**β-defensins as marker for male fertility: a comprehensive review**

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**Abstract**

Male fertility in animals directly influences the productivity of dairy herds. The epididymal sperm maturations involve extensive sperm surface modifications to gain the fertilizing ability, especially by absorptions of the plethora of biomolecules, including glycoprotein beta-defensins (BDs), enzymes, organic ions, protein and phospholipids. Defensins are broad-range non-specific antimicrobial peptides that exhibit strong relations with innate and adaptive immunity, but their roles in male fertility are relatively recently identified. In the course of evolution, BD genes give rise to different clusters with specific functions, especially reproductive functions, by undergoing duplications and non-synonymous mutations. BDs polymorphisms have been associated with milk compositions, disease resistance, antimicrobial activities, sperm motility, membrane integrity, sperm trapping in the cervical mucus, immunostimulatory, oviduct cell attachment, and egg interactions. The reproductive-specific glycosylated BDs (CA-BDs) have shown age and sex-specific expressions in male reproductive organs, signifying their physiological pleiotropism, especially in the sperm maturation and sperm transport in the female reproductive tract (FRT). By considering adult male reproductive organ-specific BDs expressions, importance in sperm functionalities and bioinformatic analysis, we have selected two bovine *BBD126* and *BBD129* genes as novel potential biomarkers of bovine male fertility. Despite the importance of BDs, however, genomic characterization of most BD genes across most livestock and non-model organisms remains predictive/incomplete. The current review discusses our understanding of BDs pleiotropic functions, polymorphism, and genomic structural attributes concerning the fertilizability of the male gamete in dairy animals.

**Keywords**

Beta-Defensin, epididymis sperm maturations, fertility, genetic marker, reproduction

1. **Introduction**

In the last few decades, the traditional ways to profile semen qualities, i.e., sperm motility, sperm morphology, and in-vitro fertilization (IVF) rate, have been replaced by novel high throughputs methods such as genomics, transcriptomics, and proteomics studies based on characterization [1]–[5]. Reproduction is a regulated and synchronized complex process involving the functioning of numerous genes. Polymorphisms or abnormalities in these genes or any defect in their coded proteins may influence the gametes production, travelling of spermatozoa in the female reproductive tract (FRT), fertilization, and embryo development [6]–[8]. The testicular spermatozoa complete their maturation in the epididymal where they come in contacts with many molecules such as phospholipids, energy components, enzymes, proteins, and glyco-proteins such as β-defensins (BDs) [9], [10]. These modifications on the sperm surface proteins defend sperm from early capacitation, acrosomal reaction, immune evasion and cervical mucus penetration, oviduct epithelial cells attachment and sperm-oocyte interactions [11]–[15].

Defensins are a broad range of non-specific cysteine-rich cationic antimicrobial peptides produced by neutrophils, tracheal mucosa, epithelial cells, lungs, intestine, mammary gland, and the reproductive organs [16]–[24]. Defensins are salt-sensitive and eliminate pathogens by membrane aggregation, depolarization, membrane pore formation and host immune modulation [25]–[28]. Defensins are categorized into three types (i.e., α, β and θ) based on their secondary structures and disulfide linkages (Figure 1) [29]. BDs are the most ancient class of defensin, and BDs bear conserved three disulfide linkages (C1-C5, C2-C4, C3-C6) and three antiparallel beta-sheets, which are necessary for their tertiary structure formation and functions [16], [30], [31]. The BD genes have a conserved signal sequence encoded by the first exon, while the mature functional peptides are generally encoded by the second exon [16], [32]. The tail region of BDs is known for its high glycosylation, making them unique for various roles. BD genes have undergone duplications & mutations in the course of evolution, resulting in various clusters with unique uses for various BDs, including reproductive functions. BDs like *DEFB1*, Enteric beta-defensin (*EBD*), lingual antimicrobial (*LAP*), *BnBD4*-5, bovine neutrophils beta-defensin 10 (BnBD10), BnBD12-13, bovine beta-defensin 118 (BBD118), *BBD119-124*, *BBD122a* were reported in the cow milk and FRT in response to the bacterial infections or diseases [16], [31], [33]–[35]. The pleiotropic functions of BDs have gotten much attention after the report on the expression of rat Bin1b (antimicrobial peptide) in the non-infectious male reproductive tracts [36]. Many studies have reported the importance of reproductive tissue-specific expressions of the BD genes in the sperm functionalities and their responsibility in the delivery of the paternal genome to the oocyte by crossing the long hostile FRT journey [31], [37]–[40].

In bovines, major studies have focused on the BD polymorphisms associated with milk content compositions, milk somatic cell counts, and diseases [41]–[44]. However, in primates, the genotypic and polymorphism studies have reported a relationship between BD gene's structural abnormalities and male infertility, such as decreased sperm motility, increased immune cells in semen, and altered surface glycosylation [14], [45]–[48]. Knockout of BD genes in the mice's genome leads to complete male sterility, and BDs polymorphism affects the protein abundance and functioning [46], [49], [50]. Deficient spermatozoa's motility, viability, and antibacterial activity are markedly improved when various amounts of exogenous recombinant-BDs were added. [44], [51], [52]. The sialicoglycoproteins (BDs) of sperm surface bind to the different proteins on the oviduct epithelial cells (OECs) and help in sperm reservoir formation [53]. During the capacitation, these BDs move out from the sperm surface, and new antigens or proteins emerge, essential for egg recognition and fertilization [54]. The BD's expressions in the reproductive tract and their functional activities make them suitable molecular markers for fertility. Despite their importance, the genomic characterization of most class-A BDs (CA-BDs) genes across most livestock and non-model organisms, including bovines, remains incomplete. The number of the BDs explored in bovine physiological studies, but characterizations of their genomic structural limit their further uses. In the bovine, the addition of prokaryotically expressed predictive BDs have improved sperm motility but failed to improve sperm fertilizibility to oocytes [55], [56]. This could be due to incomplete/predicted gene sequences and prokaryotic expressions because bacterial hosts lack post-translational modifications machinery. This review aims to drag out the importance of male reproductive BDs from different mammalian species in relevance to emerging fertility biomarkers and summarize their current genomic characterizations, especially in male bovines, because bovines are the main economical agricultural livestock animals for many countries.

