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Comparison of DNA extraction methods for genotyping equine histidine-rich glycoprotein insertion/deletion polymorphisms using oral mucosa swabs and feces

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ABSTRACT

Previously, we demonstrated unique insertion/deletion polymorphisms of equine histidine-rich glycoprotein *(eHRG)* with five genotypes composed of 45-bp or 90-bp deletions in the histidine-rich region of *eHRG* in Thoroughbred horses. Although leukocytes are typically used to collect DNA for genotyping, blood sampling from animals is sometimes difficult and invasive. Moreover, the method for extracting DNA from blood leukocytes involves complicated steps and must be performed soon after blood sampling for sensitive gene analysis. In the present study, we performed *eHRG* genotyping using DNA, isolated from oral mucosa swabs collected by rubbing the mucosa on the underside of the upper lip of horses and 100 mg of freshly excreted feces obtained by scraping their surface. In the present study, we performed *eHRG* genotyping using DNA isolated from oral mucosa swabs and feces of horses (18 Thoroughbreds, 17 mixed breeds, 2 warm bloods), and compared the accuracy of this method with that of the method using DNA from leukocytes. The DNA derived from oral mucosa swabs was sufficient in quantity and quality for *eHRG* genotyping. However, DNA derived from fecal samples requires a more sensitive detection system because of contamination with non-horse DNA, and the test quality is low. Collection of oral mucosa swabs is less invasive than blood sampling; further, oral swabs can be stored for a longer period in a specified high-quality solution. Therefore, collecting DNA samples from oral mucosa swabs is recommended for the genetic analysis of not only horses but also other animals that are not accustomed to humans.

1. Introduction

Histidine-rich glycoprotein (HRG) is a 75-kDa single-polypeptide chain plasma protein present in many species, from mammals to aquatic invertebrates (Nair & Robinson, 1999; Poon et al., 2011). Due to its ability to interact with various ligands, including heparin, phospholipids, plasminogen, fibrinogen, immunoglobulin G, C1q, heme, and zinc ions, HRG is thought to be involved in the immune response, coagulation, and angiogenesis (Jones et al., 2005; Leung, 1986; Lijnen et al., 1983; Manderson et al., 2009; Priebatsch et al., 2017; Silverstein et al., 1985). HRG is associated with the pathogenesis and severity of systemic inflammatory response syndrome and sepsis in humans, and its plasma concentration decreases in severely ill patients, indicating that HRG is a promising biomarker (Kuroda et al., 2018; 2021). Previously, we reported polymorphisms in five genotypes composed of 45-bp or 90-bp deletions in the histidine-rich region of equine *HRG* (*eHRG)* using DNA samples collected from thoroughbred horses (Muko et al., 2019; 2023). Due to their various biological functions, genetic polymorphisms in *eHRG* may be involved in athletic performance, productivity, and susceptibility to infectious diseases in horses.

DNA is usually collected from the blood or tissues because of its high yield and quality (Zemanova, 2020; 2021). However, blood or tissue

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sampling is sometimes invasive, with negative implications for animal welfare. Therefore, less invasive methods should be established to alleviate animal welfare problems. Moreover, more convenient methods reduce the workload of veterinarians and accelerate research. In the present study, we attempted to perform *eHRG* genotyping using DNA isolated from the oral mucosa swabs and feces of horses and compared its quantity and quality with that of DNA from leukocytes. Herein, we report that oral mucosa swabs, which are less invasive and can be stored for longer periods than blood samples, may be another option for collecting DNA from horses and other animals.

2. Materials and methods

2.1. Animals

All experiments with thoroughbred, mixed-breed, warm-blooded horses were performed according to the standards specified in the guidelines provided by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology. Horse blood samples and mucosal swabs were collected in accordance with the guidelines and regulations of the Tokyo University of Agriculture and Technology. All methods were performed in accordance with the ARRIVE guidelines. The study was approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology (approval nos. 30–104, R02–46, R03–50, R04–85, and R05–99). Samples were collected from horses kept in the research stable of the Tokyo University of Agriculture and Technology (Tokyo, Japan), Shadai stallion station (Hokkaido, Japan), and Horse Resort S (Aichi, Japan) according to the aforementioned guidelines, with the consent of each stable.

