# Comparison of nasal swabs and handmade foam cubes for detecting equine herpesvirus 5 (EHV-5) by quantitative polymerase chain reaction (qPCR)

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## Abstract

The control of equine respiratory infections is a biosecurity challenge. Respiratory viruses are often rapidly detected using quantitative polymerase chain reaction (qPCR) on nasal swabs. In the past, some laboratories developed handmade techniques to increase the amount of nasal secretions collected, without comparing them with nasal swabs when qPCR replaced the use of viral culture. The objectives of this study were to compare nasal swabs and handmade foam cubes for i) the detection of a common equine herpesvirus (EHV-5) by qPCR, and ii) their tolerability. Forty-five polyester swabs and foam cubes were used to sample 9 horses 5 times. These were then analyzed by qPCR for EHV-5. Agreement of qPCR results (positive, suspect, negative) was assessed using the intraclass correlation (ICC) and the avoidance scores were compared using a proportional odds mixed model. The ICC showed moderate agreement (0.61, $P < 0.001$). Twenty-seven percent of the samples were positive or suspect with either swabs or foam cubes, whereas 18% were strictly positive with swabs and 11% with foam cubes. Avoidance scores were not statistically different. Both methods have an acceptable agreement. Handmade foam cubes did not provide additional diagnostic value compared to polyester swabs, which is the method presently recommended.

## Résumé

Les infections respiratoires équines constituent un enjeu de biosécurité. La détection rapide des virus est fréquemment réalisée par PCR quantitative (qPCR) sur des écouvillons nasaux. Certains laboratoires ont développé des techniques pour augmenter la collecte des sécrétions nasales, sans les comparer aux écouvillons lorsque la qPCR a remplacé la culture virale. L'objectif de l'étude était de comparer des écouvillons nasaux et des cubes de mousse pour i) la détection d'un herpèsvirus commun (herpèsvirus équin (EHV-5) par qPCR et ii) leur tolérabilité. Quarante-cinq écouvillons et cubes de mousse ont été utilisés pour échantillonner 9 chevaux à 5 reprises et ont été analysés par qPCR pour EHV-5. La concordance des résultats de qPCR (positifs, suspects, négatifs) a été évaluée avec une corrélation intraclasse (ICC) et les scores d'évitement ont été comparés à l'aide d'un modèle mixte à cotes proportionnelles. L'ICC a montré un accord modéré (0,61, $P < 0,001$). Vingt-sept pourcent des prélèvements étaient positifs ou suspects avec les écouvillons ou les cubes de mousse, tandis que 18 % étaient strictement positifs avec les écouvillons et 11 % avec les cubes de mousse. Les scores d'évitement n'étaient pas statistiquement différents. Les deux méthodes ont une concordance acceptable. Les cubes de mousse n'apportent pas de valeur diagnostique supplémentaire par rapport aux écouvillons en polyester, la méthode actuellement recommandée.

(Traduit par les auteurs)

## Introduction

Respiratory diseases are common in horses and infectious viruses and bacteria are a source of widespread outbreaks (1). Accurate diagnosis and detection of horses shedding infectious microorganisms are central to biosecurity measures in large facilities and at sports events. Reliable sample collection that can be used in cooperative and less compliant patients is the first step in achieving an accurate diagnosis.

Both the site of sampling and the material used can affect the results. For example, the detection of *Streptococcus equi* subsp. *equi* by polymerase chain reaction (PCR) is greater when nylon (synthetic) nasal swabs are used instead of cotton swabs, and both are outperformed by culturing nasal lavages followed by PCR (2). In pigs, the less invasive nasal wipes (synthetic or cotton) are similar to synthetic nasal swabs for detecting influenza A by PCR, but nasal swabs are superior to wipes for detecting the virus by culture (3).

