

Analyzing trends in HSV Western Blot results at a Reference Laboratory

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Public Health

University of Washington

2021

Committee:

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Program Authorized to Offer Degree:

Epidemiology

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Abstract

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Objective: To better understand herpes simplex virus (HSV) Western Blot result trends over the past 20 years and to determine the proportion of Western Blot positivity for HSV-1 and HSV-2 and the proportion of results that are indeterminate. To examine the relationship between indeterminate results and age.

Methods: We used de-identified HSV Western Blot data performed at the University of Washington Virology Laboratory between 1999-2020. We included all results collected between 1999-2020 from persons aged 18 to 99 with a result of negative, positive, or indeterminate. We excluded results from the oncology service with the label Seattle Cancer Care Alliance (“SCCA”) but included all other referral locations. We assessed overall total proportion of negative, positive, and indeterminate for both HSV-1 and HSV-2 in the sample. Linear regression was used assess the trends in positive and indeterminate results over time. We then analyzed the proportion of Western Blots done in the 5 year time period before and after the 2015 CDC guidelines. Linear regression was utilized to assess the association between indeterminate results and age.

Results: Results from 188,342 samples collected from 1999 to 2020 were included in the analysis. 107,468 (57%) were HSV-1 positive while 35,294 (19%) were HSV-2 positive. There were 20,138 (11%) samples that were positive for both HSV-1 and HSV-2 and 61,533 (33%) that were negative for both types. Overall, 4,419 samples were indeterminate for HSV-2 (2%) and 2,152 (1%) for HSV-1. The proportion of HSV-1 positives decreased by 0.45% each year ($p < 0.001$) while the proportion of indeterminates increased by 0.08% each additional year ($p < 0.001$). For HSV-2, the proportion of positives decreased by 0.6% annually ($p < 0.001$) and indeterminates increased by 0.15% each year ($p < 0.001$). When looking at HSV-2 indeterminates by age category, the highest proportion was in the 56-65 age group with a total of 3.3% compared to the lowest at 1.7% in those 18-25 ($p < 0.003$).

Conclusion: HSV-1 and HSV-2 seropositivity has declined over the last twenty years, in parallel to national trends. The frequency of indeterminate results for both HSV-1 and HSV-2 may be rising possibly due to increased testing in the setting of declining seroprevalence leading to a decrease in the positive predictive value of testing and increase in false positives. Our analysis also showed that there was a statistically significant correlation between proportion of indeterminate results and older age.

Background

Genital herpes is a prevalent sexually transmitted infection (STI) that causes substantial morbidity, both related to physical manifestations including recurrent genital ulcers and psychological burdens related to stigma. Genital herpes is caused by herpes simplex virus type 2 (HSV-2) or HSV-1. While HSV-1 has historically been associated with oral ulcers, it has become an increasing cause of genital herpes as well¹. In the U.S, among people aged 14-49 HSV-2 seroprevalence was 11.9% and HSV-1 seroprevalence was 47.8% in 2015-2016. Current risk factors for HSV-2 seropositivity include female gender, Black race, older age, history of multiple sexual partners, history of prior sexually transmitted infections and low socio-economic status³. Notably, seroprevalence for both HSV-1 and HSV-2 has decreased over last 2 decades from 57.7% to 47.8% and 16.2% to 11.9%, respectively. However, given its continued prominence and implications, many sexually active people want to know their HSV status. Given the performance characteristics of the commercially available assays, recommendations in the US have focused on limiting serological testing in asymptomatic patients to decrease the risk of giving a false positive diagnosis. Giving people the diagnosis of an incurable infection impacts their future sexual health and creates an enormous psychosocial burden⁴ so limiting unnecessary testing is crucial. The United States Preventative Services Task Force (USPSTF) states that screening asymptomatic people (Grade D)⁵ for HSV-1 and HSV-2 is not recommended. The CDC 2015 STD Treatment Guidelines state when there is no active lesion, screening with type-specific HSV serologic assays is only recommended for certain populations, including people with a partner who has genital herpes and people with recurrent genital symptoms who have not been diagnosed with genital herpes⁶.

