Comparative genomic identification and expression profiling of a novel β-defensin gene cluster in equine reproductive tract

Abridged Title: Novel β-defensin gene cluster in equine genome

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Abstract

β-defensins are small cationic proteins with potent immunoregulatory and antimicrobial activity. The numbers of genes encoding these peptides vary significantly between and within species but have not been extensively characterised in the horse. Here, we describe a systematic search of the *Equus caballus* genome which has identified a cluster of novel β-defensin genes on chromosome 22, which is homologous to a cluster on bovine chromosome 13. Close genomic matches were found for orthologs of 13 of the bovine genes, which were named equine β-defensins (*eBD*) *115, eBD116, eBD117, eBD118, eBD119, eBD120, eBD122a, eBD123, eBD124, eBD125, eBD126, eBD127* and *eBD129.* As expression of the homologous cluster in the bovine was limited to the reproductive tract, tissue sections were obtained from the testis, caput, corpus, and cauda epididymis and the vas deferens of three stallions and from the ovary, oviduct, uterine horn, uterus, cervix and vagina of three mares. Using a quantitative Real-Time PCR approach each of the novel β-defensin genes showed distinct region-specific patterns of expression. Preferential expression in the caput epididymis of these novel defensins in the stallion and in the oviduct in the mare suggests a possible role in immunoprotection of the equine reproductive tract and/or fertility.

Keywords: Antimicrobial peptide, β-defensin, Equine, Reproductive tract, Equine genome.

Introduction

The recent availability and increasingly accurate annotation of multiple genomes from non-model organisms has facilitated comparative genomic analysis which can shed light on gene evolution across divergent species, and also identify genetic variation which may explain differences in phenotype among animals. The evolution of immune genes is of particular interest and the lineage-specific expansion of antimicrobial peptide gene families across farm animal species holds promise for targeted breeding or improved intervention strategies (Bruhn *et al.*, 2011). Multiple gene duplication events and subsequent sequence diversification in the mammalian lineage has resulted in a large family of defensin peptides with divergent amino acid sequence which are usually classified on the basis of their tertiary structure (Bauer *et al.*, 2001). Mammalian defensins are a large family of cationic cysteine-rich antimicrobial peptides (AMPs) with molecular masses ranging from 2 to 6 kDa and can be classified into three subfamilies; α-, β-, and θ-defensins, identified by their specific intermolecular disulfide-bond pattern, and cysteine positioning (Bruhn *et al.*, 2009b, Davis *et al.*, 2004). α -defensins are characterised by having disulfide bonds between cysteines 1-6, 2-4, and 3-5. In contrast, β-defensins are comprised of disulphide linkages between cysteine residues 1-5, 2-4, and 3-6 (Davis *et al.*, 2004, Ganz, 2003). The three disulfide bonds positioned in a predictable manner forms the defensin motif, which is conserved across peptides and species (Semple *et al.*, 2003). θ-defensins are rare peptides discovered in the rhesus macaque and are present only in primates, the single θ-defensin gene in humans is truncated by a stop codon creating a pseudogene (Tran *et al.*, 2008).

β-defensin genes have been found in most vertebrate genomes and encode small, cationic proteins produced by phagocytic cells, lymphocytes, and the epithelial cell lining of the gastrointestinal and genitourinary tracts, the tracheobronchial tree, and keratinocytes (Schneider *et al.*, 2005). Given their antimicrobial activity (Choi *et al.*, 2012, Narciandi *et al.*,

2011, Schöniger *et al.*, 2013), β-defensins are traditionally regarded as vital effector molecules of the innate immune system (Davis *et al.*, 2004, Ganz, 2003, Ganz, 2004). However, members of this gene family have also been shown to have immunoregulatory and chemotactic activity (Bowdish *et al.*, 2005). Recent studies in other species have shown that β-defensins are expressed by the reproductive tissues in mice, rats and cattle (Com *et al.*, 2003, Cormican *et al.*, 2008), which has led to speculation about a potential role in the protection of the reproductive tract against pathogens, or in the regulation of fertility. A cluster of 19 novel bovine β-defensin genes were shown to be expressed in a region-specific manner across the male and female reproductive tracts, suggesting a role in bovine reproduction (Narciandi *et al.*, 2011). Evidence for β-defensin mediated regulation of fertility has been supported by recent findings in primates – Macaque sperm have been shown to be coated with β-defensin 126 (BD126), which facilitates their passage through cervical mucus *in vitro* (Tollner *et al.*, 2008b). Similarly the same peptide is thought to contribute to capacitation (Tollner *et al.*, 2009), enabling sperm-binding to oviductal epithelia (Tollner *et al.*, 2008a), as well as the prevention of immunorecognition in the female tract (Yudin *et al.*, 2005a). Finally, polymorphisms in human BD126 are known to contribute to sub-fertility in males (Tollner *et al.*, 2011a) and a recent study has shown that β-defensin gene knock-out male mice are completely infertile (Zhao *et al.*, 2011).

Since the publication of the equine genome in 2007, 38 α-defensins have been identified, all of which were shown to be expressed in the gastrointestinal tract (Bruhn *et al.*, 2009a, Bruhn *et al.*, 2009b) and twenty of which have the potential to code for functional proteins (Bruhn *et al.*, 2009b). The retention of protein-coding α-defensin genes identifies the horse genome as distinct from members of the artiodactyl order, which have not retained α-defensin genes (Fjell *et al.*, 2008, Lynn and Bradley, 2007). Very limited characterisation of the β-defensin gene family has been performed in the horse (for review see: Bruhn *et al.*, 2011). The first

gene was discovered in 2004 (DEFB1; Davis *et al.*, 2004) and expression was documented across a range of tissues, including the small intestine, liver, heart and uterus (Schöniger *et al.*, 2013). Some genes show more specific patterns of expression including β-defensin 103, reported to be exclusively expressed in the tongue of the horse. Subsequent analysis identified additional β -defensin family members (Looft *et al.*, 2006), and although sequence validation was not performed, the synteny of those β -defensins was shown to be similar to genomes from other species. The aim of this study was to use a comparative genomics approach to search for evolutionary orthologs for a recently discovered cluster of bovine β-defensin genes in the equine genome.

