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ORIGINAL ARTICLE

Variabilities of Salivary HHV-6/7, SIgA Levels, and POMS 2 Scores Over Two Weeks Following Long-term Restriction from Practice in Athletes

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ABSTRACT

Background. Persistent physical fatigue (PPhF) is one of the most common issues in athletes; salivary human herpes virus 6 and/or 7 (HHV-6/7) have recently attracted attention as novel microbiological markers for its assessment. **Objectives.** We aimed to investigate potential variabilities of salivary HHV-6/7 levels in athletes, along with traditional assessments. **Methods.** We conducted an observational study over two weeks including 54 healthy male university athletes (n = 44) and non-athletes (n = 10). We used a questionnaire on daily life to monitor the participants' states and habits. The main measures were salivary HHV-6/7 and secretory immunoglobulin A (SIgA) levels, and total mood disturbance (TMD) scores in the Profile of Mood States. **Results.** In the questionnaire on daily life, subjective fatigue increased in the athletes ($p < 0.05$), while the health condition and daily habits did not change in either group ($p > 0.05$); accordingly, fatigue may be due to training-induced physical stressors rather than pathological events or changes in daily habits. Salivary HHV-6 levels increased only in the athletes after one week ($p < 0.05$), whereas salivary HHV-7 and SIgA levels, and TMD scores remained unchanged ($p > 0.05$). **Conclusion.** These findings suggested that salivary HHV-6 may be a more sensitive marker of PPhF than others.

KEYWORDS: ***Fatigue, Saliva, Biomarker, Infection, Immune Function.***

INTRODUCTION

Physical fatigue accompanied by daily intensive training is one of the most common problems in athletes. Athletes often experience transient physical fatigue owing to high-intensity training; however, adaptation occurs following sufficient recovery (1). Nevertheless, if recovery is insufficient, fatigue will accumulate and lead to "prolonged maladaptation" (2), which results in persistent underperformance, overreaching, and overtraining syndrome (OTS) (3). Therefore, to improve performance, it is necessary to properly assess persistent physical fatigue (PPhF) accumulated over a certain period of time, rather than transient one occurring immediately after exercise.

Traditional assessments for PPhF include subjective and objective types of measures. A typical subjective assessment is the Profile of

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Mood States 2nd Edition (POMS 2) (4). As this questionnaire can be administrated in a short time and also in large groups, it is practical in the sports field. Several previous studies have reported its usefulness in assessing physiological fatigue and diagnosing OTS (5, 6). For objective assessments, liquid biopsies of blood, urine, and saliva are often applied. Saliva is the most preferable biofluid for assessment in the sports field because it can be collected easily, non-invasively, rapidly, and frequently without a medical license (7). One of the representative salivary biomarkers is secretory immunoglobulin A (SIgA) (8). Its levels decrease after high-intensity training (9, 10), and are associated with upper respiratory tract infection (11, 12); thus, it has been regarded as a marker of the physical condition of athletes. Despite many studies on subjective and objective assessments for PPhF, no proper markers exist according to the current consensus (2).

In contrast to physiological markers produced in the human body (e.g., SIgA, cortisol, and testosterone) that have been mainly used (7), salivary human herpesvirus 6 and/or 7 (HHV-6/7) have recently attracted attention as novel microbiological markers. Salivary HHV-6/7 can be considered highly applicable markers because both viruses are known to establish latent infection at a high rate of more than 90% in the general adult population (13). Salivary HHV-6/7 was detected in 100% of judo athletes (14). Furthermore, they can be considered sensitive and specific markers of PPhF. This is because several previous studies have reported that salivary HHV-6/7 levels were increased in response to physiological fatigue accompanied by physical stressors (14-17). Meanwhile, those levels were not different between healthy individuals and patients with pathological fatigue not accompanied by physical stressors such as chronic fatigue syndrome (17).