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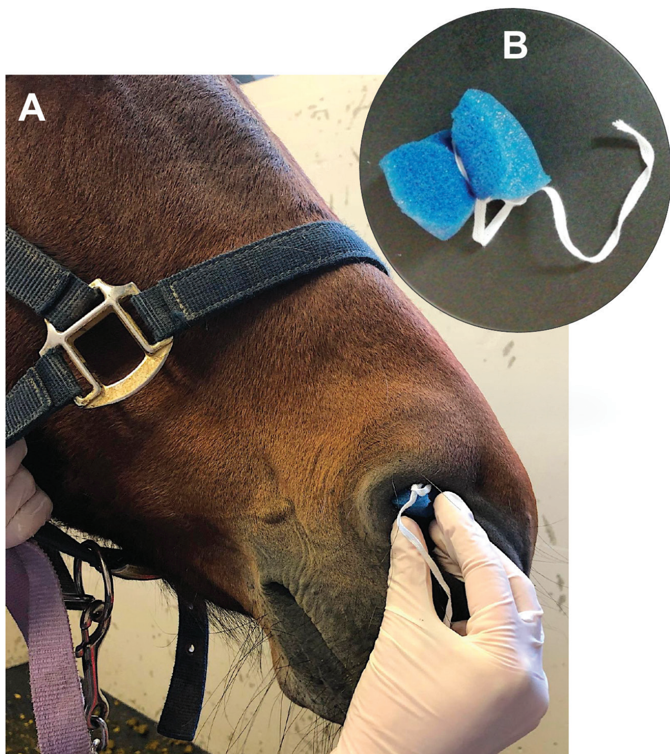


**Figure 1. Artisanal nasal foam cubes. Polyester foam cut in 2.5-cm-sided cubic pieces and tied with a polyester umbilical tape, leaving 10-cm tails to help in recovery from nostrils.**

Prior to the development of PCR analysis, the diagnosis of viral diseases was based on serology and virus isolation. The sensitivity of the latter was improved by collecting larger amounts of biological material and some laboratories developed handmade methods to increase the amount of nasal secretion collected.

In that context, the diagnostic laboratory of the Faculty of Veterinary Medicine of the Université de Montréal developed nasal foam cubes that are still used to this day. They consist of 2.5-cm-sided cubic pieces of synthetic foam fixed by umbilical tape that are sterilized in pairs before use (Figure 1). The foam cubes are well-tolerated by horses, but their preparation is time-consuming and uses non-medical material. Their large size also calls for dedicated material in the diagnostic laboratory and they create confusion among newly graduated veterinarians as the product is not commercialized.

This study was therefore intended to determine whether the handmade foam cubes were superior to commercialized nasal swabs that are currently recommended for diagnosing equine herpesvirus (EHV-1) (4). To complete the study in standardized conditions and over a short period, respiratory viruses that are frequently excreted were targeted. Equine herpesviruses 2 and 5 are widespread, including in horses without respiratory disease. In 2 previous studies, which included mainly healthy horses, EHV-2 was detected in 77% of horses and foals (using nasopharyngeal brushes or nasal swabs) and EHV-5 was detected in 47% (using nasal swabs) and 83% (using nasopharyngeal brushes) of the same 2 populations (5,6).

The present study was therefore initially carried out on nasal swabs tested for both EHV-2 and EHV-5, but the preliminary analyses showed that our research herd had a very low prevalence of EHV-2 and the study was continued testing only for EHV-5, a gammaherpesvirus commonly reported in asymptomatic horses, but also associated with cases of equine multinodular pulmonary fibrosis (7).

The objectives of this study were to compare i) viral detection of EHV-5 with the 2 sampling methods (nasal swabs and handmade foam cubes), and ii) their tolerability during sample collection. Hypotheses were that there is a good agreement between both methods and a better tolerability of the nasal swabs due to their smaller size.

## Materials and methods

### Sample size calculation

Based on preliminary results and published literature (5,6,8,9), it was assumed that between 25% and 50% of the samples would be positive for EHV-5. Assuming that a 15% difference between methods would be acceptable, it was determined that 32 to 43 samples would be necessary using the formula:

$$n = t^2 \times p \times (1 - p)/m^2$$

where: t is the alpha threshold (1.96), p is the estimated prevalence, and m is the difference. Convenience sampling of research horses was carried out using 9 horses sampled 5 times for a total of 45 samples. Since the concordance between methods was analyzed, and not the prevalence in the population, the samples were considered independent and analyzed as such.

### Horses

Nine adult horses (2 Quarter Horses, 1 Thoroughbred, 1 Paint Horse, 1 Canadian horse, and 4 crossbreeds), consisting of 7 mares and 2 geldings, 6 to 22 years of age (median age: 17) were enrolled in the study. The horses were part of the herd of the Equine Asthma Research Laboratory and all but one (the youngest) were horses which had severe asthma and were in remission at the beginning of the study. The horses were otherwise clinically healthy and had not received medication within 15 d before sample collection. Horses were sampled every 5 to 7 d for a total of 5 samplings each. The order of sampling (horse, method, and nostril sampled first) was determined randomly.