Figure: 1

1. **Association of Polymorphisms in Beta-Defensins with male fertility**

Human subfertility or infertility is defined as the inconceivable ability of couples even after one year of unprotected sexual intercourse (WHO) [57]. In contrast, in the livestock, subfertility is evaluated based on the sperm counts, sperm motility, sperm morphology and successive numbers of artificial inseminations [58]. The biological system is most affected by genomics central dogma alterations, infectious diseases, and adoptions to the new habitats or new functions [59], [60]. In the United States, the selection of breeding bulls based on genomics markers has increased a 2-4 fold improvement in the genetic makeup of Holstein cattle [3], [61]. The selective environment expression of the BD genes in the reproductive organs, which indicates their roles in diverse ways beyond from conventional antibacterial actions, is caused by the polymorphism, duplication, and mutations in the BD genes. [6], [14], [24]. Any changes in BD's expressions and protein functionalities affect male fertility [13]. Most studies on the bovine have concentrated on BD polymorphisms linked to milk production, immunological function vulnerability, and parasite resistance or illnesses. [20], [21], [24]. The bacterial mastitis in the livestock animals has affected the herding economy worldwide by reducing milk production and increasing health management costs. The increased expression of BDs during mastitis explains their importance in broad-spectrum antimicrobial properties. However, genome-wide polymorphism analysis between high and low sire conception rates revealed that most SNPs and gene copy number variations (CNVs) of BDs are linked with male fertility, such as spermatogenesis, epididymal sperm maturation, and acrosome membrane integrity. These selective polymorphism pressures have brought a wide diversity in the reproductive expressions of β-defensins genes in livestock animal genomes during evolution [24], [27], [29], [31]. The cattle genome sequencing has discovered around 186112 gene variants (including frame-shift, splice-sites, gain and loss of stop codons, missense and synonymous polymorphism) associated with disrupted proteins and lethal embryo abnormalities [1], [3]. The *BNBD4* and *DEFB103* cattle's CC/AA and CT/AC genotypes are linked to milk contents, somatic cell counts, and diseases resistance [62]–[70]. The polymorphisms in the bovine neutrophil beta-defensin 4 (*BNBD4*) gene are associated with milk production, compositions, and udder health in the Holstein-Friesian cattle [71]. The addition of recombinant human *DEFB-1* to the leukocytospermia or asthenozoospermia patients has increased their bacterial activity and sperm motility [52]. The polymorphisms in the BD genes on the cattle chromosome 27 are associated with their qualitative (QTL) traits, such as milk and meat production [71]. Bovine reproductive β-defensin haplotypes variants of *BBD115-116, BBD123-128,* and *BBD142* are associated with their fertility [59]. In rats, RNA silencing of the *Defb15* gene impacts about a 50% reduction in mRNA production and sperm motility [62]. Human *DEFB126* mutations (rs1406851490 and rs11467497) have not to effect on sperm motility and morphology; however, these mutations result in truncated *DEFB126* mRNA production, premature termination of translated proteins, and altered post-translational modifications such as glycosylation patterns on the sperm surface which cause a problem in the cervical mucus penetration lead to human sub-fertility [13], [72]. Research shows that rs1406851490 mutated sperm have unstable *DEFB126* protein, but rs11467497 mutated sperm do not have *DEFB126* proteins on the sperm surface. Human *DEFB126* mutation rs1406851497 is linked to round cells in the semen and low sperm motility, suggesting that *DEFB126* could be the most effective genetic marker of human infertility [47]. Further studies on oviduct epithelial cells (OECs) and oocyte sperm binding assays with anti-*DEFB126* antibody and neuraminidase treated sperm have found that *DEFB126* inhibits significantly sperm binding with OECs and oocytes. At the same time, the addition of externally *DEFB126* protein to deficient spermatozoa restored sperm binding properties [14]. Iranian male sperm donors with normal *DEFB126* gene show more success rate of the intrauterine insemination and *DEFB126* protein abundance on sperm surface than mutated *DEFB126* sperm donors suggesting that del/del homozygous allele mutation is the major cause of failure of IUI treatment of sub-fertility [73]. The abnormalities in mice BDs cluster present on the chromosome 8 (*Defb1, Defb2, Defb9-11, Defb13, Defb15, Defb35,* and *Defb50*) are strongly associated with sperm premature acrosomal reaction, increased intracellular calcium level, and defect in microtubules of the tail and reduced sperm motility [36], [67], [68]. The BD polymorphisms in the Norwegian Red and Holstein Friesian cattle are associated with meat production [74]. Bovine *ITGB5* (*integrin subunit beta 5*), *SPATA1*, and *PRM1-2* (protamines) polymorphism are associated with bull fertility [3]. The haplotypes of the *CATHL2* gene, which code for the antimicrobial cathelicidins family protein in the Holstein-Friesian cattle, are linked to higher milk yield and somatic cells count in milk [75]. The QTL-linked BDs polymorphism on cattle chromosome 20 could be valuable markers for selecting higher and more profitable dairy cattle [3], [71]. The copy number of the Preferentially expressed antigen in melanoma, Y-linked (*PRAMEY)* gene is negatively associated with the scrotal size, the number of normal spermatozoa, and the non-return rate in the cattle [76], [77]. The bovine CA-BDs (*BBD125-30* and *BBD132*) express in a region-specific manner in the epididymis; among them, *BBD126* & *BBD129* have been reported for their higher expression in corpus and cauda regions [29], [38], [40], [74]. The expressions of cattle *BBD115, BBD125*, and *BBD126* genes have been significantly associated with bull fertility [68]. The primates (human, chimpanzee, gorilla, orangutan, gibbon, and macaque) evolutionary study of cluster 8p23.1 (*DEB1, DEFB4* and *DEFB103-7*), 20p1.1 (*DEFB118-20* and *DEFB23*), and 20p1.3 (*DEFB125*-*29* and *DEFB132*) have shown an association between variations in their copy number and male fertility [78]. The twelve missense or non-synonymous SNPs in the *BBD129* gene in Bos taurus have been documented, but studies have not disclosed any relationships with bovine fertility [74]. In our previous study, we found two conserved non-synonymous SNPs (nsSNP) in the *BBD129* gene (T169G and A329G) in the Indian cattle (Bos indicus x Bos taurus) genome, suggesting the conservation nature of *BBD129* snSNPs across the territory. The sequencing showed that Indian cattle bulls generate a diverse population of spermatozoa with distinct *BBD129* gene haplotypes, including *BBD129*-TA (169T & 329A combined), *BBD129*-GA (T169G only), *BBD129*-TG (A329G only), and *BBD129*-GG (169G & 329G together). Sequencing revealed that the double-mutated *BBD129*-GG haplotype was strongly related with low fertile bulls, whereas the *BBD129*-TA haplotype was mostly distributed in the group of high fertile bulls.. A comprehensive analysis has found that nsSNPs negatively affect the *BBD129* protein conformations, stability, post-translational modifications and biological functionalities [79]. These reports show that BD polymorphism, CNVs, mutations, and transcripts abundance emerged as new markers to represent bull's fertility. The expression analysis of male reproductive organs of many species reports that CA-BDs, especially *DEFB126* and *DEFB129* genes, have shown their maximum expressions during adult age and regions specific manners in the different regions of the MRT, suggesting their various physiological roles and important fertility associated markers. The polymorphisms in these reproductive-specific BDs influence protein functions, which may directly or indirectly affect male fertility. Reports suggest that deficiency or lower expressions of BDs in the male reproductive organs affect the sperm functions like motility and sperm fertilization ability.

