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Full Length Research Paper

Determination of human herpes simplex virus in clear cerebrospinal fluids by multiplex polymerase chain reaction (PCR) in Rwanda

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The purpose of this study was to test CSF obtained from different regions of Rwanda for herpes simplex viruses (HSV) type 1 and 2 using a commercial multiplex PCR kit. CSF samples were obtained from patients with clinical suspicion of meningitis and encephalitis which may be caused by different microorganisms including herpes viruses. More than 80% of clear cerebrospinal fluid (CSF) submitted to two referral hospitals (King Faisal Hospital and University Teaching Hospital of Kigali) and to the National Reference Laboratory of Rwanda, were found to be negative for *Cryptococcus neoformans* and *Mycobacterium tuberculosis*, the only microorganisms tested at these facilities.

Key words: Herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2), cerebrospinal fluids, multiplex polymerase chain reaction (PCR), meningitis, encephalitis, *Cryptococcus neoformans*, *Mycobacterium tuberculosis*, Rwanda.

INTRODUCTION

Human herpes viruses, including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella-zoster virus (VZV) and human herpesvirus 6 (HHV-6) commonly cause infections of the central nervous system (CNS) and the urinary tract. Clinical signs and symptoms resulting from these six viruses are often indistinguishable from other neurological conditions (Baskin and Hedlun, 2007). While clinical outcome is favourable when therapy is administered in early stages of disease, this requires early detection of herpes virus in clinical specimens obtained from the patient. Herpes simplex viruses (HSV) persist in various cells of the host following primary infection. Infection of CNS by HSV is often associated with severe disease including encephalitis and meningitis with recorded mortality (Minjolle et al., 2002; Baskin and Hedlun, 2007; Ruzek et al., 2007). HSV-1 is the most common cause of sporadic encephalitis in adults where the seroprevalence is widespread in the population. Meningitis resulting from infection with HSV-2 is usually self-limiting and seldom causes severe CNS symptoms (Momméja-Marin, 2003). As compared to HSV-1 induced encephalitis, the symptoms associated with HSV-2 encephalitis have been described as milder, often diagnosed as meningoencephalitis (Omland et al., 2008). Symptoms of HSV-2 CNS infection can arise without typical genital herpes eruptions and also in patients who have never experienced genital herpes before.

Encephalitis is an inflammation of the brain, normally caused by virus infection (Sindic et al., 2003). In infected individuals with encephalitis, symptoms such as headache, fever, vomiting, confusion and light-sensitiveness are common and can in severe cases also cause unconsciousness, seizures and paralysis. Menin-

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Abbreviations: CSF, Cerebrospinal fluid; **HHV,** human herpes virus; **HSE,** herpes simplex encephalitis; **HSV,** herpes simplex virus; **PCR,** polymerase chain reaction; **CMV,** cytomegalovirus; **EBV,** Epstein-Barr virus; **VZV,** varicella-zoster virus; **CNS,** central nervous system.

gitis, on the other hand, has bacterial or non-bac-terial origin and leads to inflammation of the mem-branes covering the brain and spinal cord. Common symptoms of meningitis include stiff neck, light-sensitivity, severe headache, nausea, fever and mental status changes (Sindic et al., 2003). Early specific antiviral therapy will significantly reduce the mortality and serious morbidity of cases. Since the therapy must be started as possible, before the patient progresses into more lethal forms of the disease, timely diagnosis of HSV infection in the CNS must be done for proper treatment. With the introduction of PCR, virus DNA can be detected in the CSF and is the method most widely used for diagnosing viral CNS infections today (DeBiasy et al., 2002).

In Rwanda, the National Reference Laboratory (NRL) and referral hospitals routinely examine clear CSF for *Cryptococcus neoformans* and *Mycobacterium tuberculosis* in patients suspected to have CNS disease. However, the majority of clear CSF (more than 80%) from clinically suspected cases is negative for these infections. The NRL and referral hospitals do not examine CSF for HSV infection due to lack of capacity to diagnose the virus. The purpose of this study was to use a multiplex PCR-based approach to examine the presence of HSV-1 and HSV-2 infections in clear CSF from people with clinical suspicion of meningitis and encephalitis. Results obtained in the study not only give the NRL the opportunity to provide leadership to Rwanda with respect to HSV diagnostics but also point the direction for PCR-based viral diagnostics at NRL for any other disease. Furthermore, the implementation of multiplex PCR for the detection of HSV will benefit Rwandan population and its neighbouring countries as treatment with acyclovir is easily available and highly effective against these viruses.

MATERIALS AND METHODS

A total of 196 participants from three referral and nine district hospitals from five provinces in Rwanda were enrolled for this study.

