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Prevalence of Herpes Simplex Virus type 2 IgM among Women of Reproductive Age in Zaria, and Demonstration of its Cytopathogenicity in Whole Egg

Ameh, E.R., Aminu, M., and Ella, E. E*.

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Department of Microbiology, Ahmadu Bello University, Zaria

ABSTRACT

Egg culture has long been considered the diagnostic gold standard for Herpes Simplex Virus type 2 (HSV-2) and would serve as complementary to Immunoglobulin M determination to establish acute infection in a given population. The study determined the prevalence of HSV-2 IgM and the cytopathology of the virus isolated from women of reproductive age in Zaria. The study recruited 450 consenting women attending selected hospitals from which blood and cervical swab samples were obtained. Sera were analysed for HSV-2 IgM while suspension from swabs were inoculated into 12 days old embryonated chicken eggs. A total of 330 (73.3%) women screened tested positive for HSV-2 IgM and 79 (23.94) out of 330 IgM positive samples produced cytopathic effect. The highest prevalence of 88.7% (133/150) was recorded among women recruited from St. Lukes Anglican hospital, while those attending Gambo Sawaba General Hospital-Kofan Gaya, had the lowest prevalence of 50%. Furthermore, women in age group of 16-20 years, were most infected with a prevalence of 83.5% while those aged 45 years and above had the lowest prevalence of 50%. Women who had more than one sexual partner, those that did not use protection during sex and women that were pregnant had higher prevalence of 88.5%, 74.9% and 77.6% respectively and were more at risk, as compared with those with single partner (69.2%), who used protection (61.5%) and that were not pregnant (71.3%). About 22.4% (101) of the women reported to have heard about HSV-2 while 349 (77.6%) were not aware of the virus prior to this study. The symptoms found to be significantly associated with the virus were blisters/ulcers on genital area ($p=0.000$; $OR=3.000$) and dysuria ($p=0.006$; $OR=0.504$). Fever ($OR=1.659$), vaginal discharge ($OR=1.867$) and itching/burning sensation on genital area ($OR=1.431$) were other symptoms associated with the virus. The study demonstrated a high prevalence of HSV-2 among the women, indicating that, genital herpes is highly endemic in the study area. In addition, a substantial number of the women lacked knowledge of genital herpes and were actively harbouring the virus.

Keywords: Prevalence, Cytopathic effect, HSV-2 IgM, Zaria

Introduction

Egg culture has long been considered the gold standard for HSV-2 with specificity of 100%. However, levels of virus shedding, specimen

quality and transport conditions may influence its sensitivity (Paz-Bailey *et al.*, 2007). HSV-2 establishes a lytic infection in the epithelial cells,

Corresponding Author: Ella, E. E.

Department of Microbiology, Ahmadu Bello University, Zaria; Phone: +2348023634081; 07010454860;

Email: elijahella33@yahoo.com; eeella@abu.edu.ng



eliciting tissue destruction which is characterized by complex cell death pattern and latency within the neurons (Nguyen and Blaho, 2007; Pellett and Roizman, 2007). Systemic and local stress factors as occasioned by hormonal changes, pregnancy, fever, exposure to direct sunlight and several underlying diseases, can interfere with the viral latency thereby inducing viral reactivation and recrudescence infections. The cytopathogenic mechanism of diseases caused by HSV-2 involves indirect immune processes and direct virus-mediated events (Kaye and Choudhary, 2006; Fatahzadeh and Schwartz, 2007). Viral infection induces ballooning formation in cells with condensed chromatin with the nuclei and nuclear degeneration within parabasal intermediate cell of the epithelium. This results in the formation of multinucleated giant cells caused by loss of plasma membranes. These cells form vesicles between the epidermis and dermal layer of the skin containing clear fluid with large quantity of viral particles and cellular debris. The cellular debris results from inflammatory response in the corium of the skin in the course of primary herpes. In the process of recovery and healing the vesicular fluid becomes pustular due to the accumulation of inflammatory cells and sometimes forms scabs. The vesicles become replaced by shallow ulcers in cases involving the mucous membranes, however, scarring is uncommon (Richman *et al.*, 2017).

