RESEARCH ARTICLE

**Revision** Seroprevalence and characterisation of herpes simplex virus from human immunodeficiency virus in samples collected from two provinces in South Africa: a retrospective study [version 3; peer review: 2 approved]

Previously titled: Seroprevalence and characterisation of herpes simplex virus from human immunodeficiency virus in samples collected from two provinces in South Africa: a retrospective study

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**Abstract**

**Background:** Herpes simplex virus (HSV) is a widely distributed human pathogen that is known for its ulcerative lesions at the infection site. HSV can cause persistent infection in the host that is often followed by a period of latency within the neurons. Considering the high rate of HIV infection in South Africa, it is important to assess the seroprevalence of HSV with a focus to determine the epidemiological association between HSV-DNA and HIV-1 in the population.

**Methods:** A total of 44 sera samples were screened for HSV and HIV-1 using the highly sensitive enzyme-linked immunosorbent assay (ELISA). The ELISA positive samples were characterized using polymerase chain reaction (PCR) to confirm the positivity of both viruses and to further differentiate HSV into HSV-1 and -2. Thereafter, the samples were analysed for relatedness using phylogenetic analysis.

**Results:** Of the 44 samples, 36 (81.8%) were positive for HIV-1, while 35 (79.5%) were positive for HSV when screened with ELISA kits. The PCR results, with the use of type specific primers, showed that 4/35 (11.4%) samples were specific for HSV-1 while 30/35 (85.7%) were specific for HSV-2. Statistical analysis performed using the chi-squared goodness-of-fit test showed that there is a significant relationship between HSV-2 and HIV-1 transmission.

**Conclusions:** There is a significant relationship between HSV-2 and...
HIV-1 in the study population. Our study shows that some of the HSV-2 isolates are not related to the clinical isolate SD90e from South Africa, suggesting diversity in HSV-2 viral transmission.

**Keywords**
Co-infection, Enzyme Linked Immunosorbent Assay, Herpes Simplex Virus, Human Immunodeficiency Virus, Polymerase Chain Reaction.
Introduction

Herpes simplex virus (HSV) is a prevalent organism that belongs to the sub-family of alpha Herpesviridae (Greninger et al., 2018). The virus is transmitted either through oral or genital route. The routes of transmission of HSV are responsible for its differentiation into two types; herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) (Looker et al., 2008; Schiffer et al., 2014). HSV-1 is transmitted orally and it is the cause of blisters and sores around the mouth, while HSV-2 is sexually transmitted and it is traditionally associated with blisters or ulcers around the genitals (Grinde, 2013; Modi et al., 2008).

Conversely, most HSV infections are asymptomatic, a factor that is partly responsible for the high prevalence of HSV infection worldwide. However, some HSV infections show visible signs of infection in the host (Looker et al., 2015), which is often associated with the increase in virus titer that further fuels its transmission (Jaishankar & Shukla, 2016; Mazzarello et al., 2018).

It is important to determine the epidemiology of HSV-1 and HSV-2 because of the disease burden associated with their infection (Chemaitelly et al., 2019). The global prevalence for HSV-1 and HSV-2 is approximately 3.7 billion and 500 million respectively (Feng et al., 2013; WHO, 2015). In 2018, McQuillan and colleagues conducted a survey in the United States and estimated the prevalence of HSV-1 and HSV-2 as 47.8% and 11.9% respectively (McQuillan et al., 2018). However, Africa has a higher rate of HSV-2 prevalence (20–80% and 49.7%) and HSV-1 (10–50% and 50.3%) in women and men, respectively (Looker et al., 2015; WHO, 2015). HSV-2 is the most common virus responsible for genital ulcer diseases (GUD) (Johnston & Corey, 2016); however, research has shown that GUD may also be caused by HSV-1 particularly in the industrialized nations (WHO, 2015). The sudden increase in GUD caused by HSV-1 is attributable to the downward shift in trend of HSV-1 acquisition before sexual relations in this population. In addition, children who do not have HSV-1 antibodies in the early stages of life are vulnerable to genital HSV-1 infection when exposed (Bradley et al., 2014; Forward & Lee, 2003). Daniels et al. (2016), evaluated the incidence of HSV-2 and its risk factors within a cohort of HIV-1 negative women in KwaZulu-Natal, South Africa. They observed that 84% female commercial sex workers in the study population were infected with HSV-2.

