Review Article

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British Biomedical Bulletin
ISSN 2347-5447

2017
Vol.5 No.1:299

Effects of *Fusobacterium Necrophorum* on Pathogenesis and Potential Disease-Associated Factors in Plasma in Cattle with Footrot

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Received date: December 16, 2016; **Accepted date:** February 20, 2017; **Published date:** February 25, 2017

Citation: Xiao J, Sun D, Wang H (2017) Effects of *Fusobacterium Necrophorum* on Pathogenesis and Potential Disease-Associated Factors in Plasma in Cattle with Footrot. Br Biomed Bull, 5: 299.

Abstract

Footrot is a highly contagious disease of the feet of animals, characterized by the separation of keratinous hoof from the underlying tissue. Co-infection of *Dichelobacter nodosus* and *Fusobacterium necrophorum* is the main reason of footrot in clinical. Environmental factors, such as warm and wet weather and pasture quality, etc. all are conducive for indirect transmission of bacteria among individuals. *Fusobacterium necrophorum*, which product several virulence factors such as leukotoxin, hemolysin, hemagglutinin, play an essential role in the infection process. Moreover, a lot of potential differential expression (PDE) proteins were found in plasma samples from dairy cattle with footrotsome of which may be valuable for use as diagnostic biomarkers, the possible mechanism which these proteins involved in the pathogenesis of footrot were analyzed in this paper.

Keywords: Footrot; Pathogenesis; *Fusobacterium necrophorum*; Virulence factors; Potential differential expression proteins (Pde)

Introduction

Footrot is one of the major causes of lameness in cattle, sheep and goats. It is a highly contagious and debilitating disease of the feet of animals, characterized by the separation of keratinous hoof from the underlying tissue [1,2]. The primary pathogen was first identified as *Dichelobacter nodosus* in 1941 [3]. Footrot not only infect among one kind of ruminant, but also between different ruminants, one study indicates that cross-infection of virulent *Dichelobacter nodosus* between sheep and co-grazing cattle have occurred [4]. In general, *Dichelobacter nodosusis* considered the primary pathogen causing footrot since elimination of virulent strains of *Dichelobacter nodosus* appears to prevent footrot [1]. However, *Dichelobacter nodosus* is unable to replicate the symptoms of this disease on its own, as a second pathogen *Fusobacterium necrophorum* is reported to be important and indispensable to induce footrot symptoms in sheep [5]. *Fusobacterium necrophorum* was detected in 79 of total 148 bovine hoof scrapings from September 2005 to May 2006 in dairy herds of New Zealand by polymerase chain reaction [6]. Huitong et al. found four *lktA* genes of *Fusobacterium necrophorum* strains in hooves of footrot infected cattle [7]. *Fusobacterium necrophorum* was detected from footrot in ovine [8], sheep [9], and associated with hoof diseases in pigs [10] and hoof thrush in horses [11]. Beside footrot in animal, *Fusobacterium necrophorum* is involved in many diseases in human. It is the causative agent of the invasive disease Lemierre's syndrome [12,13] anaerobic endocarditis [14,15] tonsillitis [5] empyema necessitates [16]. It is also associated with peritonsillar abscess formation and otitis media in children [17,18] *Fusobacterium necrophorum* was found in majority of patients with acute tonsillitis or peritonsillar abscess by one 10 years epidemiology study in an academic hospital [19].

Aetiology and Pathogenesis of Footrot

Generally, advanced footrot usually involve the sole and hoof wall, but new infections often commence in the interdigital skin. The interdigital skin is normally resistant to bacterial infection and is susceptible if it is pre-disposed by prolonged exposure to wet and warm conditions [20]. So, the prerequisite for the initiation of footrot is the damage of interdigital skin epithelium [3]. Bacterial replication in this damaged skin leads to Interdigital Dermatitis at first, which the superficial epidermal layers are inflamed and slough off irregularly. The interdigital skin of infected feet was usually swollen, smelly [21]. Following the development of the interdigital lesion, the infection may extend into the sensitive laminae underlying the sole [22] which then progressively result in the separation of keratinous hoof capsule from the underlying tissue. Ultimately, interdigital skin of infected feet was then covered with necrotic material [21].