HSV diagnostics.

DNA from CSF was isolated using Zymo spin (Hartfield, South Africa) kit according to manufacturer's instructions. DNA was amplified using Seeplex HSV2 ACE (Seegene, Hartfield, South Africa) kit according to manufacturer's instruction. PCR was carried on a Geneamp 9700 thermocycler and resolved on a 2% agarose gel.

RESULTS

Multiplex detection of HSV-1 and HSV-2

The expected bands of 300, 473 and 981 bp corresponding to HSV-1, HSV-2 and internal control respectively were observed in both the molecular weight markers and positive control samples (Figure 1). Figure 1 is a representative gel which shows results for a sec-a section of samples that were scored positive for either HSV-1 alone (e.g. lanes 6, 7 and 14 (bottom panel)), HSV-2 alone (lanes 6 to 8, 10 and 12 all on top panel; and lanes 9, 10 and 15 on bottom panel), or dually infected with HSV-1 and HSV-2 (lane 9 (top panel)). An example of a sample in this gel (Figure 1) that had an indeterminate result is the sample loaded in well 5 (top panel) where a faint HSV-1 band is observed but there was no internal control band. This sample was subsequently repeated and found to be positive (data not shown). Majority of the samples tested were negative for both HSV-1 and HSV-2. Only the internal control (981 bp) bands were amplified, indicating that DNA was well extracted and PCR reactions were adequate (data not shown).

Proportion of HSV-1 and HSV-2 as compared to *C. neoformans* and *M. tuberculosis* in clear CSF samples

Data on *C. neoformans* was obtained from hospitals that participated in sampling of clear CSF. In all Rwandan hospitals, testing for *C. neoformans* is routinely done using India ink. Culture of these microorganisms on SDA media was also performed but only in referral hospitals and at the NRL. *M. tuberculosis* was done by Ziehl-Nelson staining technique.

A summary of data obtained for *C. neoformans* from different provinces that participated in this study is shown in Table 1. Only 11 of 139 clear CSF samples (7.9%) from Kigali City Province were positive for *C. neoformans*– samples from all other provinces had no *C. neoformans*. When the entire sample size (196) for the study is considered, this translates to only 5.6% of samples as *C. neoformans* positive. For Kigali City Province, the prevalence of *C. neoformans* (7.9%) and HSV-1 or HSV-2 (4.3 and 8.6%, respectively) for the same population is not statistically different (Figure 2).

When all samples were analyzed by multiplex PCR, a total of 7 samples were positive for HSV-1. Of these, 6 were from Kigali City Province while one was from South Province. The total number of CSF samples from these two provinces was 174 (139 from Kigali City and 35 from South Province). This represented a prevalence of 4.0% of HSV-1 for these two provinces alone and 3.6% when all the five Rwandan provinces were considered (Table 1). On the other hand, 12 out of 38 CSF samples were positive for HSV-2 alone in Kigali City Province, while two samples from Kigali City Province were positive for both HSV-1 and HSV-2 (1%). This study, therefore, identified a total of 21 out of 196 CSF samples (10.7%) as being HSV positive. The majority of CSF samples were negative for HSV (Table 1). Of all the clear CSF collected and analyzed, no *M. tuberculosis* infection was detected (data not shown).

DISCUSSION

In the present study, both HSV-1 and HSV-2 were detected in clear CSF, indicating that some of the neurological conditions from individuals where the samples were collected could be attributable to HSV, even though other viral agents may be responsible. The prevalence