Materials and Methods

Bioinformatic Identification of Equine Beta Defensin Ortholog

Homology searches with the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) were performed using gene sequences for the 19 known bovine β-defensins on chromosome 13, to find homologous genes in the equine genome (Version Broad equ/Cab2 Sep 2007). The bioinformatic tool, BLAST-Like Alignment Tool, (BLAT) was used to determine the chromosomal position of equine genes homologous to other mammalian β-defensin genes. BioEdit software was used to perform multiple sequence alignments between the equine and bovine protein sequences. To annotate the putative β-defensin-encoding sequences identified from our analysis, a phylogenetic analysis was performed using MEGA software, version 5.2 (Ram *et al.*, 2014). Bootstrap resampling was carried out 1,000 times. Phylogenetic analysis was performed to investigate the evolutionary relationships among the thirteen novel β-defensins in the equine and their bovine homologs. Equine β-defensin genes were annotated on the basis of sequence similarity and phylogenetic relationships to

previously described β-defensins in cattle to maintain consistency in the comparative analysis of β-defensins with other species. Nucleotide sequences were aligned using T-coffee (Notredame *et al.*, 2000) and annotated using Jalview (Waterhouse *et al.*, 2009).

Reproductive Tissue Collection

Reproductive tract tissues including testis, the different segments of epididymides (caput, corpus, cauda) and vas deferens (Figure 1) were collected from 2-4 years old sexually mature Connemara stallions (n=3) immediately post-castration. Reproductive tract tissues including the ovary, oviduct, uterine horn, uterine corpus, cervix, and vagina (Figure 1) in the luteal phase (confirmed by the presence of a corpus luteum; n=3) were retrieved from 5-8 year old Connemara non-pregnant mares (n=3) post-mortem, at a local abattoir, within 20 min of slaughter. All tissue samples were placed in RNAlater (Qiagen, Crawley, UK), held at 4°C overnight, and subsequently stored at -20°C.

RNA Extraction and cDNA Synthesis

Total ribonucleic acid (RNA) was extracted from all tissues using a homogenizer to disrupt cells in the RLT buffer, supplied with the RNeasy® mini kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. All samples were DNA digested to remove genomic DNA using Qiagen's on-column DNase, and eluted with water. RNA quantity was assessed using a Nanodrop spectrophotomer (Thermo Fisher Scientific, Waltham, MA, USA), while the quality was determined with the use of an Agilent Bioanaylser (Agilent Technologies, California). cDNA was synthesised using an Applied Biosystems cDNA reverse transcription kit (Life Technologies, California), and an Eppendorf Mastercycler (Eppendorf, Hamburg).

Primer Design, qRT-PCR and Data Analysis

Nucleotide sequences were retrieved from the University of California, Santa Cruz (UCSC) Genome Browser, and entered into Primer3 (Rozen and Skaletsky, 2000) to determine the best nucleotide sequence of both a forward and reverse primer, for each gene (Table 1). Primers were designed, where possible, to be intron spanning and commercially synthesised (Sigma Aldrich, MO, USA). Equine β-defensin 132 was not amplified, as only one exon was found in the genome sequence; and therefore, an intron-spanning primer could not be designed. Quantitative real time polymerase chain reaction (qRT-PCR) was performed using a 20 µL reaction mix containing: 10 µL SYBR green PCR Master Mix (Invitrogen Ltd, Paisley, UK), 2.5 µL primer and dH_2O mix, 5.5 µL dH_2O, and 2 µL sample. Plates were run in an ABI 7500 Fast Thermocycler. The cycle parameters were as follows; UNG activation was run for 2 minutes at 50°C, DNA polymerase activation for 10 minutes at 95°C, the melt cycle was run for 15 seconds at 95°C, and the annealing/extending cycle for 1 minute at 60°C. A no-template control (NTC) was run in each 96-well plate to confirm the absence of contamination and all products were run on an agarose gel to confirm the presence of a single PCR product, of the correct size. A 1% agarose gel was stained with 5 µL ethidium bromide, while each 20 µL PCR product was stained with 3 µL blue tracking dye. Three microlitres of 1kb ladder was used, while 5 µL of sample was added into each well, and gels were run at 100mV, for 40 min. Gel electrophoresis images were acquired using a FluorChem system (Alpha Innotech, CA, USA). Levels of gene of interest expression were determined using fold changes, calculated using the ΔCt (cycle threshold) method (Livak and Schmittgen, 2001), compared with the average of the two reference genes-GAPDH and ACTβ. Normalising gene expression to multiple reference genes in order to give a more reliable baseline for the calculation of relative gene expression using qRT-PCR is common practice especially when small changes in gene expression are being reported (Vandesompele et al.,

2002). Gene expression data among the tissues (within gender) were examined for normality of distribution, transformed where appropriate using an arctan transformation, and analysed using analysis of variance in the Statistical Package for the Social Sciences (SPSS, Version 21.0; IBM, USA). Post-hoc tests were carried out using the Bonferroni correction, and a P value < 0.05 was considered statistically significant.

