

Specimen-Specific and Age-Related Differences in Detection of *Herpesviridae* by Multiplex qPCR

Abstract

Background. Human *Herpesviridae* establish a lifelong latent infection after initial exposure to viruses shedding from oral mucosa. Primary infection is often asymptomatic but can present with mild respiratory symptoms and/or skin rashes. Detection of viral shed from a direct source, such as the oropharynx, may be more clinically relevant than serological testing since seropositivity reaches 50-95% by adulthood. The goal of this study was to determine specimen-specific and age-related detection rates of cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), human herpesvirus 6 (HHV-6), and varicella-zoster virus (VZV) in clinical oropharyngeal specimens using multiplex qPCR.

Methods. Respiratory specimens consisting of throat and nasal swabs were collected from symptomatic patients (1:1 male to female ratio) in five representative age groups (0-1, 1-2, 2-10, 10-20, n=80 each; 20-60, n=140) with an equal number of nasal and throat swabs per age group (n=40). Nucleic acids were extracted using the KingFisher™ Flex with an optimized MagMAX™ (Thermo Fisher Scientific, Waltham, MA) extraction protocol. A novel multiplex qPCR method was developed for simultaneous molecular detection of CMV, EBV, HSV-1, HSV-2, HHV-6, and VZV.

Results. Cumulative detection rates for herpesviruses in throat and nasal swabs were 43.0% and 12.6%, respectively, with the highest in the 1-2 year age group. Detection of HHV-6 and EBV in throat swabs was significantly higher than in nasal swabs ($p < 0.0001$). HHV-6 detection in throat swabs was highest in the 1-2 age group (55%); whereas, EBV detections in throat swabs were consistent from age one to sixty. No specimen-specific differences were observed for detection of CMV and HSV-1, and both targets had an average detection rate of 3% in each specimen source. HSV-2 was detected in one throat swab, and VZV was not detected in any tested specimen. In throat swabs, HHV-6 detection was significantly higher in younger age groups when compared to the 20-60 age group ($p \leq 0.0002$). While EBV was not detected in the 0-1 age group, the EBV detection rates ranged from 15-17.5% in pediatric groups to 25.7% in the 20-60 age group. No significant differences were observed for detection of viruses between genders in either specimen source.

Conclusions. *Herpesviridae* infections are spread through close contact with mucosal secretions, with the throat being a main source of viral replication and shed during acute infection. These data suggest that exposure to *Herpesviridae* occurs early in life as the highest detection rate for both sources was observed in the 1-2 year age group. The differences in detection rates for each virus can be explained by virus-specific host cell sites during latency. HSV-1, HSV-2, and VZV maintain latency in neurons, while EBV and HHV-6 persist in epithelial cells and salivary glands during latency. These data also indicate that nasal swabs may not be an appropriate specimen type when testing for *Herpesviridae*. This study provides evidence supporting the utility of multiplex qPCR for the detection of *Herpesviridae* from a single specimen, as well as the importance of evaluating appropriate types of clinical specimens for use with novel diagnostic tests.

Introduction

- Herpesviruses are typically transmitted through respiratory secretions and can cause respiratory symptoms and/or rashes.
- Initial exposure is followed by lifelong latent infection, and reactivation with viral shed is common.
- Approximately 95% of the world's population has been exposed to herpesviruses. The diagnostic utility of serological tests for active infections may not be appropriate since seropositivity reaches 50-95% by adulthood.¹
- Detection of viral shed from a site of active infection, such as respiratory secretions and dermal lesions, may be more clinically relevant.
- Due to latency of CMV, EBV, and HHV-6 in lymphocytes or salivary glands, detection rates in respiratory secretions may be higher and require clinical correlation for diagnostic determination.
- This study evaluated specimen-specific and age-related differences in the detection rates of herpesviruses in respiratory specimens using the Diatherix Viral Skin Rash Panel (VSRP).