Although salivary HHV-6/7 may be applicable markers of PPhF in athletes, the weekly variabilities of salivary HHV-6/7 in athletes have remained unknown. Moreover, to apply these novel markers, it is necessary to have higher sensitivity and/or specificity than other traditional assessments. Therefore, the present study aimed to investigate the weekly variabilities of salivary HHV-6/7 levels in athletes and examine the difference with the variability of the POMS 2 scores or salivary SIgA levels. We hypothesized that the salivary HHV-6/7 demonstrates higher responsivity to PPhF than traditional assessments.

MATERIALS AND METHODS

Ethical Consideration. This study was approved by the ethical committees of the Faculty of Health and Sport Sciences of the University of Tsukuba (Tai 019-95). Under the Declaration of Helsinki, all participants were informed, in writing and verbally, about the purpose and method of the study, including its possible risks. Written consent was obtained from all participants. None of the participants complained of discomfort or adverse physical conditions during the survey.

Participants. A total of 54 healthy male university athletes (baseball and rugby players) and non-athletes (student staff who belongs to the team but do not train) as controls volunteered to participate in this investigation. The characteristics of the participants in each group are as follows: Athletes (n = 44); 20.8 ± 0.2 yr, 175.1 ± 0.8 cm, 81.0 ± 1.6 kg, non-athletes (n = 10); 21.2 ± 0.2 yr, 174.9 ± 1.7 cm, 70.1 ± 1.5 kg. At the time of recruitment, we confirmed their health status and habits. None of the participants had smoking habits or drank alcohol for more than three days a week. Two participants took medication, but since both were analgesics and not antivirals, we considered that these drugs did not affect this investigation. The participants were instructed to refrain from the following before collecting saliva: consuming alcohol and caffeine within 12 h, eating and brushing teeth with dental paste within 1 h.

Study Design. This observational study was conducted over two weeks, from August 7th to 21st, 2020. Before the investigation period, the practice had been prohibited for over a month because of the spread of COVID-19; thus, the participants were presumed to have taken sufficient rest. The practice schedules followed during the investigation period are presented in Table 1. The practice time per day was limited to 2 h by their university to keep preventing the infection. Typical examples of practice programs are as follows: in baseball players, warm-up 15 min, play catch 15 min, fielding practice 30 min, batting practice 30 min, fitness training 30 min; in rugby players, warm-up 30 min, speed agility and quickness training 10 min, skill training 10 min, game style practice 30 min, contact training 10 min, and positional practice 30 min. Baseline measurements were undergone on August 7th for baseball players and non-athletes, and on August 10th for rugby players; this discrepancy stems

from the difference in the resuming day of practice. Thereafter, the measurements were conducted on August 14th (week 1) and 21st (week 2) for all participants. They consisted of questionnaires and saliva collection and were conducted before breakfast (7:00–8:00 AM for baseball players and non-athletes, 8:00–9:00 AM for rugby players).

Questionnaires. a) Questionnaire on Daily Life. Daily life can influence the fatigue state; thus, we monitored it using a questionnaire. The items included health conditions, sleep time, appetite, and subjective fatigue. For sleep time, participants answered the weekly average time. The other items were rated on a 5-point scale from "Strongly disagree (1)" to "Strongly agree (5)."

b) POMS 2. The POMS 2 is accepted as a traditional subjective assessment (4). Before conducting the POMS 2, the participants were informed how to fill in the form. The researchers were present during the entire survey to answer potential questions from participants and to check for missing items in the form. Moreover, coaches were not allowed to be present to enable athletes to give honest answers. After the results were obtained, we calculated the total mood disturbance (TMD) score by subtracting vigor-activity from the total score of tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia, and confusion-bewilderment. A lower TMD score indicates a better condition.

Saliva Collection. Saliva samples were collected as previously reported (18). The participants sat for 5 min and rinsed their oral cavity three times with distilled water for 30 sec. After 5 min, they swallowed the saliva stored in the oral cavity and chewed the sterile swab (Salivette, Sarstedt, Nümbrecht, Germany) once per second for 1 min. The Salivette swabs were then centrifuged at 5000 rpm for 15 min to extract the saliva. The extracted samples were weighed to determine the salivary secretion rate and stored at −80 °C until assays were performed.