The high prevalence of human immunodeficiency virus type 1 (HIV-1) in South Africa (about 7.7 million) has made the Joint United Nations Programme on HIV/AIDS (UNAIDS) to regard the country as the epicenter of HIV-1 in the world (UNAIDS, 2019). Considering the high prevalence of HIV-1 in South Africa and the role that HSV plays in its transmission, the need to determine the prevalence of HSV and HIV-1 co-infection is of great importance. In addition, HSV disrupts the epithelial surface at the infection site, which serves as a port of entry for HIV recruitment that progressively facilitates its transmission by two to three fold. This characteristic makes HSV an important co-factor in HIV acquisition (Looker et al., 2017; Munawwar & Singh, 2016). HSV infection makes the transmission of HIV effortless through a transmission that disrupts the epithelium. Moreover, dual infection with HIV increases the rate of HIV replication, which further suppresses the immune system, thus enhancing disease progression. The effect of HSV in HIV acquisition has been observed in Western Asia, Europe and Africa where the prevalence of HSV-2 in HIV infected populations was 60–90%, 30–70% and 50–90%, respectively, which is three times the rate of infection in normal populations (Anaodebo & Ajani, 2019; Des Jarlais et al., 2014; Mohraz et al., 2018).

It is apparent that a sturdy interaction exists between HSV-2 and HIV-1 infection (Barnabas & Celum, 2012; Kolawole et al., 2016; Todd et al., 2013), although, a contrasting opinions was reported that HSV-2 co-infection with HIV has no role in increasing the transmission of HIV (Mohraz et al., 2018). However, Freeman and colleagues conducted a systematic review on the gender-based effect of HSV-2 in the transmission of HIV infection (Freeman et al., 2006). In their study, they discovered that HSV-2 is a significant facilitator of HIV transmission in both men and women. Another study reported a three-fold risk of HIV-1 acquisition in HSV-2 infected persons in the sub-Saharan Africa (Barnabas & Celum, 2012). Most studies that discovered the association between HSV-2 and HIV-1 were conducted outside of South Africa. Albeit, one study reported an incidence of 41% of the co-infection in South African women (Abbai et al., 2015).

Accordingly, the current study aims to establish the prevalence of HSV antibodies and HSV-DNA in HIV-1 sera and further assess the evidence of HSV-2 and HIV-1 co-infection within the study cohort.

Methods

Sample collection criteria and study population

The sera samples that were used in the study were previously stored sera collected from patients who visited Bophelong Provincial Hospital in Mafikeng, North-West Province, and
Inkosi Albert Luthuli Central Hospital (IALCH-NHLS) in KwaZulu-Natal Province, both in South Africa. The study participants visited the hospitals for HIV screening and management in their respective provinces. Only 25 sera samples from each hospital (50) were selected for use in the current study. However, six of the samples were lysed and were excluded, reducing the sample number to forty-four (44). The samples were anonymized with no additional data except age and gender of the study participants.

**Laboratory analysis**

The enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of HSV and HIV-1 antibodies in the sera. Polymerase chain reaction (PCR) was used to confirm that the samples were infected with HSV and HIV-1 and to differentiate the HSV samples into HSV type 1 and 2.

**ELISA**

A highly sensitive ELISA test kit for HSV, Platelia HSV (1+2) (Bio-Rad, Marnes-la-Coquette, Paris, France) was used to measure HSV IgG antibody in the samples. The Genscreen Ultra HIV Ag-Ab test kit (Bio-Rad, Marnes-la-Coquette, Paris, France) was used to detect HIV-1 p24 antigen in the sera. The ELISA plate was pre-coated with biotinylated polyclonal antibody to p24 HIV-1 Ag. The manufacturer’s instructions were followed with slight modifications. Briefly, the sera were diluted in a ratio 1:10 and 100 µl of the diluted sera, blank, positive, and negative controls were added to a flat-bottom 96-well plate. The plate was incubated at 37°C for 45 min and washed four times. Thereafter, the plate was incubated at room temperature for 15 min with 100 µl of substrate followed by 100 µl of stop solution to terminate the reaction. The plate was read on a microplate reader at 450 nm. The samples were run in duplicate and were considered positive for HSV IgG antibody if the ratio of the average of serum OD and the cut-off value was greater than 1.2, and negative if the ratio was less than 0.8.