Table 1. Primary sites of herpesvirus infection and latency and modes of transmission.^{1, 2, 3}

Acronym	Common Name	Target Host Cell	Site of Latency	Mode of Transmission
HHV-1 (HSV-1)	Herpes simplex virus type 1	Mucoepithelial	Neurons	Genital and respiratory secretions
HHV-2 (HSV-2)	Herpes simplex virus type 2	Mucoepithelial	Neurons	Genital and respiratory secretions
HHV-3	Varicella-zoster virus	Mucoepithelial	Neurons	Respiratory secretions
HHV-4	Epstein-Barr virus	Mucoepithelial to B-lymphocytes	B lymphocytes	Respiratory secretions
HHV-5	Cytomegalovirus	Mucoepithelial to T-lymphocytes	Monocytes	Respiratory secretions, urine, breast milk
HHV-6	Roseolavirus	Mucoepithelial to T-Lymphocytes	Monocytes, salivary glands	Respiratory secretions

Materials & Methods

Clinical specimens. Respiratory specimens collected in Copan ESwab™ tubes were submitted to Diatherix Laboratories, LLC for routine Target Enriched Multiplex Polymerase Chain Reaction (TEM-PCR™) respiratory testing between October and December 2017. A total of 460 specimens were selected retrospectively across specific age ranges (0-1, 1-2, 2-10, 10-20 years, n=80 each; 20-60 years, n=140) with equal distribution of throat and nasal swabs and female to male patients (1:1 ratio). Specimens were collected for the study after the clinical laboratory's seven day hold. TEM-PCR™ results were blinded during sample selection and processing.

Materials & Methods (continued)

Nucleic acid extraction. Nucleic acids (NAs) were extracted using the KingFisher™ Flex platform with a commercially available extraction kit (MagMAX™ Pathogen RNA/DNA Kit, Thermo Fisher Scientific, Waltham, MA). Samples were pre-treated with lysozyme (0.1 mg/mL) and incubated at 37°C for twenty minutes. Following incubation, samples were treated with proteinase K (0.5 mg/mL) and MagMAX™ reagents according to manufacturer's protocol.

qPCR amplification and detection. Extracted NAs were amplified in triplicate using the Diatherix VSRP (Table 2). Briefly, 10 µL reactions were prepared with 2.5 µL TaqPath™ 1-Step Multiplex Master Mix (Thermo Fisher Scientific), 1.5 µL primer mix, 2.9 µL nuclease-free water, and 3.1 µL of template NA. Primer mixes contained four nested TaqMan® Gene Expression assays (three herpesviruses and one internal control, Figure 1) developed and optimized in-house. Amplification and detection was performed on the QuantStudio 5 (Thermo Fisher Scientific) with in-house cycling protocols.

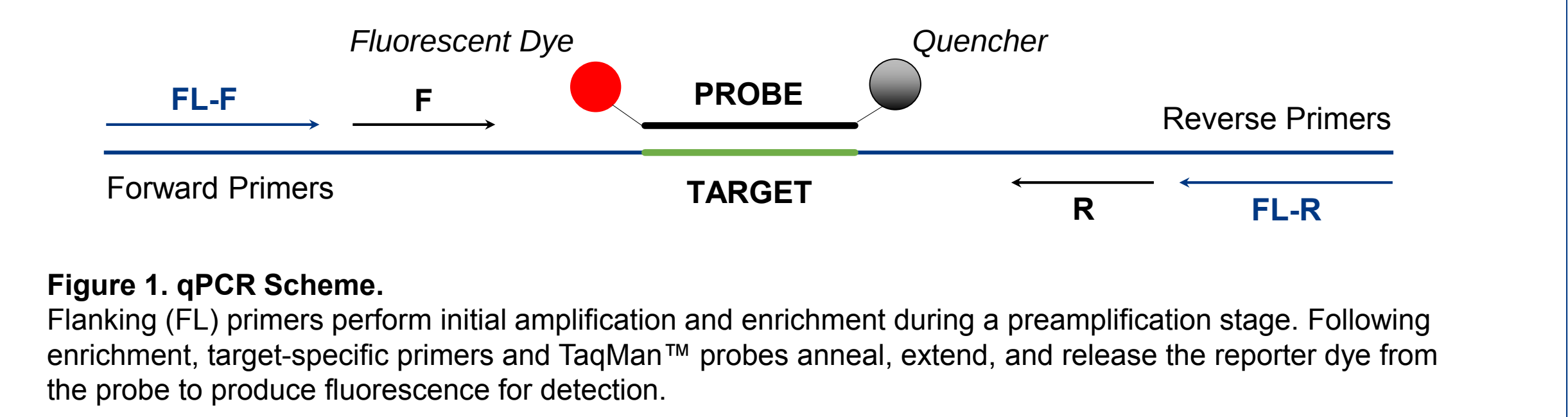


Table 1. Viruses detected by the Diatherix VSRP.

