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Equus caballus papillomavirus Type 7 is a rare cause of equine penile squamous cell carcinomas

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ABSTRACT

Penile squamous cell carcinomas (SCCs) are common, potentially life-threatening neoplasms of horses. They are well-recognized to be caused by Equus caballus papillomavirus (EcPV) type 2, although EcPV2 cannot be detected in all cases. A 23-year-old standardbred gelding developed multiple penile *in situ* and invasive SCCs that contained histological evidence of PV infection. By using both consensus and specific PCR primers, these lesions were found to contain EcPV7 DNA, but not DNA from EcPV2 or any other PV type. To determine how frequently EcPV7 is present in equine penile SCCs, specific primers were used to detect EcPV2 and EcPV7 in a series of 20 archived samples. EcPV7 was the only PV detected in one, both EcPV2 and 7 were detected in five, and only EcPV2 was detected in 14 SCCs. EcPV7 DNA was also detected in three of 10 archived oropharyngeal SCCs, although only as a co- infection with EcPV2. This is the first report of EcPV7 causing disease in horses. These results suggest EcPV7 could cause a subset of equine penile SCCs, and this is the first evidence that PV types other than EcPV2 can cause these neoplasms. The detection of EcPV7 in the oropharyngeal SCCs suggests a potential role of this PV type in the development of these SCCs. There were no clinical or histological features that differentiated lesions containing EcPV7 DNA from those containing EcPV2 DNA. If EcPV7 causes a proportion of equine penile SCCs, vaccines to prevent EcPV2 infection may not prevent all equine penile SCCs.

1. Introduction

Papillomaviruses (PVs) are small circular double-stranded DNA viruses. They are generally epitheliotropic and, with notable exceptions, are species specific (Munday, 2014). While most PV infections are asymptomatic (Greenwood et al., 2020b; Knight et al., 2013), PVs produce proteins that promote cell growth and differentiation as part of their normal life-cycle (Doorbar et al., 2012). These proteins can result in the development of hyperplastic papillomas (warts) and, less commonly, can cause neoplasia (Munday et al., 2022b).

Papillomaviruses are classified based on the sequence of the highly conserved *ORF L1* with PVs within the same genus sharing over 60 % nucleotide similarity and PVs of the same type having over 90 % similarity (Bernard et al., 2010). Papillomaviruses within the same genus typically cause similar lesions within closely related species (Bernard et al., 2010). There are currently 10 fully sequenced Equus caballus papillomavirus (EcPV) types contained within the papillomavirus episteme (https://pave.niaid.nih.gov, accessed 22 May 2024). These are divided into three genera. The *Zetapapillomavirus* genus contains only EcPV1. This PV type causes 'grass warts' which are small hyperplastic warts that are common in young horses (Munday et al., 2022a). The *Dyoiotapapillomavirus* genus contains EcPV2, EcPV4, and EcPV5. Of these, EcPV2 has been most studied, and this PV is thought to cause papillomas of the genitals and squamous cell carcinomas (SCC) of the genitals, oropharynx, and stomach (Alloway et al., 2020; Armando et al., 2024; Knight et al., 2011; Luff et al., 2023; Scase et al., 2010). In contrast, EcPV4 and EcPV5 cause aural plaques (Bromberger et al., 2023; Lange et al., 2013b). While most aural plaques remain small and are of predominantly cosmetic concern, an EcPV4-induced aural plaque was reported to progress to SCC (Peters-Kennedy et al., 2020). There are currently three PV types within the *Dyorhopapillomavirus* genus including EcPV3, EcPV6, and EcPV7. Both EcPV3 and EcPV6 cause

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equine aural plaques (Bromberger et al., 2023; Lange et al., 2011; Lange et al., 2013b; Mira et al., 2018), although EcPV3 was also detected as a co-infection with EcPV2 in a penile SCC (van den Top et al., 2015). There is only a single report of EcPV7 (Lange et al., 2013b). As this PV was detected in a swab of a penis, this PV type has not been associated with any disease in horses. EcPV8, EcPV9, and EcPV10 are currently unclassified within the papillomavirus episteme, although EcPV10 has been reported to be closely related to EcPV2 and is therefore likely within the *Dyoiotapapillomavirus* genus (Turco et al., 2023).