Results

Discovery of Novel β-defensins in the Equine Genome

Of the 19 novel β-defensin genes recently reported in the bovine, thirteen were found to be present on chromosome 22 in the equine genome (Table 1). Close genomic matches were found for orthologs of the bovine genes, which were named equine β-defensins (*eBD*) *115, eBD116, eBD117, eBD118, eBD119, eBD120, eBD122a, eBD123, eBD124, eBD125, eBD126, eBD127* and *eBD129.* Incomplete sequences were retrieved for *eBD119* and e*BD132*, which were found to only have one conserved exon. No close matches to bovine β-defensins *bBD121, bBD122, bBD125a*, *bBD128* or *bBD142* were detected in the equine genome. It should be noted that bBD122 and bBD122A are 75% identical in their amino acid composition resulting from duplication and subsequent divergence of the bovine 122 locus, all within the second exon. eBD122a is the ortholog of BBD122a, and the similarity between these peptides is 65%.

Bioinformatic Analysis

Multiple sequence analysis was performed on novel defensin sequences with a complete second exon. Despite the high sequence variation between genes, the conserved cysteine

signature was clearly visible in each (Figure 2). Phylogenetic analysis revealed two semi-distinct groups with a bootstrap value of 22, indicating 78% resemblance (Figure 3). Likewise, a bootstrap value of 100 indicates that these genes map to one another 100% of the time (through resampling of the data) and therefore, due to the very high degree of sequence similarity they are definitely orthologs. The smaller of the two groups consisting of *eBD117, eBD125, eBD126,* and *eBD127;* whilst the larger group was made up of the remaining eight genes; *eBD115, eBD116, eBD118, eBD119, eBD120, eBD122a, eBD123, eBD124,* and *eBD129.*

Expression of Novel Defensins Across Equine Reproductive Tissues

Expression of the twelve of the novel bovine β-defensin genes was investigated in a panel of reproductive tissues collected post-mortem from a panel of healthy stallions and mares (Figure 1), *eBD118* is not shown. Expression profiles for a selected panel of genes (which had the most animal to animal variation) in various tissues across the stallion and mare reproductive tracts were generated using qRT-PCR and are shown in Figures 4 and 5, respectively. Expression of *eBD116* was lower for all three stallions in the testes than in the remaining 4 regions of the reproductive tract ($P < 0.01$). The expression profile for this gene was similar among stallions and highest expression was detected in the corpus of the epididymis. In contrast, expression of *eBD117* showed more divergence among stallions, with high relative expression in the testes. *eBD117* expression was consistently higher in samples from stallion 1, than stallion 2 and 3. Gene expression in the caput of the epididymis was over 1000 fold higher than the reference genes in this tissue, making *eBD117* the highest expressed gene in this analysis (Figure 4). Clear differences between stallions were apparent in the *eBD120* expression profile, where for stallion 1, expression was highest in the corpus of the epididymis. Expression of this gene was at its lowest in the corpus for stallions 2 and 3.

Expression for *eBD125* was consistent between stallions and showed lowest expression in the vas deferens. Variability of *eBD126* was seen among the corpus epididymis samples of the three stallions, while the expression was relatively homogeneous across the other sections of the stallion reproductive tract (qRT-PCR data not shown).

In general, expression in the mare reproductive tract was lower than in the stallion reproductive tracts and also exhibited higher inter-animal variation (Figure 5). Mare 2 showed consistently lower expression across most genes than mare 1 and 3. Similarly to that detected in the stallion tract, *eBD117* was the highest expressed gene in this analysis. Expression was over 200 fold higher than the reference genes in the oviduct of mare 1 and 3. The oviduct and the uterus were found to be the predominant sites of β-defensin expression in the mare, with the exception of *eBD119*, which had significantly lower expression than all other genes, in all regions. Unlike the stallion, *eBD116* had notably lower expression in the mare's reproductive tract, in particular in the cervix and uterine horn in the mare, as did *eBD126*, which displayed extremely low expression in the ovary and uterine horn. Equine β-defensin *115* and *117* had differential regional expression across the mare reproductive tracts. Both were highly expressed in the common body of the uterus of the mare; however, they had a much lower expression in the anatomically adjacent uterine horn.

Some β-defensins (*eBD129* and *eBD122a*) were found to be homogeneously expressed across the genital tract in both stallions and mare, whereas, others (*eBD115* and *eBD116*) were more highly expressed in the stallion reproductive tract ($P < 0.01$). *EBD119* was found to be expressed at a low level across all tissue samples (both mare and stallion), while *eBD117* was shown to have the overall highest expression in the stallion ($P < 0.01$). Defensin-like 2 and 3 (DEFL 2 & 3) were expressed at low levels in the reproductive tracts of both the mare and the stallion. In general, highest expression for the majority (10 of 13) of these genes was in the caput epididymis (Figure 6).

Discussion

The advent of more completely annotated genomes from farm animal species is facilitating gene discovery at an unprecedented level. As immune genes with potent antimicrobial and immunomodulatory functions, β-defensins hold significant interest for understanding the immune response but also in the design of novel therapeutics.

The current study was designed to examine if the β-defensin genes are expressed along the reproductive tract of the mare and stallion (as they are in the bovine) and to demonstrate the variation between animals in selected EBDs. Using a comparative genomics approach, we searched the *Equus caballus* genome for homologs of 19 recently discovered bovine β-defensins. Thirteen novel genes were found on chromosome 22 in the equine genome and bioinformatic analysis revealed that the equine defensins are present in a similar syntenic sequence to those of the bovine. Orthologs for the remaining 6 genes were not found which may reflect the loss of these genes over the 100 million years of evolution since the horse and cow last shared a common ancestor.

Multiple sequence alignment showed the conservation of the characteristic six cysteine residues (Ganz, 2003) in the equine β-defensin genes. Phylogenetic analysis of these equine genes in conjunction with their bovine orthologs (Narciandi *et al.*, 2013), show a high degree of sequence similarity which suggests functional conservation of these genes over the course of evolution. The percentage of sequence identity ranged from 37% (*BD118*) to 95% (*BD119*). The ortholog pairs generally cluster together with high boot-strap values but the evolutionary relationships, among the genes was quite flat and unstructured, as expected from such short input sequences.