1. **Bovine β-defensins and Their Unique Expressions**

The digestive tracheal antimicrobial peptides (*TAP*) were the first bovine β-defensin identified in the respiratory tract's mucosal membrane. It was associated with strong antimicrobial activities against rich different bacterial species [17], [33], [80]. Phylogenetic analysis of ruminants and non-ruminants species has shown that diversity exists in the β-defensin orthologous genes [29], [31]. The result of computational genomics, bioinformatics, expression, synteny, and direct sequencing analysis has identified 57 β-defensin genes in the four clusters on the Bos taurus chromosomes 8, 13, 23, and 27 [24], [31], [74], [80], [81]. Our previous study has reported that in Buffalo, these four clusters exist on the different chromosomes: chromosome 1, 2, 3 and 14 [29]. In humans, β-defensins genes are present on four cluster sets (6p12, 8p23.1, 20p13, and 20q11.1) and are related to immune, reproductive and diseases [82]–[85]. Bovine chromosomal 27 BD genes were associated with the milk-QTL and anti-mammary infections [31], [37], [39]. Analyses of sheep and cattle β-defensins genes have shown that all these four clusters are highly conserved in relation to chromosomal positions. Sheep *oBD136, oBD134, oBD135* and *oBD131* have shown homology with cattle BD genes (chromosome 8) and reported their region-specific expressions in the epididymis [40]. The comparative analysis of Porcine with humans and cattle found 29 BDs orthologs genes, including 17 novels in the four clusters on chromosomes 7, 14, 15, and 17. The relative quantification analysis of human *DEFB1* and *DEFB3* suggested a correlation between CNVs and their respective mRNA abundance [24]. Bovine BDs genes present on chromosome 13 are highly conserved in humans and dogs. Most of them are reported for their age and sex-specific expressions in the non-infected male reproductive organs suggesting their reproductive and fertility-related physiological roles [29], [31], [33], [38], [40], [74]. Comparative in-silico mapping analysis of cattle BD genes present on chromosome 13 and buffalo BDs genes on chromosome 14 have shown the conservation of *BBD118-119, BBD122-124,* and *BBD128-129* orthologous genes [31]. The buffalo BDs (BuBDs) expression analysis has shown region-specific expressions of seven BDs genes, viz. corpus (*BuBD125, BuBD126, BuBD128,* and *BuBD129*), caput (*BuBD127, BuBD128,* and *BuBD132*), cauda (*BuBD126*), and vas deferens (*BuBD126* and *BuBD129*). *BuBD126* and *BuBD129* genes were most abundant in the matured buffalo epididymis, suggesting their involvement in sperm maturation as studied in other species [29]. In cattle, CA-BDs are only restricted to the reproductive organ-specific expressions. However, in Buffalo, CA-BDs expressions in the other body tissues have suggested that BDs still hold their traditional antimicrobial properties [29], [74]. Similarly, in ovine CA-BDs expression patterns were reported as cauda (*oBD126* and *oBD115*), corpus (*oBD132, oBD125* and *oBD125a*), caput (*oBD142, oBD116-117, oBD120-121, oBD122a* and *oBD124*), and testis (*oBD119* and *oBD122*-*123*) [40]. Equine BD genes (*eBD115-20, eBD122a, eBD123-29,* and *eBD132*) were also reported for their expressions in the epididymis [26]. These studies show that distributions and the organizations of the BDs in most species are more or less similar, and most studies focused on BDs expression dynamics patterns and their traditional antimicrobial activities in response to infections or non-infectious organs, including reproductive organs. However, the expressions of reproductive-specific class-A beta-defensins in an age-specific and regions specific depict their importance in the reproductive processes. Here, below, we have explored more about BDs roles in reproduction.