Equine penile SCCs are common neoplasms that are typically infiltrative and can necessitate amputation of the penis or even euthanasia (van den Top et al., 2008). Less frequently horses also develop oropharyngeal SCCs. These neoplasms are generally only detected after the neoplasm has invaded surrounding tissue, preventing a surgical cure. EcPV2 DNA is frequently present in both penile and oropharyngeal SCCs and this PV type is thought to cause a significant proportion of these cancers (Munday et al., 2022b; Sykora & Brandt, 2017). Herein is described a horse with penile SCCs that only contained EcPV7. Additionally, to determine how frequently EcPV7 is detectible in equine SCCs, archived penile and oropharyngeal SCC samples were investigated using specific PCR primers to detect EcPV2 and EcPV7.

2. Materials and methods

2.1. Initial case summary

A 23-year-old standardbred gelding developed multiple sessile plaques and exophytic masses on the penis. Biopsy samples of three lesions were taken and submitted for histology. The horse was monitored over the next 10 months. Over this time, an exophytic lesion on the tip of the penis enlarged to approximately 5 cm in diameter (Fig. 1). Smaller lesions that varied in appearance from sessile and darkly pigmented to pale and exophytic were also visible on the penis (Fig. 2). The penis was amputated and submitted for histology. No lesion recurrence nor metastatic disease was observed in the 9 months following amputation of the penis.

2.2. Initial PCR and DNA sequencing

DNA was extracted using a NucleoSpin DNA FFPE XS kit (Macherey-Nagel, Düren, Germany) from all three initial biopsy samples as well as from the larger mass on the tip, and samples of two smaller masses from the shaft, of the amputated penis. All DNA was extracted from 10um thick scrolls taken from a formalin-fixed paraffin histology block.

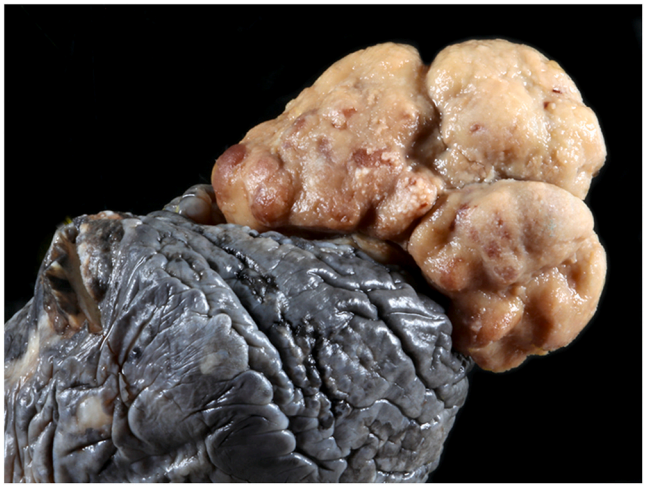

Fig. 1. The initial case presented with a mass on the tip of the penis that was 5 cm in diameter and exophytic.

Fig. 2. In addition to the large exophytic mass on the tip of the penis, the initially identified horse had multiple smaller masses on the penis. These masses ranged from exophytic papillomatous masses to mildly raised sessile pale or pigmented plaques (arrows).

Papillomaviral DNA was amplified using the MY09/11 and CP4/5 consensus primers as previously described (Munday et al., 2020). DNA extracted from an equine penile SCC that had previously been found to contain EcPV2 was used as a positive control while no template DNA was added to the negative controls. The amplified DNA was sequenced as previously described and compared to sequences in GenBank using the BLAST tool.

2.3. Detection of PV DNA in archived equine SCCs

Samples archived at the University of Calgary were used to determine how frequently EcPV7 is present in equine penile and oropharyngeal SCCs. DNA was extracted from shavings of the histology tissue blocks as before. The presence of amplifiable DNA in the samples was confirmed by amplifying a short section of the equine beta actin gene as previously described (Bogaert et al., 2006). The presence of EcPV2 and EcPV7 DNA was then determined using specific PCR primers that were designed using Geneious Prime 2019.2.3. The specific primers for EcPV2 amplify a 104 bp section of the EcPV2 *ORF L1* (EcPV2F 5'-GGCGAGGTGGGGGAAAAGGC and EcPV2R 5' - AGCGATCCACTTGGCGTGGC) while the specific primers for EcPV7 (EcPV7F 5'-GAAACCCGGGTGAGCTTGAT and EcPV7R 5'- AAGTTCATCGCCCCATAGCC) amplify a 225 bp EcPV7 *ORF L1* section. DNA was amplified by both primers using Hot FirePol® Master Mix (Solis BioDyne OÜ, Tartu, Estonia) with an annealing temperature of 60°C. DNA extracted from a penile SCC that was known to contain EcPV2 and DNA extracted from a penile mass known to contain EcPV7 were the positive controls while no template DNA was added to the negative control. DNA amplified by the EcPV7F/R primers and EcPV2F/R primers was sequenced as previously described.

