Characterization of Antimicrobial Peptides Expressed by the Equine Ocular Surface and Amniotic Membrane

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Background

Antimicrobial Peptides

- The ocular surface expresses key effectors of the innate immunity called antimicrobial peptides or AMPs to provide protection against many pathogen types including bacteria, viruses and fungi.[1]
- These peptides are continually expressed or they are upregulated by pathogenic stimuli.[2,3]

Location

- Human β-defensin 1 (*DEFB1*), 2 (*DEFB4*), 3 (*DEFB103A*) and cathelicidin are expressed by the human ocular surface.[4]
- AMPs have been characterized in the reproductive tract of horses,[5] but no studies have assessed the AMP expression pattern of the equine ocular surface.

Clinical Relevance

- AMPs help maintain ocular health when normal physical barriers to infection are disrupted, for example when the cornea is ulcerated.
- Infected corneal ulcers can be challenging to manage both medically and surgically, with one retrospective analysis identifying that 25% of eyes with mycotic keratitis had to be enucleated due to nonresponse to treatment.[6]
- As amniotic membrane has been shown in humans to have antimicrobial properties,[7] it has been used as a surgical graft in horses.[8] However, studies on the antimicrobial properties of equine amniotic membrane have not been performed.

Hypothesis and Aims

Hypothesis: Putative orthologs of both cathelicidin and defensin gene families will be expressed in equine cornea, conjunctiva and amniotic membrane.

Aim: Determine and validate the AMP expression patterns in equine ocular tissues and amnion.

Sub Aim 1: Define the stability of mRNA expression between individual horses and different biological sites for multiple housekeeping genes.

Sub Aim 2: Compare gene expression patterns of cathelicidins and defensins between conjunctiva, cornea and amniotic membrane in the horse.

Materials and Methods

Tissue Collection

- The corneal surface was scraped with a #15 blade and epithelial cells were placed directly in cell lysis buffer (**Fig. 1**).
- Conjunctiva, amniotic membrane, testis and epididymis were collected, placed into RNAlater and frozen at -20℃ (**Fig. 1**).

RNA extraction

- RNA was extracted from 30-150 mg of tissue using the GeneJET RNA Purification kit (ThermoFisher Scientific, Waltham, MA). The manufacturer's protocol was followed, except that reagent volumes prior to the column purification were doubled.

cDNA synthesis

- 300-600 ng of total RNA was reverse transcribed using the Maxima First Strand cDNA synthesis kit for RT-PCR with dsDNase (ThermoFisher Scientific).

Primer Design

- Putative orthologs of functionally relevant human AMPs were identified in the equine genome.
- Interexonic primers were designed for the orthologs using MacVector (MacVector Inc., Cary, NC).

qPCR

- Reactions were performed in triplicate.
- Testis and epididymis were used as positive controls.
- NormFinder assessed housekeeping gene stability.[9]
- The $2^{-\Delta Ct}$ method was used to calculate the relative gene expression.[10]
- Amplicons were verified with Sanger sequencing.

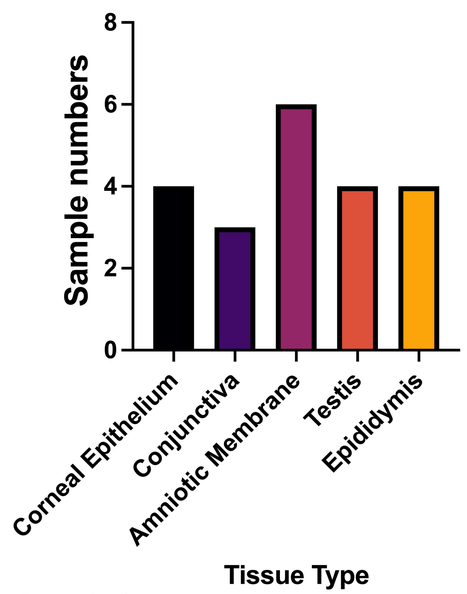

Figure 1. Sample numbers of each tissue type collected. Corneal epithelium and conjunctiva were prospectively collected from horses euthanized for reasons unrelated to this study. Amniotic membrane was collected after parturition from healthy pregnancies. Testis and epididymis were collected from routine castrations.

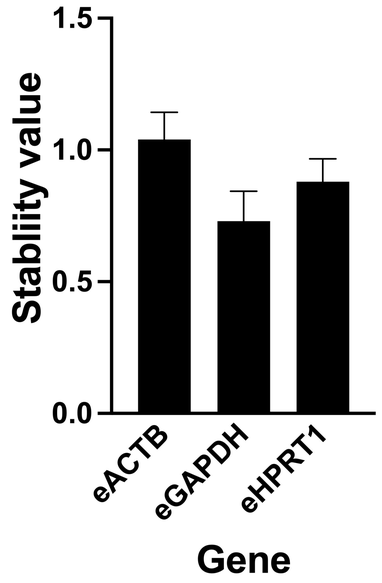

Figure 2. Housekeeping gene stability between samples and across tissue types. This analysis identified that there was variation in the stability of the different housekeeping genes assessed in these tissues and that *β-actin* showed the most stable expression.

