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Identification of putative orthologs of clinically relevant antimicrobial peptides in the equine ocular surface and amniotic membrane

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Abstract

Objectives: This study aimed to define the antimicrobial peptide (AMP) expression pattern of the equine ocular surface and amniotic membrane using a targeted qPCR approach and 3′Tag-sequencing. It will serve as a reference for future studies of ocular surface innate immunity and amniotic membrane therapies.

Procedures: A targeted qPCR approach was used to investigate the presence of orthologs for three of the most highly expressed beta-defensins (*DEFB1, DEFB4B*, and *DEFB103A*) of the human ocular surface and amniotic membrane in equine corneal epithelium, conjunctiva, and amniotic membrane. 3′Tag-sequencing was performed on RNA from one sample of corneal epithelium, conjunctiva, and amniotic membrane to further characterize their AMP expression.

Results: Equine corneal epithelium, conjunctiva, and amniotic membrane expressed *DEFB1, DEFB4B*, and *DEFB103A. DEFB103A* was expressed at the highest amounts in corneal epithelium, while *DEFB4B* was most highly expressed in conjunctiva and amniotic membrane. 3′Tag-sequencing from all three tissues confirmed these findings and identified expression of five additional beta-defensins, 11 alpha-defensins and two cathelicidins, with the alpha-defensins showing higher normalized read counts than the beta-defensins.

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CONFLICT OF INTEREST

The authors have no financial interests to disclose.

Conclusions: This study identified AMP expression in the equine cornea and conjunctiva, suggesting that they play a key role in the protection of the equine eye, similar to the human ocular surface. We also determined that equine amniotic membrane expresses a substantial number of AMPs suggesting it could potentiate an antimicrobial effect as a corneal graft material. Future studies will focus on defining the antimicrobial activity of these AMPs and determining their role in microbial keratitis.

Keywords

amniotic membrane; cathelicidin; corneal epithelium; conjunctiva; defensin

1 | INTRODUCTION

Ulceration of the cornea is one of the most common ophthalmic disorders seen in horses.[1] These ulcers are further complicated by infectious organisms, including *Aspergillus* spp., *Fusarium* spp., *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), and *Streptococcus* spp,[2–5] thus jeopardizing the health of the eye. Once infection has taken hold, both medical and surgical therapeutics may have limited efficacy. For example, one retrospective study reported that 36% of equine eyes with mycotic keratitis lost vision despite medical intervention and enucleation was necessary in 25% of affected eyes due to nonresponse to treatment.[2] Thus, investigations defining the native antimicrobial environment of the equine ocular surface is of vital importance.

The epithelial lining of the cornea and conjunctiva comprises the physical and biochemical barriers against infection. One of the main forms of biochemical protection is provided by key effector molecules of the innate immune system called antimicrobial peptides (AMPs). AMPs are small cationic peptides expressed by epithelial cells and leukocytes of most mammals.[6–8] There are two main AMP subclasses, cathelicidins, and defensins, which have broad-spectrum antimicrobial activity against many pathogens, including bacteria and fungi.[9] Additionally, the defensins are subclassified into alpha-, beta-, and theta-defensins depending on their disulfide bonding array. While there are multiple proposed mechanisms of action, these peptides are typically thought to attach and incorporate into the membranes of pathogenic or infected cells where they disrupt the normal membrane structure and induce pore formation.[10,11]

AMPs have been shown to play a functional role in the defense of the ocular surface in humans and mice. For example, mice deficient in murine cathelicidin-related antimicrobial peptide have an increased risk of corneal infections and have an impaired innate immune response against *P. aeruginosa* translating to prolonged infections and delayed healing times.[12] Human beta-defensin 1 (peptide: hBD1, gene: *DEFB1*), hBD2 (*DEFB4*), and hBD3 (*DEFB103A*) have been shown to provide protection against *S. aureus* and *P. aeruginosa* on the human ocular surface.[13,14] In horses, AMP expression and activity of the alpha-defensins, beta-defensins, and cathelicidins have been most thoroughly characterized in the reproductive tract[15] along with the identification of beta-defensins in the gastrointestinal and respiratory tracts.[16] However, no studies have defined the AMP expression patterns of the equine ocular surface.