1. **Roles of β-Defensins in Reproductions**

**4.1 Beta-Defensin: Sperm Maturation**

The male accessory organ epididymis, prostate secretion, seminal vesicle secretions, and hormone regulations majorly contribute to sperm maturation, protection, selection, and storage [86]–[90]. The epididymis is generally divided into three segments; the proximal antigen tolerogenic caput region responsible for initial motility and antimicrobial activity; the middle corpus region responsible for sperm motility, antibacterial activity, and major surfacial modifications and; the distal cauda regions responsible for maintenance of pro-inflammatory circumstances, antimicrobial activity, final modifications of sperm with fertilizing ability and sperm storage (Figure 2) [91], [92]. The different segments of epididymis have been reported for different transcriptomics and protein profiles, including expressions of BDs proteins [91], [93]. The testicular spermatozoa are infertile and immotile, but as they pass through the epididymis, they mature and gain fertilizing ability by surface modifications, including glycoproteins such as BDs [7], [94]. These sperm surface modifications are significant for sperm motility and sperm protection from epididymal immune systems [95]. The exogenous addition of reproductive Bin1 defensin-like peptide to caput immature spermatozoa impacts the sperm motility and sperm-egg interactions suggesting its pleiotropic role in male reproduction [36], [96]. The expression of human and mice epididymal BDs (*hBD5, hBD6, mBD11, mBD12,* and *mEP2e*) have been reported in the male reproductive tract; they protect sperm cells from pathogens by stimulating the innate immunity [38], [68], [97]. Castration of macaque's testis has reduced BDs mRNA expressions [98]. The expression of macaque *DEFB118* and *DEFB123* were observed only in matured males depicting their roles in the sperm protection and maturation as similar to rat *Bin1b* and human *DEFB126* [99]. The transcriptomics analyses of the epididymis in the distinct fertility bulls have revealed differential expressions of genes associated with biological functions such as sperm glycosylation, sperm membrane modifications, and sperm calcium transport [100]. In the Yak bovine, the expressions of BDs (*DEFB106A, DEFB109, DEFB119, DEFB123, DEFB125,* and *SPAG11*) have been found associations with male gamete quality, antimicrobial activity and immune responses in a region-specific manner in epididymis [101], [102]. All these reports suggest that epididymal glycosylated BDs protect the sperm from epididymis immune recognition by forming a thick glycocalyx coating on the outer surface of sperm and proving antimicrobial activities. The BDs expressions analyses have suggested that regional epididymis secretions of BDs provide suitable environments for the region-specific maturation of spermatozoa. The transcriptomics and proteomics analysis of epididymis BDs have revealed that BDs perform pleiotropic functions in reproductive organs, making spermatozoa more immune evaders and functional in the MRT and FRT.