Moreover, a typical lesion of bovine digital dermatitis is found on the plantar surface of foot which presents as a circumscribed moist ulcerative erosive mass along the coronary band or interdigital space [23]. Though footrot is not non-infectious, environmental factors, such as warm moist conditions, high temperatures and pasture play an essential role in the initiation and development of the disease [24]. Histologically, Interdigital Dermatitis and footrot presenting progressive chronicactive pododermatitis, gradually from a mild form in clinically normal feet to a focally severe form with frequent areas of purulence in footrot [25]. There is a loss of stratum corneum and granulosum, invasion of stratum spinosum by reactive inflammation (infiltration of neutrophils, plasma cells, lymphocytes, and eosinophils in dermis) [26]. Over time, lesions can become larger, prone to ulceration or physical trauma [27].

The pathogenesis of footrot is very complex and multifactorial. In most cases, co-infection of *Dichelobacter nodosus* and and *Fusobacterium necrophorum* have the most important roles in footrot [28] and *Dichelobacter nodosus* plays the primary role in disease progression, with *Fusobacterium necrophorum* playing a secondary role [29]. The viewpoint that footrot is dependent on a mixed bacterial infection is supported by the pharyngotonsillitis in human [17]. Hoof horn separation does not occur without the involvement of *Dichelobacter nodosus* [25]. Early histopathological observations of tissue sections from footrot lesions described little inflammatory response in areas with *Dichelobacter nodosus*, but severe inflammation in response to invasion by *Fusobacterium necrophorum* [30]. Even though, some studies have indicated that *Dichelobacter nodosus* appears to be the primary invader of the epidermal matrix and to initiate the process of hoof separation, providing a necessary environment where *Fusobacterium necrophorum* can flourish [30,31]. The infection appears to be the result of the synergistic action of two kinds of particular bacterial species, of which *Dichelobacter nodosus* is the causative transmitting agent and *Fusobacterium necrophorum* appears to be necessary for the induction and development of the disease [31]. This was confirmed by the results that lesion was infiltration by polymorphonuclear leucocytes and a dense population of filamentous bacteria, visually identified as *Fusobacterium necrophorum* in Interdigital Dermatitis sections [32]. Most studies indicate that both *Fusobacterium necrophorum* and *Dichelobacter nodosus* are essential for the invasion of the epidermal matrix of the hoof and neither bacterial species alone will cause a footrot lesion [22]. Firstly, *Fusobacterium necrophorum* colonises the stratum corneum, and then facilitating infection with *Dichelobacter nodosus*. The established *Dichelobacter nodosus* infection allows *Fusobacterium necrophorum* to penetrate more deeply into the tissue, causing further inflammation and destruction of epidermal tissue [22]. *Fusobacterium necrophorum* establish infection, resulting in the development of Interdigital Dermatitis. While this condition is itself relatively mild, it provides the necessary pre-disposing conditions for infection with *Dichelobacter nodosus* [31,32]. Additionally, there is evidence that *Dichelobacter nodosus* is not a major agent of lameness in New Zealand dairy cattle, while *Fusobacterium necrophorum* possibly could be [6]. Anyhow it seems that *Fusobacterium necrophorum* either facilitates disease development by increasing the damage to the interdigital skin and promoting Interdigital Dermatitis that subsequently permits replication of *Dichelobacter nodosus* [6,31] or follows *Dichelobacter nodosus* or exacerbate the severity and persistence of footrot [33,34]. In quite large extent, we consider that *Fusobacterium necrophorum* plays an opportunistic or secondary role, because it is consistent with understanding of the role of *Fusobacterium* spp. in other diseases. *Fusobacterium necrophorum* is present in lesions and abscesses in many polymicrobial infections, where they are considered to enhance disease severity through synergistic relationships with other pathogens [35,36].