Human amniotic membrane expresses high amounts of AMPs with demonstrated antimicrobial effect.[17] This is clinically relevant as equine amniotic membrane is used as a graft for ulcerative keratitis to restore the structural integrity of the cornea.[18] In addition to reestablishing the physical barrier, these grafts also provide anti-inflammatory, antifibrotic, antiangiogenic, and antimicrobial effects,[18] features which are especially important for the return of normal vision after corneal injury. The use of equine amniotic membrane transplantation has been successful in treating equine eyes with corneal ulcers infected with either bacteria or fungi.[18,19] Equine amniotic membrane homogenates have also be applied topically for the treatment of corneal ulcers ex vivo and in dogs, providing similar clinical effects as grafts.[20,21] However, a study on the use of amniotic membrane homogenates in horses with surgically induced corneal ulcers did not identify an increase in healing time.[22] As these animals had clean, epithelial wounds, further work is needed to determine if there is a therapeutic effect of amniotic membrane homogenates in patients with infected stromal wounds in this species. Despite the use of amniotic membrane for the treatment of infected equine corneal ulcers, the AMP expression pattern, and therefore antimicrobial effect, of this tissue remains unknown.

This study aims to characterize the role of AMPs in normal tissues to better inform our understanding of their role in health We hypothesized that putative orthologs of both the cathelicidin and defensin gene families would be expressed by the equine cornea, conjunctiva, and amniotic membrane. As there is no literature on the AMP expression of these tissues in horses, we first investigated the presence of the equine orthologs of the three most highly expressed beta-defensins of the human ocular surface, then we further characterized the AMP repertoire of these tissues with 3′Tag-sequencing. These findings lay the foundation for future investigations on microbial keratitis, a condition horses are especially prone to, and on the benefits of amniotic membrane as a corneal grafting material and topical adjunctive therapy.

2 | MATERIALS AND METHODS

2.1 | Tissues

Equine corneal epithelium ($n = 5$) and conjunctiva ($n = 4$) were prospectively collected from horses with no history or clinical evidence of ocular surface disease. Horses were euthanized for reasons unrelated to this study and owner consent was provided for their unrestricted use in research. Tissue was collected from horses within 1 hour of euthanasia. Corneal epithelium was scraped using a #15 blade or excimer spatula and submerged directly into cell lysis buffer.[23] Sections of conjunctiva (temporal bulbar conjunctiva due to accessibility) were sharply excised with Stevens tenotomy scissors. Amniotic membrane samples ($n = 6$) were collected after parturition from normal pregnancies of university-owned animals. Testis and epididymal samples ($n = 4$) were collected from client-owned horses presenting to the UC Davis Veterinary Medical Teaching Hospital for routine castrations to serve as positive controls. The conjunctiva, amniotic membrane, testis, and epididymal samples were submerged in RNAlater and stored at −20°C until further processing. The number of tissues procured was based upon tissue availability and prior studies defining AMP expression from different tissues.[9]

2.2 | RNA isolation

Tissue samples stored in RNAlater were thawed and 30-150 mg of tissue depending on the sample type was used for RNA extraction. Total mRNA was extracted using the GeneJET RNA Purification Kit (ThermoFisher Scientific) according to the manufacturer's protocol with the exception that reagent volumes prior to transitioning the lysate into the columns were doubled. RNA quantification was performed using a Nanodrop spectrophotometer.[23]

2.3 | Quantitation of AMP expression

A targeted qPCR approach was utilized to determine the expression of three of the equine orthologs of the most highly expressed beta-defensins of the human ocular surface. Interexonic primers were designed using MacVector (MacVector Inc.) for the putative orthologs of human beta-defensins 1, 4, and 103, which corresponded with equine *DEFB1, DEFB4B* and *DEFB103A*, respectively (Table 1). Depending on the tissue type, 300–600 ng of total RNA was reverse transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-PCR with dsDNase (ThermoFisher Scientific). The resulting cDNA was used directly for qPCR using the SYBR green PCR master mix (Applied Biosystems). Amplification was performed using the StepOne Real-Time PCR System (Applied Biosystems/Life Technologies) with the following parameters: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 59°C for 1 minute followed by a melting curve stage of 95°C for 15 s, 60°C for 1 min, and a continuous ramp up of 0.3°C until 95°C was reached then 95°C was maintained for 15 s. All reactions were performed in triplicate to control for internal variability. As it has been shown that testis and epididymal tissue express the most AMPs in other species,[24] both tissues were used as positive controls for all qPCR primer sets. Relative expression was determined using the $2^{-\Delta Ct}$ method.[25]