HSV-2 is prevalent in several developing countries of Asia, North America and Northern Europe (Weiss, 2004). The reported prevalence HSV-2 among women in the African continent indicates 22% in Tanzania, 68% in Kenya and 90% among commercial sex workers in Zaire (Agabi *et al.*, 2010). Furthermore, age- and sex-specific prevalence of 59% was reported for HSV-2 among commercial sex workers within ages 25 and 26 years in Lagos, Nigeria (Dada *et al.*, 1998). Several risk factors have been associated with HSV-2 and prominent among them are older

age, multiple sexual partners, working in brothels and hotels, persistent HIV-1 infection, recent Genital Ulcer Disease (GUD) or Sexually Transmitted Infections (STI) symptoms, lower educational level and female gender (Watson-Jones *et al.*, 2007; Chohan *et al.*, 2009; Arama *et al.*, 2010; Okuku *et al.*, 2011; Mugo *et al.*, 2011; Ivete *et al.*, 2014). The study aimed at determining the prevalence of HSV-2 IgM, cytopathic pattern of the virus in egg culture and the possible factors that predisposes the women to HSV-2 in the study area.

Materials and Methods

Study Area

Zaria metropolis where the study was conducted is located on latitude 11° 04'N and longitude 7° 42'E. It is home to the old Zaria Emirate in Kaduna State in North and comprises of Zaria and Sabon Gari local Government Areas. The city is largely heterogeneous city with a population of about 1,490,000 of people drawn from different ethnic mix in which Muslim the Hausas and Fulanis predominate. The city houses several Federal Institutions thus attracting people from different states as well as others who come from different parts of the world. It is the gateway to several states in the North and thus provides the unique and multicultural population ideal for the conduct of the study (Oladimeji and Ojibo, 2012).

Study Population

The study was conducted among women of reproductive age within the ages of 15-49 years old, attending selected secondary hospitals spread across two local Government Areas (LGAs) in Zaria metropolis.

Study Design

A cross sectional, hospital-based study where equal number of samples were collected from consenting women attending selected hospitals, using a simple randomized sampling technique

The study population was drawn from three hospitals located in Zaria metropolis. Two of the hospitals, St. Luke's Anglican Hospital Wusasa and Gambo Sawaba Hospital, Zaria City are located in Zaria LGA while Major Ibrahim Bello Memorial Hospital is located in Sabon-Gari LGA. These hospitals serve a large population of women seeking various gynaecological services. Women that were not within the defined age bracket as well as those that declined consent were excluded from the study. A structured questionnaire was administered to obtain information on socio-demographic variables and possible factors that predisposes the women to HSV-2 in the study area. Ethical approval was granted by the Kaduna State Ministry board of Ethics (Ref-MOH/ADM/744/Vol.1)

Determination of Sample Size

The sample size was arrived at using the equation by Naing et al. (2006), and a prevalence of 44.3% (Kalu, 2013) at 95% confidence interval.

$$n = z^2 pq/d^2$$

$$n = (1.96)^2 \times 0.443 \times 0.557 / (0.05)^2 = 379.17$$

The calculated minimum size was 379.17; however, 450 blood samples were collected for the assay.

Collection of Samples

Venous blood samples were collected from 450 women aseptically into 5ml plain vacutainer tubes through venipuncture with the assistance of a qualified and certified medical personnel. The blood was allowed to clot and the serum was separated into plain sample bottles using a micropipette. The sera obtained were refrigerated (-20°C) till required for use.

Vaginal swabs were also collected aseptically from the women with the assistance of the nurse in the ward using a sterile cotton swab stick. Thereafter swab sticks were transferred into sterile transport medium (2ml of Balanced Salt Solution)

in Bijou bottles and transported in icepacks to the Laboratory for further analysis.