Biomarker Assays. a) SIgA Assay. Salivary SIgA, an immunological marker, was used for traditional objective assessment. Its levels were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (18). Salivary SIgA levels were calculated by multiplying the concentration by the amount of secreted saliva; this value was expressed as μg/min.

b) HHV-6/7 Assays. Salivary HHV-6/7 levels were used as novel markers of PPhF. Viral DNA was extracted from 200 μL saliva using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The DNA was eluted in 25 μL of elution buffer. HHV-6/7 DNA copies were quantified by TaqMan quantitative polymerase chain reaction (qPCR) assay with an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The TaqMan qPCR method was carried out as previously described (14).

Amplifications were performed in a total volume of 10 μL containing 5 μL of premix, TaqPath qPCR Master Mix, CG (Applied Biosystems), 0.04 μL of PCR forward primer (50 μM), 0.04 μL of PCR reverse primer (50 μM), 0.25 μL of TaqMan probe (10 μM), 2 μL of viral DNA, and 2.67 μL of distilled water. The primer sequences used for qPCR were as follows: HHV6 forward primer, 5'-CAA AGC CAA ATT ATC CAG AGC G-3'; HHV-6 reverse primer, 5'-CGC TAG GTT GAG AAT GAT CGA-3'; HHV-6 probe, 5'-FAM-CAC CAG ACG TCA CAC CCG AAG GAA T-MGB-3'; HHV-7 forward primer, 5'-ATG TAC CAA TAC GGT CCC ACT TG-3'; HHV-7 reverse primer, 5'-AGA GCT TGC GTT GTG CAT GTT-3'; and HHV-7 probe, 5'-FAM-CAC GGC AAT AAC TCT AG-MGB-3'. The cycle conditions were 95 °C for 20 sec, followed by 45 cycles of 95 °C for 3 sec and 58 °C (for HHV-6) or 60 °C (for HHV-7) for 30 sec. All samples were assayed in triplicates, and the median of the three values was used as the representative value since some low-concentration samples often show large deviations; this method helps to avoid eliminating them artificially. The salivary HHV-6/7 levels were calculated by multiplying the DNA copy number by the amount of secreted saliva; this value was expressed as $\log_{10}$ transformed copies/min.

Statistical Analysis. All data are expressed as mean ± SEM. Statistical analyses were performed using SPSS Statistics version 26 (IBM, Armonk, NY, USA). A two-way repeated analysis of variance (ANOVA) was used to examine the group × elapsed week interaction and to calculate the *p*-value, *F*-value, and partial eta squared (η_p^2) value for each marker. If a significant interaction was found, individual differences were determined using the Bonferroni post-hoc test. For all tests, a value of $p < 0.05$ indicated a statistically significant difference.

RESULTS

Questionnaire on Daily Life. Figure 1 shows the data of the questionnaire on daily life. We found no interaction in health conditions (p = 0.173, F = 1.787, η_p^2 = 0.033), and there were no significant changes over time (p = 0.104) or differences between groups (p = 0.163) regarding this parameter. In addition, there were no significant interactions in sleep time and appetite (p = 0.662, F = 0.372, η_p^2 = 0.007 and p = 0.823, F = 0.195, η_p^2 = 0.004, respectively), but the differences between groups were significant (p = 0.006 and p = 0.049, respectively). In subjective fatigue, we identified a significant interaction (p = 0.012, F = 3.818, η_p^2 = 0.068). The Bonferroni post-hoc test showed significant differences between baseline and weeks 1 and 2 in the athletes (p = 0.002 and p = 0.018, respectively), as well as between groups at weeks 1 and 2 (p = 0.015 and p = 0.005, respectively).

Table 1. Training Schedule.