Expression analysis of β-defensins in the reproductive tracts of other species demonstrated quantitative variation in gene expression in various sections of the genital tract,

thus suggesting that site-specificity of gene expression may reflect differences in the biological role of these β-defensins (Com *et al.*, 2003, Jelinsky *et al.*, 2007, Zhou *et al.*, 2004). All novel equine genes examined in this study were found to be expressed across the reproductive tracts of all three stallions and the basal expression of these β-defensin genes in the absence of infection suggests that in addition to being antimicrobial and capable of protecting sperm from infections, these molecules may constitute an essential component in maintaining the normal reproductive process. The high expression found in the caput epididymis for all genes, in all stallions, and the low gene expression patterns found in the vas deferens, for the majority of the genes (8 out of 13) in stallions, is in agreement with studies which suggest that initiation of sperm maturation, and the induction of progressive motility are associated with β-defensin absorption, which occurs in the epididymis of humans (Tollner *et al.*, 2011b), macaques (Tollner *et al.*, 2008b), rats (Zhou *et al.*, 2004), mice (Yudin *et al.*, 2008), pigs (Choi *et al.*, 2012) and cattle (Narciandi *et al.*, 2013). This consistent and high expression of β-defensins in the caput epididymis, is suggestive of a potential role in final sperm maturation for these genes.

β-defensin *126* is a gene of particular interest as it has been documented to regulate human male fertility. Coating the sperm glycocalyx in humans (Tollner *et al.*, 2011b) and macaques (Tollner *et al.*, 2011b, Tollner *et al.*, 2008b), *DEF126* has been found to be critical for the movement of sperm through macaque cervical mucus (Tollner *et al.*, 2008b). Furthermore, the removal of *DEF126* from the sperm surface is essential for the completion of capacitation in the female reproductive tract of macaques (Tollner *et al.*, 2009, Yudin *et al.*, 2003). This is essential for the biochemical events leading to the recognition, and subsequent fertilisation of the oocyte in macaques (Tollner *et al.*, 2004). Following treatment of macaque sperm, with anti-*DEF126* antibodies Tollner *et al.*, demonstrated that sperm had significantly reduced ability to penetrate cervical mucus (Tollner *et al.*, 2008b), while upon

add back of *DEF126*, penetration ability was restored. Furthermore, humans that are homozygous for the *DEF126* gene (del/del) have been shown to have reduced fertility; more specifically this mutation causes reduced mucus penetration ability in sperm (Tollner *et al.*, 2011b). In this study, *eBD126* had relatively homogenous expression across the stallion genital tract, with the exception of the corpus epididymis, which was more varied, suggesting that it has a similar role in sperm maturation as that described in other species (Tollner *et al.*, 2008a, Tollner *et al.*, 2008b, Yudin *et al.*, 2005a, Zhou *et al.*, 2004). *eBD117* was found to have the overall highest expression across all reproductive tissue samples in both the stallion and mare. Interestingly, in the cow, the expression of the β-defensin gene cluster is limited to the reproductive tract, with the exception of *eBD117* (Narciandi *et al.*, 2011).

Based on their location in the testis and epididymis, and in consideration of recent and associated studies (Tollner *et al.*, 2009, Tollner *et al.*, 2008a), β-defensins could potentially mediate the binding of the sperm to the equine female oviductal epithelia. In addition, defensins could be important for sperm maturation in the male tract: this process is known to occur by the attachment of multiple proteins (*DEFB126, cAMP;* Yudin *et al.*, 2003, Yudin *et al.*, 2005b) to the sperm surface during its transit through the epididymis (Acott and Hoskins, 1981, Narciandi *et al.*, 2011, Yudin *et al.*, 2003). Alternatively the regional distribution of the β-defensins could be explained by the various groups targeting specific pathogens only present in that region, or to prevent infection in the female reproductive tract following mating (Sorensen *et al.*, 2003), however, the lack of inflammation or infection in tissues sampled in this study suggests a role outside that of solely defence. However, it has been demonstrated in pregnant women that there is an increase in expression of β-defensins in the amniotic fluid of women with intrauterine infection, and preterm labour (Mitchell *et al.*, 2013). Interestingly, women with bacterial vaginosis, a condition in humans similar to endometritis in the mare, had an up regulation of β-defensins in the uterine environment,

where the concentration of human β-defensin and bacteria were positively correlated (Mitchell *et al.*, 2013). Similar findings have also been reported in hens, where CpG-ODN derived from microbes upregulates the expression of *IL1B* and *IL6* by interaction with TLR21 and then IL1B induces *AvBD1* and *-3* to prevent infection in the vagina (Sonoda *et al.*, 2013). Finally, the annotation of the equine genome is at an early stage and more thorough characterisation may show that some of these β-defensin genes are actually alternatively spliced isoforms rather than distinct genes, as has been shown for orthologs in the human genome (Radhakrishnan *et al.*, 2005).

Conclusion

In conclusion, this study is the first to genomically identify and validate the expression of a novel β-defensin gene cluster in equine reproductive tract. The evolutionary orthologs of these genes have been shown to play a pivotal reproductive-immunobiological role, across a range of species including mice, macaques and men. Shedding light on the evolution of these pleiotropic molecules, these genes can now be targeted for population genetic analysis, to identify functional polymorphisms that may contribute to higher fertility in individual horses, and between horse breeds.

This could help generate new assisted reproductive technologies, as well as the development of alternative treatments, for conditions such as post breeding induced endometritis, which is one of the largest causes of infertility in mares. Sequence variants in these genes could explain some of the phenotypic variation in susceptibility to infection in mares. A study with a much higher number of animals now needs to be done to investigate if variation in the β-defensin expression can explain differential susceptibility to infection in mares.

Competing Interests

None of the authors have any competing interests.