As there have been no qPCR analyses of equine corneal epithelium, conjunctiva, or amniotic membrane performed to date, an appropriate reference gene needed to be determined for these tissues. In this study, *beta-actin* (*ACTB*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *hypoxanthine phosphoribosyl-transferase 1* (*HPRT1*) were assessed for stability within and across different tissue types (Table 1). To assess the stability of the different reference genes, the C_T values for each gene were assessed across the samples using Normfinder as the model-based approach utilized by this software provides the most robust measure of gene expression stability.[26,27]

To validate the qPCR products, Sanger sequencing was performed on two cDNA samples. PCR was performed using the MyTaq HS Red Mix (Meridian Bioscience, Cincinnati, OH) following the manufacturer's protocol. PCR conditions were as follows: 95°C for 1 min; 95°C for 15 s, 60°C for 15 s, 72°C for 10 s, repeat 35X; 72°C for 7m. PCR products were purified using the GeneJET PCR Purification Kit (ThermoFisher Scientific) following the manufacturer's protocol. The purified PCR products were submitted to the UC Davis Genome Center for Sanger Sequencing. Chromatograms were assessed using the SnapGene Viewer (Insightful Science).

2.4 | 3′Tag-sequencing

One RNA sample each from corneal epithelium, conjunctiva and amniotic membrane were submitted for 3′Tag-sequencing to broaden our investigation of the AMP expression in these tissues. The submitted samples were chosen based on sample concentration and RNA quality. Prior to submission, RNA samples were treated with dsDNase (ThermoFisher Scientific) to remove genomic DNA and column purified with the RNeasy MinElute CleanUp Kit (Qiagen, Hilden, Germany) following the manufacturers' protocols. The samples were then submitted the Genome Center at the University of California, Davis for 3′Tag-sequencing. The unique molecular identifiers (UMIs) and spacers were trimmed from the reads using UMI-tools (https://github.com/CGATOxford/UMI-tools/blob/master/doc/QUICK_START.md). The trimmed reads were then aligned to the EquCab3.0 transcriptome (https://www.ncbi.nlm.nih.gov/genome/?term=equine) using MacVector. The Bowtie tool was utilized to perform the alignment allowing for gaps in the first 10 base pairs, 3 threads, discarding reads with less than 25 base pairs, trimming ends with quality scores of less than 20, and allowing up to 12 alignments per read. The transcripts per kilobase million (TPM) were assessed for each run as this method normalizes expression within a run, allowing for comparison between different runs. The TPM is calculated by dividing the read counts by the length of the gene in kilobases (reads per kilobase). Then, these values are added and divided by one million to identify the scaling factor. Finally, the read per kilobase value is divided by the calculated scaling factor. These data are available in the Sequence Read Archive (PRJNA882825).

3 | RESULTS

3.1 | Reference gene analysis

As an appropriate reference gene had not been identified previously for equine corneal epithelium, conjunctiva, or amniotic membrane, this study aimed to identify one. We investigated the stability in and across corneal epithelium, conjunctiva, amniotic membrane, testis, and epididymis of three commonly used reference genes, *ACTB, GAPDH*, and *HPRT1*. This analysis showed that there was variation in these traditional reference genes between tissue types and across different samples from the same tissue type. Our analysis identified that *ACTB* was most stably expressed between different tissues and between individuals and was thus used in the remaining qPCR experiments performed in this study (Figure 1).

3.2 | AMP mRNA expression

In the five corneal epithelial samples assessed, expression of *DEFB1, DEFB4B*, and *DEFB103A* was identified (Figure 2A). These data show that *DEFB103A* was expressed at about 35 times that of *DEFB1*. In conjunctiva, the three beta-defensin genes were also expressed, but at different amounts (Figure 2B). Here, *DEFB103A* was expressed at the lowest amount and *DEFB4B* was the most highly expressed at over 600 times that of *DEFB103A*. In the six amniotic membrane samples assessed, all three genes were expressed relatively equally (Figure 2C). In aggregate, expression of the three defensin genes was identified in all ocular surface tissues and amniotic membrane samples, suggesting that they play a functional role in the innate protection of those tissues.

In addition to the relevant ocular surface tissues, equine RNA from testis and epididymis were included in this experiment as positive controls as they are known to express the entire repertoire of defensins encoded by the genomes of other species.[9,24] In testis, a similar trend in expression to conjunctiva was identified where *DEFB4B* was the most highly expressed and *DEFB103A* was expressed at the lowest amount (Figure 2D). In the current study, one testis sample had undetectable expression of *DEFB103A* and was not included in the figure. However, in epididymis, the expression of *DEFB103A* was undetectable in all four tissue samples assessed (Figure 2E).

