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# Detection of 5 Latent *Herpes Viruses* and *Pneumocystis Jirovecii* in Saliva of Healthy Children by PCR

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**Background:** *Herpes virus* group can persist in the latent form in the body, *Pneumocystis jerovecii*, on the other hand, is a fungus which is ubiquitous in the respiratory tract of mammals, with 90% of adult human beings possessing antibodies to this organism. Concomitant presence of different microorganisms, may result in one microorganism altering the activity of another.
**Objectives:** To determine the frequency of 5 latent *Herpes viruses* and *Pneumocystis jirovecii* and to ascertain the rate of concomitant presence of these microorganisms in saliva of healthy children.
**Patients and Methods:** This cross-sectional study was performed on 150 immune-competent children 1 to 15 years old visited in the outpatient clinics at a university-affiliated children's hospital from 22nd May to 21st August 2011. Samples of saliva obtained from the children were subjected to DNA extraction by polymerase chain reaction.
**Results:** At least one microorganism was isolated from saliva of 148 children, (98.6%). In 13 cases only 1 microbe was detected, (8.6%), 2 pathogens were isolated from 24 children, (16%), 3 from 20 cases, (13.3%), 4 from 54 individuals (36%), and 5 from 37 subjects (54.4). HSV was the most frequent organism detected in 80.7%, and *P. jirovecii* the least frequent, (32.7%). Other viruses were HHV6, 66.7%, CMV 66.0%, HHV7 64.7%, and EBV 37.3% in order. No significant difference was found in the frequency of microorganisms in the two sexes or in different age groups, except for CMV which was significantly less frequent in age group of 6-10 years (P value = 0.001).
Regression analysis revealed an association between HHV6 and HHV7 (OR = 1599.4; P value < 0.001); a correlation was also found between EBV and CMV (OR = 3.1; P value = 0.012). In contrast, a negative correlation was found between *P. jirovecii* and three viruses of the *Herpes virus* group including EBV (OR = 0.27; P value = 0.006), HSV1 (OR = 0.36; P value = 0.028), and CMV (OR = 0.48; P value = 0.075).
**Conclusions:** Shedding of latent *Herpes viruses* and concomitant presence of multiple inactive micro-organisms is common in saliva of immune-competent children.

*Keywords:* Virus Latency; *Simplexvirus*; Pneumonia, Pneumocystis

## 1. Background

Viruses belonging to the *Herpes virus* group can persist in the latent form in the body; common sites for persistence and latency are the salivary glands, the mononuclear cells in the peripheral blood, and the central nervous system. According to some studies viral persistence and shedding may continue for many years and even throughout life (1).

Concomitant presence of different microorganisms, which has been observed by some researchers, may result in one microorganism altering the activity of another. Virus-virus interaction, (VVI), is a significant alteration in the pattern of illness caused by one virus as a result of simultaneous or previous infection by a different virus; it appears to be a common phenomenon. DaPalma et al. have suggested a framework that provides a systematic approach for studying VVI. They categorized VVI into 3 different types: direct exchanges of genetic products, indirect effects due to changes in the surroundings, and the immunological interactions (2). An example of VVI has been observed in patients chronically infected with *Hepatitis C virus*, (HCV); while, the clinical course of chronic hepatitis C is thought to be worsened by HBV, it has been suggested that HBV suppresses replication of HCV and vice versa. Similarly, infection with *Hepatitis A virus*, (HAV) causes a reduction in HCV-RNA, leading to infection alleviation in some patients with chronic HCV infection (3).

*Pneumocystis jirovecii*, on the other hand, is a fungus which is ubiquitous in the respiratory tract of mammals, with 90% of adult human beings possessing antibodies to this organism. However it is a known cause of oppor-

**Implication for health policy/practice/research/medical education:**
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tunistic and often life-threatening infection in the immune-compromised host (4).

Sato et al. have isolated *Herpes viruses* by Multiplex polymerase chain reaction from the adenoids of 35 young children after adenoidectomy. Most common viruses, detected in more than 15% of specimens, included *Adenoviruses*, HHV-7, *Epstein-Barr viruses*, *Enteroviruses,* and *Rhinoviruses*. Multiple viruses were isolated from 28 adenoids (5).

In another study by Berger et al. EBV, *cytomegalovirus*, (CMV), HHV-6, and HHV-7 were isolated from the tonsils and/or adenoids of young children by quantitative PCR. They observed that beta- and gamma *Herpes viruses* exert mutual influences on each other; for example it was seen that lower levels of HHV-6 DNA were detected in adenoids with positive results for CMV DNA than in adenoids with no CMV DNA. On the other hand higher levels of CMV and HHV-7 were found in adenoids that did not harbor EBV DNA (6).

## 2. Objectives

We performed this study to find out the frequency of 5 latent *Herpes viruses* and *Pneumocystis jirovecii* in the saliva of asymptomatic immune-competent children, and also to determine the rate of co-infection with different pathogens.