2.2. DNA extraction

Genomic DNA (gDNA) from Thoroughbred horses (13 males and 5 females, aged 3–10 years, mean age ± SD = 5.9 ± 1.9), mixed breed horses (15 males and 2 geldings, aged 1–22 years, mean age ± SD = 5.9 ± 5.0), and warm blood horses (2 geldings, aged 21 years each) was extracted from blood leukocytes, oral mucosa swabs, or feces. gDNA was collected from blood leukocytes as described previously (Muko et al., 2023). Briefly, blood was collected from the left jugular vein using a 22-gauge needle and a 20-ml syringe. Blood was transferred to a heparinized-centrifuge tube soon after the collection and centrifuged at 1500 rpm for 15 min at 4 °C. After centrifugation, the plasma was removed and the buffy coat was then used for gDNA extraction using the Maxwell RSC Tissue DNA Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The kit is optimized for the use with the Maxwell RSC Instrument (Promega) which enables the automated extraction of nucleic acids. An oral mucosa swab sample was collected by rubbing the mucosa on the underside of the upper lip five to seven times with a specified cotton swab using Isohelix DNA/RNA buccal swabs (SK-2; Isohelix, Harrietsham, Kent, UK) and each collected swab head was soaked with 500 μl DNA/RNA Shield (Zymo Research, Irvine, CA, USA). Cotton swabs were stored at room temperature for more than one week in the dark. Each tube containing a cotton swab was then vortexed for 30 s, and the cotton swab was removed from the solution using tweezers. gDNA was purified using the remaining solution and the Maxwell RSC Tissue DNA Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. Fecal samples were collected immediately after defecation and placed in the plastic bag for transport to the laboratory. One hundred milligrams of the feces were suspended in 400 μl DNA/RNA Shield. The solution was mixed by vortexing, and visible detritus was removed using tweezers. The gDNA was then purified using the solution and Maxwell RSC Tissue DNA Kit (Promega). Collected DNA yield and purity of the collected DNA (260/280 nm or 260/230 nm) were assessed using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). In the present study, DNA yield was calculated using leukocytes from 1 ml of whole blood, 1 cotton swab of the oral mucosa, and 1 g of feces per unit.

2.3. eHRG genotyping

Polymerase chain reaction (PCR) analysis of *eHRG* was performed using GoTaq Master Mix (Promega) (Muko et al., 2023) and PrimeSTAR Max DNA polymerase (TaKaRa Bio, Shiga, Japan). Initially, each gDNA (leukocytes [40 ng], oral mucosa [20 ng], feces [50, 100, or 500 ng]) was added to 10 μl of a reaction mixture containing GoTaq Master Mix (Promega) and a pair of primers (leukocytes gDNA: 0.5 μM, oral mucosa and feces gDNA: 0.25 μM, Forward: 5′-ACTCTGGTCGGCATGAGCATA-3, ′ Reverse: 5′-TTTGTGTTTATTACTGGTCACATT-3′). PCR was performed with TaKaRa PCR Thermal Cycler Dice Touch (TaKaRa Bio) according to the following protocol: Initial step of 2 min at 95 °C; followed by 25 (leukocytes gDNA) or 40 (oral mucosa and feces gDNA) cycles of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C.

PrimeSTAR Max DNA polymerase (TAKARA Bio) was used for more sensitive detection. Briefly, each gDNA (leukocytes [40 ng], oral mucosa [20 ng], feces [100 ng]) was added to 10 μl of a reaction mixture containing PrimeSTAR Max DNA polymerase and 0.4 μM of the same pair of primers previously described. PCR was performed with TaKaRa PCR Thermal Cycler Dice Touch (TaKaRa Bio) according to the following protocol: Initial step of 2 min at 98 °C; followed by 25 (leukocytes gDNA), 30 (oral mucosa gDNA), or 35 (feces gDNA) cycles of 15 s at 98 °C, 15 s at 60 °C, and 10 s at 72 °C. The PCR products from all experiments were separated by 2 % agarose gel electrophoresis and visualized using ethidium bromide. Predicted band of *eHRG* can be detected at 812 bp in each PCR analysis.