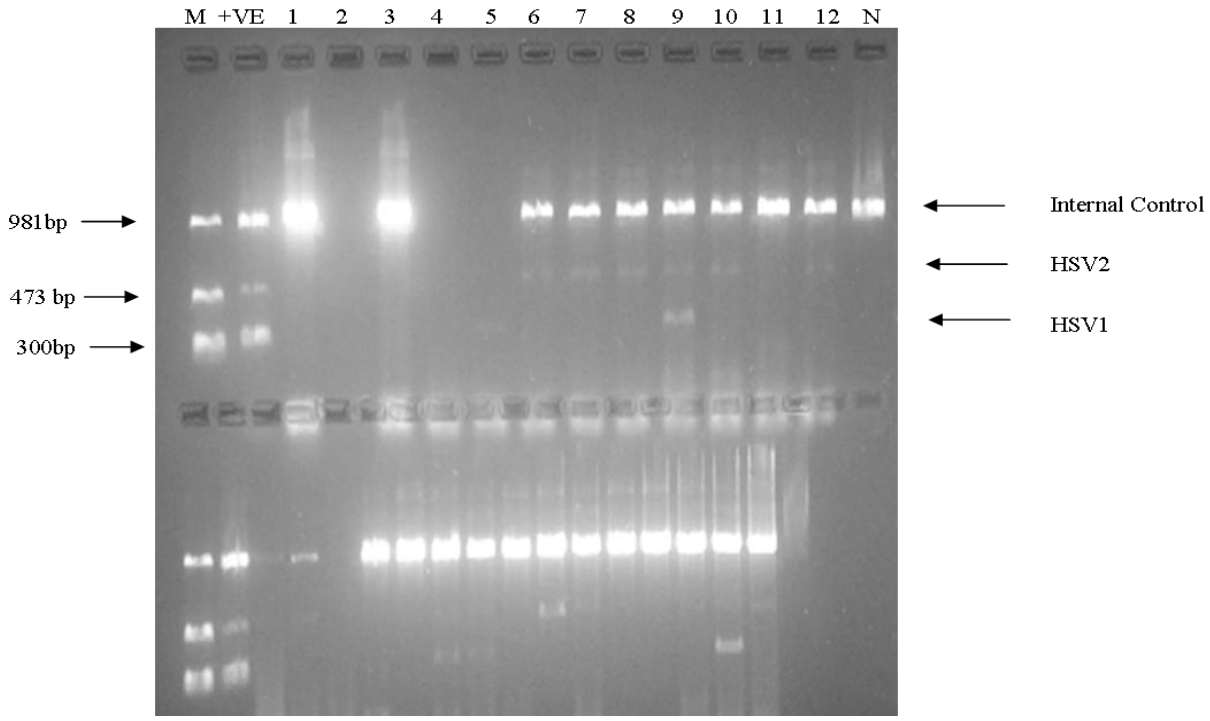

Figure 1. Multiplex detection of HSV1 and HSV2 in clear CSF. Samples were DNA extracted using Zymo spin (Hartfield, South Africa) kit. DNA was amplified using Seeplex HSV2 ACE (Seegene, Hartfield, South Africa) kit according to manufacturer's instruction and resolved on 2% agarose gel and stained using green nucleic acid stain. First two wells (top and bottom panel) were loaded with molecular weight markers (M) and with positive control (+ve), respectively. 'N' designates negative control (top panel). All other wells were loaded with PCR products except for wells 17 and 18 (bottom panel) which were empty.

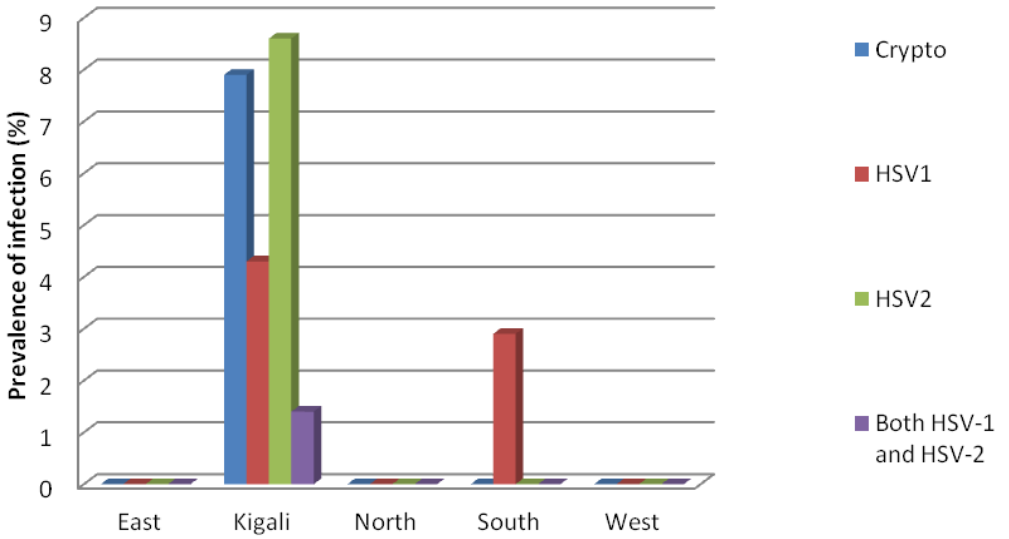

Figure 2. Summary of the prevalence of *C. neoformans* and HSV by province. The y-axis shows the prevalence of *C. neoformans* and HSV, while the x-axis shows the provinces within Rwanda where clear CSF samples were collected. The bars are shown in color as follows: Blue– proportion of *C. neoformans* (as determined by Indian ink microscopy), red– proportion of HSV-1, green– proportion of HSV-2 and purple– proportion of samples with dual infection (both HSV-1 and HSV-2).