Determination of HSV-2 IgM in the Patient's Sera Using ELISA

Sera were analyzed using the Enzyme Linked Immuno-Sorbent Assay-ELISA (Diagnostic Automation / Cortez Diagnostics USA) according to the manufacturer's instruction. An appropriate dilution (1:40) of the patient's samples and controls (negative, positive control, and calibrator) were prepared and 100 µl was dispensed into the appropriate wells. Well A1 was set as blank into which 100 µl of the sample diluent was added. The plates were tapped to effect proper mixing and removal of bubbles after which they were incubated at room temperature (28°C) for 30min. The wells were then aspirated and repeatedly washed thrice with washing buffer followed by the addition of 100 µl of enzyme conjugate and incubation for about 30 minutes at room temperature. At the end of incubation time, the enzyme conjugate was aspirated once again and washing was repeated as done before. Thereafter, 100 µl of Tetra Methyl Benzidine (TMB) chromogenic substrate was dispensed in to the wells and the plates were further incubation as before. At the end of incubation, the reaction was stopped by the addition of 100 µl of (2 N) HCl and the plates were read at 450 nm with a microwell reader and interpreted as either negative or positive. To obtain the cut off optical density (O.D) value, the O.D of calibrator was multiplied by the Factor (f) printed on label of calibrator. The IgM Index of each sample was calculated by dividing the O.D values of each sample by the obtained O.D value of cut off calibrator

Cultivation of HSV-2 in Whole egg and demonstration of Cytopathic Effect

Egg preparation and candling: Eggs for viral cultivation were obtained from the National Animal Production Research Institute, Shika. The eggs were transported to the Virology Laboratory

of the Department of Microbiology Ahmadu Bello University Zaria and were incubated at 37°C for 12 days, preparatory to egg inoculation. Prior to inoculation, the eggs were candled in a process where the eggs were placed at the orifice of a candling box and the airspace as well as the veins marked to determine the route of inoculation and to prevent piercing the veins, which may lead to bleeding and death of the embryo (Rodgers, 1981).

Egg Inoculation: The vaginal swabs were brought to room temperature, vortexed in 2mls of balanced salt solution to suspend the viral particles present in the swab before inoculation into the chorioallantoic membrane of the eggs. Briefly: the egg was placed on its side in a plate; the surface of the egg was disinfected with 70% alcohol and iodine tincture before inoculation. Using an egg punch / egg borer, the airspace was punctured as well as the route for the chorioallantoic membrane. A slight pressure was placed on the airspace, to allow for the collapse of the chorioallantoic membrane, after which 100µl of the viral suspension was placed on the chorioallantoic membrane of the egg using a sterile needle and syringe and the punch was sealed, using candle wax. The inoculated eggs were incubated in a humid incubator at 37°C for 5days (Rodgers, 1981).

Observation of Inoculated Eggs for Cytopathic / Plaque Formation: The eggs were observed for formation of plaques/growth after 5 days of incubation and plaques were indicated as viral particle and were correlated with the results of the IgM antibodies present in the study population.

Results

The result showed that 330 women representing a prevalence of 73.3% were IgM positive and 79 out of 330 (23.94%) samples produce cytopathic effect as evident in plaque formation (Fig. 1). The prevalence observed among the women from St. Lukes Anglican Hospital Wusasa, was 88.7% (133/150) which was the highest, while those that

attended Gambo Sawaba General Hospital-Kofan Gaya, had the least prevalence of 50% and this variation was statistically significant ($\chi^2=64.705$, $p=0.000$) (Table 1). Furthermore, a statistically significant association ($p=0.011$) was observed in relation with the ages of the women with women within age group 16-20 years, being most infected with a prevalence of 83.5% while those aged 45years and above had the least prevalence of 50% (Fig.2).

Women who had more than one sexual partners, who did not use protection during sex and were pregnant had higher prevalence of 88.5% , 74.9% and 77.6% respectively and were more at risk, as compared with those with single partner (69.2%), who used protection (61.5%) and were not pregnant (71.3%). Number of sexual partners ($p=0.000$) and use of protection ($p = 0.041$) were significantly associated with the virus while pregnancy status was not ($p = 0.159$) (Table 2.)