Fusobacterium necrophorum, a gram-negative, non-spore-forming, obligate anaerobe, is part of the normal flora of the oral cavity, genital tract, and alimentary tract [36] and is a normal inhabitant of the gastrointestinal, respiratory, and genitourinary tract of cattle [37,38]. *Fusobacterium necrophorum* is present as a primary or secondary opportunistic pathogen in numerous necrotic diseases conditions generally termed '*necrobacillosis*' in humans, domestic and wild animals [36,38]. In cattle, *Fusobacterium necrophorum* induces abdominal abscesses, often in hepatic tissue, [36,39] it has been reported that this bacterium causes calf diphtheria, foot rot etc. [31,40,41] and necrotic laryngitis in beef and dairy cattle which are of significant economic importance to the cattle industry [38]. In humans, *Fusobacterium necrophorum* is associated with Lemierre's syndrome, a condition that primarily affects young and healthy persons [42,43]. Furthermore, it causes pharyngotonsillitis, pharyngitis, parotitis, dental abscesses, and middle ear infections, including mastoiditis [44-46].

In cattle, *Fusobacterium necrophorum* is classified into two subspecies, ss. necrophorum and ss. fundiliforme, also called biotype A (*Fusobacterium necrophorum* subspecies necrophorum) and biotype B (*Fusobacterium necrophorum* subspecies funduliforme) respectively [36,47]. These two subspecies can be distinguished by their growth, morphological, biochemical and molecular characteristics [38,48,49]. Subspecies necrophorum is more frequently encountered in infections than subsp. Funduliforme, and the latter tends to occur more frequently in mixed infections [37,50,51]. Subspecies necrophorum is the predominant and primary etiological agent of bovine liver abscesses [37,52]. While subsp. funduliforme is less frequently isolated from liver abscesses [51] despite their reported predominance in the rumen contents [53]. Subspecies funduliforme is the predominant biotype isolated from ruminal lesions [37,38]. The *Fusobacterium necrophorum* subsp. necrophorum is more virulent than *Fusobacterium necrophorum* subsp. funduliforme because of more potent or increased production of virulence factors [38].

Main Virulence factors Producted by Fusobacterium Necrophorum

Recent studies have revealed that subsp. necrophorum can produce several virulence factors such as leukotoxin; endotoxic lipopolysaccharide (LPS), hemolysin, hemagglutinin, capsule, adhesins or pili, platelet aggregation factor, dermonecrotic toxin,

and several extracellular enzymes, including proteases and deoxyribonucleases and one new molecular collagenolytic cell wall component (CCWC), with collagenase activity, which was separated in past [54-56]. All these factors contribute to entry, colonization, proliferation, establishment of the organism and to the development of lesions on the ruminal wall (ruminal abscesses or rumenitis) and abscesses in the liver [52,57]. However, leukotoxin which is encodes by genome of *Fusobacterium necrophorum*, is considered to be the major virulence factor involved in the pathogenesis of fusobacterial infections [58] as indicated by a correlation between toxin production and ability to induce abscesses in laboratory animals, [59,60] and feedlot cattle [36,58].

Animal experiments have illustrated the ability of a leukotoxin-producing strain of *Fusobacterium necrophorum* to induce morbidity and mortality, and the inability of non-leukotoxin producers or low toxin producers to cause infections [59]. Huitong Zhou isolated a new variant of the leukotoxin gene of *Fusobacterium necrophorum* from the hoof of a sheep with ovine footrot [61]. The strains of *Fusobacterium necrophorum* without the ability of leukotoxin-producing cannot induce foot abscesses in cattle following intradermal inoculation [59,60]. Furthermoreseveral investigators have reported that type A produces more leukotoxin and is isolated more frequently from liver abscesses. Type B produces less leukotoxin, so subsp. funduliforme was less pathogenic [59,60,62].