For an unrelated research project, horses were initially kept outside (first sampling) and later housed indoors. Although no procedures were carried out for that other project, clinical signs of exacerbation of equine asthma appeared in some horses during the study. All animal manipulations were carried out in accordance with the guidelines of the Canadian Council for Animal Care and the protocol was approved by the Animal Care and Use Committee of the Université de Montréal (21-Rech-2148).

### Sample collection

Six-inch (15-cm) sterile polyester-tipped swabs (Puritan Medical Products, Guilford, Maine, USA) were used as nasal swabs following the American Association of Equine Practitioners (AAEP) recommendations for EHV-1 and 4 (4). Two swabs were introduced together in a nostril, advanced into the ventral meatus to at least 10 cm and withdrawn after a 360° rotation.

Polyester foam (foam cushioning; Sun-Glo Products, Mississauga, Ontario) was cut into 2.5-cm-sided cubic pieces of synthetic foam and tied with a polyester umbilical tape, leaving 10-cm tails to avoid losing the foam in the nostrils (Figure 1). The handmade foam cubes were sterilized in pairs. For each horse, 1 foam cube was inserted as far as possible (5 to 6 cm) into each nasal passage and withdrawn a few seconds later.

Disposable gloves were worn by the operator (AC; the same operator for all sampling) and the gloves were changed between swabs and foam cubes. Nasal swabs were placed in sterile dry tubes, whereas nasal foam cubes were put into a plastic sample container. Both were stored at 4°C until DNA extraction, which was carried out within 18 h of collection. The swabs and foam cubes were made from synthetic materials to avoid inhibition by natural fiber such as cotton (10).

## qPCR analysis

Nucleic acid was extracted at the Molecular Diagnostic Laboratory of the Université de Montréal using the same protocol for swabs and foam cubes, with minor modifications to account for the size of the foam cubes. The foam cubes were put into 60-mL syringes and 1 mL of phosphate-buffer saline (PBS) was added directly to the open syringe before closing it. Another 1 mL of PBS was put into the submitted original foam containers, swirled around, and aspirated in the syringe, in order to recover as much material as possible.

After the foam cubes were incubated for at least 2 min at room temperature, the PBS-secretions mixture was transferred into a cryovial. The swabs were placed in tubes containing 1 mL of PBS, then vortexed, and left at room temperature for at least 5 min. For both foam cubes and swabs, 100 μL of resuspended PBS was used to extract the nucleic acids.

Nucleic acids were extracted in a KingFisher Flex (ThermoFisher, Waltham, Massachusetts, USA) using the BioSprint 96 One-For-All Vet Kit (Qiagen, Hilden, Germany), following the American Association of Veterinary Laboratory Diagnosticians (AAVLD) accreditation protocol PON-MOL-0107 and the manufacturer's protocol.

Detection of EHV-5 was done by a qPCR-validated diagnostic assay targeting the ORF8 gB gene, following the AAVLD accreditation protocol PON-MOL-0143. Briefly, the sequences of the primers and probe used within the EHV-5 qPCR assay were adapted from Hue *et al* (11): forward 5′-AACCCGCCGTGCATCA-3′; reverse 5′-AGGCGCCACACACCCTAA-3′; and probe: 5′-/6-FAM/ACAACACCA/ZEN/CCAACCCCTTTCTGCTG/ 3IABkFQ/-3′. The EHV-5 qPCR is expected to produce a 66-bp size amplicon.

qPCR reactions were done in a RotorGene Q apparatus (Qiagen) following this protocol: 95°C incubation for 5 min and 45 times cycling at 95°C for 15 s, then 60°C for 15 s. Samples were considered positive with qPCR cycle threshold ($C_t$) results of $< 33$, suspect with $C_t \geq 33$ and $< 40$, and negative with $C_t \geq 40$.

## Tolerability: Avoidance score

An avoidance score was established to evaluate the tolerability of the 2 sampling methods. A score of 0 = no movement of the head during sampling; 1 = a slight head movement, with 1 person easily able to collect the sample; 2 = head movement and some resistance, with 1 person still able to collect the sample; 3 = moderate resistance, with 2 people needed for collection; and finally, a score of 4 = high resistance with sedation possibly necessary. The same observer (AC) did the scoring but was not blinded to the method.