3.3 | 3′Tag-sequencing

As the equine genome contains approximately 70 predicted defensins and two cathelicidins, 3′Tag-sequencing was utilized to fully interrogate the AMP expression of corneal epithelium, conjunctiva, and amniotic membrane. This investigation identified that alpha-defensins, beta-defensins, and cathelicidins were all expressed in these three tissues (Table 2). Additionally, similar trends in expression of the three beta-defensins utilized in the targeted approach were identified in this data set (Table 3). However, there were two notable differences between the qPCR results and the 3′-Tag sequencing, namely that expression of *DEFB4B* was not detected in the 3′-Tag sequencing data from the corneal epithelial sample and that *DEFB103A* was not detected in the 3′-Tag sequencing data from the conjunctival sample. In all tissues, the alpha-defensins showed higher normalized read counts than the beta-defensins.

4 | DISCUSSION

The equine orthologs of three of the most highly expressed and functionally relevant human AMPs were identified in equine ocular surface tissues, suggesting that they play a vital role in the protection of the equine ocular surface. Additionally, equine amniotic membrane expresses AMPs, further supporting their antimicrobial role when used as a corneal surgical grafting material and/or topical therapy. Finally, the equine corneal epithelium, conjunctiva, and amniotic membrane all express alpha-defensins and cathelicidins in addition to beta-defensins.

There is no universal reference gene across species or even within one organism, thus, identification of a suitable reference gene is recommended prior to performing qPCR experiments. As a suitable reference gene had not previously been identified for equine corneal epithelium, conjunctiva, or amniotic membrane, this study aimed to determine suitable reference genes for subsequent analyses. This study investigated the stability of *GAPDH, ACTB*, and *HPRT1* mRNA expression between tissue types and individuals due to their success in other epithelial tissues.[28,29] In this study, differences within and across tissues in the expression of the three reference genes assessed were identified. Despite this variation, *ACTB* had the most stable expression across the tissues of this study, suggesting that this would be the best reference gene for these tissues in this and future studies.

In the qPCR and 3′Tag sequencing analyses of corneal epithelium, *DEFB103A* was identified as the most highly expressed beta-defensin, which is consistent with the findings in other species. *DEFB103* is documented to be a highly potent AMP of the ocular surface

in humans.[13] Thus, the identification of a high *DEFB103A* expression in the equine corneal epithelium suggests that it also plays a major role in the protection of the ocular surface of this species against pathogens such as *P. aeruginosa, S. aureus*, and Aspergillus spp.[2,3] As horses are appear to be predisposed to infectious keratitis, particularly fungal keratitis,[2] further work investigating peptide expression and activity, and comparing those directly with the human peptide expression could identify species-specific differences leading to this predisposition.

DEFB4 and its protein product, hBD2, have been identified in human conjunctival specimens.[14,30] However, there are some reports that demonstrate inconsistent expression.[31,32] This is likely due to a low basal expression of *DEFB4* with higher expression being induced by pathogenic or inflammatory stimulation.[33] Additionally, hBD2 peptide expression compared to other AMPs is also variable in human conjunctiva. For example, Lehmann *et al.* 2000 identified that the conjunctiva expresses more hBD1 than hBD2.[34] In contrast, Garreis *et al.* 2010 identified higher expression of hBD2 compared with hBD1.[30] In the present study, consistently high expression of *DEFB4B* was identified in all equine conjunctival samples assessed. As this study had a small sample size with only four conjunctival samples, it is difficult to determine if these findings present a true difference between horses and humans, though the magnitude of the expression at approximately 600 times that of *DEFB103A* suggests that this difference is true. Further studies comparing *DEFB4B* expression from horses with healthy ocular surfaces and horses with microbial keratitis are warranted to determine the inducible nature of this AMP in the equine ocular surface.