Target Virus	Target Acronym
Enterovirus*	EV
Enterovirus D68*	EV-D68
Human parechovirus*	HPeV
Epstein-Barr virus	EBV
Parvovirus B19*	B19
Herpes simplex virus type 1	HSV-1
Herpes simplex virus type 2	HSV-2
Cytomegalovirus	CMV
Human herpesvirus 6	HHV-6
Human herpesvirus 7*	HHV-7
Varicella-zoster virus	VZV

*Detections were not evaluated in this study.
*Human herpesvirus 7 was not included in the VSRP at the time of the study.

Controls. Extrinsic extraction controls were prepared using titrated HSV-1 and VZV stock viruses (Zeptomatrix, Buffalo, NY) diluted in Amies Medium to 1.0E5 and 1.0E3 pfu/mL, respectively. For an intrinsic control, the elution buffer was spiked with plasmid DNA (5.0E3 copies/µL) synthesized to contain an *Arabidopsis thaliana* gene sequence. No template controls utilizing nuclease-free water were included for each plate. All controls were evaluated in triplicate.

Results

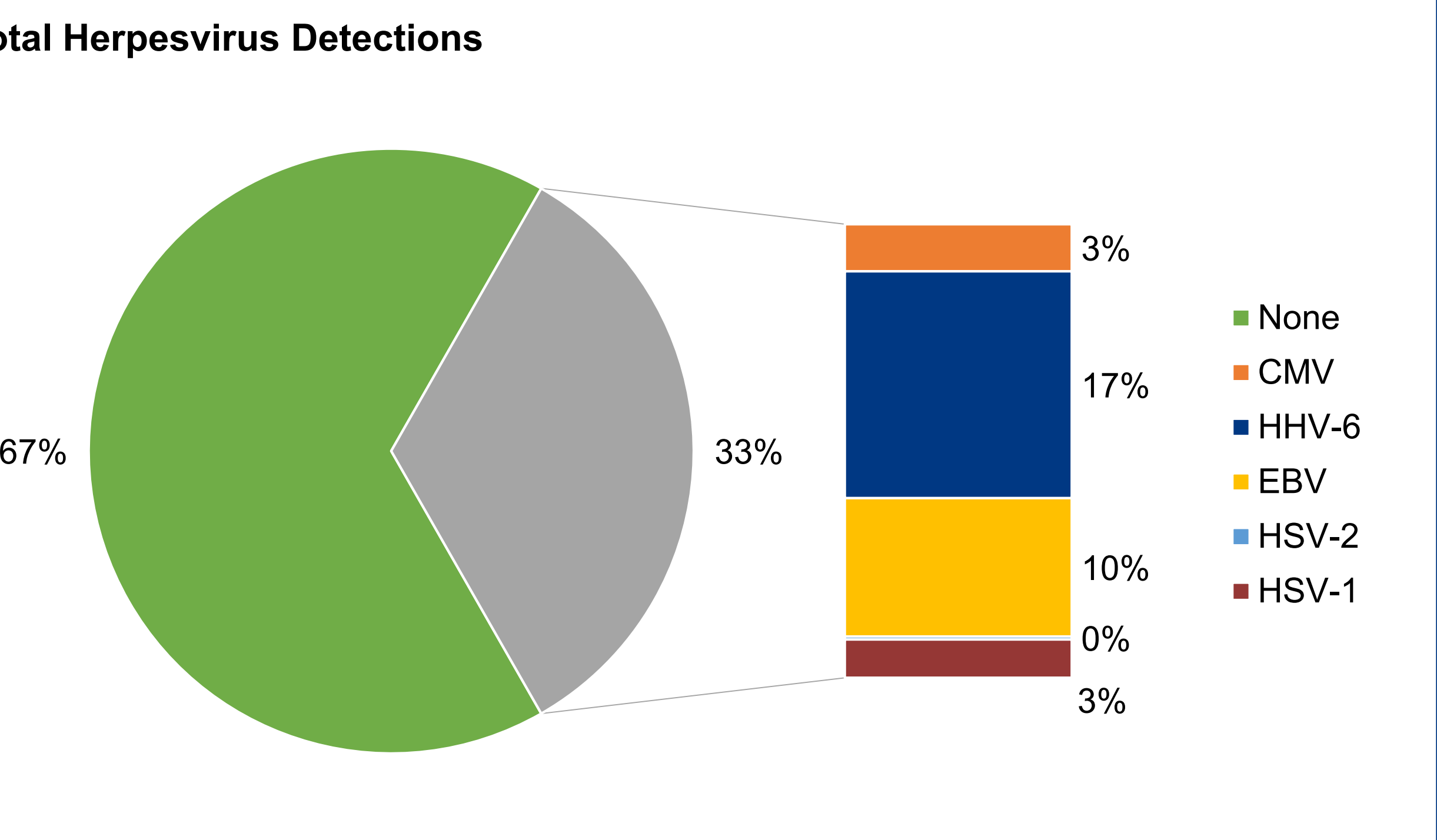


Figure 2. Herpesviruses detections in respiratory samples. Clinical respiratory specimens (throat and nasal, n=460) were retrospectively selected and evaluated for detection of herpesviruses. Nucleic acids were extracted and amplified using multiplex target enriched qPCR to detect cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), Epstein-Barr virus (EBV), herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), and varicella-zoster virus (VZV). No VZV detections were observed. None, no detections.

Results (continued)