The *Fusobacterium necrophorum* leukotoxin is a large secreted extracellular protein of high molecular weight that is cytotoxic to neutrophils, macrophages, and hepatocytes [59,63]. The complete nucleotide sequence of the tricistronic (lkt BAC) leukotoxin operon of *Fusobacterium necrophorum* has been determined [40,64] and the *lktA* (leukotoxin) gene is 9726 bp long and encodes a protein of 3241 amino acids [40]. The sequence diversity of the promoter region of the leukotoxin operon explains the different levels of leukotoxin production between the two subspecies [58,63]. Moreover, the *lktA* gene appears to be unique to *Fusobacterium necrophorum*, as it is reportedly not present in other *Fusobacterium* species [64]. In the past, it was thought that *Fusobacterium necrophorum* leukotoxin does not share sequence homology with any other known bacterial leukotoxin, but a recent study suggests the presence of a homologue of *lktA* in the F. equinum genome [53]. The full-length recombinant leukotoxin has been shown to be toxic to bovine polymorphonuclear (PMN) leukocytes, and the toxin is more active against PMNs than against lymphocytes [65,66]. *Fusobacterium necrophorum* leukotoxin is cytotoxic to neutrophils, macrophages, hepatocytes, and possibly to ruminal epithelial cells [63]. The toxin induces apoptosis at low concentrations and lyses the cells at higher concentrations [50]. The cytotoxicity appears to be specific to ruminant (cattle and sheep) and human neutrophils, but not those from pigs or rabbits and only moderately toxic to neutrophils of horses [63]. The toxin induces apoptosis at low concentrations and lyses the cells at higher concentrations and is more active against PMNs than against lymphocytes [65]. The ability of leukotoxin to modulate the host immune system by its toxicity, including cellular activation of PMNs and apoptosis-mediated killing of phagocytes and immune effector cells, represents a potentially important mechanism of its pathogenesis [65].

Hemagglutinin is a heat-labile, low molecular weight protein (19 kDa) rich in amino acids alanine, glutamine and histidine. It is not known whether hemagglutinin is an outer membrane protein or a secreted protein that agglutinates erythrocytes. Kanoe et al. demonstrated that hemagglutinin was present on the cell surface and suggested that they may be bacterial appendages [67]. Animal isolates of subsp. necrophorum with hemagglutinin were more virulent than animal and human isolates of subsp. funduliforme lacking hemagglutinin [68]. It has long been recognized that *Fusobacterium necrophorum*, particularly subsp. necrophorum, agglutinates erythrocytes from chicken and other animal species [50] Shinjo et al. reported that both subspecies of *Fusobacterium necrophorum* varied in their ability to agglutinate erythrocytes from different animal species [69]. Shinjo and Kiyoyama [69] reported that a hemagglutinin-lacking mutant strain of *Fusobacterium necrophorum* was not as lethal to mice as the wild type strain. Enhanced virulence of subsp. necrophorum, compared to subsp. funduliforme, may in part result from better adherence to the ruminal epithelium [68]. Bacterial adherence was inhibited by pre-treatment with either anti-hemagglutinin serum or trypsin and pepsin [70]. Pretreatment with lipase and neuraminidase had no effect on bacterial adherence to the ruminal epithelium [70]. Therefore, hemagglutinin may play a significant role in adherence to and invasion of ruminal epithelial cells by *Fusobacterium necrophorum*, an initial step in the pathogenesis of liver abscesses in cattle [62].

As with any other Gram-negative bacterias, the outer membrane of *Fusobacterium necrophorum* contains endotoxic LPS and the effect of LPS on the genesis of hepatic abscesses in laboratory animals has been studied by several investigators [71]. The chemical composition of *Fusobacterium necrophorum* LPS varies depending on the subspecies [72]. Nakajima Y demonstrated that injection of a mixture of *Fusobacterium necrophorum* and its LPS induced hepatic necrosis and abscess formation in mice [73]. Garcia et al. reported that the differences in virulence associated with endotoxin from different *Fusobacterium necrophorum* subspecies were due to the changes in leukocyte trafficking and endotoxin content in mice [74]. The purified *Fusobacterium necrophorum* LPS was able to activate peritoneal macrophages in synthesizing the pro-inflammatory cytokine, interleukin-1 [75]. But the role of LPS in the development of footrot needs to be evaluated further. Besides LPS, very recently, two studies identified several outer membrane proteins, which play an important role in adhesion to the bovine endothelial cells surface, which is a critical initial step in the pathogenesis [76,77].