## Statistical analysis

For the 3-level analysis, the samples were classified as negative, suspect, or positive as previously described. To refine the analysis, results were also dichotomized in 2-level analyses. In the broad definition 2-level analysis, all samples with a $C_t < 40$ were considered positive, as could be justified in the management of an outbreak for example. In the narrow definition of 2-level analysis, only samples with a $C_t < 33$ were considered positive.

The effect of sampling order (swabs or foam cubes first) on the qPCR results was evaluated using a proportional odds mixed model (POMM) for the 3-level analysis (positive, suspect, negative) and generalized linear mixed model (GLMM) for the 2-level analysis (positive, negative), with the horse identity as a random factor, to avoid pseudoreplication bias.

The agreement between the swab and foam methods was evaluated by estimating the inter-method, single rater, intraclass correlation (ICC 2.1) (12), using the *psych* package in *R* (13). Agreement with ICC $> 0.90$ is considered excellent, ICC above 0.75 and up to 0.90 is considered good, ICC between 0.50 and 0.75 is considered moderate, and ICC below 0.50 reflects poor agreement.

Samples for which both methods yielded $C_t$ values $< 40$ were compared using a paired Student's *t*-test and the correlation between the 2 was evaluated using a Pearson correlation test, after testing for normality and for variance homogeneity with Shapiro-Wilk and Levene's tests, respectively. The avoidance score was evaluated with a proportional odds mixed model using horse identification as a random factor. Statistical analysis and figures were done with *R* version 4.0.3 and GraphPad Prism 9.3.1.

# Results

## Effect of sampling order on qPCR

There was no significant effect of the order of sampling in the 3-level analysis (POMMs, $P > 0.68$) and the 2-level analysis (GLMMs, $P > 0.49$).

## Agreement and correlation between methods for qPCR

Eight swabs and 5 foam cubes were positive, 4 swabs and 7 foam cubes were suspect, and 33 swabs and foam cubes were negative. Twenty-nine samples were negative with both methods, 2 were suspect, and 4 samples were positive. Seven of the disagreements were "weak," *i.e.,* one method was suspect and the other was negative or positive. Only 3 samples "strongly" disagreed, and they were all positive with the swabs ($C_t$ between 28.24 and 32.29) and negative with the foam cubes (Figure 2).

The 3-level ICC was 0.61 [95% confidence interval (CI): 0.43 to 0.74], the broad 2-level ICC was 0.55 (95% CI: 0.36 to 0.70), and the narrow 2-level ICC was 0.56 (95% CI: 0.37 to 0.71), which corresponds to a moderate agreement between both methods. The

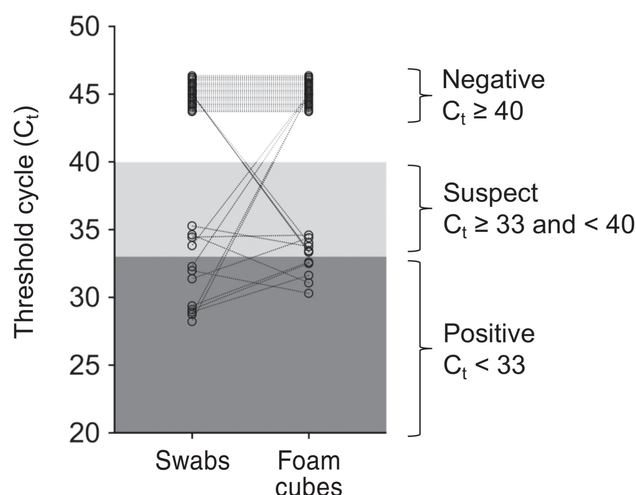


**Figure 2. Cycle threshold. Cycle threshold ($C_t$) values for each pair of nasal swabs and foam cubes (45 pairs). Negative samples were given an arbitrary value of approximately 45 for the purpose of visual representation. The dark shaded area represents positive samples ($C_t$ values < 33) and the light gray area represents suspect samples ($C_t$ values ≥ 33 and < 40). Pairs are connected by dotted lines.**

$C_t$ values of positive samples were 30.01 (mean) ± 1.61 [standard deviation (SD)] and 31.63 ± 0.97 for swabs and foam cubes, respectively, and were 34.56 ± 0.59 and 33.86 ± 0.51 for suspect samples.