2.4. Quantitative real-time PCR (qPCR) assay

The proportion of horse-derived DNA in oral mucosa swabs or fecal DNA samples was measured by qPCR, according to a previously described method (Köppel et al., 2011). gDNA derived from leukocytes in the buffy coat of six Thoroughbred horses (three males and three females) was mixed and used as a standard, representing 100 % equine-origin DNA. A seven-point, four-fold serial dilution series was prepared for the standard, ranging from 100 to 0.024 ng per reaction. DNA extracts of oral mucosa (50 ng) or feces (100 ng) were added to 20 μl of a reaction mixture containing TB Green® Premix Ex Taq II (TaKaRa Bio), ROX Reference Dye II, and 0.2 μM of primers against the horse growth hormone receptor (GenBank accession number: AF392878, Forward: 5′- CCAACTTCATCATGGACAACG C-3,′ Reverse: 5′-GTTAAAGCTTGGCTCGACACG-3′). qPCR was performed with QuantStudio 3 (Thermo Fisher Scientific) according to the following cycling protocol: Initial step of 30 s at 95 °C; followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C. Assuming uniform distribution of DNA within the samples and consistent PCR amplification efficiency, a standard curve was constructed using the Ct values of the standards (the equation is shown in Fig. 1). The amount of equine DNA in the samples (oral mucosa: 50 ng, feces: 100 ng) was calculated by interpolating the Ct values into the standard curve. This DNA amount was then divided by the total DNA amount (oral mucosa: 50 ng, feces: 100 ng) of the samples to calculate the proportion of equine DNA in the sample. This proportion was multiplied by the amount of extracted total DNA to calculate the equine DNA yield for each sample (Köppel et al., 2011).

2.5. Statistical analysis

Using JMP version 14.0 software (SAS Institute, Cary, NC, USA), comparisons between multiple groups were performed through one-way analysis of variance, followed by the Steel–Dwass test. Due to the high variability and non-normal distribution of the biological samples in this study, the Steel-Dwass test was selected. This test does not require an assessment of whether the distribution is normal or not. For the comparison of data between two groups, the Wilcoxon rank-sum test was

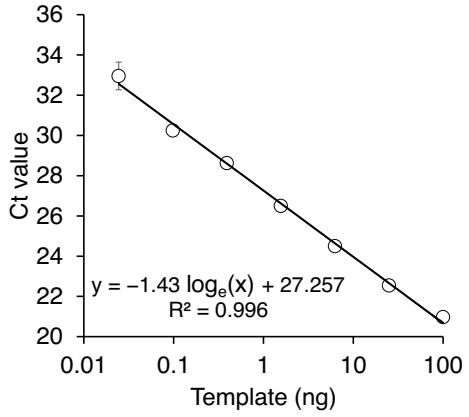

Fig. 1. Standard curve of quantitative real-time polymerase chain reaction (qPCR) assay. Genomic DNA derived from peripheral leukocytes of six Thoroughbred horses was used as the standard and a seven-point dilution series was prepared (100 ng to 0.024 ng). qPCR was performed using TB Green Premix Ex Taq II and primers against the horse growth hormone receptor. A standard curve was constructed using the Ct values of the standard. The quantity of horse-derived DNA was calculated using the equation ($y = -1.43\log_e(x) + 27.257$) and the calculated correlation coefficient (R^2) was 0.996 (y was Ct value and x was the quantity of horse-derived DNA).

used for similar reasons, as it does not require the assumption of normal distribution and is robust against outliers in the data.