The Correlation between Plasma Proteins from Dairy Cattle with Footrot and Virulence Factors

In one recent study, which based on proteomic analysis of plasma proteins from dairy cattle with footrot plasma [16], potential differential expression (PDE) proteins were found in

plasma samples from dairy cattle with footrot (Table 1)seven of which may be valuable for use as diagnostic biomarkers, including Haptoglobin, SERPINA 10 protein, afamin precursor, haptoglobin precursor, predicted peptidoglycan recognition protein L (PGRP-L), apolipoprotein D, keratan sulfate proteoglycan (KS-PG) [78] 3 of 16 PDE proteins, haptoglobin, haptoglobin precursor, and afamin precursor are response of innate immunity [79-81].

Table 1: Possible effect of seven potential differential expression proteins on pathogenesis of footrot.

Categorization of protein	Protein	Possible effect in pathogenesis of footrot.
innate immune recognition molecules	predicted peptidoglycan recognition protein L(PGRP-L)	Recognition of the innate immune activators of the Gram-negative anaerobic bacterium F. necrophorum
acute phase proteins	haptoglobin	Response of innate immunity. Inhibits its oxidative activity. Prevent iron-utilizing bacteria from. Benefiting from hemolysis. Acute-phase protein.
	haptoglobin precursor	It may same with above.
	afamin precursor	Response of innate immunity. Affect metabolic syndrome. Involve in inflammation. Acute-phase protein.
regulatory proteins	SERPINA 10	Involve in blood coagulation. Involve in complement activation. Involve in fibrinolysis. Involve in angiogenesis. Involve in inflammation.
	apolipoprotein-D	Relate to metabolism and lipid transport. Inhibite oxidative stress. Inhibite apoptosis.
cell adhesion and cytoskeletal proteins	keratan sulfate proteoglycan	Correlation with the vesicles. Marker of cartilage catabolism Caused by suppuration, necrosis, and corruption

Haptoglobin (abbreviated as Hp) is the protein that is encoded by the HP gene. Inblood plasma, haptoglobin binds free hemoglobin (Hb) released from erythrocytes with high affinity and thereby inhibits its oxidative activity. In the process of binding hemoglobin, haptoglobin can prevent iron-utilizing bacteria from benefiting from hemolysis. So haptoglobin is considered as one of acute-phase protein [82]. In clinical, Haptoglobin was a biomarker for mastitis [83], Interdigital Dermatitis [84], arthritis [85] and hoof disease [86] was verified as plasma biomarkers of footrot in dairy cattle [78]. In the footrot plasma, iron ion binding proteins which lead to the increase of the iron ion concentration in the footrot plasma [78]. The changes of the iron ion concentration may correlate with erythrocyte hemolysis caused by *Fusobacterium necrophorum* infection [87,88]. Accordingly, the emergency of haptoglobin in plasma can be considered as a response of innate immunity to hemolysis.

SERPINA 10 protein, namely Protein Z-dependent protease inhibitor, is involved in blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, and tumor suppression [79] and the deficiency will lead to thrombosis [89]. The emergence of SERPINA 10 protein in plasma from footrot-affected cattle may indicate the defense response of the host against footrot caused by Fusobacterium necrophorum infection. The effects of SERPINA 10 including complement activation, fibrinolysis, angiogenesis, and inflammation may all implicate in footrot. Therefore, SERPINA 10 is likely to be the main reason that *Fusobacterium necrophorum* enhance platelet aggregation, leading to intravascular thrombus formation [17].