Samples for which both methods yielded $C_t$ values < 40 ($n$ = 8), had no significant difference between groups [swabs (mean ± SD): 31.90 ± 2.64; foam cubes: 32.62 ± 1.57, $P$ = 0.48] and there was no significant correlation between the $C_t$ obtained with both methods (r = 0.17, $P$ = 0.70).

## Tolerability: Avoidance score

Scores ranged from 1 to 4 and there was no obvious habituation or worsening of the horses' compliance over time. There was no statistically significant effect of the sampling method on the avoidance score (Figure 3, $P$ = 0.184).

## Percentage of positive samples and intermittent excretion

Although this was not the main goal of the study, a few observations were made on the excretion of EHV-5 in this closed research herd. Overall, 27% (12 out of 45) of samples (swabs or foam cubes) were positive or suspect ($C_t$ < 40) and 18% (8 out of 45) of swabs and 11% (5 out of 45) of the foam cubes were strictly positive ($C_t$ < 33). Two horses were negative at all time points with both methods and 2 other horses were only "suspect" at one time point. The other 5 horses were positive on at least one time point and all of them were suspect or positive on at least another time point.

# Discussion

## General findings

The comparison of these 2 methods showed a moderate agreement. Among positive and suspect samples, $C_t$ values were not significantly different between methods. There were, however, fewer than expected positive samples and the power for this comparison is limited. Contrary to our second hypothesis, swabs were not better tolerated than foam cubes.

## Advantages and disadvantages of both methods

In addition to their ease of storage and use, it was noted that nasal swabs could be inserted deeper into the ventral meatus than the foam cubes, which were limited by the length of the operator's fingers. When using swabs, it was also easier to see whether or not the samples collected were covered in biological material.

Swabs were subjectively less well-tolerated, but this impression was driven mainly by 2 horses (Horses 7 and 8; Figure 3) and did not translate into statistically significant differences. Despite this and a moderate agreement with nasal swabs, factors against the use of handmade foam cubes include the tedious process and the potential for non-medical material to be modified by the manufacturer over time, with the addition of PCR inhibitors that could go unnoticed.

## Comparing swabs and foam cubes without a reference method

In this study, we compared 2 methods and elected not to use the swabs as the reference method. Nasal swabs are recommended by the AAEP for detecting EHV-1 and EHV-4 (4), but they are not universally considered the "gold standard." Nasopharyngeal swabs could theoretically be considered the reference method of sampling for detecting upper airway pathogens, but Pusterla *et al* (14) showed that nasal swabs were not less likely to yield a positive result in an outbreak of EHV-1 (24 positive results with nasopharyngeal swabs, 28 with nasal swabs) and both methods yielded similar nucleic acid recovery. The authors speculated that the drainage of mucus and exfoliated epithelial cells from the upper airways *via* the ventral meatus could increase the sensitivity of swabs for some infectious agents (14).

This cannot necessarily be extrapolated to other diseases and most notably, nasopharyngeal lavage and nasopharyngeal swabs are recommended over nasal swabs for detecting *Streptococcus equi* subsp. *equi* (15). Overall, the difficulty of sampling a horse using 3 different methods without interfering with the other methods (by removing or dragging secretions, inducing snorting, or affecting compliance) reinforced the decision not to use nasopharyngeal swabbing as the reference in this study.

Without an appropriate reference method, an acceptable difference was predefined, which would make sense from a clinical point of view and would take into account the comparison of 2 imperfect methods. Recent literature on SARS-CoV-2 considered a difference of 15% between 2 sampling methods acceptable (16), and this difference was used for calculating the sample size in the current study.

Finally, comparing the $C_t$ values between methods is subject to different dilutions of extracted target DNA since the amount of recovered material from the nose is likely to be greater with foam cubes, but this amount is also diluted in twice as much PBS (to facilitate recovery from the foam). The different rate of dilution between methods was also highlighted as a potential pitfall in another study (17), but the use of the reference gene equine glyceraldehyde-3-phosphate dehydrogenase (eGAPDH) to express the targeted genes as copies per million epithelial cells would reduce this problem (18).