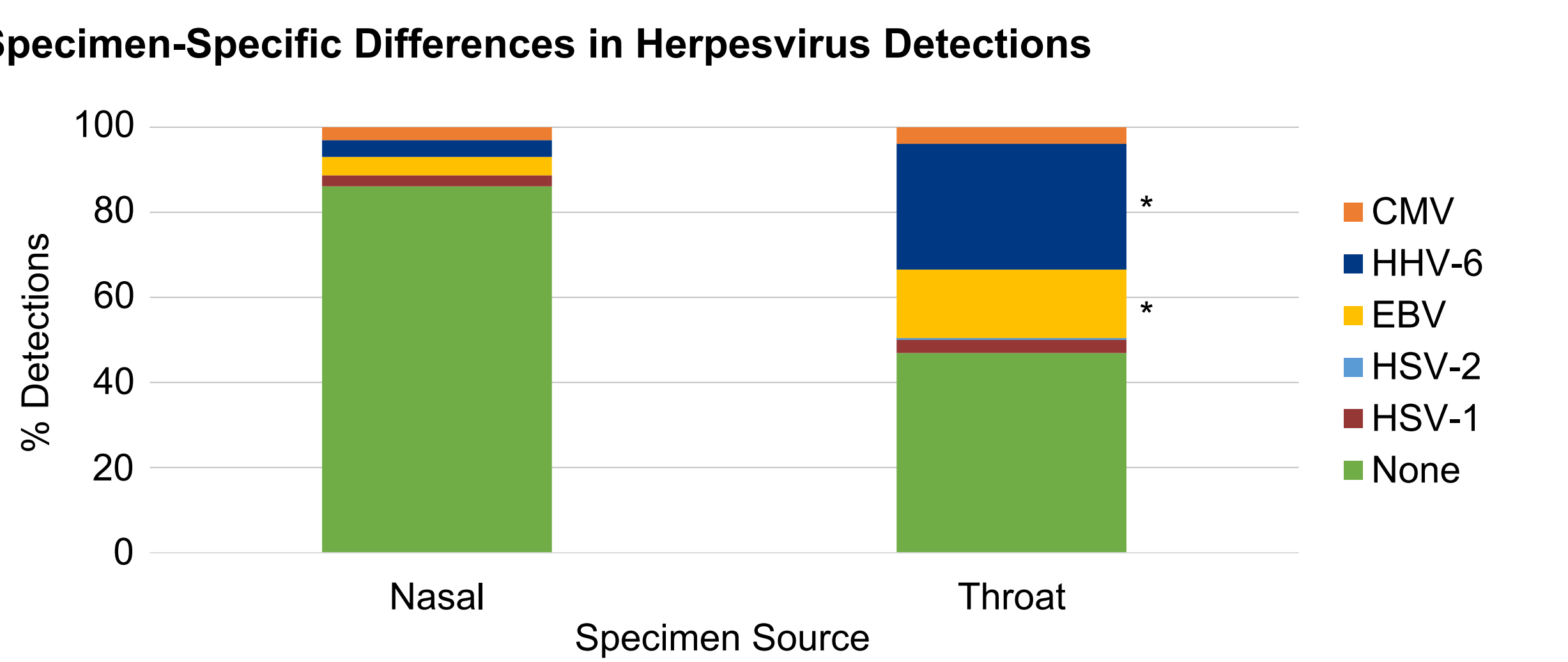


Figure 3. Herpesvirus detections in nasal and throat swabs. Nasal (n=230) and throat (n=230) swabs were retrospectively selected and evaluated for detection of herpesviruses. Nucleic acids were extracted and amplified using multiplex target enriched qPCR to detect herpesviruses. Detection rates per target in both specimen sources were compared (* $p < 0.0001$). No VZV detections were observed. None, no detections.