**DNA isolation and PCR**

DNA and RNA were extracted from the sera samples using QIAamp® MinElute® Virus Spin kit and QIAamp® Viral RNA Mini kit (Whitehead Scientific, Cape Town, South Africa), respectively, following the manufacturer’s protocols.

PCR was performed using the samples that were positive for ELISA (HSV type 1 and 2 and HIV-1) with viral gene specific primers. Previously published primers from Nie et al. (2011); Victória et al. (2005) and Schmutzhard et al. (2004) were used to amplify the integrase, glycoprotein B (gB) and glycoprotein G (gG) region of HIV-1, HSV-1 and HSV-2 respectively, as outlined in the *Extended data* (Table A). The HSV positive samples were also tested for HIV-1 co-infection using PCR. This allowed the detection of HIV-1 and HSV-1 and/or -2 co-infections from the study samples. Briefly, 25 µl PCR reaction mixture (Quick-Load® Taq 2X Master Mix kit, Biolabs) with each primer set targeting the different regions, was prepared (Extended data, Table B) and amplification was performed with the use of a T100™ thermal cycler (Bio-Rad, Hercules, California, United States). HSV-1 and -2 amplification was performed with the nested PCR cycling conditions outlined in the *Extended data* (Table B). A reverse transcription PCR was used to amplify the HIV-1 integrate gene from the extracted HIV-1 RNA samples. This was achieved with the PCR cycling conditions outlined in the *Extended data* (Table C) and the PCR products were analysed by gel electrophoreses.

**DNA sequencing**

Next-generation sequencing (NGS) was used to validate the genomes of the samples used. Only the samples that exhibited high titers of HSV-2 and HIV-1 with ELISA were sequenced. Thus, DNA sequencing was performed on four HSV-2/HIV-1 co-infected samples with the highest titers. The samples were annotated as G13, G15, G20 and G34. HSV-2 primers were used to sequence G13, G15 and G34 while the G20 sample was sequenced using HIV-1 primers since this was the only sample with a high HIV-1 titer. NGS was carried out on the Illumina MiSeq NGS platform at Inqaba Biotec (Inqaba Biotechnical Industries (Pty) Ltd, Johannesburg, SA). Phylogenetic analysis was performed by trimming and aligning the sequences using BowTie 2 v 2.3.2 (Langmead & Salzberg, 2012). All aligned data were further annotated to determine the viral genome using Prokka v 1.12 (Seemann, 2014). Thereafter, the sequence data were subjected to Molecular Evolutionary Genetics Analysis (MEGA 7) against HIV-1 and HSV-2 reference genomes obtained from National Centre for Biotechnology Information (NCBI). Phylogenetic analysis was performed to assess the evolutionary relatedness of the sequenced data in relation to the reference genomes. The HSV-2 published reference sequences that were used in the analysis were SD90e (KF781518) from South Africa, HSV-2 strain 333 (M15118), a wild-type laboratory reference strain from USA, and glycoprotein G-2 (AF141858), a European HSV-2 isolate. The HIV-1 reference sequences that were used in the analysis were two HIV-1 subtype C sequences from South Africa (HM569277 and HM569273), two HIV-1 subtype B from USA (AF203332) and Japan (LC022388), and HIV-1 subtype A (HM466997) from Europe. Thus, the reference sequences were selected to explore the relatedness of the sequenced samples with published reference strains from the country (South Africa) and from other developed countries. In addition, different HIV-1 subtypes were selected from different regions to identify the sub-type of our clinical isolate.

**Statistical analysis**

Data analyses were carried out on Statistical Package of Social Sciences (SPSS) software (version 25). The chi-squared goodness-of-fit test ($\chi^2$) was used to evaluate for an association between the categorical variables. Relationship between the demographics and the viruses was tested using Pearson correlation coefficient. The 5% significance level was considered as a significant $p$ value in this study.

**Ethical approval**

The study received ethical approval from the North-West University Research Ethics Regulatory Committee (NWU-00068-15-A9).
Results

The demographics of the study population showed that majority of the study participants were female (79.5%) with a low percentage of males (20.5%) (Table 1). The mean age and standard deviation of the study population were 33.09 ±11.94 years.

ELISA screening of the samples showed that 36/44 (81.8%) were seropositive for HIV-1 while 35/44 (79.5%) were positive for either HSV-1/2 antibody (Table 1 and Extended data, Figure A). Notably, the study participants within the age group of 21–40 years had the highest HSV and HIV-1 infection rates, as depicted in Table 1.