Authors' Contributions

Gillian Johnson completed all the laboratory and bioinformatic work. Andrew Lloyd and Cliona O'Farrelly provided guidance with the bioinformatic and laboratory work, respectively. Kieran Meade and Sean Fair designed the experiments and were the lead supervisors of Gillian Johnson.

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Table 1 Oligonucleotide sequences for primers of the genes used in this study, and the genomic coordinates of the defensin genes (UCSC version equCab2).

Gene symbol	Forward primer (5'- 3')	Reverse primer (5'- 3')	Genomic Co-ordinates
GAPDH	AAGGTCATCCCTGAGCTGAA	TGGAAGAGTGGGTGTCACTG	NM_001163856.1
ACTb	GATCTGGCACCACACCTTCT	GAGTCCATCACGATGCCAGT	NM_001081838
DEFL2	CCTTCTCATCTTTGTTGTCCTC	TTCCTCCTCAGGCAGACACT	AM039964
DEFL3	CTTGTGTGTCTCATCCAGACG	CGTGTTTTCTCCCCGTTTT	AM039964
eBD115	TATGCTGCTGGATCATTCCTC	GTTACCTGGGGAAGCCTGA	chr22:22,315,338-22,510,961
eBD116	CCATCCTTCTGATGCTGGTT	TTCTGCACATGCCTTGGTAA	chr22:22,391,758-22,396,465
eBD117	GACATTGCAGGAAAGACTGC	TGGATTGGTCTGTGTGGTTG	chr22:22,440,878-22,440,997
eBD119	CTTTCTGGCCATGGAACCAG	TGTTACCCATGCACTGAAGG	chr22:22,475,857-22,475,916
eBD120	CGGTTGGTGTGCAAAGATGA	CCAGGGGAGTTCTCCATCAA	chr22:22,474,538-22,474,687
eBD122a	GCTGGAATCTTCATGGCACC	CAGTGGTAAATTCGGCTGGA	chr22:22,510,961-22,511,080
eBD123	ACCCGAAAATGCTGGAATCT	TGGTACTTGGGCTTCACACA	chr22:22,546,361-22,546,486
eBD124	AATTCAAACGGTGCTGGAAG	TGAGTCCGTAGGGGAGACAG	chr22:22,559,089-22,562,585
eBD125	GCTGGCTGGGAAGTCAAA	AATCCTCCTCACTCGTTATGG	chr22:22,264,333-22,270,987
eBD126	GGTATGTGAGAAAGTGTGGAAACA	TCTTTGCTGCACATGCCAGT	chr22:22,207,554-22,207,679
eBD127	ACTTAAGAAATCGTGGGGTGA	GTCATTGGCTTTGGACGTGT	chr22:22,192,358-22,192,582
eBD129	TGGGGAGATGCAAAGACCAT	ACCGGTGCTTTTGATTTTGGA	chr22:22,136,194-22,138,292

List of Figures

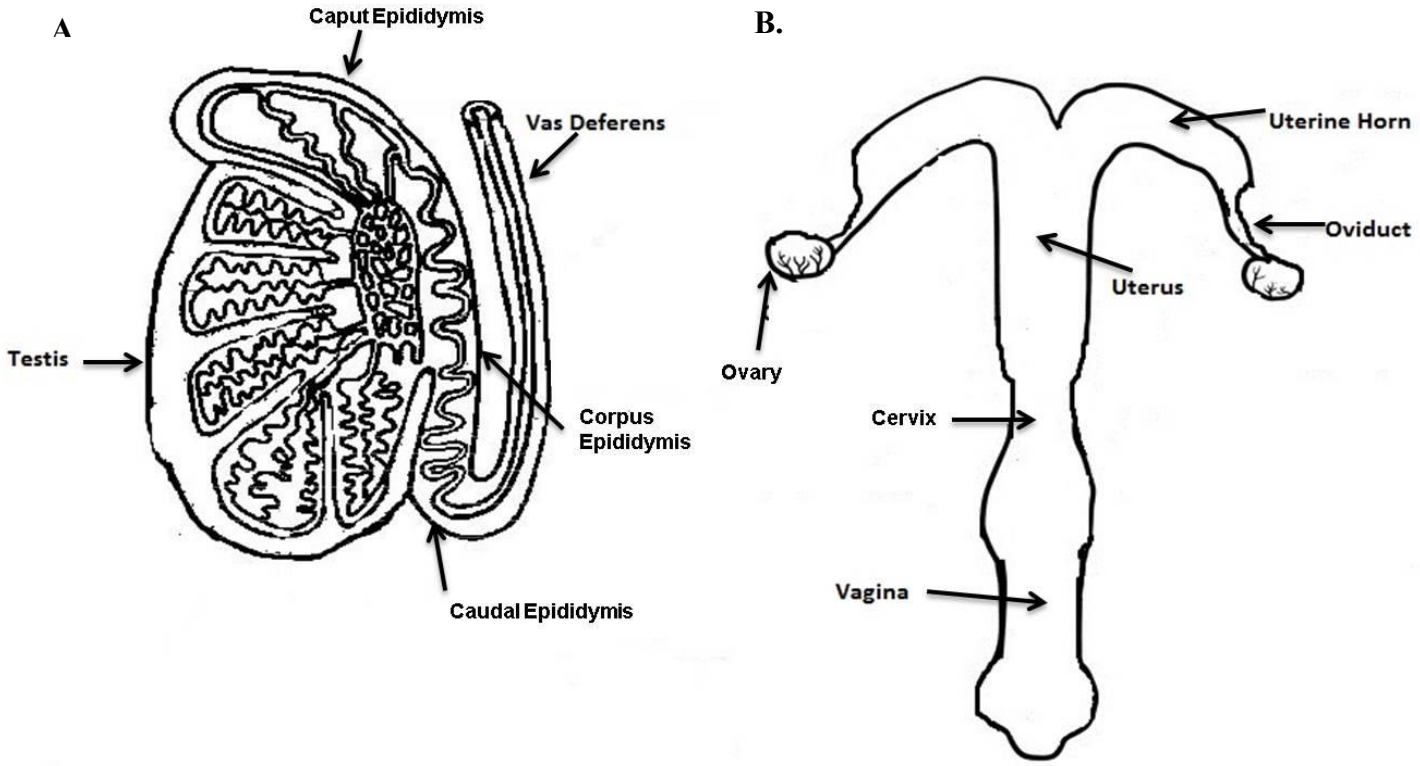


Figure 1: Sites of tissue sample collection. The sites of tissue collection in the reproductive tract of the stallion (A) and mare (B). Note that the reproductive tracts are not represented to scale.