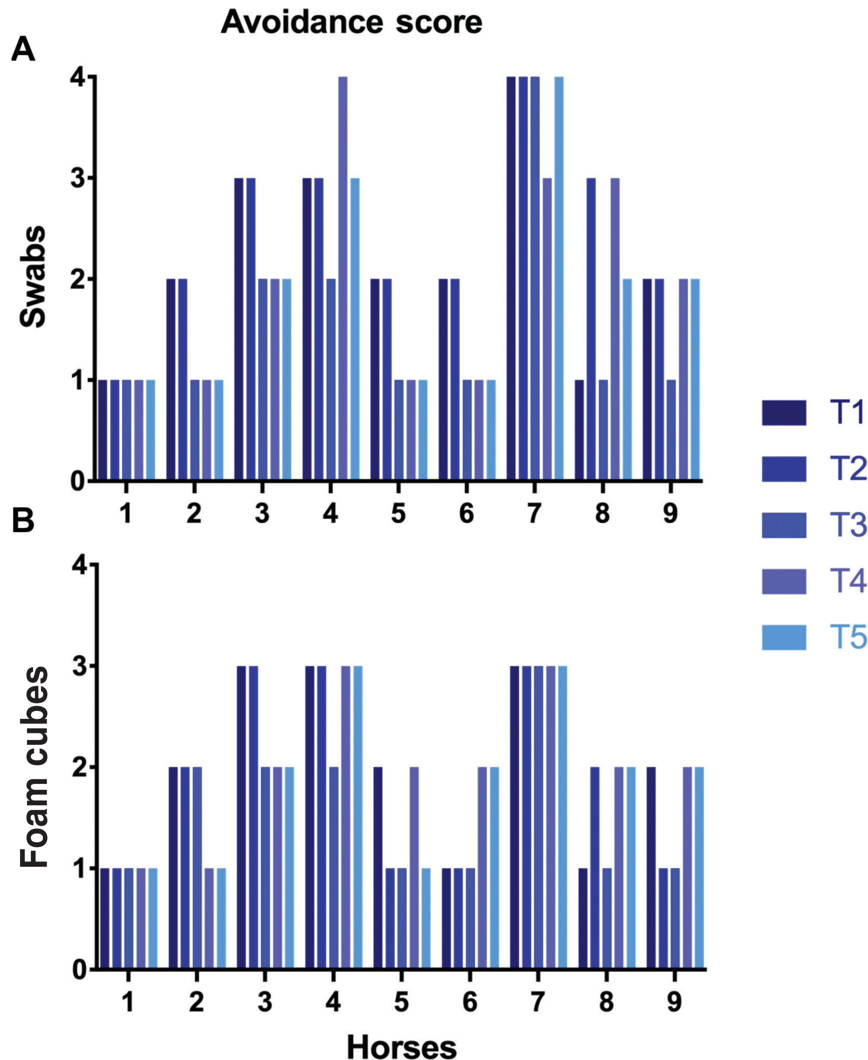


**Figure 3. Tolerability. Avoidance scores for the 9 horses sampled on 5 occasions (T1 to T5) using swabs and foam cubes. 1 — Slight head movement, 1 person can easily collect the sample; 2 — Head movement and some resistance, 1 person can still collect the sample; 3 — Moderate resistance, 2 people needed for collection; and 4 — High resistance and sedation could be necessary.**

## Percentage of positive samples

The percentage of horses that were positive at each time point was lower than expected (8) and ranged from 11 to 44% (1 to 4 out of 9 horses). This could be explained by the population selected, which consisted of adult horses in a closed herd. However, 5 horses (56%) were positive on at least one time point (7 horses or 78% if "suspects" are included), which supports the fact that EHV-5 seems to be widespread in the equine population and that transitory excretion might be common. A more in-depth understanding of the excretion kinetics in larger populations could bring valuable epidemiological information.

## Extrapolation to other pathogens and outbreaks

These results can probably be extrapolated to other EHV and upper airway viruses. It is possible that the larger amount of nasal discharge in horses with clinical signs could change the $C_t$ values and favor one of these methods, but it is unlikely to create false negative results, which are the ones most feared in managing outbreaks.

In conclusion, this study shows that results obtained with a handmade device used for years in a referral hospital were not significantly different from those obtained using the synthetic swabs currently recommended by the AAEP. Protocols developed to increase recovery of biological material before the use of qPCR should be re-evaluated in the era of molecular diagnostics.

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