Age-Related Differences in Herpesvirus Detections

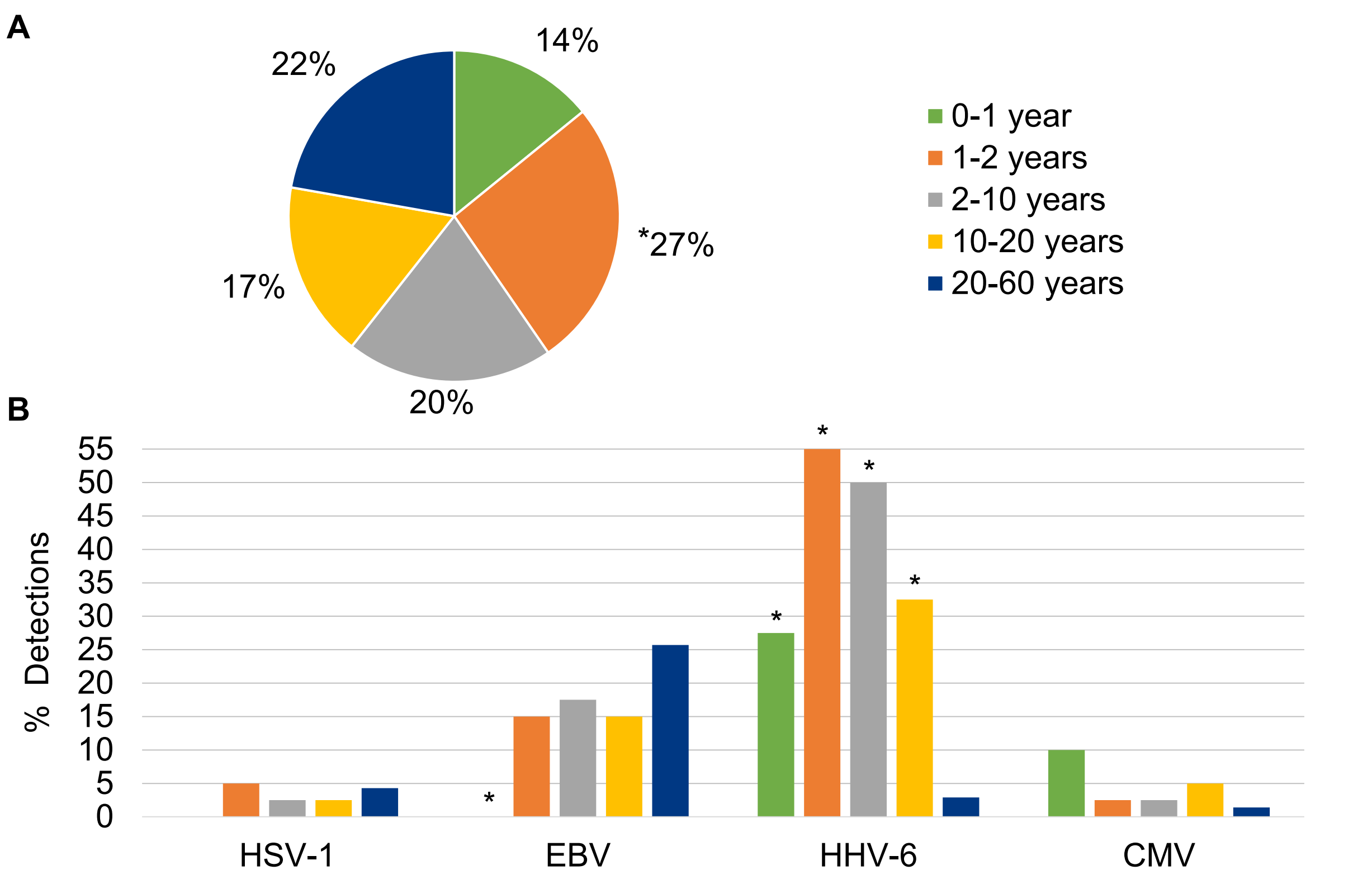


Figure 4. Herpesvirus detection rates by age. Throat swabs were retrospectively collected and evaluated for detection of herpesviruses across age ranges: 0-1, 1-2, 2-10, and 10-20 years (n=40 each) and 20-60 years (n=70). Nucleic acids were extracted and amplified using multiplex target enriched qPCR to detect herpesviruses. Detection rates in throat swabs are compared by (A) overall herpesvirus detections (* $p = 0.0067$ compared to 0-1 year) and (B) detections by age group (* $p < 0.0002$ compared to 20-60 years). HSV-2 was detected in a 24 year old patient (not shown). No VZV detections were observed.