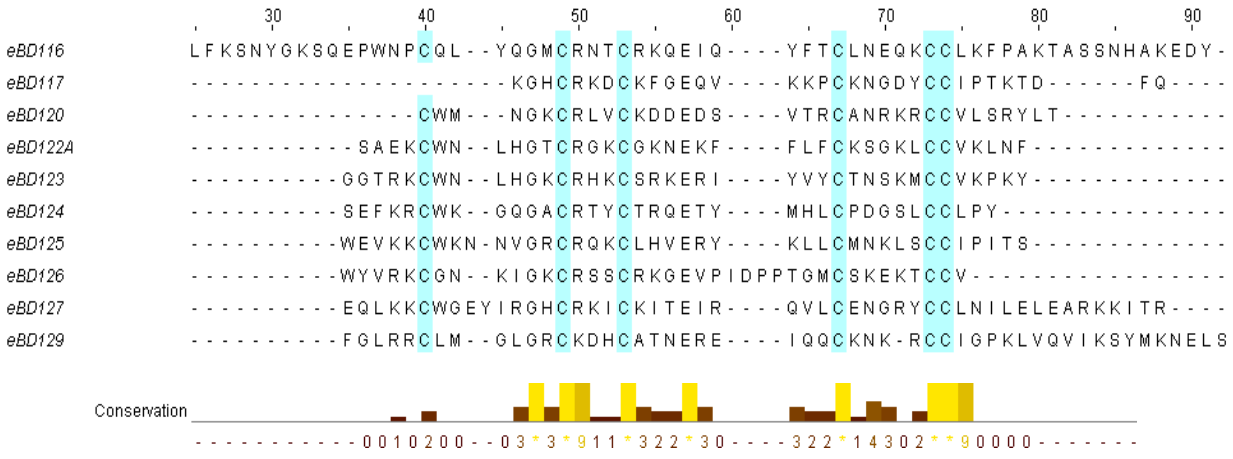


Figure 2: Multiple sequence alignment of the equine β-defensins. Multiple sequence alignment showing the conserved cysteine residues for the novel equine β-defensins (shaded). [*eBD115*, *eBD119* and *eBD132*, genes were excluded from the figure, as only first exon sequences had been recovered].