of HSV-1 was much lower than that of HSV-2 (Table 1) which is consistent with observations by other groups (Chayavichitsilp et al., 2009). Only two individuals were dually infected with both viruses from Kigali City Province. One observation made in the data (Figure 1) is that some bands (for both viruses) were more intense than others. Since the test applied here is only a qualitative test (that is, to indicate presence or absence of HSV DNA), it is likely that band intensity reflected either on the abundance of HSV in the sample relative to others

Table 1: Comparison of *Cryptococcus neoformans* and HSV infections by Province

Province	*Cryptococcus neoformans*				HSV1				HSV2				HSV1/HSV2 coinfections			
	Negative		Positive		Negative		Positive		Negative		Positive		Negative		Positive	
	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
North	7	100.0	0	0.0	7	100.0	0	0.0	7	100.0	0	0.0	7	100.0	0	0.0
East	7	100.0	0	0.0	7	100.0	0	0.0	7	100.0	0	0.0	7	100.0	0	0.0
South	35	100.0	0	0.0	34	97.1	1	2.9	35	100.0	0	0.0	35	100.0	0	0.0
West	8	100.0	0	0.0	8	100.0	0	0.0	8	100.0	0	0.0	8	100.0	0	0.0
Kigali	128	92.1	11	7.9	133	95.7	6	4.3	127	91.4	12	8.6	137	98.6	2	1.4
Total	**185**	**94.4**	**11**	**5.6**	**189**	**96.4**	**7**	**3.6**	**184**	**93.9**	**12**	**6.1**	**194**	**99.0**	**2**	**1.0**

or different efficiencies of PCR amplification. A quantitative assay (such as real-time PCR) will have to be applied to determine the relative amounts in different CSF samples perhaps in a future study.

It is well known that CSF may contain inhibitors that can partially or completely block DNA polymerase activity and cause false negative results. Due to the clinical and therapeutic implications of a false-negative PCR result, identification of inhibited PCRs is a priority. Therefore, when PCR is employed for diagnostic purposes, it is imperative to adopt adequate controls for assessing sample suitability for PCR. In the present multiplex PCR assay, occasional false-negative results for HSV DNA amplification could lead to unidentifiable false-negative results if internal control primers were not included in the assay. Thus, failure of DNA extraction or failure to remove any inhibitors of DNA amplification may be avoided by the present assay. Apart from the ability to detect two different viruses in the same reaction tube, the inclusion of a positive control sample (which is added at the stage of setting up PCR in place of CSF-derived DNA sample) is important in ensuring specificity of amplified HSV DNA from biological samples. Reactions that fulfilled the requirement for positive control but had a negative control that failed to either amplify the expected internal control band or had more than one band were repeated before individual samples could be scored as either being positive or negative for HSV.

It is interesting that HSV was detected only in Kigali City and South Provinces. Perhaps it is not too surprising though, as the majority of CSF samples analyzed were from these provinces since of 196 clear CSF collected, a total of 174 (or 89%) were from Kigali City and South Provinces. Since 20 of 21 HSV positive CSF were from Kigali City Province alone, the prevalence of HSV in this province is 14.3%, a figure much higher than the national average of 10.7% obtained in this study. Indeed, all the other 3 provinces had no detectable HSV in CSF.

As shown in Figure 1, HSV detection with multiplex PCR from CSF in Rwanda is a major finding. Even though there are no laboratories in Rwanda that attempt to detect HSV as possible causative agents of encephalitis and meningitis, the practice in many countries that have not adopted PCR techniques is an invasive and expensive brain biopsy specimen procedure which has been previously used as a benchmark for the laboratory diagnosis of HSV encephalitis, meningitis and other CNS diseases.

The prevalence of *C. neoformans* was 7.9% out of 128 samples and 11 (5.6%) out of 196 samples collected from five provinces based on data obtained from hospitals where clear CSF was collected (Desalermos et al., 2012). For patients tested for *M. tuberculosis*, all of them were negative to TB. These observations indicate that these microorganisms can still be detected in clear CSF, hence the need to analyze all clear CSF for *C. neoformans* infection.

Routinely, of all CSF samples received by the NRL from regional hospitals in Rwanda, only about 20% regularly test positive for *C. neoformans* and/or *M. tuberculosis*. Majority (about 80%) of the CSF samples were not tested for viruses such as HSV. It is quite likely (based on results from this study) that a huge fraction of these samples are positive for HSV. Since both encephalitis and meningitis are serious neurological diseases, it will be important for NRL to routinely analyze these samples for HSV; unlike most viruses, these can effectively be treated with drugs.

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