When comparing expression of the targeted beta-defensins between corneal epithelium, conjunctiva, and amniotic membrane, this study identified that the beta-defensin expression from amniotic membrane were comparable to those present in corneal epithelium and conjunctiva. This suggests that amniotic membrane could have a similar ability to protect against pathogens as the native ocular surface tissues. However, the role of AMPs in the antimicrobial protection conferred by amniotic membrane grafts in the cornea is still unknown. A potential concern with the use of these grafts for their antimicrobial properties is that the cryopreservation of equine amniotic membrane prior to its use as a graft or its processing into a homogenate product to be instilled could degrade the activity of its AMPs. Thus, future studies should include investigating the AMP expression and activity of preserved equine amniotic membrane for surgical use. However, it has been shown that cryopreserved human amniotic membrane does express viable AMPs that contribute to the protection of infected lesions.[35] In aggregate, these previous studies suggest that cryopreserved equine amniotic membrane used for corneal grafts could still express functional AMPs, thus contributing to the antimicrobial protection of corneal lesions.

Testis and epididymis were included in the qPCR experiments as positive controls as they highly express AMPs in horses and other species.[15,24] In our analysis, expression of *DEFB1, DEFB4B*, and *DEFB103A* was identified in testis, though *DEFB103A* expression was variable. Expression of *DEFB1*, and *DEFB4B* was identified in the epididymal samples assessed, but *DEFB103A* was not identified in any equine epididymal samples. However, species-specific differences in AMP expression have been identified in other studies. For

example, Com *et al.* 2003 identified expression of *DEFB1* and *DEFB4* in the epididymis and testis of rats and mice, yet *DEFB4* and *DEFB103* were not identified in human testis.[36]

3′Tag-sequencing is an economic, yet powerful screening tool to identify expression of transcripts, thus it was determined to be adequate for an initial study to identify the highly expressed AMPs of the equine ocular surface and amniotic membrane. However, this technique is not as sensitive as qPCR, which employs transcript specific primers. Thus, the absolute quantities between the qPCR study and the 3′Tag-sequencing analysis may differ, though the trends in the data should remain the same. For example, in this study, the 3′Tag-sequencing data identified similar expression patterns to the targeted qPCR analysis, but some small differences were identified, namely that no expression of *DEFB4B* was identified in corneal epithelium and that no expression of *DEFB103A* in conjunctiva was detected in the Tag-sequencing data. As these transcripts were identified at low levels in the qPCR analysis, this difference is likely due to the differences in sensitivity and specificity of these techniques. Another reason for the differences between the qPCR and the 3′Tag-sequencing is that the 3′Tag-sequencing data must be mapped to the genome to identify transcripts. Thus, sequence similarities between genes can lead to inappropriate mapping of reads leading to inaccurate read counts with this methodology. This issue is avoided in qPCR by the use of specific primers and verifying the transcripts with Sanger sequencing, as performed in the current study.

This analysis was also used to investigate the expression of additional defensins that identified expression of both alpha- and beta-defensins in all three tissue types. Interestingly, this study identified that alpha-defensins are more highly expressed in these tissues than beta-defensins. Alpha-defensins are classically described as being produced only by leukocytes and in the gastrointestinal tract. Thus, most studies on AMP expression profiles of the ocular surface focus on beta-defensin expression. However, alpha-defensins have been identified in corneal and conjunctival samples of other species. For example, expression of alpha-defensins 1 and 3 has been identified in the human cornea and conjunctiva.[32] Expression of human alpha-defensin 1 could be induced from conjunctival cells with the introduction of a viral pathogen.[37] One study assessed the efficacy of rabbit alpha-defensin 1 and 5 against *S. aureus* and *P. aeruginosa*, common equine ocular pathogens, identifying that they had antimicrobial efficacy against those pathogens.[3] Most studies investigating AMP expression of the human ocular surface have performed targeted approaches to determine their expression, which limits their ability to detect the diverse array of alpha- and beta-defensins encoded in the human genome. Due to this limitation, it is challenging to compare the results of this study to previous human studies, thus the significance of the high expression of alpha-defensins identified here is unclear. It is possible that these data could represent contamination from infiltrating neutrophils into our samples. Future studies will further define and localize the expression of alpha-defensins and their functional role in the protection of the equine ocular surface.

In addition, cathelicidin expression was present in these tissues. Specifically, we identified the expression of *equine cathelicidin 3* (*eCATH3*) in all three tissues but *eCATH2* expression was only identified in conjunctival tissue. This expression pattern is challenging to compare to other species, such as humans and mice, as many only encode a single cathelicidin gene in

their genome, whereas horses have four annotated cathelicidin genes with only two that have been shown to be expressed.[16] Thus, further work is needed to evaluate the antimicrobial activity between the two equine cathelicidins and to determine if they are modulated by the same factors.