Herpesvirus Co-Detections

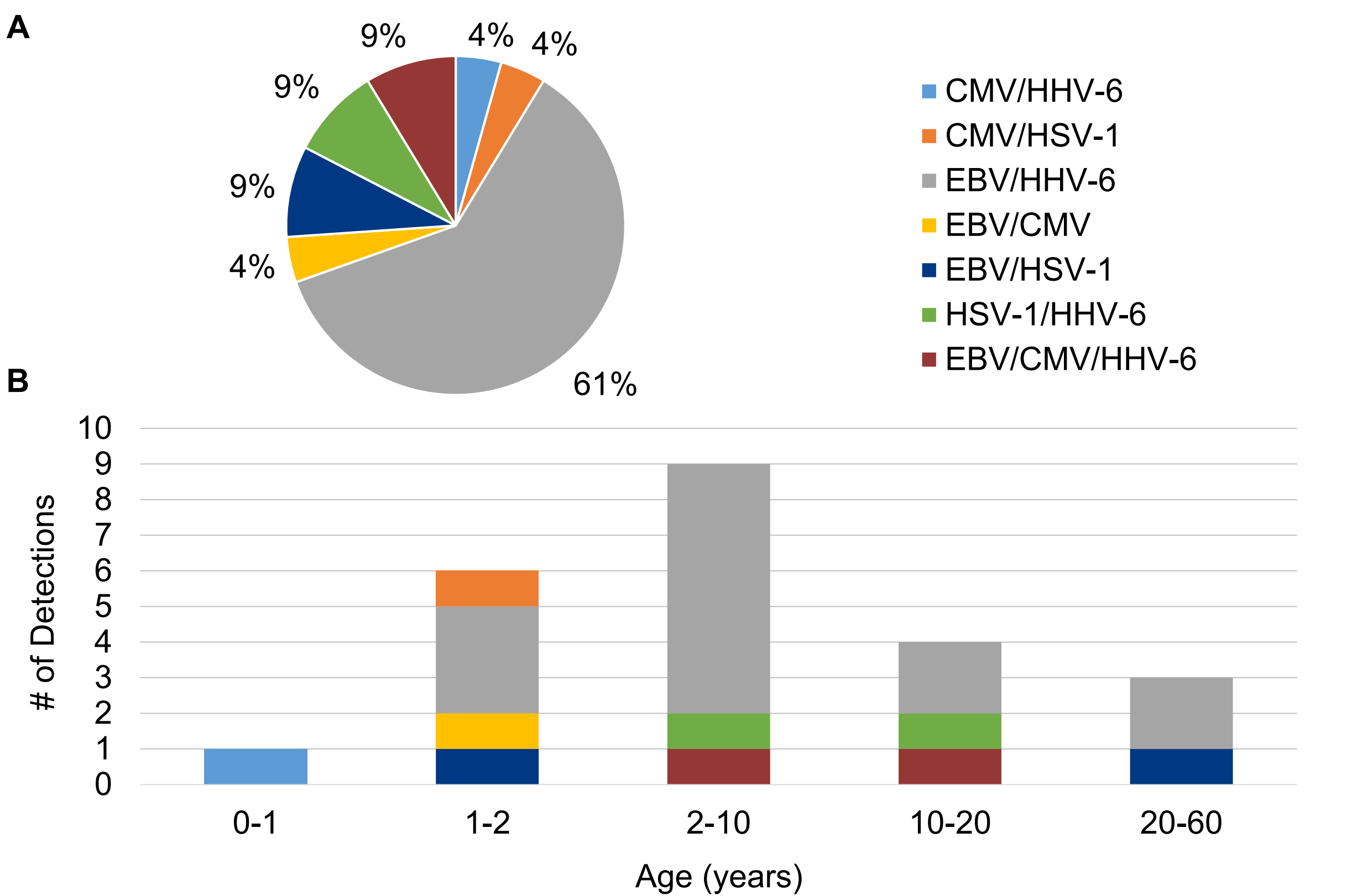


Figure 5. Herpesvirus co-detections in respiratory specimens. Clinical respiratory specimens (throat and nasal, n=460) were retrospectively selected and evaluated for detection of herpesviruses. Nucleic acids were extracted and amplified