Several FDA approved type-specific HSV IgG assays, which claim to differentiate accurately between antibodies to HSV-1 and HSV-2, are commercially available. These enzyme immunoassays (EIA) tests are high throughput and allow increased access to testing. However, these tests suffer from lack of specificity compared to the gold standard HSV Western Blot (WB). For example, a study done by Golden et al at a public health STD clinic showed that in a low prevalence group of men who were tested

and found to be seropositive for HSV-2 on FOCUS ELISA testing, only 80% of them were found to be positive (PPV of 84%) on confirmatory Western Blot testing. When the cutoff value was raised to >3.0 for the ELISA instead of the manufacturer cut off of >1.1 however, the PPV increased to 98%. This suggests that it may be necessary to have higher index cutoffs for these assays to ensure accurate results⁷.

Another recent study by Agyemang et al⁸ supported these findings in a real-world setting. It showed that, compared to the Western Blot, the FDA approved HerpeSelect test had poor sensitivity (70.1%) and poor negative predictive value for HSV-1 (70.0%) and poor specificity (57.4%) and positive predictive value (50.7%) for HSV-2. Though the study looked specifically at HerpeSelect, it did note that there was a variety of other tests being used in the USA. One of the principal concerns with the other less studied EIAs is that they were approved using HerpeSelect as the comparator. As such, they may have similar sensitivity/specificity issues compared to HerpeSelect but have not been thoroughly analyzed individually. This also creates an issue when trying to interpret the appropriate reference cut off for confirmatory testing as this may vary from test to test. Because these studies have shown that widely available FDA-approved EIAs for HSV have both poor positive predictive value for detecting HSV-2 and poor negative predictive value for HSV-1, the 2015 CDC STD Treatment Guidelines strongly recommended confirmatory testing for those with type specific EIA results in a low positive range, specifically a HerpeSelect test with index value <3.5 ⁶. The recommended confirmatory testing is the University of Washington Western Blot or the Biokit, both which have been shown to improve specificity⁹.

The Western Blot is performed by growing HSV in vitro and then putting viral lysates onto a gel, performing electrophoresis to separate proteins by weight, and then transferring to nitrocellulose paper¹⁰. The strips of paper are then incubated with a patient's serum and bands appear at certain molecular weights, indicating the presence of antibodies to specific HSV proteins. The presence of 4 bands or more is considered positive. Results are considered indeterminate when 1-3 bands are detected.

Because several antibodies to HSV proteins are detected in a positive test, the WB has very high specificity. In contrast, the enzyme-linked immunoassay (EIA) detects a single antibody to glycoprotein

G, so it may have more non-specific cross reaction, making it less specific with higher chance of false positives.

However, even though the Western Blot is the gold standard, there are still a number of results that are Western Blot indeterminate. Indeterminate results may occur after recent acquisition of HSV or due to non-specific cross-reactive antibodies.

Since the 2015 CDC STD Treatment Guidelines recommended confirmatory testing using the Western Blot and since additional serologic tests have become available over time, it will be interesting to assess whether there have been any trends in the proportion of HSV indeterminates over time. We hypothesize that this could occur in the setting of more HSV screening in the community and increased awareness about the potential for false positive results with EIA/ELISA tests. This poses a challenge when discussing diagnosis/prognosis with patients since they may be inclined to repeat testing to determine if indeterminate results either remain indeterminate or seroconvert to positive or negative serostatus. Since HSV is a chronic viral infection associated with stigma, it can have a substantial social and mental health impact on patients and our ability to provide an accurate diagnosis is essential.

Methods

Data Source

We used de-identified HSV Western Blot data performed at the University of Washington Virology Laboratory between 1999-2020. This is the only laboratory in the United States currently that provides access to the HSV Western Blot either for local providers or as a reference laboratory allowing capture of a substantial number of people who undergo confirmatory testing for HSV serostatus. The University of Washington Human Subjects Division determined that use of these data does not require approval from the University of Washington Institutional Review Board.