3. Results

3.1. Initial case histology and lesion classification

Examination of the three biopsy samples taken from the penis revealed plaque-like masses consisting of thickened and dysplastic epithelium. One of the plaques contained cells that had significant quantities of melanin pigment and macrophages containing large quantities of melanin were visible in the underlying submucosa (Fig. 3). Despite the marked epithelial disorganization, definitive evidence of invasion of the underlying submucosa was not visible. The epithelium surrounding the plaques was mildly thickened and scattered epidermal cells had expanded clear cytoplasm and shrunken nuclei (koilocytes) with surface keratinocytes showing prominent clumping of keratohyalin granules (Fig. 4). The final histological diagnosis was multiple *in situ* carcinomas and epithelial hyperplasia containing PV-induced cytopathic changes.

Three masses from the penis were sampled 10 months later when the penis was amputated. Histologic examination of the large mass on the tip of the penis revealed a SCC with cells arranged in nests. Most cells within the SCC appeared basaloid and, although some keratin pearls were visible, these were not a prominent feature within the SCC. Invasion of neoplastic cells into the underlying submucosa was visible, although considering the large size of the mass, there was less invasion than expected (Fig. 5). This mass was diagnosed as a well-differentiated SCC. The two smaller masses consisted of thickened and dysplastic epithelium. Large quantities of melanin pigment were visible in the mass that clinically appeared dark and sessile. As in the previous samples, examination of the surrounding epithelium revealed mild hyperplasia with koilocytosis and keratohyalin clumping.

3.2. Evaluation of PV DNA in the initial case

Papillomaviral DNA was amplified by the CP4/5 consensus primers from all three initial biopsy samples and all three lesions from the amputated penis. Sequencing of the amplicons from all 6 lesions revealed that the DNA amplified was identical to the EcPV7 sequence within GenBank. The MY09/11 consensus primers did not amplify PV DNA from any of the six samples taken from the penis. The primers specific for EcPV7 also amplified PV DNA from the lesions, but no PV DNA was amplified by the primers specific for EcPV2. Sequencing of the DNA amplified by the EcPV7-specific primers confirmed that this PV type was present in the lesions. For all PCR reactions, DNA was amplified as expected from the positive controls, but not the negative controls.

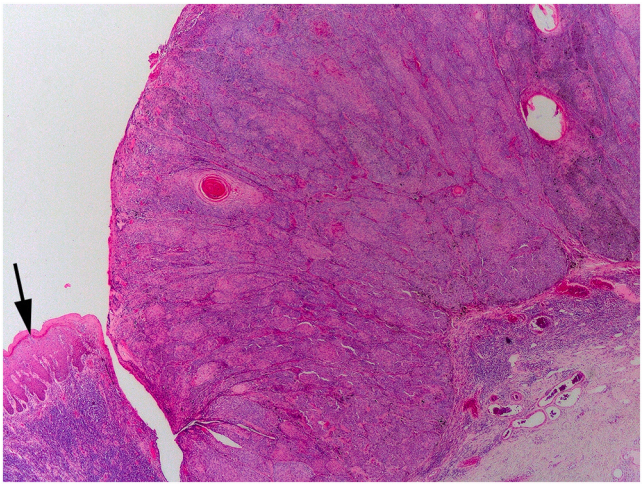

Fig. 3. Histology of a pigmented sessile lesion from the initially identified horse revealed marked thickening and dysplasia of the epithelium. Melanin pigment in visible within many of the dysplastic cells. Infiltration of the underlying mucosa is not visible. Normal mucosa is also visible (arrow). HE 25x.