Figure: 2

**4.2 Beta-Defensin: Immunomodulatory role**

The changes in hormones during the ovulation period make the female reproductive tract more immunogenic by increasing the levels of antibodies (IgA & IgG), cytokines, chemokines, immune phagocyte cells, and watery mucus backflow secretions to prevent the entry of microbial pathogens during sexual intercourse. Even sperm are also foreign bodies for the female, so it is essential to modify or mask the proteins or antigens present on the outer surface of the sperm membrane so that sperm can easily cross all the adverse barriers or hostile preparedness of the FRT during fertilization [7]. Recent studies reported that highly glycosylated BDs are coated on the sperm surface during epididymal maturation and mask most antigens or proteins to protect sperm from immune surveillance in FRT [7], [103]. Evidences have shown the importance of BDs in the immune modulations during inflammations, microbial infections and other non-inflectional events. The structural activity relationship have revealed that the hydrophobicity of BDs affects how they interact with eukaryotic cells, while side chains of basic amino acids determine the microbicidal activity [14], [46]. The BDs perform numerous immune-regulatory functions, such as promiscuous ligands for various immune receptors like *TLRs, CCRs* and *melanocortin* receptors and chemoattraction of immune cells [31], [104]. The low abundance of glycans or removal of glycans from bovine spermatozoa was led to trapping by in-vitro cultured neutrophil cells [105]. Mouse BD-14 interacts with immune *CCR2* & *CCR6* chemokine receptors and stimulates the production of the chemokines & cytokines molecules and the activation of macrophages [51], [106], [107]. The expressions of *DEFD1-2, DEFB21*, *DEFB24, DEFB27, DEFB30, DEFB36, SPAG11c, SPAG11t, TLR1,* and *SPAG11e* genes were increased during epididymis infections and associated with activation of dendric cells and neutrophils to remove the pathogens [97], [108], [109]. Human *DEFB126* core peptide shows anti-inflammatory activity by reducing mRNA expression of IL-6, TNF-α, IL-α, and IL-1β cytokines against bacterial infections [110]. Immunization of rabbits with neuraminidase-treated spermatozoa and un-treated spermatozoa has shown a strong band against human *DEFB126* in the case of un-treated spermatozoa. It has shown multiple bands against neuraminidase-treated spermatozoa, and it suggests that *DEFB126* is the major component of human sperm surface and it masks most of the human sperm surface antigens or proteins [7]. Similarly, in the macaque, the phosphatidylinositol phospholipase C (PI-PLC) treated sperm extracts were weak immunogens in female macaque than the sperm extract isolated after capacitation [29], [111]. Human *hBD2* and *hBD3* have been reported for their similarity with chemokine receptors, suggesting that BDs share similar structural mechanisms of immune system activation [112]. Human sperm surface chemokine receptors can modulate sperm motility by interacting with the *DEFB129* protein. The interference of the *CCR6* receptor reduced sperm motility and anti-bactericidal activity [113]. Interestingly, BDs have been implicated in activating NF-κB signaling in HEK cells through *TLR1* and *TLR2* receptors, thus dispelling the myth that TLR signaling is restricted to only Pathogen-associated molecular pattern (PAMP) recognition [114]. Human BDs like *hBD3* have been demonstrated to dampen the expression of pro-inflammatory genes in the *TLR4* stimulated macrophages [115]. The communication between the reproductive and immune systems through these BDs or AMPs may be involved in mitigating immune responses mounted against the allogeneic spermatozoa in the FRT. These innate effectors have extended their functional repertoire into the adaptive immune system and the reproductive system's realms. A better understanding of their expressions in the MRT and their correlation with sperm motility and its fertilizing ability will help gain insights into their roles in regulating male fertility.

**4.3 Beta-Defensin: Fence of the Female Reproductive Tract**

A highly specialized epithelial spermatozoon cell is like a spaceship carrying the paternal genome across the hostile environment of FRT. Sperm cells are foreign bodies for females, but sperm must travel a long path in FRT to fertilize an oocyte. To do that, the outer shell (glycocalyx) of sperm must be so protective and modified that sperm can ease the move, dock, and deliver its consignment to the target planet oocyte [7], [10]. We have studied above that glycoproteins secreted by epididymis are absorbed on the sperm surface and maintain the sperm membrane integrity and sperm longevity in humans, rats, cattle, sheep, goats, macaque, drosophila, fish, crab, etc. [7], [10], [56], [109]. Ruminants, rabbits, and primates are the only mammals that ejaculate millions of sperm in the female vagina during sexual coitus; however only a few thousand reach the oviduct (Figure 3). In primates, 20-60 nm thick glycans coat forests are incorporated on the sperm surface during the testicular spermatogenesis and epididymis maturation. These shields of carbohydrates on the sperm surface are significant for the number of events involved in the fertilization process, such as sperm maturation, motility, evading the immune system, cervical mucus penetration, oviduct epithelial cells binding and sperm-egg interactions [10], [14], [15], [116]. The initial incorporations of N- & O- glycoconjugates on the sperm membrane occur in the seminiferous tubules Golgi body and endoplasmic reticulum during spermatogenesis. The testicular seminiferous *GALGT1* enzyme (N-aceteylbeuraminyl-galactosylglucoceramide N- acetylgalactosaminyl transferase) adds N-acetylgalactosamine type glycans on sperm surface proteins, and most of them are fucosylated and mannose types involved in immune modulation, oviduct cell attachments, egg recognition and male fertility [117], [118]. The O-glycosylations have been observed on many glycoproteins incorporated during the spermatogenesis process, such as GPI-linked prion proteins (*CD230*), *hyaluronidase*, and *ADAM3* fertility-related proteins. Recent studies have reported that further post-translational modifications of testicular spermatozoa occur in the epididymis, and only the epididymal matured sperm can fertilize the oocyte [102], [111], [117]. Mammalian sperm membrane GPI-linked glycoproteins could provide an adhering nest for epididymal secreted proteins such as *Glycoledin-S, CD52, CD55, CD59* and beta-defensins. Seminal *glycoledin-S* glycoprotein was reported with Lewis X and Lewis Y glycans, and its involvement in epididymis immunity, sperm capacitation and zona pellucida glycoproteins interactions [111], [117], [118]. Epididymis glycoproteins provide flexible movements around the glycosidic bonds and prevent sperm non-specific interactions with cervical mucus and immune cells of the MRT and FRT [10], [111], [118]. Human, rat and macaque epididymis BDs could absorb on the entire surface of sperm and provide extensive sialio-glycocalyx coats [7], [45], [119]. The tail regions of these reproductive BDs proteins are rich in serine, threonine and asparagine amino acids (40-60%), suggesting their high potential for glycosylations [117], [119]. The glycosylations and lectins studies have reported about 60 nm dense sialic glycocalyx on the sperm surface, and this glycocalyx has been reported as absent or decreased in the capacitated sperm, suggesting their importance in crossing the mucin-rich cervical mucus and immune surveillance of the FRT [7], [48], [120]. Sperm morphological abnormalities and alterations of glycosylations could cause the trapping of sperm by barriers of the FRT, suggesting the cryptic choice of females for the specialized spermatozoa [117], [121]. The mutations in the human DEFB126 have been reported as abnormal O-glycosylations, and reduced mucus penetration leads to human infertility [13]. Similarly, the oviductal capacitated spermatozoa have reported less surfacial glycosylations, suggesting that removal of BDs during capacitation unmasks many sperm-surface proteins essential for the egg interactions [14], [50], [122]. These reports suggest that the expression of BDs in the epididymis plays a role in sperm maturation and prepare sperm to cross the unfriendly environment of the FRT.