Population

We included HSV Western Blot results collected between 1999-2020 from persons aged 18 to 99 with a result of negative, positive, or indeterminate. We excluded those with a result of “low level

antibody, unable to subtype”. There were samples that did not list sex and were not included in any sub-analysis related to sex but were included in the overall analyses otherwise. We also excluded results from the oncology service with the label Seattle Cancer Care Alliance (“SCCA”) which encompassed 12,070 (6%) samples. This was done because these samples had a higher likelihood of being indeterminates given transfusion history in oncology patients. We included results from all other referral locations, both inpatient and outpatient.

Statistical Analysis

We performed a descriptive analysis of the Western Blot (WB) data and assessed the results of HSV-1 and HSV-2 antibody tests performed over time. We looked at the overall total proportion of negative, positive, and indeterminate for each viral type tests in the sample. We then looked at the proportion of Western Blots done in the 5 year time period before and after the 2015 CDC guidelines since this is when it changed to specifically recommend performing a WB or Biokit as a confirmatory test if index value for EIA/ELISA test was ≤ 3.5 . We also compared the proportion of indeterminates to determine time trends within that time frame. We then analyzed the trends in the proportion of HSV-1 and HSV-2 positives and indeterminates by year, separately over time. We performed a linear regression to determine whether the relationship between year and proportion of positive tests was statistically significant. We set our $\alpha = 0.05$ and considered our results to be statistically significant if the 2-sided p-values < 0.05 . We repeated this linear regression analysis for indeterminate results as well. We then specifically looked at the proportion of indeterminate HSV-1 and HSV-2 tests by age category compared to total proportion of people in that category. We again utilized linear regression to look at the relationship between these two variables and assessed statistical significance. Lastly, we examined the proportion of men vs. women relative to their number in the sample with HSV-2 indeterminate results, excluding those with no gender listed. We used a Z-test to determine if there was a statistically significant association. RStudio Version 1.2.5042 was utilized for this analysis.

Results

Overall, results from 188,342 samples collected from 1999 to 2020 were included in the analysis after applying inclusion and exclusion criteria. We excluded 1,770 results with “low level antibody, unable to subtype” as well as 4 people over the age of 99. There were 4,409 samples that did not list sex and were not included in any sub-analysis related to sex but were included in the overall analyses otherwise. Additionally, 12,070 samples from the SCCA oncology service were excluded. All other 695 referral locations, both inpatient and outpatient, were included. None of these centers contributed more than 5% of the total samples. Only 2 sites contributed 5% (Dynacare labs, a reference laboratory, and UWMC Hall Health, a University-based health clinic serving students) while 26 contributed between 1-5% (Table 4) and the remaining 667 sites contributed less than 1%.

Out of 188,342 samples in this study, 107,468 (57%) specimens were HSV-1 seropositive while 35,294 (19%) were HSV-2 seropositive. There were 20,138 (11%) samples that were positive for both HSV-1 and HSV-2 and 61,533 (33%) that were negative for both types (Table 1). Overall, 4,419 samples were indeterminate for HSV-2 (2%) and 2,152 (1%) for HSV-1. Over the 5 year time period before and after the 2015 CDC guidelines were released, we found that there were 43,655 Western blots performed between 2011 – 2015 and 37,134 performed between 2016 – 2020 (Table 2). The number of indeterminates slightly varied between the two-time frames, with 1,250 indeterminate results (2.86%) between 2011 – 2015 and 2,670 (3.8%) the following 5 years.

When looking at the numbers of HSV-1 and HSV-2 positives and indeterminates by year, there were significant changes over time. The proportion of HSV-1 positive results decreased by 0.45% each year ($p < 0.001$) while the proportion of indeterminate results increased by 0.08% each additional year ($p < 0.001$). (Figure 1 and 2). A similar trend was seen in HSV-2 results with the proportion of positives decreasing by 0.6% annually ($p < 0.001$) and indeterminates increasing by 0.15% each year ($p < 0.001$). (Figure 1 and 2). When looking at HSV-2 indeterminate results, 54% were associated with HSV-1 positivity and 46% with HSV-1 negative results (Table 4).