## 3. Patients and Methods

This was a cross-sectional study performed on 150 immune-competent children, 1 to 15 years old, equally divided among three age groups of 1-5, 6-10, and 11-15 years. They were approached and enrolled from outpatient clinics for routine check-ups, minor childhood ailments or minor surgery, in Mofid children's hospital, a university-affiliated hospital, during the study period of 3 months, from 22nd May to 21st August 2011.

Children with fever, failure to thrive, chronic illnesses or congenital abnormalities were excluded from the study. After obtaining written consent from the parents, members of the study team took medical histories and examined the child to rule out comorbid conditions, then trained investigators obtained samples from the oral cavity by special foam swabs supplied by Malvern medical developments, (Worcester), which were rubbed on the gums and transferred to microtubes containing 200 mL of Saline phosphate buffer. Specimens were immediately sent to the laboratory and stored at -70°C till all 150 specimens had been collected. Samples were then thawed and subjected to the process of DNA extraction. I-genomic CTB DNA extraction mini Kit (Cat. No 17341) manufactured by Intron® was used for DNA extraction. PCR was used for isolating the 6 microorganisms including 5 latent *Herpes viruses* and *Pneumocystis jirovecii*. Samples were then submitted for electrophoresis performed on Agarose gel supplied by Bioneer® (South Korea). Electrophoretic patterns were viewed by Uvitec and the photographs saved.

### 3.1. Statistical Methods

We used STATA/SE 11.0 to calculate binomial exact confidence interval for frequency of each microorganism. Coexistence or association between any microorganism pairs, controlled for presence/absence of other three microorganisms performed by logistic regression analysis. P-values less than 0.05 were considered statistically significant.

## 4. Results

From the 150 children included in the study, 88 (58.7%) were male. At least one microorganism was isolated from the saliva of 148 children, (98.6%). In 13 cases (8.6%) only 1 microbe was detected, in 24 youngsters (16%), 2 different strains were found, 3 microorganisms were isolated from 20 children (13.3%), 4 from 54 individuals (36%), and 5 from 37 subjects (37%). HSV was the most frequent organism detected in 80.7% of children, while *P. jirovecii* was the least frequent, isolated from 32.7%. Other viruses were HHV6, 66.7%; CMV 66%; HHV7 64.7%; and EBV 37.3% in order (*Table 1*).

No significant difference was found in the rate of isolated microorganisms in the two sexes or in different age groups, except for CMV which was significantly less frequent in subjects between the ages of 6 to 10 years (P value = 0.001).

Regression analysis revealed a positive association between HHV6 and HHV7 (OR = 1599.4; P value < 0.001); positive correlation was also found between EBV and CMV (OR = 3.1; P value = 0.012). In contrast negative correlation was found between *P. jirovecii* and the three viruses of the *Herpes virus* group including EBV, HSV1, and CMV (ORs were 0.27, 0.36 and 0.48 respectively); these correlations were statistically significant for EBV and HSV1 and marginally significant for CMV; P values were 0.006, 0.028, and 0.075 respectively).

**Table 1.** The Number and Percent of HHV6, HHV7, CMV, EBV, HSV1 and *Pneumocystis jirovecii*

| | Percent | No. |
|---|---|---|
| **HHV6** | 66.7 | 100 |
| **HHV7** | 64.7 | 97 |
| **CMV** | 66 | 99 |
| **EBV** | 37.3 | 56 |
| **PCJ** | 32.7 | 49 |
| **HSV1** | 80.7 | 121 |

## 5. Discussion

Our findings revealed that the 5 *Herpes viruses* and *P. jirovecii* are common inhabitants in the oral cavity of young

children, HSV1, being the most prevalent of these organisms. Rate of detection of the microorganisms varies with the sampling and isolation techniques; we used conventional PCR for detection of these microorganisms. Asymptomatic shedding of HSV1 in saliva has been reported to vary between 0.45% and 74% in different studies (7).

In Sato's study on children *Adenovirus* was the most common isolated pathogen in 80% of children, HHV7 was found in 51.4%, EBV in 42.9%, and *Enteroviruses* in 31.4%; while, HSV1, CMV, and HHV6 were detected only in 2.9% of cases; these figures are widely different from ours (5). These differences could be due to the difference in the sampling sites in the two studies, and also by the techniques used for isolation. In addition their specimens were collected from children who had undergone adenoidectomy, presumably from infected adenoids, while our samples were obtained from the oral cavity of healthy children with no chronic respiratory infections. In Berger's study, however, EBV was isolated in 80% of tonsils and adenoids, CMV from 66%, HHV6, and HHV7 from 77% (6).

In a study on healthy adults EBV was detected in the saliva of all individuals at-least once during the 14-month study period; the rate of viral shedding varied during different seasons, being highest during spring and fall. The authors postulate that the reasons for this variation is multifactorial; one factor influencing viral shedding could be the high exposure to allergens during these seasons which may result in increased oral secretions, or it may be due to a rise in immune cells and as EBV resides in B cells, an influx of infected cells would cause increased viral shedding (8).