The PCR amplification of glycoprotein B region of HSV showed that 4/35 (11.4%) of the HSV positive samples were positive for HSV-1 (Table 2 and Extended data, Figure B). Similarly, to differentiate the HSV-1 and HSV-2 positive samples, PCR amplification of the glycoprotein G region of HSV positive samples was performed (Table 2). The data showed that 30/35 (85.7%) were HSV-2 positive [Table 2 and Extended data (Figure C)]. Another finding was that 1/44 (2.3%) males and 2/44 (4.5%) females were HSV-1/HIV-1 co-infected.

Furthermore, a significant proportion of the population, 6/44 (13.6%) males and 24/44 (54.5%) females were HSV-2/HIV-1 co-infected (Figure 1).

Furthermore, PCR was also used to amplify the integrase gene of HIV-1 genome in the samples and the data showed that 36/44 (81.8%) samples that were positive for HIV-1 p24 using ELISA were also PCR positive.

NGS was performed to confirm the genomes of HSV-2 and HIV-1 viral isolates that were detected using PCR. The samples that were sent for NGS sequencing were those that exhibited high titers with ELISA, that is, HSV-2 sequences 13, 15 and 34 and HIV-1 sequence G20. The sequenced data was compared with reference sequences obtained from NCBI that were selected based on the amplified targeted regions. A maximum likelihood phylogeny method of analysis was used with a bootstrap value of 1,000 replicates to generate evolutionary trees for HSV-2 and HIV-1, respectively. The phylogenetic analysis of HSV-2 sequence data showed that sequences 13 and 15 are more closely related to glycoprotein G2 reference strain than sequence 34, with 98–99% similarity to the G2 strain (Figure 2).

The HIV-1 phylogenetic tree in Figure 3 shows that G20 sequence is 100% closely related to subtype A (HM466997) and distantly related to subtype B (AF203332 and LC022388) and subtype C (HM569277 and HM569273) reference genomes.

The SPSS v25 statistical software was used to detect whether there is a relationship between age, HSV, and HIV-1. SPSS was also utilized to evaluate the association between HSV and HIV-1 positive samples (Table 3). The two-tailed correlation test exhibited a statistical positive link between age and HSV-1 (0.366**) as shown in Table 3. HSV-2 and HIV-1 samples are also positively correlated (0.690**). In addition, an inverse or negative relationship between HSV-1 and HSV-2 (-0.463**) was detected.

The SPSS chi-square goodness-of-fit test was also used to assess association between HSV and HIV-1 positive samples.

| AGE GROUP | HIV-1 Positive | HIV-1 Negative | HSV Positive | HSV Negative |
|-----------|----------------|----------------|--------------|--------------|
|           | Male Female    | Male Female    | Male Female  | Male Female  |
| <20       | 0 5            | 1 2            | 0 5          | 1 2          |
| 21–40     | 5 14           | 1 2            | 5 12         | 1 4          |
| 41–60     | 2 10           | 0 2            | 2 11         | 0 1          |
| TOTAL     | 7 29           | 2 6            | 7 28         | 2 7          |

| AGE GROUP | HSV-1 PCR Positive | HSV-2 PCR Positive |
|-----------|--------------------|--------------------|
|           | MALE FEMALE | MALE FEMALE  | MALE FEMALE |
| <20       | 0 0        | 0 5        |       |
| 21–40     | 0 0        | 5 11       |       |
| 41–60     | 1 1        | 1 8        |       |
The data showed that there was no significant association between HSV-1 and HIV-1 ($X^2(1) = 0.138, p>0.05$). However, there was a strong statistical association between HSV-2 and HIV-1 ($X^2(1) = 20.952, p< 0.05$), as shown in Table 4.