When looking at HSV-2 indeterminates by age category, the highest proportion was in the 56-65 age group with a total of 3.3% compared to the lowest at 1.7% in those 18-25 ($p = 0.003$) When looking at HSV-1 in comparison, the lowest prevalence was in those older than 65 and the highest in those age 18-25 years (Figure 3). Examining the samples with indeterminates by sex revealed that there was a higher proportion of men with indeterminates (2.8%) compared to women (2.1%) ($p\text{-value} < 0.001$).

Discussion

In this analysis of 1999-2020 HSV Western Blot data, we found that overall trends in our data are similar to national trends. Nationally, HSV-1 and HSV-2 seroprevalence has declined over the last decade, with HSV-1 decreasing from 59.4% to 48.1% and HSV-2 decreasing from 18% to 12.1% from 1999-2000 to 2015-2016². A more recent local study done with King County Public Health STD clinic also reported that there has been a decrease in first-episode genital HSV-2, though genital HSV-1 acquisition remained stable¹¹. Our results mirrored this decline in seropositivity over the years for both HSV-1 and HSV-2. The underlying factors leading to the decrease in seroprevalence for HSV-2 are unknown though some have hypothesized it could be due to improved education about preventative interventions and changing sexual practices¹¹. For HSV-1, this shift has been attributed to decreased acquisition during childhood¹².

Our primary aim was to determine time trends in indeterminate results for HSV-1 and HSV-2. To this author's knowledge, there have been no prior studies specifically looking at HSV Western Blot trends in indeterminate results. Indeterminate results are thought to be either true positives in the setting of recently acquired infection or false-positive, due to non-specific cross-reactive antibodies. Our analysis showed that while overall HSV-1 and HSV-2 seroprevalence were decreasing, the proportion of samples that were indeterminate for both HSV-1 and HSV-2 were increasing over the years. We hypothesize that this may be due to the fact that despite guidance from USPSTF and CDC, testing itself is still increasing in the setting of declining seroprevalence. This would then decrease the positive predictive value of testing and increase false positive ELISA. We originally theorized that this could also be because an

increasing number of people started obtaining Western Blots after 2015 due to the CDC STD Treatment Guidelines recommendation to confirm low positive results, however, our data showed that in the 5 years following that recommendation (2016 -2020), the number of Western Blots done was less than the 5 year period before (2011-2015). When looking at HSV-2 alone, our analysis also showed that there was a statistically significant correlation between proportion of HSV-2 indeterminates and increase in age. This association seemed to plateau once persons reached age 65. We also looked at HSV-2 indeterminate results in relation to HSV-1 seropositivity to assess if a positive HSV-1 result increased the likelihood of an indeterminate HSV-2 outcome. We found that about 54% of HSV-2 indeterminate samples had a concurrent positive HSV-1 and 46% did not. Given these numbers, we do not think there is enough evidence to support any significant association between HSV-1 positivity resulting in an HSV-2 indeterminate result.

Our study had several limitations. First, as there was no other specific data collected in this dataset, we were not able to assess if there were any additional confounders or risk factors that may be associated with someone having an increased likelihood of an indeterminate result. Importantly, we did not know whether people obtained the WB after having a low positive ELISA result. We also can't determine what the indications for testing are so won't be able to analyze whether there are more indeterminates in people who have more risk factors vs. routine screening (i.e., transplant setting). There may be selection bias based on availability of testing since this is the only lab performing Western Blot in the country and requires that the participant coordinate with their local provider and ship the specimen. While we attempted to exclude those who obtained the test in the oncologic setting by removing data labeled with "SCCA" as the location, we acknowledge that this was not all encompassing. Many of the inpatient floors that were included were home to oncology/transplant patients therefore we could still have some persons who do not meet our target population of those being screened for sexual health exclusively. However, as these none of these groups comprised a large proportion (>5%) of overall sample size, we elected to keep them in the analysis. We were not able to link repeat testing in samples

that may have once been indeterminate and may have seroconverted on repeat testing as we did not have individual patient identifiers.

An area of future study could be to look at repeat testing in individuals who had an indeterminate and seeing whether they seroconvert and whether they remain stable over time. More investigation in this area could help bridge this gap in knowledge and bring some clarity to the interpretation of these indeterminants and allow for clear prognosis discussions with patients.