3. Results

3.1. DNA quality and yield from each sample type

Blood samples, oral mucosa swab samples, and fresh fecal samples were collected from 37, 31, and 23 horses, respectively. gDNA was extracted from each sample, as described in materials and methods. Feces yielded the highest amount of total gDNA, followed by leukocytes from the blood. The gDNA yield per unit was the lowest from oral mucosa swabs (Table 1). Since OD260/280 is an index used to evaluate protein contamination, the protein content in total gDNA from leukocytes was the lowest, followed by that from the oral mucosa (Table 1). Protein contamination in the gDNA extracts of feces was the highest (Table 1). The OD 260/230 results showed that gDNA from oral mucosa swabs and feces contained more polysaccharides and phenol components (Cagil et al., 2011; Kagan et al., 2019; Porebski et al., 1997; Sahu et al., 2012) than that from leukocytes. To detect horse-specific genes in the samples, we used a method established previously (Köppel et al., 2011). A mixture of gDNA derived from the peripheral blood leukocytes of six Thoroughbred horses was utilized as the standard, representing 100 % horse-origin gDNA. qPCR for the horse growth hormone receptor gene was performed using samples at seven consecutive concentrations (0.024–100 ng of template). As shown in Fig. 1, a graph with high linearity was obtained by plotting the Ct values at each concentration on a logarithmic scale, demonstrating a suitable standard curve. Using this standard curve, the Ct values of DNA extracted from oral mucosa swabs and feces were extrapolated to calculate the gDNA concentration of each sample, and the percentage of horse gDNA among the total gDNA recovered from the samples was determined. The purity of the horse-derived gDNA in the oral mucosa swabs exceeded 100-fold to that in the feces (Table 2). However, there was little difference in horse gDNA yield between oral mucosa swabs and feces (Table 2).

3.2. eHRG genotyping using gDNA from each sample format

Templates were created from the extracted gene samples, and *eHRG* detection was performed using PCR (Muko et al., 2023). A clear band was detected in samples derived from oral mucosa swabs, although its intensity was slightly lower than that of samples derived from blood. In contrast, no clear bands were observed in the fecal-derived samples, even when the amount of template used in the reaction was increased (Fig. 2A). Therefore, we performed detection using PrimeSTAR Max DNA polymerase, which has a higher detection sensitivity, and found that the intensity of the bands became stronger in samples derived from oral mucosa swabs, and clear bands were detected in samples derived from feces at the predicted size (812 bp) of the PCR product. However, nonspecific laddering bands were observed in all samples (Fig. 2B).

4. Discussion

Most researchers and clinicians are aware of the importance of genetic analyses and diagnoses. Research on genes has also progressed in the field of veterinary science, and genetic testing is now commonly used to assess the risks of certain diseases and for drug selection of mast cell tumors in dogs (Giantin et al., 2012; Gil, 2015; Vozdova et al., 2019). Particularly in equine science, genetic testing is increasingly used for parent-child identification, individual identification, and doping determination in racehorses (Holl et al., 2017; Tozaki et al., 2022; 2023). However, the collection of genes from animals that do not provide consent sometimes poses problems from an animal welfare perspective. We usually collect DNA from the blood or tissues, and these methods yield high-quality DNA in sufficient quantities (Muko et al., 2023). Blood and tissue sampling are sometimes invasive and have negative implications for animal welfare. Moreover, collecting DNA from blood or tissues is a more complicated procedure that requires a familiar technician. Therefore, less invasive and simplified methods should be established to alleviate animal welfare problems and accelerate research. In the present study, we investigated a more convenient

Table 1
Genomic DNA yield and quality from each source.

DNA source	Materials	*n*	DNA yield (μg)				OD 260/280				OD 260/230			
			Mean	Median	IQR	SE	Mean	Median	IQR	SE	Mean	Median	IQR	SE
Leukocytes	1 ml blood	37	8.76	8.33	6.22–10.29	0.62	1.86	1.86[a,b]	1.84–1.88	0.00	1.85	1.86[d,e]	1.70–2.02	0.05
Oral mucosa	1 swab	31	1.02	0.88	0.69–1.24	0.09	1.64	1.64[a,c]	1.58–1.69	0.02	0.49	0.52[d,f]	0.24–0.68	0.04
Feces	1 g	23	129.64	124.74	73.33–173.08	13.71	1.51	1.52[b,c]	1.47–1.55	0.02	0.71	0.71[e,f]	0.63–0.77	0.02