As we have documented AMP expression from the equine ocular surface, modulation of AMP expression could represent a novel therapeutic approach for augmenting the antimicrobial activity of the ocular surface in patients with microbial keratitis. AMPs have been shown to be upregulated by pathogenic or inflammatory stimuli and small molecules.[38] For example, a recent study found that small molecules from medicinal plants were able to induce upregulation of *DEFB103* expression in an immortalized human cell line.[39] Investigation into whether similar agents upregulate AMP expression in the ocular surface is warranted to evaluate their therapeutic potential as better treatments for infectious keratitis are needed in both equine and human medicine.

5 | CONCLUSIONS AND FUTURE DIRECTIONS

This study aimed to characterize the AMP expression profile of the equine ocular surface and amniotic membrane. In doing so, we identified that the most functionally relevant beta-defensins of other species, namely *DEFB1, DEBF4B*, and *DEFB103A*, were expressed by the healthy equine ocular surface. Additionally, these beta-defensins were also expressed by equine amniotic membrane, helping to define the antimicrobial activity of these surgical grafts. This study also identified that the equine ocular tissues and amniotic membrane expressed high amounts of alpha-defensins, though the functional significance of these findings is still not well understood. Finally, this study demonstrated that the equine ocular surface and amniotic membrane express *eCATH3*, but that only conjunctiva expresses *eCATH2*. Future studies will focus on comparing AMP expression from healthy equine eyes and from eyes with microbial keratitis, particularly fungal keratitis. This study could define the selective upregulation of specific AMPs that are critical for protecting the equine eye from pathogens. These data could also be utilized to determine if affected animals have decreased AMP expression, predisposing them to the development of infection. Additionally, future work will be focused on the modulation of AMP expression to augment the innate immune response using small molecules.[38]

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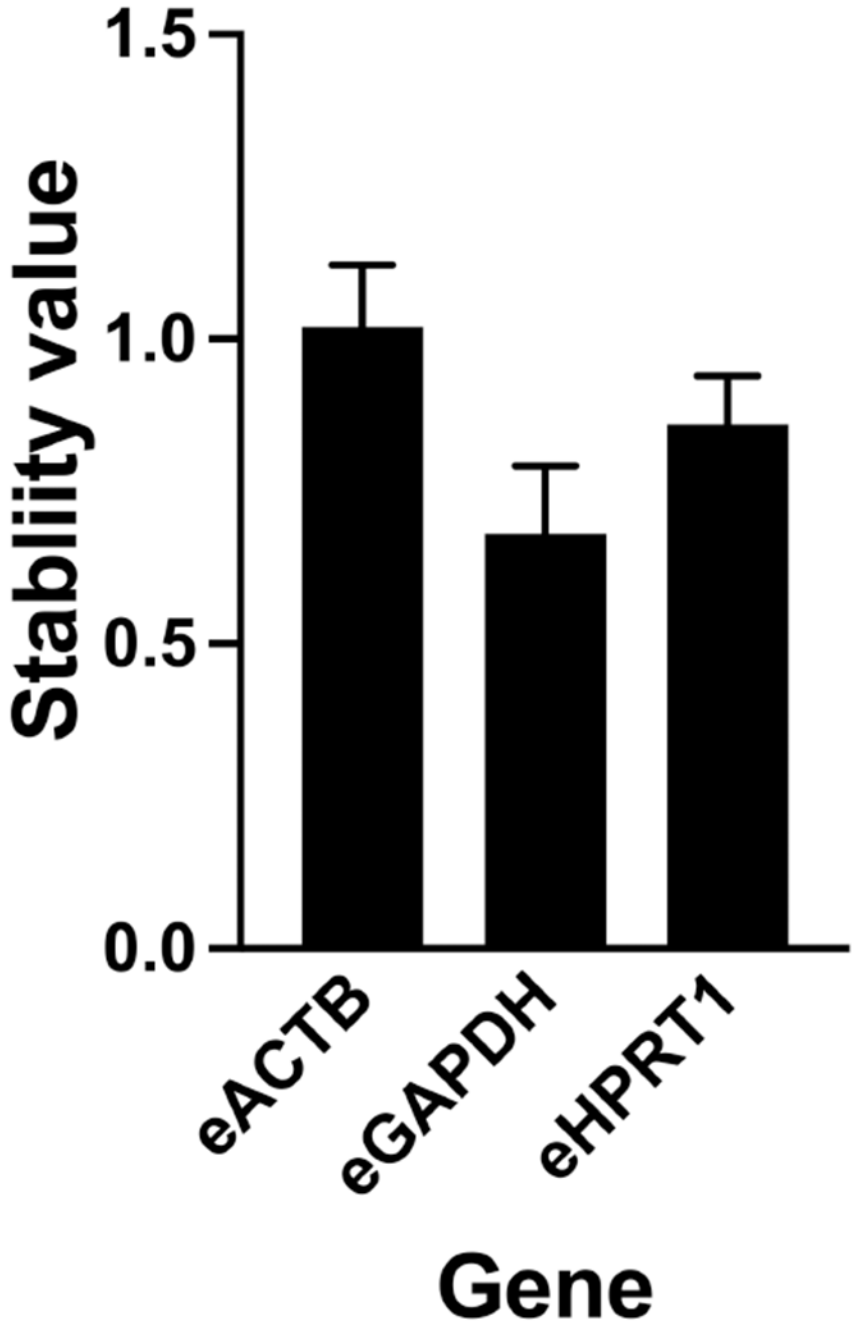