using multiplex target enriched qPCR to detect herpesviruses. Clinical specimens with two or more herpesvirus detections (n=23) were assessed by (A) co-detection type and (B) prevalence in different age groups.

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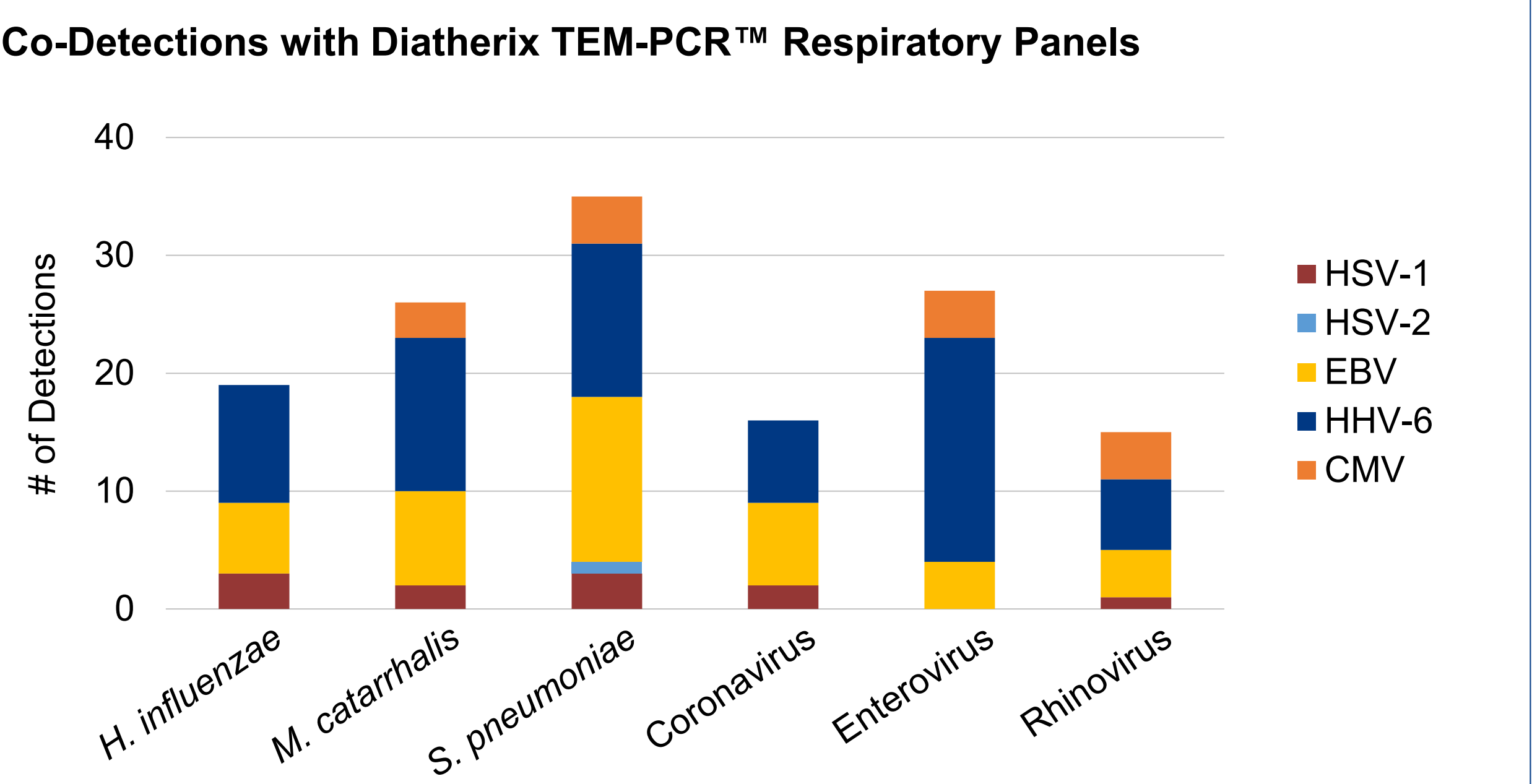