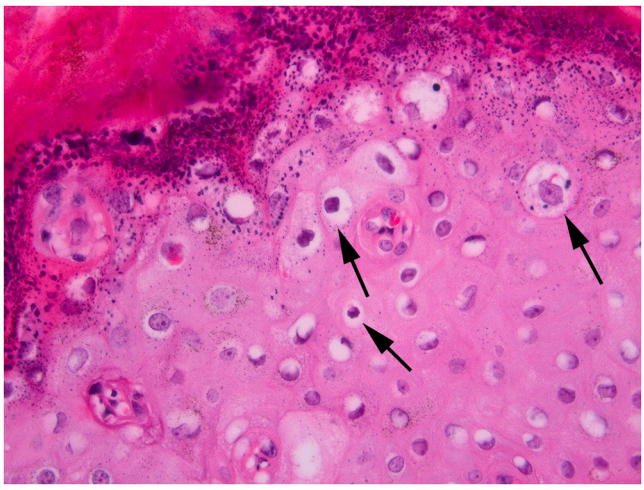

Fig. 4. Histological examination of the epithelium surrounding the neoplasms from the initially described horse revealed mild thickening with numerous cells that contained increased quantities of pale cytoplasm and darkened nuclei (arrows). Marked keratohyalin clumping was also visible. Both changes are consistent with papillomavirus infection. HE 400x.

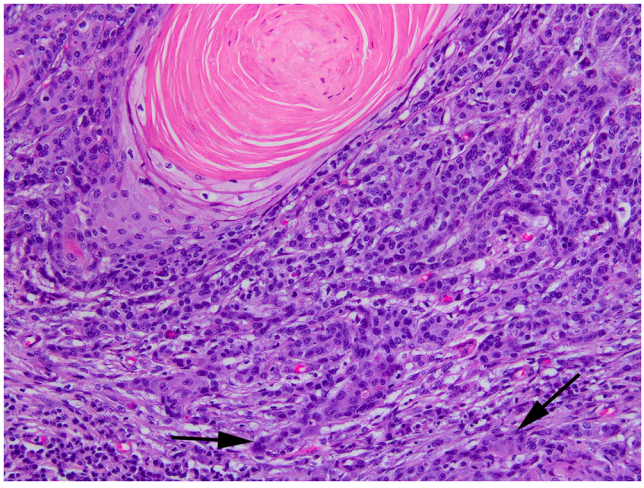

Fig. 5. The mass from the tip of the penis from the initially described horse is a well differentiated squamous cell carcinoma. Although nests of cells are visible infiltrating the underlying mucosa (arrows), the neoplasm is not as infiltrative as is typical for neoplasms of this type. HE 200x.

3.3. Detection of EcPV7 in archived equine SCCs

Thirty SCC samples were confirmed to contain amplifiable beta actin DNA. The samples included 20 penile SCCs and 10 oropharyngeal (this term is used broadly to include SCCs of the caudal oral cavity, pharynx, and larynx) SCCs (Supplemental Table). Twenty-eight samples were from horses in North America with two from horses in New Zealand. Histology confirmed the diagnosis of SCC in all cases. Within the 30 SCCs, two samples did not contain either EcPV2 or EcPV7. Both these samples were oropharyngeal SCCs (Table 1). Only one SCC contained EcPV7, but not EcPV2, DNA. This sample was a penile SCC that had been removed from a horse in North America. Histology of this sample revealed a predominance of basaloid cells; however, in contrast to the initial case, the mass appeared highly infiltrative, and the neoplastic cells were poorly differentiated. When the histology of this SCC was compared to the others in the series of SCCs, no unique histological

Table 1
Summary of 30 archived samples of equine SCCs from the penis or oropharynx. Specific PCR primers were used to detect DNA from Equus caballus papillomavirus (EcPV) type 2 and EcPV7. Results from the horse initially found to be infected by EcPV7 are not included in this table.

Lesion	EcPV2 only	EcPV7 only	EcPV2 and ECPV7	No PV DNA	Total number
Penile squamous cell carcinoma	14	1	5	0	20
Oropharyngeal squamous cell carcinoma	5	0	3	2	10
All lesions	19	1	8	2	30

features were observed. Eight of the 30 samples contained both EcPV2 and EcPV7 DNA including 5 penile SCCs and 3 oropharyngeal SCCs. EcPV2, but not EcPV7, was detected in 14 penile SCCs and 5 oropharyngeal SCCs.