August 2020							
	Mon	**Tue**	**Wed**	**Thu**	**Fri**	**Sat**	**Sun**
Date					7	8	9
Baseball					*0Practice	Practice	Practice
Rugby					-	-	-
Date	10	11	12	13	14	15	16
Baseball	-	Practice	Practice	Practice	*1Practice	Game	Game
Rugby	*0-	Practice	Practice	Practice	*1-	Practice	Practice
Date	17	18	19	20	21		
Baseball	Practice	Practice	Practice	Game	*2Practice		
Rugby	-	Practice	Practice	Practice	*2-		

-: rest day, *0: baseline measurement, *1: week 1 measurement, *2: week 2 measurement

*We conducted the sample collection and questionnaire before breakfast, leaving the data of measurement days unaffected by the practice or game of the day.

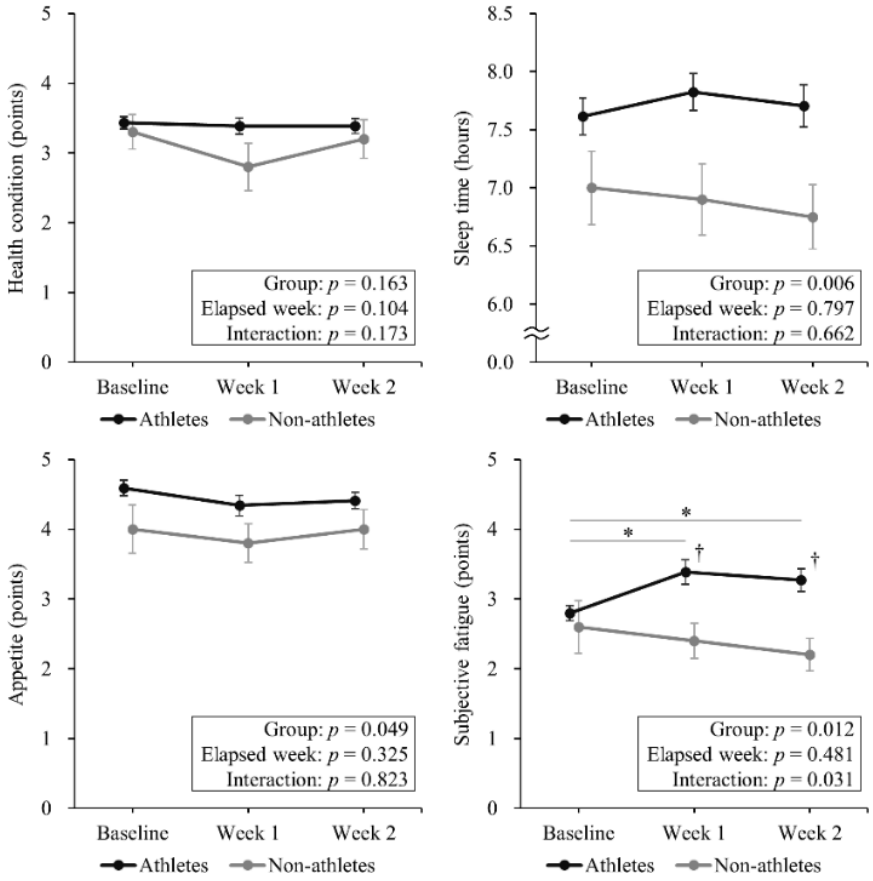

Figure 1. Variabilities of questionnaire on daily life.

The results of the post-hoc test showed *p < 0.05 in the comparisons between weeks and †p < 0.05, in the comparison between athletes and non-athletes.

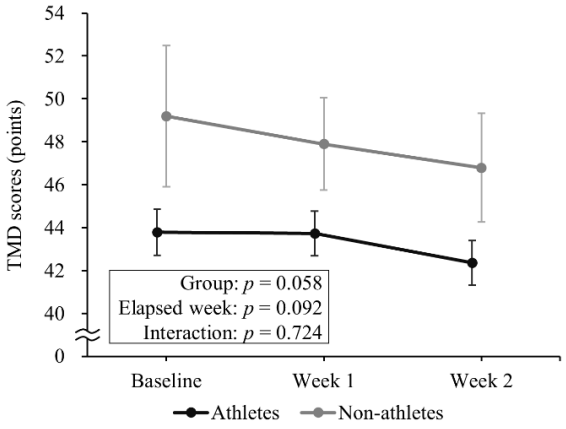

Figure 2. Variability of TMD scores.