Figure: 3

**4.4 Beta-Defensin: Sperm Cervical Mucus Penetration**

Hormones regulate all reproduction events during the ovulation period, and they make reproductive organs so compatible for fertilization between sperm and oocyte, resulting in new progeny, and any imbalances in the hormones may result in failure of fertilization or an unfavorable environment for embryo implantation [89], [90]. In mammalian animals, cervical mucus acts as a gatekeeper for sperm by filtering morphologically abnormal and less motile sperm, thus playing a curious role in the mysterious choice of sperm for females (Figure 4) [9], [121]. During ovulation, females secrete estrogen and progesterone hormones which lower the vaginal pH, looseness the cervix, increase the level of mucin glycoproteins, immunoglobulin A (IgA), lysozyme, cytokines, chemokines, and antimicrobial peptides, and provide a favorable environment for fertilization so that only superior quality of sperm pass all these adverse barriers FRT and fertilize the oocyte [7]. The interactions between the cervical mucus proteins and sperm surface glycoproteins could affect sperm progressive motility. BDs are negatively charged sialoglycoproteins that constitute a significant portion of the sperm surface and facilitate sperm traveling into negatively charged mucin-rich cervical mucus [46]. BDs mutated sperm face the problem of trapping in the cervical mucus due to their outer abnormal glycocalyx morphology [7], [13]. The treatments of sperm with capacitation factors that remove BDs proteins from the surface significantly reduce sperm cervical mucus penetration abilities [14], [46], [48], [50]. The alterations in the glycosylations on the bovine spermatozoa could cause the trapping of spermatozoa by macrophages and neutrophil immune cells [105]. Thus, adherences of BDs on the sperm surface during epididymis maturation emerged as the most important factors necessary to ease the transport of sperm in cervical mucus. The polymorphism and mutations in the BD genes could lead to structural and post-translation alterations in the resultant proteins, which could influence sperm trapping in cervical mucus and attract immune cells of the cervix.

Figure: 4

**4.5 Beta-Defensin: Sperm Oviduct Epithelial Cells Binding or Reservoir Formation**

After facing the first line of the defense of the FRT (cervical mucus and uterine immune evasion), sperm have to face the next phase of their FRT journey, i.e., oviduct epithelial cells (OECs) binding and formation of sperm reservoir. Sperm reservoir formation is the critical event of fertilization which involve sperm attachments to the OECs with the help of lectin-like sperm surface glycoproteins (including BDs), rostral sperm head surface and close contact between head membrane proteins and the ciliary surface of OECs [14], [121], [123]–[125]. The OECs ciliary projections bear the oligosaccharide carbohydrate residues, which help the sperm in binding and reservoir formation [126]. The distributions of carbs on the OEC depend on the ovulation phase could affect sperm interactions with OECs. The removal of carbs with the help of glycosidases or inhibition with the lectin mimics prevents the spermatozoa from binding to the OECs, suggesting that OECs crabs help in the sperm storing or reservoir formations [126]–[128]. In addition to cervical mucus penetration and immune evasion, BD glycoproteins on the entire surface of uterine and oviductal spermatozoa suggest that BDs still have more functions in the FRT. Bovine seminal protein-1 (*BSP1*) and *BSP5* help sperm maintain membrane integrity and act as decapacitation factors. Bovine PDC-109 and porcine defensin-like spermadhesins (*AQN*-1) are coated on the ejaculated spermatozoa and help sperm in the OECs binding. In contrast, their removal from the sperm surface during capacitation significantly impacts the OECs binding [14], [50], [53], [55]. The in-vitro removal of *DEFB126* by capacitation factors (1mM caffeine + 1mM dbcAMP, ACT), *DEFB126* anti-antibody inhibition and neuraminidase treatment have shown significantly less binding of sperm to the oviduct epithelial explants compared to control group. At the same time, add-back of exogenous BDs and other glycoproteins to the above-treated groups of spermatozoa or capacitated spermatozoa can restore sperm binding ability with oviduct epithelial explants [52], [53], [119]. The various haplotypes of bovine BDs could also impact the sperm-oviduct binding [53], [129]. Thus, BDs appear to offer sperm OECs binding and preserving sperm resources for awaiting oocytes. In conclusion, epididymis glycosylated BDs prepare sperm to cross unfavorable barriers of FRT and help in OECs attachment where sperm hyperactivity and oviduct pH cause their removal from sperm surface, leads to capacitation process and sperm movement toward oocyte [14], [53], [130].