Most of the women (349) had no knowledge of HSV -2 with 267 (76.5%) testing positive while 101 indicated that they knew about it among which, 63 (62.4%) were positive. Similarly, 383 women were not aware of their HSV status, although 291 (76.0) were positive while of the 67 that had knowledge of their status, only 39 (58.2%) were positive. The association was statistically significant ($p=0.005$) (Table 2).

Women with symptoms of fever vaginal discharges, itching sensation, blisters dysuria and pains were 273, 129, 155, 114, 86 and 261 with percentage prevalence of 77.3, 81.4, 76.8, 86.8, 61.6 and 72.4 respectively. The symptoms that were statistically associated with HSV-2 were Blisters/ ulcers on genital area ($p=0.000$; OR= 3.000) and dysuria (pain during urination) ($p=0.006$). Symptoms such as fever, vaginal discharge and itching/burning sensation on genital area were relatively associated with HSV-2 infection with Odds ratio of 1.659, 1.867 and 1.431 respectively (Table 3).

Table 1: Prevalence of HSV-2 among Women of Reproductive Age in Relation to Selected Hospitals

Hospital	No Examined	No Positive (%)	χ^2	P-Value
SLAH	150	133(88.7)	64.705	0.000*
MIBMH	150	122(81.3)		
GSGH	150	75(50.0)		
Total	450	330(73.3)		

Key: * $p < 0.05$ = Statistically Significant association exist SLAH-St. Luke's Anglican Hospital, Wusasa; MIBMH-Major Ibrahim Bello Memorial Hospital, Sabon-Gari; GSGH-Gambo Sawaba General Hospital, Zaria city all in Zaria Metropolis

Table 2: Prevalence of HSV-2 among Women of Reproductive Age in Relation to Some Risk Factors

Risk Factor	No Examined	No Positive (%)	Odds Ratio / Confidence Interval	P-Value
No of Sexual Partners				
Single	354	245(69.2)	0.291 (0.15-0.57)	0.000*
Multiple	96	85(88.5)		
Use of Protective device during sex				
Yes	52	32(61.5)	0.537 (0.29-0.98)	0.041*
No	398	298(74.9)		
Pregnancy Status				
Pregnant	147	114(77.6)	1.391 (0.88-2.21)	0.159
Not pregnant	303	216(71.3)		
Awareness of Genital Herpes/HSV-2				
Yes	101	63(62.4)	0.509 (0.32-0.82)	0.005
No	349	267(76.5)		
Awareness of HSV-2 Status				
Yes	67	39(58.2)	0.440 (0.26-0.76)	0.002
No	383	291(76.0)		
Total	450	330(73.3)		

Table 3: Prevalence of HSV-2 among Women of Reproductive Age in Relation to Some Disease Symptoms

Symptoms	No Examined	No Positive (%)	Odds Ratio / Confidence Interval	P-Value
Fever				
Yes	273	211(77.3)	1.659 (1.09-2.53)	0.018
No	177	119(67.2)		
Vaginal Discharge				
Yes	129	105(81.4)	1.867 (1.13-3.09)	0.014
No	321	225(70.1)		
Itching/Burning sensation on Genital Area				
Yes	155	119(76.8)	1.431(1.51-4.04)	0.232
No	295	211(71.5)		
Blister, Ulcers on Genital Area				
Yes	114	99(86.8)	3.000 (1.66-5.41)	0.000*
No	336	285(71.6)		
Dysuria (Pain During Urination)				
Yes	86	53(61.6)	0.504 (0.31-0.83)	0.006*
No	364	277(76.1)		
Feeling of Pain on thigh, Back, Knees and Buttocks				
Yes	261	189(72.4)	0.894 (0.58-1.37)	0.604
No	189	141(74.6)		
Total	450	330(73.3)		

Discussion

The overall prevalence of HSV-2 IgM amongst the women in Zaria was found to be 73.3%. This percentage is higher than the 59% reported in a study involving commercial sex workers conducted in Lagos-Nigeria (Dada *et al.*, 1998) and 44.3% amongst antenatal clinic attendees in Benin City, Nigeria (Kalu, 2013). Higher prevalence of HSV-2 have been reported in four sub-Saharan African cities; 90% in Cotonou,