Discussion

There are insufficient data to recount the prevalence of HSV genotypes among HIV-1 infected individuals in the Republic of South Africa. This study provides insight into an existing interrelation between HSV and HIV-1 and the potential risk that one of the viruses may have on the other. Our focus was to determine the seroprevalence of HSV among HIV-1 sample cohort and further assess for possible co-infections (HSV-1/HIV-1 or HSV-2/HIV-1). In the study, 81.8% and 79.5% of the samples were positive for HIV-1 and HSV, respectively. Of the positive HSV samples, 85.7% were positive for HSV-2. Conversely, the most prevalent type of HSV (HSV-1) as recorded by previous studies (Debrah et al., 2018; Looker & Garnett, 2005), was not highly prevalent in this study population (11.4%). This may be due to the small sample size as HSV-1 infection is often acquired during childhood and there were no childhood participants in the study (age range 1–10 years). Although, Debrah et al. used a larger sample cohort in their study, there was no record of childhood participants. Most of the high HSV-1 prevalence recorded was for participants aged 25–44 years. This low prevalence was also observed in a study conducted in the USA by Ayoub et al. (2019), where they examined the progression of HSV-1 epidemiology in the country. The study showed that more children will reach the age of sexual debut with no antibody protection against HSV-1. In addition, the low rate is attributable to the change in disease spread in the population since there are reduced viral HSV-1 antibodies at a very young age, a factor influencing HSV-2 acquisition.

Our study also revealed a higher rate of HSV-2/HIV-1 co-infection (13.6% males, 54.5% females) compared with HSV-1/HIV-1 (2.3% males, 4.5% females). Thus, one of the few observations drawn from this study was the relatively high prevalence of HSV-2 compared to HSV-1 in HIV-1 co-infected samples. The validity of the high prevalence of HSV-2/HIV-1 co-infection in this study is supported by previous studies (Looker et al., 2017; Patel et al., 2012). The increase in HSV-2/HIV-1 co-infection is attributed to the route of viral transmission since both viruses share a similar route of transmission. Furthermore, it was also discovered that females were more susceptible to HSV-2 infection 30/35 (85.7%) than their male counterparts 4/35 (11.4%) in this population. This correlates with the findings of Pebody et al. (2004) and Smith & Robinson (2002), who reported that women are more at risk of acquiring HSV-2 infection compared with men. Similar findings were also observed by Celum et al. (2004) that more than half of the female population who are HIV-1 positive suffer from HSV-2 infection. This might be due to their early exposure to sexual relations than their male counterpart (Schiffer et al., 2014). Another probable explanation for the high

| AGE | HSV-1 | HSV-2 | HIV-1 |
|-----|-------|-------|-------|
|    | 1.066** | -0.061 | 0.208 |
| HSV-1 | - | -0.463** | -0.056 |
| HSV-2 | - | - | 0.690** |
| HIV-1 | - | - | 1 |

**Correlation is significant at 0.01 level (2-tailed)
Phylogenetic analysis revealed that HSV-2 samples 13 and 15 from this study do not share the same ancestral lineage with a more virulent clinical isolate SD90e (accession number KF781518) from South Africa (Newman et al., 2015) and the HSV-2 laboratory strain 333 (M15118). However, sample 34 is closely related to SD90e. Thus, the distant relation between SD90e reference genome and samples 13 and 15 may suggest geographical diversity in viral transmission within South Africa. This was also discovered in a study conducted by Newman et al. (2015), where they explored the geographical diversity between HSV-2 sequences and observed that HSV-2 sequences from Uganda are strongly related to the HSV-2 sequence from USA. Furthermore, a close relation of the two samples (G13 and G15) was observed with the less virulent glycoprotein G-2 strain originating from Scotland, which might be attributed to international migration. This was also observed by Szpara et al. (2014) where African strains isolated in Kenya were shown to cluster closely with Europe, North America, and Asian strains.

A previous study on the relationship between age and HSV-1 prevalence by Smith & Robinson (2002) reported that global HSV-1 prevalence increases with age. The statistical relationship between age, HSV and HIV-1 infection in our study revealed a similar significant relationship between age and HSV-1 (p = 0.366*). Similarly, we discovered a discreet relationship between age, HSV-2 and HIV-1. However, HSV-1 showed an inverse correlation with HSV-2 (p = -0.463*). This may suggest that an increase in HSV-2 prevalence in the population will result in a decline in HSV-1 as supported by Ayoub et al. (2019). It was also discovered that a robust significant relationship exists between HSV-2 and HIV-1 (p =0.690**) suggesting that a steady rise in HSV-2 contributes to an increase in HIV-1 infection in the population. A probable reason for this relationship is that the viruses share a similar route of entry and the impact of one is significant on the other as seen in the micro-ulceration of the genitalia in HSV-2 patients, which provides a port of entry for HIV-1 (Sheth et al., 2008).