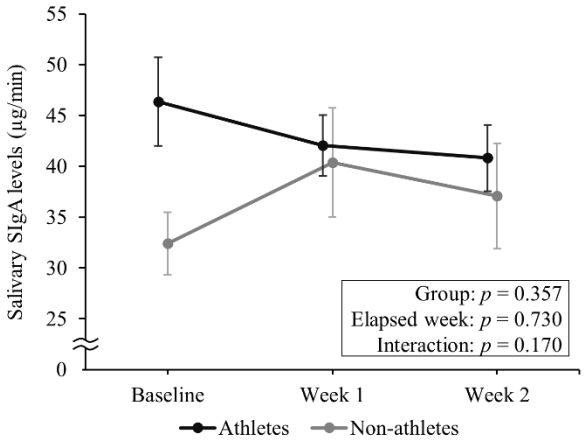

Figure 3. Variability of salivary SIgA levels.

POMS 2. Figure 2 shows the data of TMD scores. Two-way repeated ANOVA indicated that there was no significant interaction (p = 0.724, F = 0.283, η_p^2 = 0.005), and no significant change over time (p = 0.092) or difference between groups (p = 0.058).

Salivary SIgA Levels. Figure 3 shows the salivary SIgA data. Two-way repeated ANOVA revealed no significant interaction in salivary SIgA levels (p = 0.170, F = 1.857, η_p^2 = 0.034), and no significant change over time (p = 0.730) or difference between groups (p = 0.357).

Salivary HHV-6/7 Levels. The total detection rate of HHV-6/7 in saliva was 96.2% (52 of 54 subjects: 42 of 44 athletes, 10 of 10 non-athletes). Figure 4 shows the data of salivary HHV-6/7 levels. For salivary HHV-6, two-way repeated ANOVA indicated a significant interaction (p = 0.044, F = 3.215, η_p^2 = 0.060). The Bonferroni post-hoc test showed significant increases from baseline to weeks 1 and 2 in the athletes (p = 0.003 and p = 0.005, respectively), but did not show any changes in the non-athletes. Conversely, there was no significant interaction (p = 0.883, F = 0.027, η_p^2 = 0.001), and no significant change over time (p = 0.898) or difference between groups (p = 0.130) in salivary HHV-7 levels.

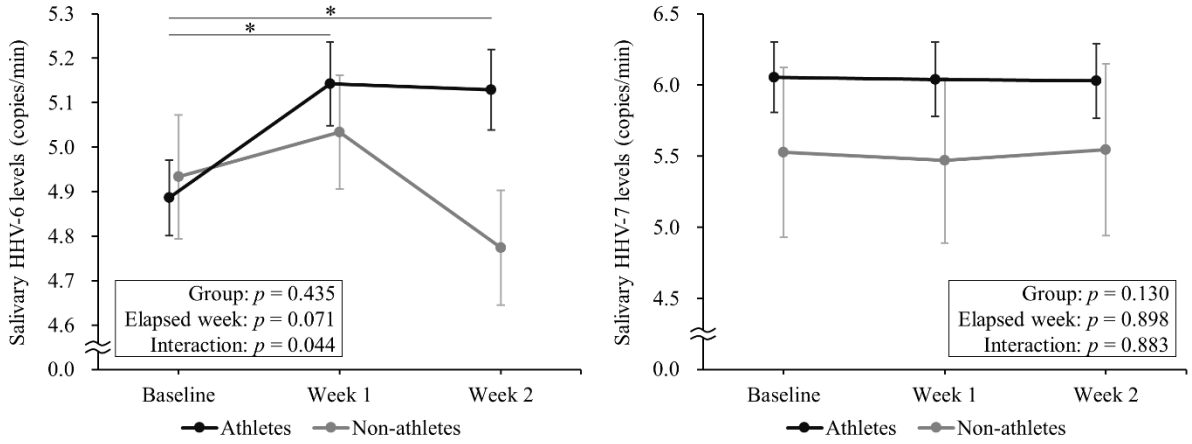

Figure 4. Variabilities of salivary HHV-6/7 levels.
The results of the post-hoc test showed *p < 0.05, in the comparisons between weeks.