Tables and figures

Table 1: Total number of HSV serologic tests included, by result

| TEST TYPE | N (%) |
|--------------------------|---------------|
| HSV 1 POSITIVE | 107,468 (57%) |
| HSV 2 POSITIVE | 35,294 (19%) |
| HSV 1 AND HSV 2 POSITIVE | 20,138 (11%) |
| HSV SERONEGATIVE | 61,533 (33%) |
| HSV 1 INDETERMINATE | 2,152 (1%) |
| HSV 2 INDETERMINATE | 4,419 (2%) |
| TOTAL # SAMPLES | 188,342 |

Table 2: Proportion of WB done the 5 year time period before and after the 2015 CDC guidelines

| Years: | 2011 - 2015 | 2015- 2020 | Total: |
|----------------|------------------|------------------|------------|
| Total # | N = 43,655 | N = 37,134 | N = 80,789 |
| Indeterminates | N = 1,250 (2.9%) | N = 1,420 (3.8%) | N = 2,670 |

Table 3: HSV-2 Indeterminates by HSV-1 Status:

| | HSV 1 Positive N (%) | HSV 1 Negative N (%) | Total |
|-------------------------|-------------------------|-------------------------|----------------|
| HSV-2 Indeterminate | 2,380 (54%) | 2,039 (46%) | 4,419 |
| Not HSV-2 Indeterminate | 105,088 (57%) | 76,683 (52%) | 181, 771 |
| Total | 107, 468 (58%) | 78,722 (42%) | 186,190 |

Table 4: Referral locations contributing more than 1% of samples:

| | |
|---|---|
| Children's Hospital (Virology) | Dynacare Labs |
| Harborview Medical Center Women's Clinic | Labcorp Seattle |
| Laboratories Northwest | Labs Northwest Mayo Aor |
| Northwest Hospital Mayo Aor | Northwest Meridian Women Health Campus |
| Northwest Meridian Women Health Outpatient Medical Center | Obstetrics - Maternal Infant Care Clinic |
| Other | Path Assoc Med Lab Mayo Aor |
| Path Associates Med Lab | Providence Med. Center, Seattle |
| Quest Diagnostics Seattle | Seattle Childrens Mayo Aor |
| The Polyclinic | University Women's Care Clinic Gynecology |
| University of Washington Medical Center Kidney/Pancreas Liver Tx Surg | University of Washington Medical Center Micc Obstetrics |
| University of Washington Medical Center Pre Kidney | University of Washington Neighborhood Clinic Belltown Clinic |
| University of Washington Neighborhood Clinic Factoria Clinic | University of Washington Neighborhood Clinic Federal Way Clinic |
| University of Washington Neighborhood Clinic Hall Health | University of Washington Neighborhood Clinic Issaquah Clinic |
| University of Washington Neighborhood Clinic Shoreline Clinic | University of Washington Neighborhood Clinic Woodinville Clinic |

Figure 1: Proportion of HSV-1 and HSV-2 positive Western Blots over time:

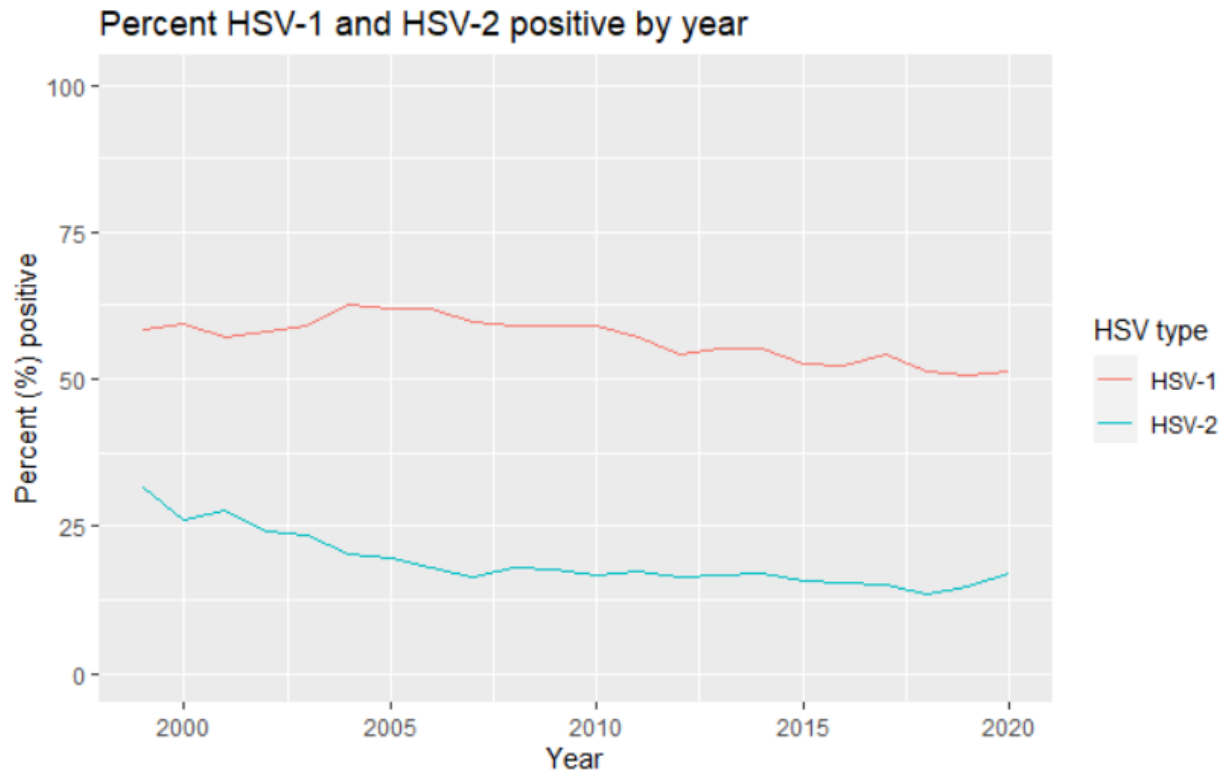


Figure 2: Proportion of HSV-1 and HSV- 2 indeterminates by year:

