLUSIONS

Incidence and existence of Enteroviruses and EV 71 in Sudan were documented through detection of Enteroviruses and EV71 RNA in CSF samples indicating high infection among meningitis patients in Sudan. Moreover, the Enteroviruses and EV71 detection using RT-PCR were established.

Generally, these findings are useful for further studies since there is little available information about EV71 infection in Sudan.

5.3. RECOMMENDATIONS

1. More research is required for further study the prevalence and characterize other Enteroviruses in Sudan. The risk factors associated with Enteroviruses infection are to be determined.

2. Improving and optimizing of RT-PCR and it is application as a routine technique for the diagnosis of EV 71 and other Enteroviruses in Sudan.

3. Further research work should be carried out to characterize EV 71 at molecular level.

4. Continues molecular surveillance of EV71 in Sudan is required to understanding of factors influencing the infection and emergence of outbreaks in the region by this virus.

5. Determination of the most common genotype in Sudan to understand the evolution of the virus and its association with outbreaks in Sudan.

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