**Figure:** 5

**4.6 Beta-Defensins: Sperm-oocyte Interaction**

It has been already known that testicular and non-capacitated spermatozoa cannot fertilize an oocyte, only matured and capacitated sperm can do this. After crossing the second phase of FRT (i.e., oviduct epithelial cell binding and sperm reservoir formation), now, sperm have to face the third phase, which involves recognition and oocyte fusion. The releasing of spermatozoa from oviductal reservoirs occurs due to physiological conditions switching in the oviduct, such as changes in hormones, inorganic ions, pH, altered sugars level, sulfatedglyco-conjugates, and glutathione molecules [131]. FRT hormones generally act on the sperm cation channels and induce sperm hyperactivation by in-fluxing extracellular calcium ions, which facilitate sperm detachment from oviduct epithelial cells [131]. Seminal plasma proteins maintain sperm membrane integrity, and the removal of seminal and epididymal glycoproteins during sperm hyperactivation help in sperm capacitation and oocyte interactions [48]. Porcine seminal plasma defensin-like spermadhesin proteins *AQN-1, AQN-2, PSP-1,* and *PSP-2* protect sperm acrosome membrane, and these proteins are takeoff from sperm surface in capacitated spermatozoa which assist sperm-oocyte interactions (Figure 5). In contrast, adding these proteins to capacitated spermatozoa has shown inhibition of sperm-oocyte interactions [116]. In primates, bovine and porcine epididymal BDs are coated on the sperm surface. They get off the sperm surface during the capacitation process, unveiling new antigens or proteins (glyco) involved in egg interactions [13], [24], [130], [132]. In cattle, it has been reported that increased numbers of thiol groups of glycosylated proteins on the sperm membrane are linked to the detachment of sperm from OECs and sperm traveling toward oocyte [13], [15], [53], [133]. The polymorphism in BDs genes has been reported to cause sperm defective functionalities, including lower oocyte binding ability [134]. All these reports suggest that the roles of sperm surface BDs are to help sperm in the cross hurdle of the FRT where they are removed from the surface and lead to the unmasking of new sperm surface proteins essential for the sperm interactions with the egg or egg recognition.

**4.7 Beta-Defensin: Sperm Motility and Capacitation**

Human asthenospermia or abnormal sperm motility responsible for around 18 % of male sub-fertility and complete infertility. Sperm capacitation involves the influx of calcium ions and alterations of sperm membrane components such as levels of cholesterol, phosphatidyl-inositol-3-kinase, cyclic adenosine monophosphate, adenylyl cyclase, actin polymerization, protein kinase A, phospholipase D, Src family kinase, protein kinase C, epididymal glycoproteins, phospholipids, and many other molecules [135], [136]. Capacitation and acrosome reactions are associated with increased kinase activities and hyper phosphorylations of serine, tyrosine, and threonine amino-acids [137]–[140]. Sperm decapitating molecules include seminal plasma proteins and epididymal secretions that prevent sperm early or inappropriate capacitation and maintain acrosomal membrane integrity before the conference with the zona pellucida membrane [45], [50], [140], [141]. The crossing of hostile and anatomical restrictions of FRT by sperm and then interaction with oocyte are not a matter of chances, but it is a pre-program process and generally regulated by the chemotaxis environment of FRT. The menstrual cycle-dependent expression of *CCL20* chemotactically attracts sperm toward the oocyte by binding with the sperm surface *CCR6* receptor, which influences the calcium ion influx and enhances sperm motility so that sperm reach the oocyte [142], [143]. The epididymis BDs are well known for their contributions to antimicrobial activity, sperm maturation, sperm viability and sperm motility in several mammalian species, including humans, macaque, rat, mouse, cattle, porcine, sheep, goats and many others [45], [50]. As we studied above, BDs are well known as chemokine-inflammatory intermediates, and their age-specific expressions influence the MRT's and FRT's physiological parameters. The seminal human *hBD*-*1* binds to the *CCR6* chemokine receptor on the sperm surface and increases sperm motility by triggering the calcium ions influx [144]. The addition of recombinant *hBD-1* to deficient spermatozoa has considerably increased sperm movements, sperm viability, and bacterial activity [44], [51], [52]. Rat *Bin1b* defensin-like antimicrobial peptide protects sperm cells from epididymis tract infections and helps initial sperm maturation and sperm motility. The miRNA interference of Bin1b leads to reduced mRNA expression, sperm surface abundance, and sperm motility. At the same time, the addition of exogenous recombinant Bin1b or co-culture with caput epithelium secretions have been reported to improve sperm motility [145]. Similarly, human epididymis specific *DEFB129* interacts with sperm surface chemokine receptor *CCR6*, consequences elevation of a sperm cytosolic calcium ion by influencing pore-forming *CatSper* channel, and it could affect sperm functions required for fertilization such as motility and acrosomal reaction [142], [144], [146]. The epididymis *BD-108* expression impact sperm motility in Vulpes logopus [147]. The polymorphism and deletions of mice β-defensin gene cluster result in complete sterility and reduce straight sperm motility and oocyte binding abilities [134]. The addition of bovine recombinant-*BBD126* has improved sperm motility of non-motile caput spermatozoa [55]. Rat *DEFB22* protein could maintain sperm in decapacitation conditions and protects sperm immune surveillance [119]. The presence of *DEFB126* (human & macaque), *DEFB22* (rat) and *PDC-109* (bovine) proteins on the oviduct spermatozoa during fertilization suggest that BDs help in the maintenance of premature capacitation and acrosome reaction bursting before sperm-egg interactions [119]. These reports suggest how BDs adhered to the sperm surface in the epididymis programmed and controlled physiological environments. The attachments of these proteins (glyco) to the sperm plasma membrane help the sperm to maintain membrane integrity, non-capacitated and non-acrosomal reacted conditions during cauda storage and transportation through FRT. In the oviduct, where the physiological conditions changes, sperm surface proteins are detached from the surface and lead to unmasking the hidden antigens or new proteins essential for egg recognition. The angiotensin-converting-enzyme on the cattle and boar spermatozoa surface helps remove the glycosylphosphatidylinositol-anchored glycoproteins, leading to sperm capacitation [15], [148]. These findings explain the importance of BDs in male fertility that BDs are not only restricted to antimicrobial or immune-modulatory activities but also associated with the sperm's other functionalities essential for the fertilization (Figure 6) and also suggest that the exogenous addition of recombinant BDs proteins to sub-fertile asthenospermia and leukospermia or deficient spermatozoa could be a practical diagnostic approach to improve male fertility.