Abbreviations: IQR, interquartile range; SE, standard error.
Statistical analysis of median values was performed by a Steel–Dwass test.
[a] $P < 0.0001$: leukocytes vs. oral mucosa; protein contamination was lower in samples from leukocytes than in those from the oral mucosa.
[b] $P < 0.0001$: leukocytes vs. feces; protein contamination was lower in samples from leukocytes than in those from feces.
[c] $P < 0.0001$: oral mucosa vs. feces; protein contamination was lower in samples from the oral mucosa than in those from the fecal samples.
[d] $P < 0.0001$: leukocytes vs. oral mucosa; polysaccharide or phenol contamination was lower in leukocyte samples than in the oral mucosa samples.
[e] $P < 0.0001$: leukocytes vs. feces; polysaccharide or phenol contamination was lower in samples from leukocytes than in those from feces.
[f] $P < 0.005$: oral mucosa vs. feces; polysaccharide or phenol contamination was lower in the fecal samples than in the oral mucosa samples.
Owing to the different amounts of each sample used for detection, statistical comparisons of the extraction volumes were not performed.

Table 2
Comparison of equine DNA quantity between oral mucosa and feces derived samples.

DNA source	*n*	Horse-derived DNA (%)				Horse DNA yield (μg)			
		Mean	Median	IQR	SE	Mean	Median	IQR	SE
Oral mucosa	10	3.784	3.229[a]	1.065–5.878	0.954	4.85	3.24	0.74–8.78	1.47
Feces	10	0.030	0.018[a]	0.012–0.020	0.010	5.08	2.79	1.80–3.28	2.07

Abbreviations: IQR, interquartile range; SE, standard error.
Statistical analysis of median values was performed by a Wilcoxon rank sum test.
[a] $P < 0.0005$: oral mucosa vs. feces; horse-derived DNA (%) was lower in fecal samples than in oral mucosa samples.
Owing to the different amounts of each sample used for detection, statistical comparisons of the extraction volumes were not performed.

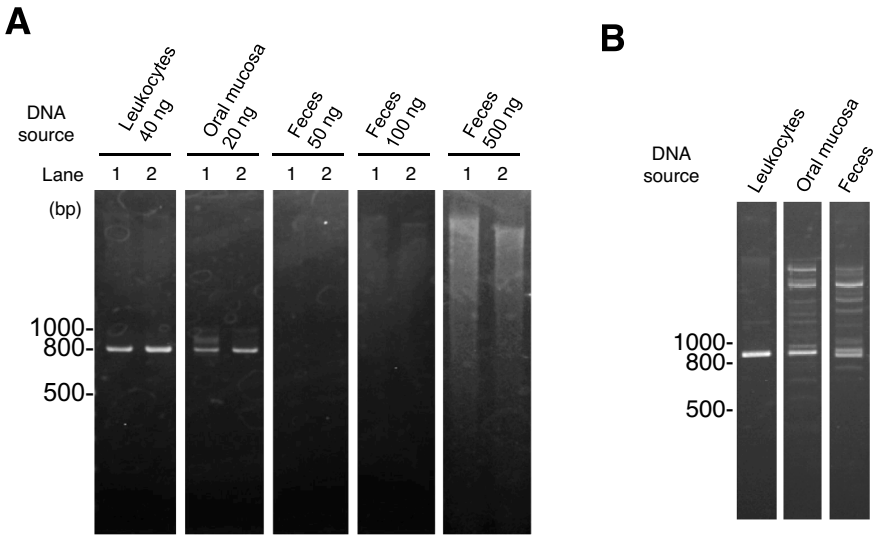

Fig. 2. Equine histidine-rich glycoprotein genotyping using genomic DNA from each source. The histidine-rich region of equine histidine-rich glycoprotein was amplified by polymerase chain reaction using genomic DNA extracted from leukocytes, oral mucosa swabs, and feces of wild type horses. Two kinds of DNA polymerase were used: GoTaq Master Mix (A) or PrimeSTAR Max DNA polymerase (B). For the GoTaq Master Mix (A), typical results of different horses were shown as lanes 1 or 2 in each detection. The polymerase chain reaction products were separated on 2 % agarose gel and stained with ethidium bromide.