Afamin (*AFM*) is the member of the albumin gene family, was discovered in 1994, which is known to be present in plasma, cerebrospinal etc [90]. Afamin comprises four genes which encode structurally related serum transport proteins. In clinical, it was found that afamin was associated with metabolic syndrome and cancer in ovarian [91], bladder [92]. Blood concentrations of afamin have been found to be associated with a variety of disease phenotypes including metabolic syndrome and related pathologies such as obesity, pregnancy complications, type-2 diabetes and dyslipidemia [93], which suggest that metabolic disorder may occurred during footrot. In addition, afamin showed a rather strong inverse association with the inflammatory biomarkers C-reactive protein and interleukin-6 [94], these result was opposed to the effects of other factors in infection. Future research regarding the effects of afamin on footrot need be done.

Peptidoglycan recognition receptor proteins (PGRPs) are a family of pattern recognition receptors (PRRs) [95]. Based on molecular weight, PGRPs are classified into three types, i.e., short, intermediate, and long PGRPs (PGRP-S, PGRP-I, and PGRP-L, respectively) [96] PRRs are considered as one of the important innate immune molecules, can recognize peptidoglycan (PGN) of the bacteria cell wall and play an important role in host immune defense against pathogen infection [97]. So the up-regulation in footrot cattle plasma of PGRP-L may reflect its effect on Gram-negative anaerobic bacterium *Fusobacterium necrophorum* by binds to its compounds, such as LPS of gram-negative bacteria [98].

Apolipoprotein D is a secreted glycoprotein, member of the lipocalin superfamily, with a related beneficial role in metabolism and lipid transport [99]. Unlike other **lipoproteins**, it is mainly produced in the liver, apolipoprotein D is mainly produced in the brain and testes [100]. In clinical, Apo D expression is up regulated in several human neuropathologies [101] situations where Apo D overexpression seems to be directly related with increases in oxidative stress and apoptosis [102]. It seems that several authors have suggested that Apo D has an important function expression of Apolipoprotein D in footrot cattle may act as one neuroprotective protein and an antioxidant defense system as previous experiments [103,104]. In spite of the correlation between oxidative stress and increased gene expression, the mechanisms that Apo D exerts protective function need to be fully elucidated.

Keratan sulfate (KS) is a glycosaminoglycan (GAG) type consisted of a sulfated poly-N-acetyl lactosamine chain that have been found especially in the cartilage, and bone [105,106]. The PDE protein keratan sulfate proteoglycan (KS-PG) identified in the plasma from footrot-affected dairy cattle may reflect catabolism of hoof cartilage and the damage of joint space. Additionally, Keratan sulfate has important effects in leukocyte recruitment and activation [107,108] therefore, the up-regulation expression of it may to some extent consistent with suppuration, necrosis, and corruption of the hoof tissue.

Four of sixteen PDE proteins in footrot plasma, including keratan sulfate proteoglycan, centromere protein F, desmoplakin and similar to superficial zone protein, involving cell adhesion and cytoskeletal proteins, exhibit a certain correlation with the vesicles in cellular components GO categories [78]. Centromere protein F (CENPF) is an essential nuclear protein associated with the centromere-kinetochore complex and plays a critical role in chromosome segregation during mitosis. Up-regulated CENPF expression was positively correlated with venous invasion in tumor [109]. Desmoplakin is a critical component of desmosome structures in cardiac muscle and epidermal cells, which function to maintain the structural integrity at adjacent cell contacts by interacting with keratins and vimentin [110]. The vesicles are a basic tool used by the cell for organizing cellular substances, and perform a variety of functions, including metabolism, transport, buoyancy control, enzyme storage, and acting as chemical reaction chambers [111]. Emergence of the vesicle-related proteins could represent a special change of the cellular components during the development phase of footrot [78].

Conclusion

The pathogenesis of footrot is very complex and multifactorial. In most cases, footrot is dependent on a mixed bacterial infection. *Fusobacterium necrophorum* either promote disease development by increasing the damage, subsequently permits replication of *Dichelobacter nodosus*. Many virulence factors such as leukotoxin, endotoxic lipopolysaccharide, hemolysin, hemagglutinin involve in footrot infected by fusobacterial. Seven potential differential expression (PDE) proteins from dairy cattle plasma implicated in the pathogenesis of footrot.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgment

Research reported in this study was supported by Chinese National Key Research and Development Plan, (Grant No. 2016YFD0501106).

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