The association between HIV-1 and HSV was analysed using chi-square goodness-of-fit test and it was discovered that no significant association exist between HSV-1 and HIV-1 (X² (1) = 0.138, p > 0.05) but a strong statistical association was found between HSV-2 and HIV-1 (X² (1) = 20.952, p < 0.05). Although, there has been contrasting opinions on the association of HSV-2 and HIV-1, the current study is consistent with the findings of Lurie et al. (2003), demonstrating that HSV-2 is a risk factor for HIV infection in South Africa, as also suggested by Szpara et al. (2014).
with Sudenga et al. (2012) and Omori & Abu-Raddad (2017) who suggested that the infection of one virus may fuel the transmission of the other. In their studies, Sudenga et al. (2012) observed that HIV-1 positive individuals with higher CD4+ counts at baseline and those with lower viral load were associated with HSV-2 acquisition, while Omori & Abu-Raddad (2017) used the sexual network determinants as components to determine the prevalence of HIV-1/HSV-2. They deduced that HIV is an agent of HSV-2 transmission in the population. However, another study led by Kouyoumjian et al. (2018) discovered a robust association between HSV-2 and HIV-1, with HSV-2 prevalence being consistently higher than HIV-1 in the global population. The data from this study suggests that contracting one virus (either HSV-2 or HIV-1) will influence the acquisition of the other. This highlights the importance for data collation on HSV-2 and HIV-1 infected persons in South Africa.

Limitations
The study is a retrospective study with the aim to determine the prevalence of HSV-DNA in HIV-1 sera. However, there were certain limitations. Primarily, the study population was small when compared to the size of the general population. In addition, the sera samples were collected exclusively from North-West and KwaZulu-Natal Provinces, poses a challenge to generalize the outcomes of the study to the South African populace.

Conclusion
This study revealed a clustering variation of HSV-2 sequences. The clustering differentiated the sequences from the prominent sequence (SD90e) found in South Africa, which suggests diversity in the transmission of the virus. Similarly, a different HIV-1 sub-type was isolated from the study population, different from other HIV-1 sub-type C isolates in South Africa. Despite the small sample size, a high prevalence of HSV-2/HIV-1 co-infection (68.2%) was recorded which shows that a positive association exists between HSV-2 and HIV-1, suggesting that an increase in one of the viruses may influence the spread of the other. Thus, acknowledging that a relationship exists between these two viruses, and to identify how the transmission of one could affect the other, requires a larger cohort that is well described with longitudinal measurements of HSV-1, HSV-2, and HIV-1 as well as measurements of potential confounders such as condom use, partner change and other sexually transmitted diseases.

Data availability
Underlying data
SRA: gG sequencing of HSV-2 (sample G15), Accession number SRX9590097: https://www.ncbi.nlm.nih.gov/sra/?term=SRX9590097

SRA: gG sequencing of HSV-2 (sample G34), Accession number SRX9590139: https://www.ncbi.nlm.nih.gov/sra/?term=SRX9590139

SRA: gG sequence (sample G13), Accession number SRX9590139: https://www.ncbi.nlm.nih.gov/sra/?term=SRX9590139

SRA: HIV-1 sequencing targeting integrase region (sample G20), Accession number SRX9531105: https://www.ncbi.nlm.nih.gov/sra/?term=SRX9531105

Dryad: HSV AND HIV RAW DATA, https://doi.org/10.5061/dryad.zs7b44j7g (Obisesan et al., 2020).

This project contains the following underlying data:
- CSV spreadsheet containing demographics and ELISA and PCR results for all 44 sera samples.

Extended data
Dryad: F1000 SUPPLEMENTARY FILE, https://doi.org/10.5061/dryad.zs7b44j7g (Obisesan et al., 2020).

This project contains the following extended data:
- Table A: Primers for the detection of HSV-1, HSV-2 and HIV-1.
- Table B: PCR reaction mixture and thermocycling conditions for HSV-1 and -2.
- Table C: Thermocycling conditions for HIV-1 PCR.
- Figure A: ELISA results of HSV as recorded on the microplate reader.
- Figure B: Age variation and the frequency distribution of HSV-1 in the study population
- Figure C: Age variation and the frequency distribution of HSV-2 in the study population

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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Version 3

Reviewer Report 11 November 2021

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Bongiwe Ndlovu
HIV Pathogenesis Programme, University of KwaZulu-Natal, Durban, South Africa

I am happy with the revised manuscript. Although, a few points should be addressed before the study is indexed. Conclusions should be revised, the authors mentioned that "There is a significant relationship between HSV-2 and HIV-1 in the study population". Are they referring to a positive association? This should be clarified.