Benin Republic, 84.1% in Yaounde, Cameroun, 93.9% in Kismu, Kenya and 87.7% in Ndola, Zambia (Weiss, 2004). Furthermore, a study among women with STIs in Jos, Nigeria reported a prevalence of 87% (Agabi *et al.*, 2010). The high HSV-2 prevalence reported in this study among pregnant women contrast reports from sub-Saharan Africa (40%), Latin America (30%), North America (17 – 22%) and Europe (2 – 24%) (Anzivino *et al.*, 2009). Studies from Sweden, Switzerland and China have also shown

HSV-2 prevalence especially among antenatal women to be 20.7% and 10.8% (Berntsson *et al.*, 2000; Chen *et al.*, 2005)

The high prevalence obtained in this study could be due to sexual activities engaged in by the women and other stressors such as pregnancy, hormonal disturbances that reactivates the virus/infection. Infection was found to be significantly associated with the selected hospital location ($p= 0.006$).

Sex remains one of the means of transmission of HSV-2; although genital herpes is not classified as a sexually transmitted disease but regarded as a sexually transmitted viral infection. These women were within the sexually active age and were pregnant. Pregnancy is characterized with hormonal disturbances and other stressors which could predispose these women to the virus or reinfection (Kaye and Choudhary, 2006; Fatahzadeh and Schwartz, 2007). The virus was highly endemic in the communities where the hospitals were situated and could have resulted from to lack of knowledge and awareness about the mode of transmission of the virus. Many cell culture techniques have been employed in the cultivation of HSV-2. The use of the traditional whole egg proved useful in this work which is cheap and easy to use. The study demonstrated cytopathogenic effect (CPE)/plaques characteristic of the virus in 23.9% of the IgM positive samples. This is within the range of 19 – 27% for crusted lesions but lower than that for vesicles (52-93%) and ulcers (41-72%) (Scoular *et al.*, 2002).

Immunity decreases with increase in age leading to increase in susceptibility to infectious agents. Increase in sexual activities and negative life style increases the chances of contracting STDs including HSV-2. These observations could also be responsible for the statistically significant association ($p = 0.011$) observed between HSV-2 and women within age group 16-20 years. This observation is in agreement with the report of

previous studies in Jos and Zaria (Agabi *et al.*, 2010; Ameh *et al.*, 2016).

Number of sexual partners was found to be significantly ($p= 0.001$) associated with HSV- 2 infection in this study, as also reported by several findings which reported higher prevalence of HSV-2 infection among patients with multiple sexual partners (Auvert and Buve, 2001; Weiss, 2004; Mehta *et al.*, 2008). The use of protection was also found to be statistically associated with the infection, ($p= 0.041$) which is in agreement with the report of Biswas *et al.* (2011). In contrast, Agabi *et al.* (2010) and Dada *et al.* (1998) reported that the use of protection during sexual activity, had no statistical significance to HSV – 2 infection. Pregnancy was also found not to be statistically associated with the infection ($p = 0.159$). Pregnancy has been reported by several workers to be a stressor on women which could lead to or cause reactivation of the virus from its latent state and primary exposure to the virus (Biswas *et al.*, 2011; Bochner *et al.*, 2013).

The symptoms found to be statistically associated with HSV-2 were blisters/ ulcers on genital area and dysuria (pain during urination) ($p=0.006$). This result agrees with the reports of Biswas *et al.* (2011) and Bochner *et al.* (2013). Women with fever, vaginal discharge and itching/burning sensation on genital area were more likely to be infected with HSV-2 with Odds ratio of 1.659, 1.867 and 1.431 respectively; however, the associations were not statistically significant.

Conclusion

The study established that a significant number of women were positive for HSV-IgM and were actively harbouring the virus as demonstrated by the cytopathic result. This is an indication that HSV-2 is highly endemic in the communities where the hospitals were situated and could have resulted from lack of knowledge and awareness about the virus and its mode of transmission. It

was also found that women in age group 16-20 years were most infected.

Furthermore, pregnant women who had multiple sexual partners and who did not use protection during sexual intercourse had higher prevalence and were more at risk of HSV-2.

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