Figure 6. Herpesvirus co-detections with seasonal respiratory bacteria and viruses. Respiratory specimens processed with a Diatherix TEM-PCR™ respiratory panel for detection of the selected seasonal bacteria (*Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*) and viruses (human coronavirus, enterovirus, and rhinovirus) were evaluated for herpesvirus co-detections (n=138).

Discussion

- One or more herpesvirus was detected in less than 30% of specimens with 43.0% and 12.6% in throat and nasal specimens, respectively.
- Detection rates for HSV-1 and HSV-2 were similar between specimen sources. Since these herpesviruses maintain latency in neurons, detection in a nasal or throat specimen may suggest an active infection at the collection site.
- Detection of EBV and HHV-6 was significantly higher in throat swabs, which may be attributed to viral latency in lymphocytes or salivary glands. Clinical correlation would be suggested since EBV and HHV-6 may be detected in respiratory secretions during latent infection.
- Overall herpesvirus detection rates in throat swabs at 1-2 years old was significantly higher than detection rates in the 0-1 year old group. Data suggested that exposure to herpesviruses may occur early in life as the highest rate of detections was in the 1-2 year age group compared to the other tested groups.
- Although not statistically significant, CMV detections were higher in the 0-1 year age group. CMV can potentially be transferred through the breastmilk of a mother with an active infection which could contribute to the elevated detection rate in this age group.
- EBV and HHV-6 were accounted for in 70% of all herpesvirus co-detections. Synergy has been reported between the two viruses leading to reactivation of EBV into the lytic phase during acute HHV-6 infections.⁴ Although age differences were observed for EBV and HHV-6 co-detections, the sample size was too small to draw any conclusions.
- No correlation was observed for herpesvirus co-detections with frequently detected respiratory bacteria and viruses.
- This study evaluated herpesvirus detection rates in symptomatic respiratory samples with no clinical correlation of skin rash symptoms. A herpesvirus detection in a specimen taken from an active infection site from a patient presenting with a rash may be more indicative of active herpesvirus infection.

Conclusions

- Exposure to *Herpesviridae* occurs early in life as indicated by higher detection rates in symptomatic respiratory specimens from patients less than 10 years of age.
- Increased detection rates for herpesviruses may occur with specimens collected from sites harboring virus-specific latency cells.
- Throat swabs may be more appropriate than nasal swabs as a specimen source due to viral shed from throat epithelia and salivary glands during an active infection.
- The Diatherix multiplex VSRP is a novel diagnostic tool for the detection of *Herpesviridae* in a single specimen source.

References

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