DISCUSSION

We conducted an observational study over two weeks to investigate the weekly variabilities of salivary HHV-6/7 levels and to examine the potential difference in the variability of the TMD scores or salivary SIgA levels. Salivary HHV-6 levels increased significantly in the athletes, whereas those of HHV-7 did not change. In addition, there were no significant changes over time and no differences in salivary SIgA levels and TMD scores in either group.

At first, we considered the factors related to fatigue by the results of the questionnaire on daily

life. The lack of changes in health conditions in either group suggests that no participants experienced pathological fatigue. In daily habits, the athletes showed significantly longer sleep times and higher appetite; however, there was no change over two weeks. Thus, it seemed that the participants were not affected by the changes in their daily habits. There was a significant increase in subjective fatigue from baseline to weeks 1 and 2 in the athletes, unlike in non-athletes. Therefore, athletes may be affected by physical stressors due to practice rather than by pathological factors or lifestyle changes.

Based on the above assumptions, we considered the results of traditional assessments. In the subjective assessment, the TMD scores tended to be lower in the athletes than the non-athletes and tended to decrease gradually in both groups. A possible explanation could be that the activity improved their mood states regardless of any physical fatigue. A previous study reported that the vigor-activity subscale increased, while other negative subscales decreased following exercise (19). In the present study, since the investigation was started after a long-term restriction of outdoor activities, the resumption of practice or physical activity probably allowed the participants to improve their psychological condition. This would suggest that the POMS 2 has a risk of overlooking the actual PPhF because it may be affected by the motivation or life events of the athletes, as pointed out previously (17).

In the objective assessment, salivary SIgA levels demonstrated no change over weeks or difference between the two groups. This observation does not correspond with the previous report that repetitive training decreases the resting levels of salivary SIgA (20, 21). The discrepancy can be attributed to the good psychological condition of the athletes, as it has been reported that salivary SIgA levels are also affected by psychological stressors (22, 23). The tendency towards lower TMD scores in the athletes suggests their good psychological conditions; thus, it may have mitigated the effects of physical stressors due to training.

Here, we focused on the novel markers salivary HHV-6/7. In contrast to the abovementioned traditional assessments, salivary HHV-6 levels significantly increased only in the athletes. Considering the practice schedule, time, and typical examples presented in the material and methods section, it is surmised that the physical load was not high. Nonetheless, salivary HHV-6 levels increased significantly. Moreover, this result is in line with previous studies (14-17); thus, salivary HHV-6 may have high responsivity to PPhF. In particular, a cross-sectional study on nurses reported that the salivary levels of HHV-6 were higher in the frequency of night shifts, and the salivary SIgA levels were not associated (15). Therefore, salivary HHV-6 might have independent meaning from measures of salivary SIgA.

The reason why HHV-6 can reactivate independently of immunity is considered because HHV-6 is affected by endoplasmic reticulum (ER) stress rather than immuno-suppression. ER stress is induced by exercise-related factors such as the rapid flow of Ca^{2+} or oxidative stress (24). The previous study reported that salivary HHV-6/7 levels increased after judo training along with oxidative stress markers (14). Hence, salivary HHV-6 may be a marker with unique implications that differ from salivary SIgA. Incidentally, ER stress also results in the production of interleukin (IL)-6. IL-6 well known as a representative "myokine," increases rapidly in blood levels after exercise and causes underperformance (25-27). Thus, salivary HHV-6 may reflect physiological fatigue directly or indirectly.