and less invasive method for gene collection from horses to analyze *eHRG* polymorphisms. Initially, we attempted to extract gDNA from horse coat hair. Gene extraction requires hair roots; it was necessary to collect many skin hairs to ensure sufficient DNA because the amount of DNA that could be extracted from each hair root was small (Tozaki et al., 2020; Watts et al., 2012). Since this procedure is extremely invasive, we decided to use oral mucosa swabs and feces from horses. Using DNA isolated from oral mucosa swabs and feces of horses, we compared the DNA yield and quality with those of DNA from leukocytes. Although sufficient amounts of DNA were extracted from both samples for PCR analysis, contamination of DNA from microorganisms and feed is a serious concern. Therefore, we applied this method to detect horse meat contamination in food products (Köppel et al., 2011) and identified horse-specific genes in both samples. The ratio of horse genes in each sample was calculated using horse leukocyte-derived DNA as the standard. The results revealed that the number of horse genes that could be recovered from feces was very low, and that there was a high contamination of non-horse genes. In contrast, the purity of horse genes recovered from oral mucosa swabs was found to be adequate for PCR analysis, although not as high as that of leukocytes. Interestingly, OD 260/230 showed a large contamination of polysaccharide and phenol components in gDNA from both the oral mucosa and feces. Since the same extraction method was applied to leukocyte samples, the composition of diets for horses might influence the OD 260/230 results (Cagil et al., 2011; Kagan et al., 2019; Porebski et al., 1997; Sahu et al., 2012). We also attempted to extract DNA from horse saliva using an even less invasive method and found that the total DNA yield was not statistically different from that of oral mucosa swabs (data not shown). However, possibly because of non-horse DNA contamination, gDNA from saliva was not suitable for accurate PCR analysis.

PCR analysis of each sample for *eHRG* showed that the DNA derived from oral mucosa swabs was almost as detectable as that from leukocytes. In contrast, no PCR product was obtained from fecal DNA, even when the amount of template used in the reaction was increased. Even though collecting fresh fecal samples, they were inevitably contaminated due to environmental exposure. Drying of feces and bacterial adhesion degraded the quality of horse gDNA, and there was a significant amount of non-horse DNA, which likely prevented accurate PCR amplification. Attempts were made to detect DNA using a more sensitive DNA polymerase; however, DNA from the feces was evaluated as unsuitable for accurate PCR analysis because of the large amount of non-horse gene contamination and the formation of many nonspecific products.

Here, we report that oral mucosa swabs are a simple, less invasive, stable for long-term storage, and animal welfare-friendly method, as well as a convenient procedure for veterinarians, and may be another option for collecting DNA from horses.

5. Conclusions

In the present study, we evaluated a less invasive and more accurate method for *eHRG* genotyping using gDNA from blood leukocytes, oral mucosa swabs, or feces. The quantity and quality of gDNA vary by source, with the highest amount of gDNA being recovered from feces, although enough for PCR were obtained from all samples. In terms of quality, gDNA from feces and swabs tended to have more contamination

(such as proteins, polysaccharides, or phenol) compared to leucocytes gDNA. Although *eHRG* genotyping using fecal samples was difficultt, oral mucosa swabs provided an adequate gene recovery rate and purity, leading to clear PCR results. In future research, modifying the gDNA extraction method from fecal samples or using rectal swabs could potentially reduce contamination and increase the recovery efficiency of horse gDNA derived from intestinal epithelial cells. Based on animal welfare, oral mucosa swabs may become an option for gene sample collection from not only horses but also other animals.

Animal welfare/ethical statement

The experimental protocol was approved by the standards specified in the guidelines provided by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology as well as those in the Science Council of Japan's guidelines for the use of experimental animals (approval nos. 30–104, R02–46, R03–50, and R04–85).

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CRediT authorship contribution statement

Ryo Muko: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Yoshinobu Ojima:** Validation, Writing – review & editing. **Hiroshi Matsuda:** Conceptualization, Validation, Writing – review & editing, Supervision. **Yuko Toishi:** Formal analysis, Writing – review & editing. **Masa-aki Oikawa:** Validation, Writing – review & editing. **Taekyun Shin:** Validation, Writing – review & editing. **Hiroaki Sato:** Validation, Writing – review & editing. **Akane Tanaka:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare no potential conflicts of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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