FIGURE 1.

Reference gene stability across tissues. The stability values and their standard deviations are presented here from the NormFinder reference gene stability analysis performed. This analysis identified that there was variation in the stability of these reference genes in corneal epithelium, conjunctiva, amniotic membrane, testis, and epididymis. However, it did identify that equine *beta-actin* (*eACTB*) was the most stable

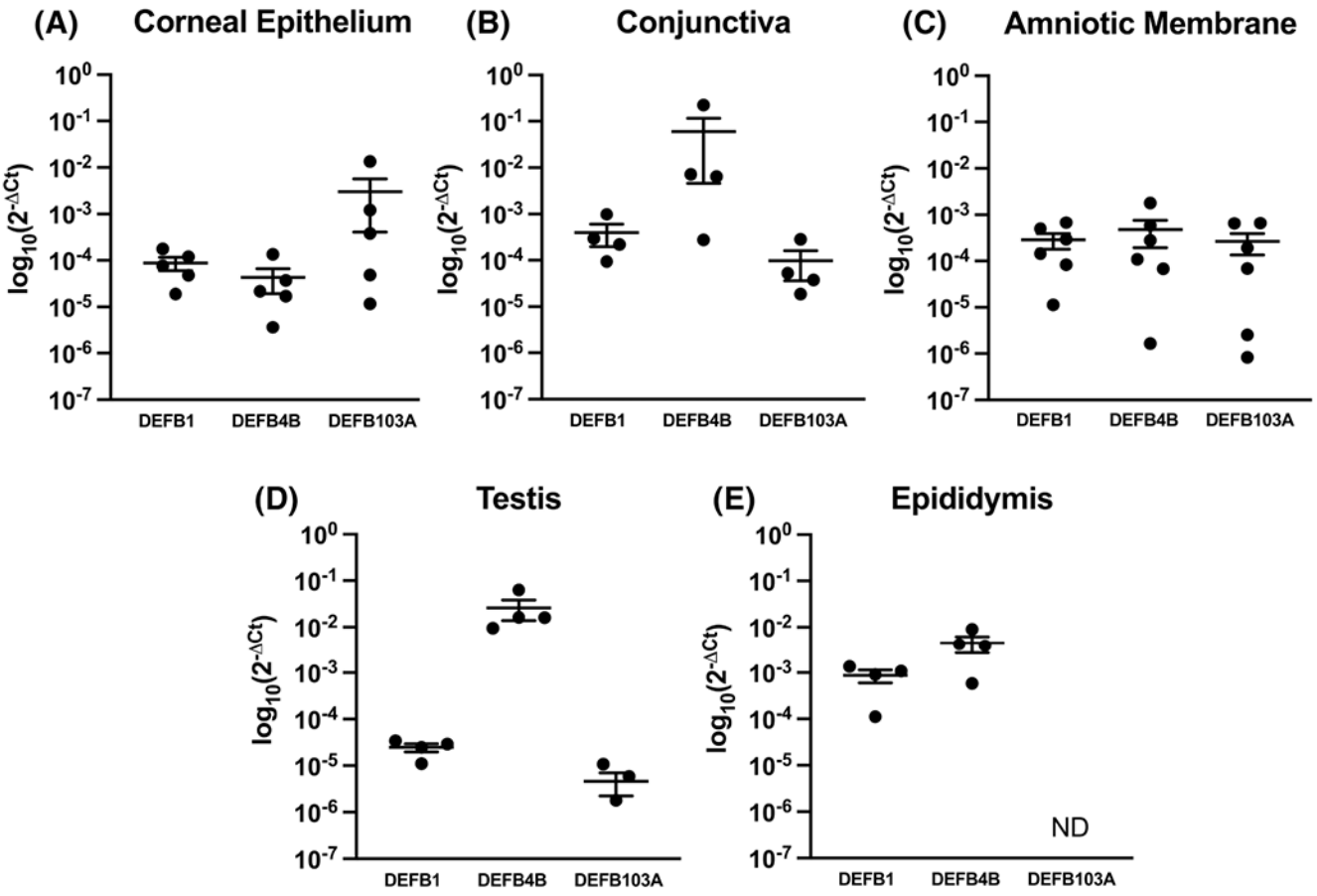

FIGURE 2.

Targeted qPCR assessment of equine beta-defensin expression across tissues. The relative expression of *beta-defensin 1* (*eDEFB1*), *eDEF4B*, and *eDEFB103A* was determined, identifying that all three genes are expressed in the tissues of interest. The mean expression (line) and standard error (error bars) are presented for corneal epithelium (A), conjunctiva (B), amniotic membrane (C), testis (D), and epididymis (E). These data were normalized to the expression of *eACTB*. One testis sample and all epididymal samples showed no expression of *eDEFB103A*. The results from the single testis sample were not incorporated into the figure due to the use of a logarithmic scale in this graph. ND: not detected

TABLE 1

Primers for qPCR amplification

Gene	Sense Primer (5′-3′)	Antisense Primer (5′-3′)
ACTB	TCACCAACTGGGACGACATG	CGGGGTGTTGAAGGTCTCAA
GAPDH	CATCAAATGGGGCGATGCTG	GGTTCACGCCCATCACAAAC
HPRT1	ATGGTCAAGGTCGCAAGCTT	CTACTAAGCAGCTGGCCACA
DEFB1	ACACTTCAGCCTCTGGAAGC	GAGGGTCAGCAGCAGAAAGT
DEFB4B	AGGGTCAAAACAGATCGGCA	TCACAGCAGTTTCTCCGCTT
DEFB103A	CCAGTCTCAGCGTGCTACAA	CTTCAAGGAGGCATTTTTGC

TABLE 2