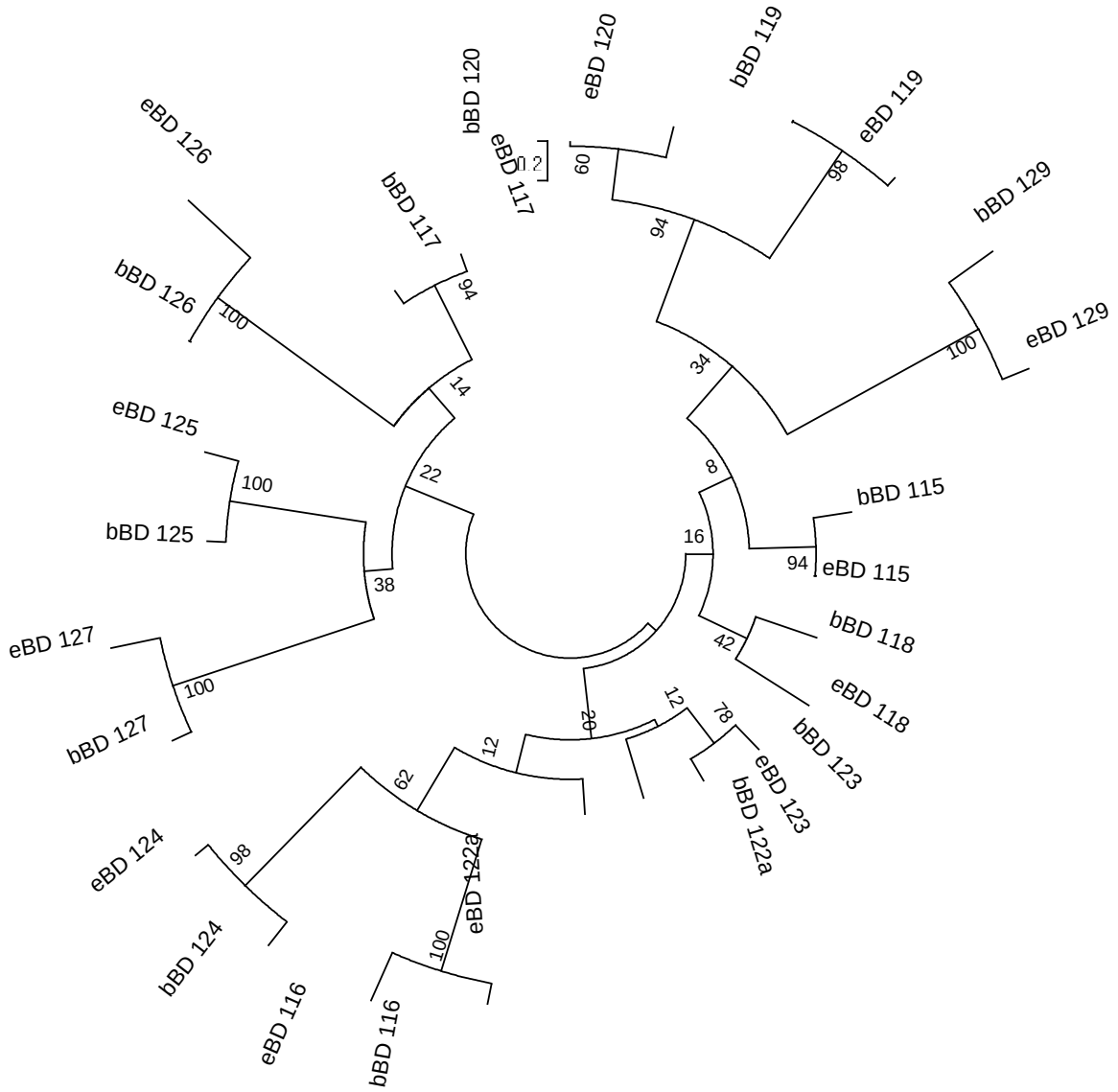


Figure 3: Phylogenetic tree between the between the thirteen novel β-defensins in the equine genome with the bovine orthologs. Phylogenetic tree showing the evolutionary relationship between the thirteen novel β-defensins in the equine genome with the bovine orthologs. The numbers at the nodes are % bootstrap values.

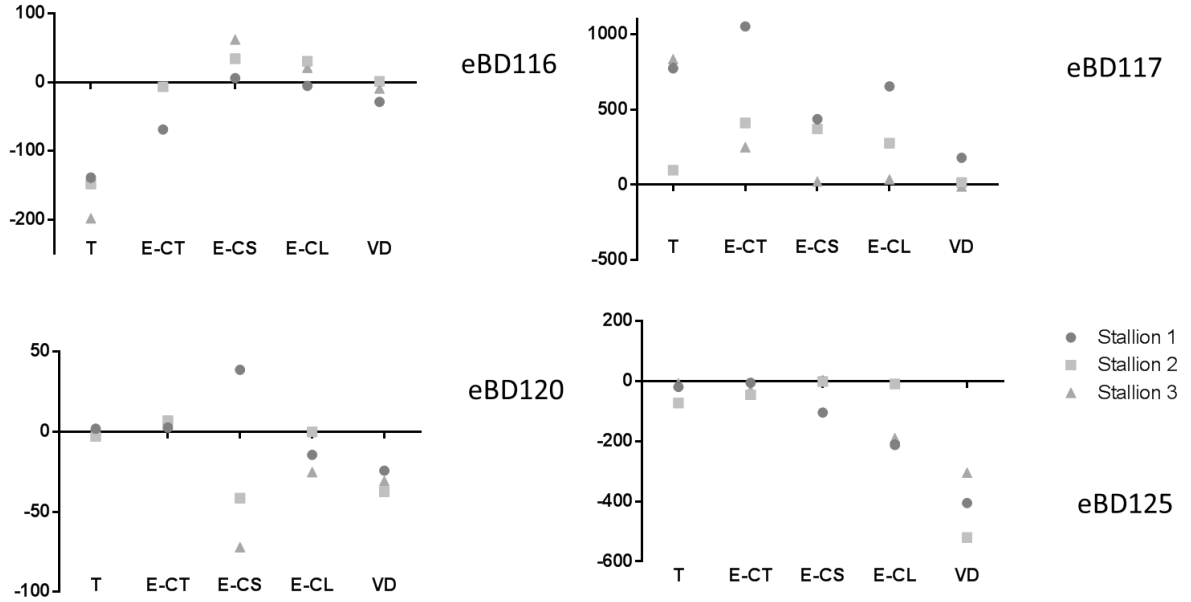
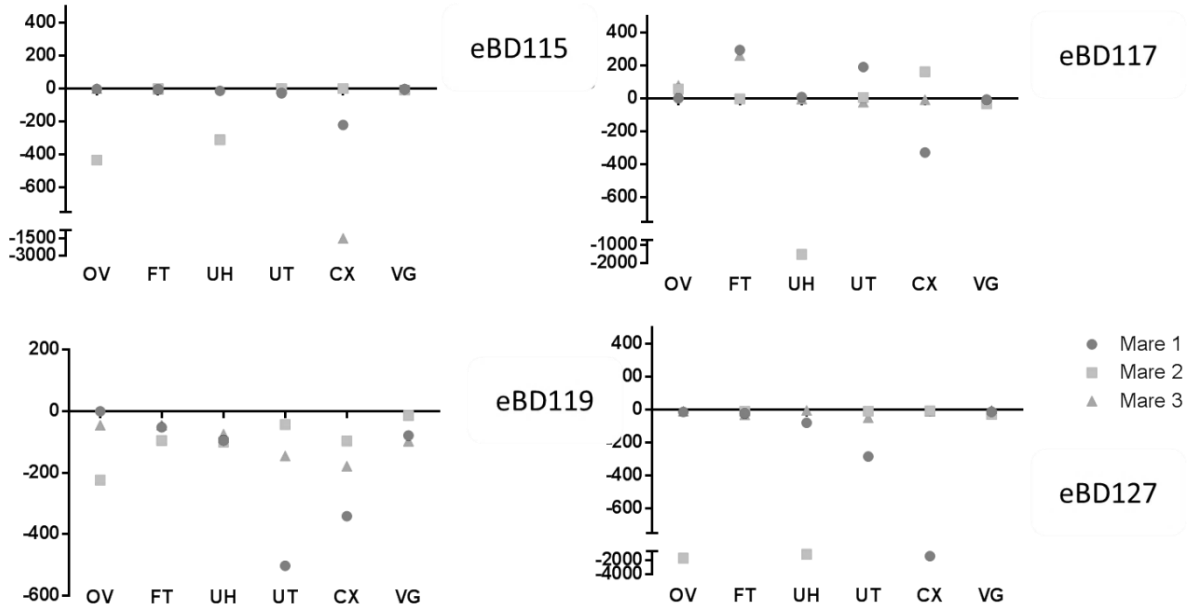


Figure 4: Expression of four representative β-defensins in the reproductive tracts of 3 stallions. Variation in expression of selected equine β-defensin genes (*eBD116, eBD117, eBD120* and *eBD125)*, across the reproductive tracts of 3 stallions. Expression was normalised to the average of *GAPDH* and *ACTβ* gene expression (represented by 0 on graph). Tissue sections identified as T, testis; E-CT, caput epididymis; E-CS, corpus epididymis; E-CL, cauda epididymis; VD, vas deferens.