Results

Housekeeping genes

- The stability of three commonly used housekeeping genes, *β-actin* (*eACTB*), *Glyceraldehyde-3-phosphate dehydrogenase* (*eGAPDH*) and *Hypoxanthine phosphoribosyltransferase 1* (*HPRT1*), was compared in the tissues of interest (**Fig. 2**).

β-defensin mRNA expression

- Corneal Epithelium: *β-defensin 103* (*eDEFB103*) was expressed at a level 35X higher that of *β-defensin 1* (*eDEFB1*) (**Fig. 3A**). This is functionally relevant as *DEFB103A* has been identified to be the most potent defensin in the human cornea.[4]
- Conjunctiva: *eDEFB103* was expressed the least in this tissue and *β-defensin 4B* (*eDEFB4B*) was the most highly expressed in this tissue showing an expression level 180X that of *eDEFB1* (**Fig. 3B**).
- Amniotic membrane: the three β-defensins assessed were relatively equally expressed in this tissue (**Fig. 3C**).
- Testis: this tissue showed a similar expression pattern to conjunctiva (**Fig. 3D**). One sample was excluded from the *eDEFB103* analysis for this tissue because its expression was undetectably low.
- Epididymis: No expression was detected for *eDEFB103* suggesting that testis was a better positive control for the genes of interest in this study (**Fig. 3E**).

Sanger Sequencing

- Sequencing confirmed the identify of amplicons of the three housekeeping genes and the three β-defensins.

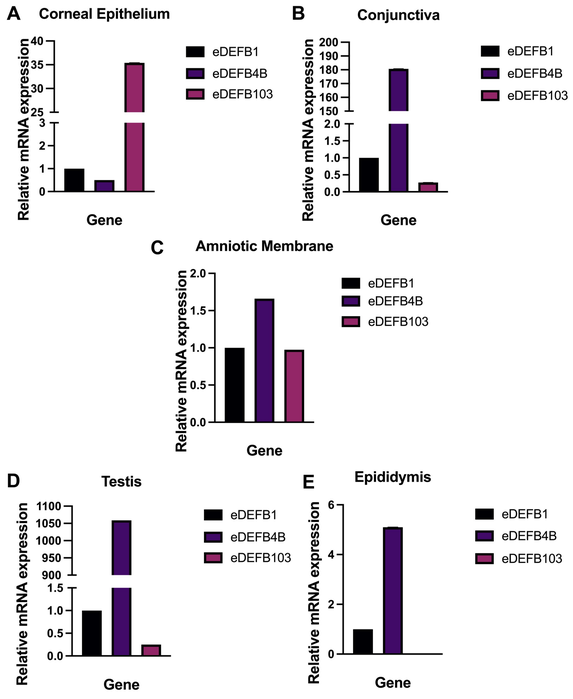

Figure 3. Relative equine β-defensin mRNA expression. In these graphs, the expression levels were normalized to the expression of β-defensin 1 (eDEFB1) for corneal epithelium (A), conjunctiva (B), amniotic membrane (C), testis (D), and epididymis (E).

Conclusions

Conclusions

- *β-actin* was the most stable housekeeping gene for the tissues in this study.
- Expression of the three β-defensins was identified in the ocular surface tissues and amniotic membrane (**Fig. 4**).
- Equine corneal β-defensin expression parallels that of humans suggesting a similar functional role.

Future Directions

- Optimize cathelicidin qPCR investigation.
- RNA-sequencing to identify additional AMPs.
- Compare these normal results with samples affected with infectious keratitis to determine if differential gene expression is present.

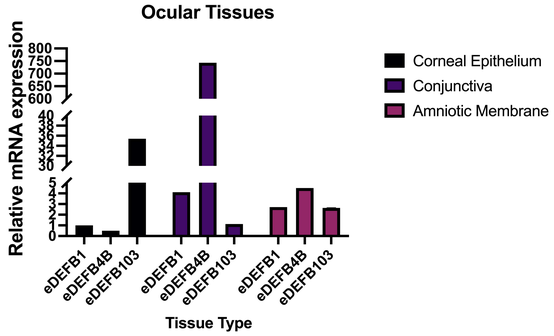

Figure 4. Relative equine β-defensin mRNA expression in corneal epithelium, conjunctiva and amniotic membrane. The expression levels in this figure were normalized to the expression of *β-defensin 1* (*eDEFB1*) for corneal epithelium.

References

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