Meanwhile, no interaction was observed in salivary HHV-7 levels. A previous study on the Japan Self-Defense Forces has reported that salivary HHV-7 levels significantly increased over five weeks (17). Moreover, Kondo mentioned the possibility that salivary HHV-7 may reflect longer-term fatigue than HHV-6 (28). Accordingly, the experimental period in the present study may not have been sufficient to observe the variability in salivary HHV-7 levels.

In addition, salivary HHV-6 and -7 levels showed different variabilities, although they have very similar characteristics. This was probably caused by the differences in their types of infection. While HHV-6 has been confirmed to infect latently, HHV-7 has been reported to infect persistently rather than latently (29). This may also be the reason for the difference in the DNA copy number between salivary HHV-6 and -7 in the present study. Given the paucity of studies on both salivary HHV-6 and -7 in athletes, these novel markers must be carefully investigated including underlying mechanisms in further studies.

There were two main limitations in this study. One is the particular set of after a long-term rest

due to the spread of COVID-19. Athletes are not restricted from activities for more than a month normally; thus, different results might be obtained if the athletes had done usual (high intensity and frequency) training. Paradoxically, we believe that this study provides valuable findings because of the few opportunities which can target well-rested athletes. The other is that we were unable to determine how much physical stressor can induce reactivation of HHV-6/7. To address the issue, exercise intervention studies are needed to examine sufficient physical stressors to increase the salivary HHV-6/7 levels from various perspectives such as exercise intensity, time, frequency, and modality. We predict that HHV-6 may be reactivated by a lower stressor than HHV-7 based on the results of this study.

In further studies, it will be noteworthy to examine the relationship between salivary HHV-6 and OTS. One of the distinctive symptoms of OTS is depression. Interestingly, a previous study reported that a protein produced by HHV-6, named small protein encoded by the intermediate stage transcript of HHV-6-1 (SITH-1), is strongly associated with the development of depression (30). In combination with the findings of the present study, it is possible that athletic training induces increasing in salivary HHV-6 levels, and it may cause the development of OTS. Since the pathogenesis of OTS is still unclear, this hypothesis should be tested.

In practical implication, it is necessary to pay attention to physical fatigue after long-term rest, regardless of the subjective condition in athletes. The results of this study showed that the TMD scores tended to decrease in contrast to the salivary HHV-6 levels increased in the athletes. This indicates that there is a risk of the potential accumulation of physical fatigue, even if the athletes feel in good condition. To avoid overlooking this risk, salivary HHV-6 might be useful.

CONCLUSION

We conducted an observational study on athletes and non-athletes over two weeks to investigate the variabilities of salivary HHV-6/7, SIgA levels, and TMD scores in POMS 2. As a result, salivary HHV-6 showed a significant increase in the athletes, but salivary HHV-7 levels did not. Moreover, there were no changes in TMD scores or salivary SIgA levels. These findings suggest that salivary HHV-6 may be a more sensitive PPhF marker than others; it may also be an independent marker from the immune marker. The association between the increase of the salivary HHV-6 levels and the development of the OTS should be examined in future studies.

APPLICABLE REMARKS

- The use of salivary HHV-6 is expected to allow for earlier detection of PPhF which other markers fail to do.
- Salivary HHV-6 levels increased independently of salivary SIgA levels; thus, the viral marker has a different meaning from the immune marker.

AUTHORS' CONTRIBUTIONS

Study concept and design: Shinsuke Tamai. *Acquisition of data:* Shinsuke Tamai, Ryota Sone, Akari Kitahara. *Analysis and interpretation of data:* Shinsuke Tamai, Kai Aoki, Takehito Sugasawa, Kazuhiro Takekoshi. *Drafting the manuscript:* Shinsuke Tamai, Ryota Sone, Akari Kitahara. *Critical revision of the manuscript for important intellectual content:* Koichi Watanabe. *Statistical analysis:* Shinsuke Tamai. *Administrative, technical, and material support:* Kai Aoki, Takehito Sugasawa, Kazuhiro Takekoshi. *Study supervision:* Koichi Watanabe.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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