**Figure 6**

1. **Beta-Defensin: Current Genomics Characterization Status across the Species**

Although the pleiotropic functions of BDs have emerged as fertility biomarkers, but their genomic structural characterizations across domestic animal species remain predictive and incomplete except for a few species (Table 1). We have collected genomic structural data of BDs from different mammalian species from the NCBI databases by searching the term "beta-defensin" [149]. Most completely characterized BDs are reported as antimicrobial activity, immune stimulations, protecting sperm membrane integrity, and sperm functionalities; on the other hand, the incomplete or predictive BDs have been limited to only their mRNA expressions and polymorphisms studies. Most reproductive class-A BDs proteins have importance in male fertility due to their high glycosylation potentials and surface adherence properties. Human *DEFB126* has emerged as a most potent biomarker for male fertility as exogenous addition improves sperm functionalities, while polymorphisms in *DEFB126* negatively affect sperm functions and lead to male subfertility. An exogenous addition study was conducted on bovine; however, prokaryotically expressed *DEFB126* or *BBD126* protein has increased sperm motility but failed to improve fertility. There could be two reasons behind this failure to improve bovine fertility; 1) lack of post-translational machinery in prokaryotes host and; 2) incomplete genomic status or characterization of *BBD126*. Thus the genomic sequence of *BBD126* has been removed from the NCBI database [149].

Similarly, the bovine *BBD129* gene has emerged as another BD candidate, the most prominent expressing BDs in the MRT. Also, its bioinformatic characterization has found a long C-terminal tail with high potential for O- & N- linked glycosylations compared to other members of the reproductive CA-BDs [79]. By collecting data and the importance of reproductive class-A BDs, we have given attention to two bovine *BBD126* and *BBD129* genes and summarize their current genomic characterization of different species in table 2. This data suggests that the bovine BBD129 gene is almost predictive in all species, including the bovine family, while in the case of the bovine family, there are no sequences of BBD126 (DEFB126) on NCBI, thus limiting fertility-related research use. We have done an exercise on the O-linked & N-linked glycosylation prediction analysis on BBD126 and BBD129 genes and summarized their glycosylation potentials in table 2 [150], [151]. We also have done an exercise on phylogenetic conservation analysis by using amino acid sequences of *BBD126* and *BBD129* genes from different mammalian species. The multiple amino acid sequence alignments analysis has shown that both genes are conserved at cysteine amino acids in mammalian species and these cysteine amino acids form disulfide linkages, a unique feature of BDs (Figures 7, Figure 8). The phylogenetic analysis of BBD126 and BBD129 genes has shown that both genes are highly conserved within different species of the bovine family and closely related to other ruminant species (Figure 9). The evolutionary study of the DEFB126 gene and DEFB129 gene were tested on a total of 17 and 33 distinct species, respectively, using the maximum likelihood approach and the Tamura-Nei model (Figure 9). Thus, to explore more on BDs, firstly, we need to work on their genomic characterizations. We also need to design new strategies such as expressing them in appropriate host systems and supplement additions to semen straws to improve the fertility of domestic male animals.

Table 1

Table 2

Figure: 7

Figure: 8

Figure: 9

**6. Conclusion**

β-defensins antimicrobial peptides are emerging genomic makers for selecting superior quality livestock breeding animals. The BDs orthologs are highly conserved across the species, and their expressions in age-specific and sex-specific manners in the reproductive organs suggest their pleiotropic roles in addition to their traditional antimicrobial and immuno-stimulatory activities. The polymorphisms in the BDs are associated with interferences in proteins' structural conformations, stability, post-translational modifications and biological functionalities. The expression analysis of male reproductive organs of many species reports that CA-BDs, especially *DEFB126* and *DEFB129* genes are important fertility-associated markers. Despite the significance of BD, most β-defensin genes across species' genomic characterization is still incomplete & predicted incomplete & only predicted. So, here, we need to work on BDs genomic characterizations and design strategies for augmented sperm fertilizing ability.

**Highlight Points:**

Reproductive pleiotropic functions of β-defensins are emerging as attractive biomarkers for male fertility.

The expressions of β-defensins in an age-specific and region-specific in epididymis suggest their importance in male fertility.

The polymorphisms in β-defensins could affect sperm functionalities, and exogenous additions of β-defensins could improve sperm functions.

Epididymis β-defensins have been reported for their pleiotropic functions in sperm maturations, antimicrobial activity, sperm motility, cervical mucus penetration, oviduct epithelial cells attachment/sperm reservoir formation, and egg recognitions.

Despite their importance, their genomic structural characterizations limit further exploration of reproductive functions.

BBD126 and BBD129 genes emerged as new potential biomarkers of bovine male fertility.

**Conflict of interest:**

Authors show no conflict of interest.

**Author Contributions**

SS has drafted the whole manuscript; SS, VK, PK, RK, and TKD have contributed to scientific idea/editing; SS, RK, and TKD have contributed to the final manuscript arrangement. All authors contributed to the article and approved for submission.

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