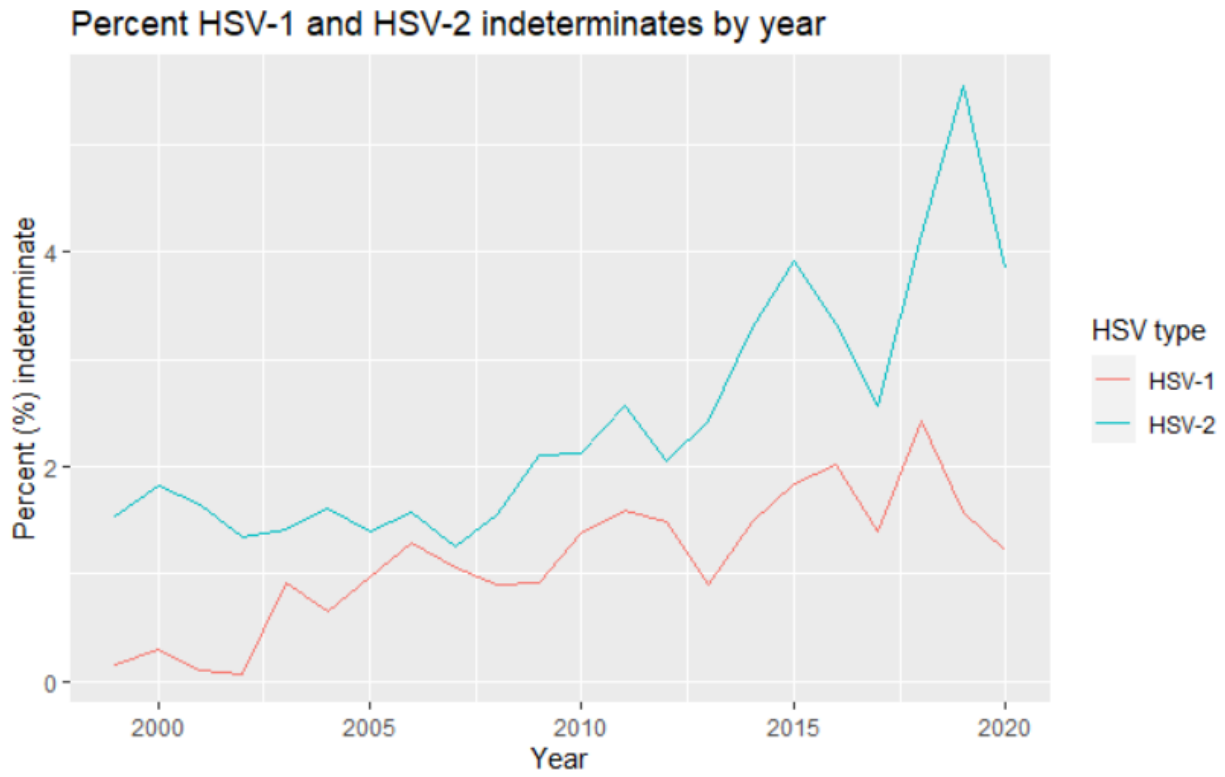
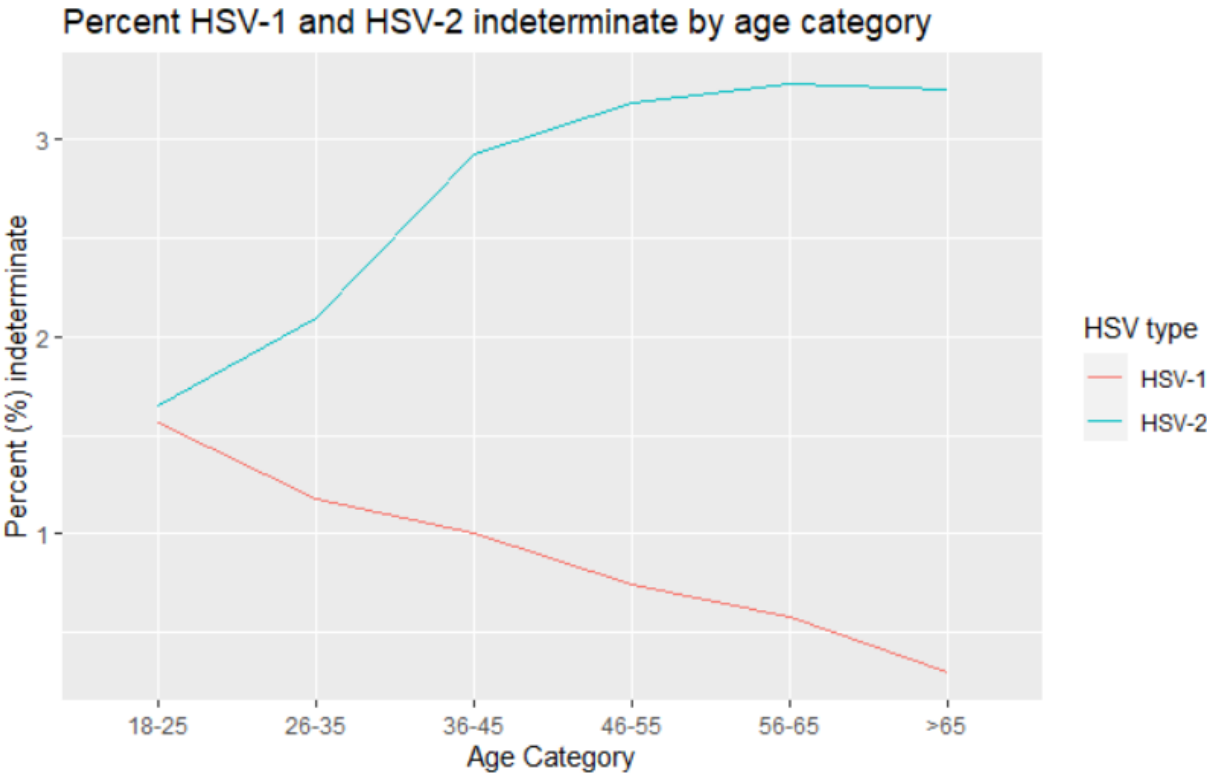


Figure 3: Indeterminate HSV-1 and HSV-2 tests by age category



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