The presence of EcPV7 within a lesion was confirmed by sequencing all DNA amplified by the EcPV7-specific primers. This confirmed that EcPV7 DNA was present within nine of the 30 SCCs including six penile SCCs and three laryngeal SCCs.

4. Discussion

These results provide the first evidence that EcPV7 may cause a subset of equine penile SCCs. A role of EcPV7 in equine penile SCCs was supported by the detection of only this PV type in all three biopsy samples of the penile masses from the initial case. Additionally, EcPV7 remained the only PV type detectible in the penile lesions when additional samples were taken 10 months later. Furthermore, one of the 20 archived samples of penile SCCs contained only EcPV7 DNA. While the results from the archived samples supported a potential role for EcPV7 in causing penile SCCs, consistent with previous reports (Sykora & Brandt, 2017), penile SCCs that contained EcPV2 were more common than those containing EcPV7 in the archived samples.

As PVs can asymptomatically infect the equine penis (Greenwood et al., 2020b; Knight et al., 2013), the detection of PV DNA within a lesion does not prove causality. However, a role of PV infection in the development of the penile SCCs in the initial horse was supported by the development of multiple *in situ* and invasive carcinomas over the penis. The development of multiple neoplasms is consistent with an infectious cause in contrast to sporadic cancers that usually develop as a single lesion. Additionally, the lesions contained koilocytes and clumping of keratohyalin granules which are considered evidence of a PV etiology in equine penile neoplasms (Ramsauer et al., 2019). The development of multiple lesions that contained histological evidence of PV infection therefore supports a PV etiology. As EcPV7 was the only PV type detected in these lesions, this supports a causal association between EcPV7 and penile neoplasia in horses.

Infection by both EcPV2 and EcPV7 was detected in a quarter of the series of archived penile SCCs. The detection of mixed infections makes it difficult to determine which of the PVs was causative. Considering the well-established role of EcPV2 in equine penile cancer (Sykora & Brandt, 2017), it is likely the SCCs that contained a mixed infection were predominantly caused by EcPV2. However, it cannot be excluded that EcPV7 was the predominant cause of some of the SCCs that contained a mixed infection. If so, EcPV7 could cause a higher proportion of equine penile SCCs that the 5 % of archived SCCs that were found to contain only EcPV7 DNA. It is also possible that both PV types contribute to SCC development with coinfection by EcPV2 and EcPV7 more strongly promoting neoplasia than infection by just one PV type. Quantitative PCR analysis of these, and additional samples, may provide further evidence of the roles of each PV type in neoplasms containing a mixed infection.

The samples from the initial horse were evaluated for the presence of PV DNA using both the MY09/11 and CP4/5 consensus primers. The use of these primers was important as, while both primers amplify EcPV2 DNA, only the CP4/5 primers amplify EcPV7 (Lange et al., 2013b). In contrast, most previous studies of equine penile SCCs have used methods that specifically detect EcPV2 (Armando et al., 2021; Bogaert et al., 2012; Greenwood et al., 2020a; Lange et al., 2013a; Miglinci et al., 2023; Scase et al., 2010; Tuomisto et al., 2024; van den Top et al., 2015). While smaller numbers of studies did use consensus primers, these studies used the MY09/11 primers to detect PV DNA in penile SCCs (da Silva et al., 2022; Knight et al., 2011). As neither the methods specific for EcPV2 nor the MY09/11 primers amplify EcPV7 DNA, it is possible that EcPV7 was present, but undetected in previous studies of equine penile SCCs. Therefore, it is possible that some of the SCCs thought to not be caused by PV infection in previous studies (Zhu et al., 2015) were caused by an undetected EcPV7 infection. The methods used in previous studies probably also explains why EcPV7 has not been previously identified in equine penile SCCs, despite this type being detected in 30 % of the archived penile SCCs in the present study.

Prior to this study, there was only one report in which EcPV7 was detected. This PV type was detected in a swab of the penis of a horse that had visible masses (Lange et al., 2013b). As no diagnostic evaluation of the penile masses was performed, neither the nature of the masses nor the relationship between the masses and EcPV7 could be determined. Therefore, this is the first report of disease in a horse associated with EcPV7 infection. While additional cases are required, evidence suggests infection by EcPV7, like EcPV2, causes hyperplastic and neoplastic lesions in horses.