AMPs identified in 3′Tag-sequencing data from equine corneal epithelium, conjunctiva, and amniotic membrane

Family	Transcript	Corneal Epithelium	Conjunctiva	Amniotic Membrane
Alpha-defensins	*eDEFA1*	●	●	●
	eDEFA5	●	●	●
	eDEFA5L	●	●	●
	eDEFA11	●	●	●
	eDEFA12	●	●	●
	eDEFA16	●	●	●
	eDEFA20	●	●	●
	eDEFA22	●	●	●
	eDEFA31L	●	●	●
	eDEFA35L	●	●	●
	eDEFB36L	●	●	●
Beta-defensins	***eDEFB1***	●	●	●
	eDEFB4B		●	●
	eDEFB103A	●		●
	eDEFB115	●	●	●
	eDEFB124			●
	eDEFB127	●	●	●
	eDEFB128	●		
	eDEFB135	●	●	●
Cathelicidins	*eCATH2*		●	
	eCATH3	●	●	●

Note: Circles represent that expression was identified. All alpha-defensins (*eDEFA*) were identified in all tissues with a higher read count than most beta-defensins (*eDEFB*). Trends identified in the qPCR analysis of three selected beta-defensins (bold) were replicated here. Equine cathelicidin 2 (*eCATH2*) was only identified in the conjunctival tissue.

TABLE 3

Selected AMP expression of functionally relevant AMPs

Transcript	Corneal Epithelium	Conjunctiva	Amniotic Membrane
DEFB1	2.38	79.38	19.04
DEFB4B	0	369.89	0.96
DEFB103A	42.93	0	1.24
eCATH2	0	8.94	0
eCATH3	70.61	294.03	275.71

Note: In order to normalize the data between experiments, the transcripts per kilobase million (TPM) were calculated. This is a normalization technique in which the read counts are divided by the length of the gene in kilobases (reads per kilobase). These values are then added and divided by one million to identify the scaling factor. Then, each read per kilobase is divided by the scaling factor. The TPM values for selected AMPs are presented here for one sample from corneal epithelium, conjunctiva, and amniotic membrane.