In sample collection criteria and study population, the authors mentioned that "six samples were lysed and were excluded". This is confusing, are they referring to poor sample quality? This should also be clarified. Which stop solution did they use to terminate ELISA experiments?

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: HIV Immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 18 October 2021

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Debrah Oksana
Department of Molecular Medicine, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana
The manuscript has been improved during the last revision. I commend the authors for carefully addressing the reviewers' feedback. The manuscript can be accepted for indexing.

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**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Infectious diseases of public health concern, medical biochemistry, chemical pathology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Reviewer Report 01 October 2021**

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Debrah Oksana
Department of Molecular Medicine, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

**General Comments:**
The manuscript has been improved during the revision. For the results section, I have some suggestions:

1. Due to small sample size, author should categorize age on three categories only: >20 years, 21-40 years & 41-60 years.

2. Table 1 & Table 2: kindly specify that the data is number of positive/ negative cases/ male & female. You can put near the parameter “N”.

Conclusion section should be revised. The sample size is very small to make the statement such as “...HSV is highly prevalent, and women are mainly affected. Moreover, HSV-2 infection in the study cohort was robustly associated with HIV-1”. Similarly review the conclusion in Abstract.

Limitation section should be revised. Sample size is very small to make statement such as “The study contributed to literature by exploring a broad group of participants between 11 to 60 years”.
It is suggested that authors focused in their conclusion more on the sequencing analysis of the viruses, and present the prevalence of co-infection as side data, due to small sample size.

**Comments:** Recommend for indexing after revisions are made.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Infectious diseases of public health concern, medical biochemistry, chemical pathology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Oct 2021

**oluwafemi obisesan**, North-West University, Mafikeng, South Africa

Dear Reviewer,

We appreciate the efforts, comments, and concerns in making the manuscript better. We have gone through the comments and edited the manuscript accordingly in the revised manuscript. The inputs made in response to your comments are listed below.

**General comments:**

1. Due to the small sample size, author should categorize age on three categories only: <20 years, 21-40 years & 41-60 years.

**Response:** We have categorized the age as suggested in Tables 1 & 2 on pages 6 and 7 of the revised manuscript.

1. Table 1 & Table 2: kindly specify that the data is number of positive/ negative cases/ male & female. You can put near the parameter “N”.

**Response:** We have made clear demarcations of positive and negative HIV-1 and HSV infections in Table 1. The same has been done for Table 2 to differentiate HSV-1 and HSV-2 PCR positive males and females.

1. Conclusion section should be revised. The sample size is very small to make the statement such as “…HSV is highly prevalent, and women are mainly affected. Moreover, HSV-2 infection in the study cohort was robustly associated with HIV-1”. Similarly, review the conclusion in Abstract

**Response:** The conclusion section has been revised, focusing more on the sequence analysis of the viruses as outlined on page 12.

1. Limitation section should be revised. Sample size is very small to make statement such as “The study contributed to literature by exploring a broad group of participants between 11-60 years

**Response:** Thank you for noting this. We have excluded the statement from the limitation section on page 12.

**Competing Interests:** No competing interests to disclose.
Study Title: The title of the paper should be revised, it is unclear. The location of samples collected should be removed from the title and properly explained in the Methods section.

The introduction: The authors should restructure the sentences. Some sentences are long and they have typographical errors. Some statements are inaccurate, for example: they mentioned that transmission is asymptomatic.

Methods: The methods section should be revised. The authors should indicate the type of samples used for the study (blood, plasma, or serum), the country of origin, and the hospitals/institutions where the samples were collected. They should also define convenience sampling.

Laboratory Analysis: The authors should restructure the sentences. They also used abbreviations like EIA & PCR without describing them first/writing in full.

ELISA method was not properly described. Which HIV-1/HSV proteins did they use to measure antibody responses? The authors also did not specify which genes of interest and primers were used to amplify the HIV-1 genome with nested PCR. This should be clearly explained and referred to supplementary information.

Sequencing: It is not clear why G13, G15, G34 were sequenced with HSV-2 primers while G20 was amplified with HIV-1 primers.