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547 Figure 5: Expression of four representative β-defensins in the reproductive tracts of 3 mares.
548 Variation in expression of selected equine β-defensin genes (*eBD115, eBD117, eBD119* and
549 *eBD127)*, across the reproductive tracts of 3 mares. Expression was normalised to the average
550 of GAPDH and ACTβ gene expression (represented by 0 on graph). Tissue samples
551 identified as OV, ovary; OvD, oviduct; UH uterine horn; UT, uterine corpus; CX, cervix and
552 VG, vagina.

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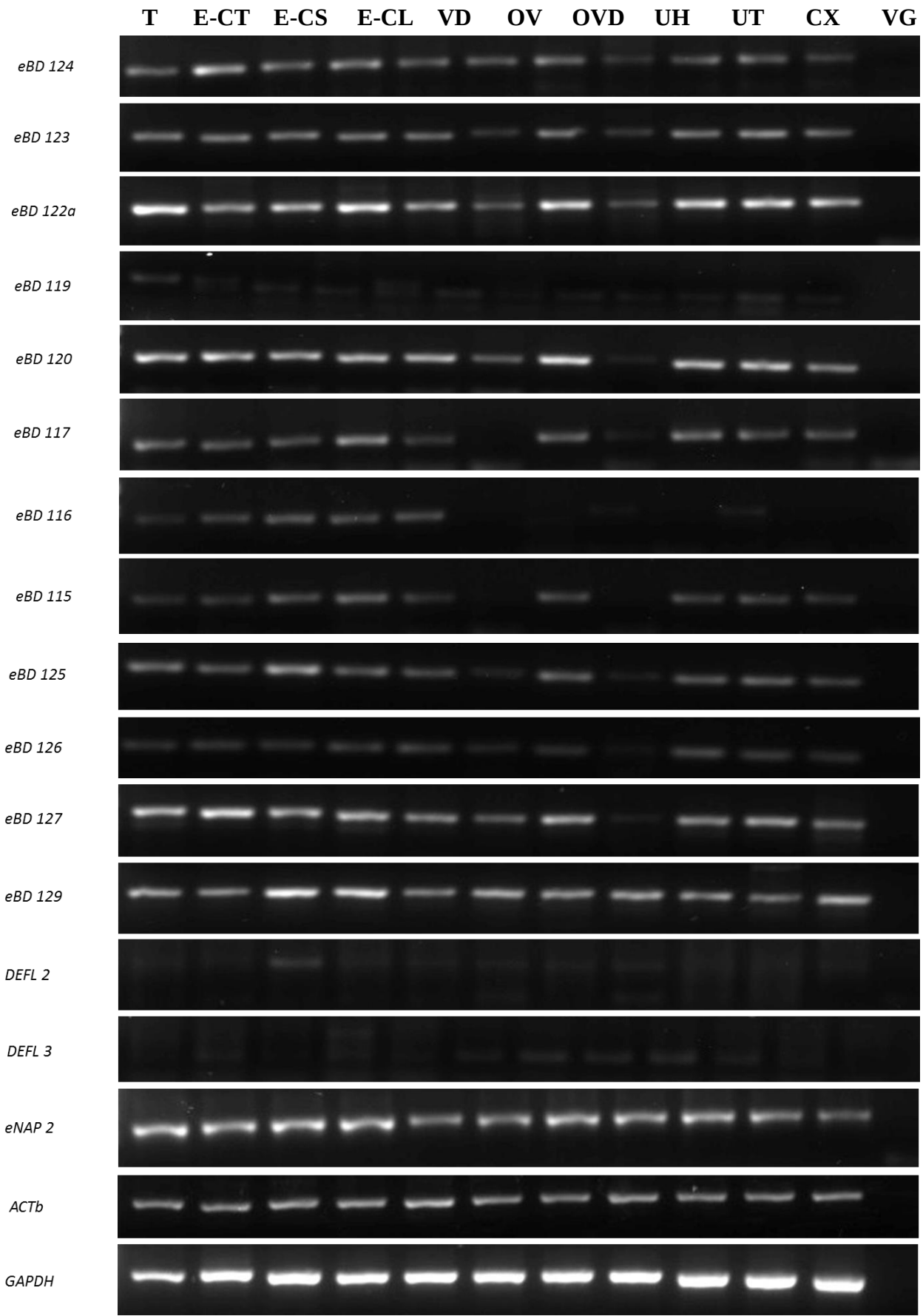
555 Figure 6: Tissue expression profiles of novel equine β-defensins shown using gel
556 electrophoresis following qRT-PCR. Tissue expression profiles of novel equine β-defensins,



DEFL 2 & 3, and reference genes (ACTβ and GAPDH) across the stallion and mare reproductive tracts, shown using gel electrophoresis following qRT-PCR. Results shown are from a representative stallion (lanes 1-5; T, testes; E-CT, caput epididymis; E-CS, corpus epididymis; E-CL, cauda epididymis and VD, vas deferens) and a representative mare (Lanes 6-11; OV, ovary; OvD, oviduct; UH, uterine horn; UT, uterine corpus; CX, cervix; VG, vagina). A no-template control (NTC) is shown in lane 12.