Penile lesions caused by EcPV2 have been reported to have a variable appearance ranging from hyperplasia to *in situ* carcinoma and invasive carcinoma (Ramsauer et al., 2019). While no lesions in the initial case were consistent with hyperplastic viral papillomas, both *in situ* and invasive carcinomas were present. This suggests that lesions caused by EcPV7 cannot be differentiated clinically from lesions caused by EcPV2. Histologically, the SCC that was examined from the initial case appeared less invasive than is typical for equine penile SCCs. This could suggest that SCCs associated with EcPV7 have a less aggressive biological behavior than those associated with EcPV2. However, the SCC that contained only EcPV7 in the archived series of equine penile SCCs did not show any unique histological features with significant invasion of the surrounding tissue by the neoplastic cells. Additional SCCs containing just EcPV7 DNA are required before firm conclusions can be made regarding differences in biological behavior in SCCs caused by the different types of PV.

While EcPV7 appears to be a rare cause of equine penile SCCs, the ability of this PV to cause penile SCCs is important due to the current interest in using vaccines against EcPV2 to prevent equine penile neoplasia (Hainisch et al., 2023). The present study suggests that, while preventing EcPV2 infection of horses would have prevented the 14 of the 20 SCCs in the archived series that only contained EcPV2, it may not have prevented the 5 SCCs that contained a mixed infection and is unlikely to have prevented the SCC that contained only EcPV7. Therefore, the results of this study suggest that vaccines containing both EcPV2 and EcPV7 would be required to fully protect horses against penile SCCs.

Infection by EcPV7 appears to result in similar proliferative lesions on the penis of horses to those caused by EcPV2. PVs within the same genus typically result in similar lesions (Bernard et al., 2010). Considering the apparent similarity of the penile lesions caused by EcPV2 and EcPV7, it would therefore be expected these PV types are within the same genus. However, EcPV2 is a Dyoiotapapillomavius and EcPV7 is a Dyorhopapillomavirus. While this could be evidence that EcPV7 does not cause penile lesions, aural plaques are caused by PV types within both the *Dyoiotapapillomavius* and *Dyorhopapillomavirus* genera (Bromberger et al., 2023; Mira et al., 2018). Therefore, as PV types within both genera can cause aural plaques, it is less surprising that PV types within both genera can also cause hyperplastic and neoplastic lesions of the penis.

As oropharyngeal SCCs have also been associated with EcPV2 in horses (Luff et al., 2023), it was hypothesized that a proportion of these tumors could be caused by EcPV7. However, while infection by EcPV7 was detected in a three or the 10 oropharyngeal SCCs in the series of archived neoplasms, this PV type was only detected as a coinfection with EcPV2. Therefore, no firm conclusions regarding a role of EcPV7 in the development of oropharyngeal SCCs can be made. However, this is the first time that EcPV7 has been detected in a sample from the oral cavity and the ability of this PV to infect the oral mucosa suggests EcPV7 may have the potential to cause SCCs of the mouth and throat of horses. As EcPV2 has also been associated with gastric and vulval neoplasms in horses (Alloway et al., 2020; Armando et al., 2024), it is possible that EcPV7 may also be present in a proportion of these neoplasms.

5. Conclusion

EcPV7 may cause hyperplastic and neoplastic diseases of the equine penis that are indistinguishable from those caused by EcPV2. EcPV7 can also be present as a co-infection in equine penile SCCs, although the role of EcPV7 in these co-infections is unknown. EcPV7 can infect the oral mucosa, suggesting the potential for this PV type to cause neoplasia of the mouth and throat of horses. This is the first evidence that EcPV7 causes disease of horses.

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CRediT authorship contribution statement

Michael Hardcastle: Writing – review & editing, Investigation. **John Munday:** Writing – original draft, Investigation, Formal analysis. **Cameron Knight:** Writing – review & editing, Methodology, Investigation. **Christa Bodaan:** Writing – review & editing, Investigation. **Camille Codaccioni:** Writing – review & editing, Methodology, Investigation.

Declaration of Competing Interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2024.106155.

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