Results:
- Did you measure p24 antibody titers?
- Figure 1 is confusing, the authors used too many colors. They should consider simplifying the figure by showing a bar graph with positive and negative responses for each age group. The figure legend is too brief.
- The authors did HSV1/2 PCR. However, the results are not shown in the article. The bar graphs are only shown in the supplementary information. These should be simplified and added as figure 2 of the manuscript. Where is the figure that shows HIV-1/HSV co-infections? These can be summarized clearly in a pie chart.
The interpretation of the sequencing data needs improvement. Which HIV-1 subtypes are HIV LC201873, 4533/11 and KU609428? Are they South African strains? What was the aim of selecting these strains?

HIV-1 LC022388 and 3793/15 KU609388) were used in constructing the phylogenetic tree. However, it is not clear why these strains were selected for analysis.

The relationships between age and HSV-1, HSV-2, and HIV-1 or HSV-1 and HSV-2 are not clearly explained. Are they positive or negative associations?

Discussion:
- The authors mentioned that HSV-1 is the most prevalent type of HSV as recorded by previous studies. However, they did not cite those studies.
- In this study, they only found 4 patients with HSV-1. They should explain the differences between these studies that could lead to this difference in the findings.
- The differences in HSV prevalence between males and females, could be due to their anatomy? It is unclear whether the studies conducted by Cunningham 2006 and Ayoub 2019 were done on South African participants or not.

Conclusion: The conclusion should be revised. The study was done on a small sample size.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: HIV Immunology
I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 03 March 2021

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Debrah Oksana

Department of Molecular Medicine, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

General comments:
1. Majority of the sentences can be restructured for better representation and understanding of information.

2. The text contains some grammatical and punctuation errors, they should be corrected before indexing.

3. It is suggested that the authors focus more on the sequencing analysis of the viruses, and present the prevalence of co-infection as side data, due to small sample size.

Specific comments:
Below are more specific comments by section:

1. Title: The title of the article should be revised to make it more specific. For example, the country where the study took place should be included. This is an international journal and it is important to know from the title where these provinces can be found.

2. Abstract: It is advised to revise the structure of the “Abstract” to improve the quality of the paper (or example, background, conclusion etc.).

3. Introduction: Some sentences are very long and can be restructured for better representation and understanding of information (for example, first and second sentence, etc.). Objectives can be better expressed.

4. Methodology: Generally, this section can be improved. The details can be added to some laboratory analysis, even though the publication was done and the authors used the manufacturer's instructions.

   - Sample collection criteria and study population: this section needs clarification. According to the authors, there was a retrospective study, but the methodology states that the sample was collected from the patients using convince sampling
approach. Where later it was mentioned that the sample was collected blindly from the facilities. Please, kindly write in details the sampling collection approach so that it would be easy to understand. Secondly, why were different numbers of sera samples collected from two provinces (20 sera samples from North-West and 24 from KwaZulu-Natal Provinces)? It would be appropriate to explain the sampling approach that was used.

- **Laboratory analysis**: the structure of this paragraph can be improved.
- **ELISA**: It was mentioned that the kits used in research were not specific. This statement needs some clarification, is it applied for HSV test kit or for HIV-1 test kit also?

5. **Results**:
- Table: Presentation can be improved (for example, in table 1 there was no data for age group less than 10 years and etc.).
- Table 1 & figure 1 represent the same data. One should be removed, as it is a duplication of data representation.
- It was mentioned in the methodology section, that the PCR was used to confirm the non-specific ELISA method even for HIV-1. There is no data in the result section. Please, kindly include this data.
- Some information on result section should be sent to methodology section.

- **Discussion**: It is very difficult to draw the conclusion on the co-infection prevalence of HSV type 1 and type 2 and HIV-1, due to the small sample size and small co-infection in the study population.

- **References**: Some references in the text were wrongly introduced, for example, Debrah et al., 2018 was cited to present prevalence of HSV in Gambia, whereas Debrah and colleagues did the research in Ghanaian population.

- **Limitation**: The main limitation of the study is small sample size. This section mentioned that the age of study participants is from 14-49 years, whereas the results (table 1) introduce the data up to 60 years.

- **Conclusion**: It is very difficult to draw the conclusions with such small sample size. The authors took a risk to make such conclusions.

**Conclusion**: major revision is needed.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Partly

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Infectious diseases of public health concern, medical biochemistry, chemical pathology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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