In our study concomitant presence of two or more microorganisms was noticed in most children; we also found a positive correlation between HHV6 and HHV7 and between CMV and EBV; while a negative association was revealed between *P. jirovecii* and the *Herpes viruses*, EBV, CMV and HSV1.

It has been shown in previous studies that viruses mutually influence each other (2, 9). Katasafanas et al. have shown that HHV7 can reactivate HHV6 in vitro, which after reactivation starts replicating, and results in disappearance of HHV7, showing that sequences of activated HHV6 can replicate by using the genetic material belonging to HHV7 (9).

Although we observed a positive correlation between CMV and EBV in our samples, but Ling et al. found no correlation between these two viruses; EBV was isolated from 80% of their subjects and CMV from only 13% (8). However, their subjects were healthy adults, and we got our samples from children. This association is important in transplant patients as simultaneous presence of these 2 viruses is accompanied by a 4 to 6 fold increase in the occurrence of lympho-proliferative illnesses, as compared to EBV infection alone. It appears that CMV may modulate cytokines and TNF α to increase the replication of EBV (10). We do not know whether this association between these 2 viruses, seen in our cases, is an indication of transactivation like that seen with HHV6 and HHV7 in other studies, or just due to simultaneous shedding of the 2 viruses. It has been documented that transactivation, a process by which genes of one virus may be activated by the gene products of another virus, may happen between 2 heterologous viruses; for example HHV6 is known to activate the EBV Zebra gene (2). Concomitant infection with EBV and CMV in patients with infectious mononucleosis results in blunting the immunological response, and may lead to persistence of the infection, resulting in longer duration of hospitalization (11, 12). PCR method appears to be the optimal method for detecting CMV in saliva with a sensitivity of 100% and a specificity of more than 99%.

In our study *P. jirovecii* was isolated from the saliva of about one-third of asymptomatic children; these figures are comparable with those of some other researchers (13). Asymptomatic infection with *P. jirovecii* is very common in humans; antibodies against this pathogen can be detected in 90% of adults. DNA of *P. jirovecii* has been isolated from the nasopharyngeal secretions in 32% of immune-competent infants with mild upper respiratory infections in Chile (4). DNA extraction by Nested PCR on samples collected from the oral cavity was shown to have a sensitivity of 78%, and a specificity of 100% for detection of *P. jirovecii* (14).

We found a negative association between, *P. jirovecii* and the 3 latent *Herpes viruses*, (HSV1, EBV and CMV). However we did not find similar reports in our literature search and we do not know the significance of this finding. It could be due to our method of isolation conventional PCR as opposed to nested PCR. In addition, as noted previously, pathogens exert mutual influence on each other; presence of latent viruses causes an increase in CD4 and CD8 T cells, and it is postulated that reactivation of these viruses, though asymptomatic, may activate the immune system and modulate the host response to other pathogens through different mechanisms. The response of CD4 T cells to a viral infection leads to activation of the memory cells which are long-life circulating lymphocytes which recognize epitopes of that specific microorganism. These memory cells may cross-react with the antigens of a different pathogen; thus, the immune response to a new infection would be determined by the immune memory attained by the host from previous infections, (heterologous immunity) (15). In addition, T cell activated by bystander mechanism mediates production of cytokines (TNF α, Interferon gamma and interleukin). Bystander T cell activation has been observed in studies of *Herpes simplex virus* and other viruses leading to production of cytokines which can contain infection with other pathogens (16).

Since cellular immunity plays a major role in inhibiting *P. jirovecii* infection; it may be surmised that simultaneous infection with *Herpes viruses* and *P. jirovecii* would suppress the latter microorganism through the mechanisms discussed above, and this could be one reason for

the negative association observed between these pathogens in our study.

As mentioned above, infection with *Herpes viruses* at a young age leads to persistence of these viruses in a latent form, a process that activates the immunologic host response and may modulate and even suppress infections with heterologous pathogens; this phenomenon may be used to advantage in preventing infections with other pathogens by devising new vaccines comprising live attenuated viruses of the *Herpes virus* group. It would also be of great use in preventing opportunistic infections such as those with *P. jirovecii* in immune-compromised patients.

Our findings revealed that viral shedding of *Herpes viruses* and simultaneous presence of different pathogens is prevalent in immunocompetent children. Further studies are needed to clarify the implications of these findings, and the mutual influence of multiple co-infecting microorganisms.

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## Authors' Contribution

Dr. Abdollah Karimi: idea formulation, research methodology guidance and critical review, Dr. Mehran Ahmadi : research progression, Dr. Farideh Shiva : paper writing and literature review , Dr. Fatemeh Fallah : laboratory supervision, and Dr. Sedigheh Rafiei Tabatabaei : paper editing and literature review.

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