CHAPTER I

Histophilus somni (*Haemophilus somnus*)

ABSTRACT

Histophilus somni is a commensal and an opportunistic bacterial pathogen associated with multisystemic diseases in cattle and sheep. Some strains of *H. somni* isolated from the urogenital tract of cattle are biochemically and serologically similar to the pathogenic strains, but are relatively innocuous. A close genetic relationship between bovine isolates of *Histophilus somni* (previously known as *Haemophilus somnus*) and the ovine isolates of *Histophilus somni* (previously known as *Histophilus ovis* and *Haemophilus agni*) has been documented. Several virulence factors/mechanisms have been identified in *H. somni* of which the phase-variable lipooligosaccharide, induction of apoptosis of host cells, intraphagocytic survival, and immunoglobulin Fc binding proteins have been well characterized. Although commercial bacterins have been available to vaccinate cattle, their efficacy has not always been reliable. An understanding of the virulence factors of *H. somni* will be beneficial in developing new and improved diagnostic tests and vaccines. This chapter provides a comprehensive description of *H. somni* in terms of various features that may facilitate survival and pathogenesis.

Species characteristics

Histophilus somni is a Gram-negative, cocco-bacillary, facultatively anaerobic, non-spore forming, non-motile, non-capsulated, fastidious bacterium within the family *Pasteurellaceae* (Kilian and Biberstein, 1984). This commensal bacterium has been known to inhabit the reproductive tract (Bisgaard, 1993; Corbeil et al., 1995) and the lower (Gogolewski et al., 1989) as well as upper (Humphrey and Stephens, 1983) respiratory tract of cattle. In the laboratory, unlike some of the other important members of the *Pasteurellaceae*, *H. somni* does not require nicotinamide adenine dinucleotide (factor V) or hemin (factor X), but does require a complex, enriched medium for growth (Asmussen and Baugh, 1981; Inzana and Corbeil, 1987). Whereas blood or blood factors and incubation in 5-10% CO_2 (capnophile) are essential for growth on agar plates, growth in broth does not require these conditions (Inzana, 1999). However, thiamine monophospate (TMP) supplementation is required for growth in media such as Columbia broth or brain heart infusion (BHI) broth (Asmussen and Baugh, 1981). A synthetic medium supplemented with cysteine, cystine, and IsoVitaleX for culturing *H. somni* strains has also been described (Merino and Biberstein, 1982).

Colonies of *H. somni* on blood agar plates incubated for 24-48 hours are small and grayish (Fig. 1.01). Colonies of *H. somni* from BHI agar plates incubated for 48-96 hours are small and light yellow (Fig. 1.02). The characteristic yellow pigment of *H. somni* is very obvious on cotton swabs (Fig. 1.03). A weak β-like hemolytic activity can be observed in some *H. somni* strains on blood agar (Figs. 1.01, 1.04, and 1.05), and a zone of clearing may be found around *H. somni* colonies on BHI agar (Fig. 1.06). Strains of *H. somni* can ferment D-glucose producing acid without gas, are oxidase and indole positive, but catalase and urease negative (Kilian and Biberstein, 1984).

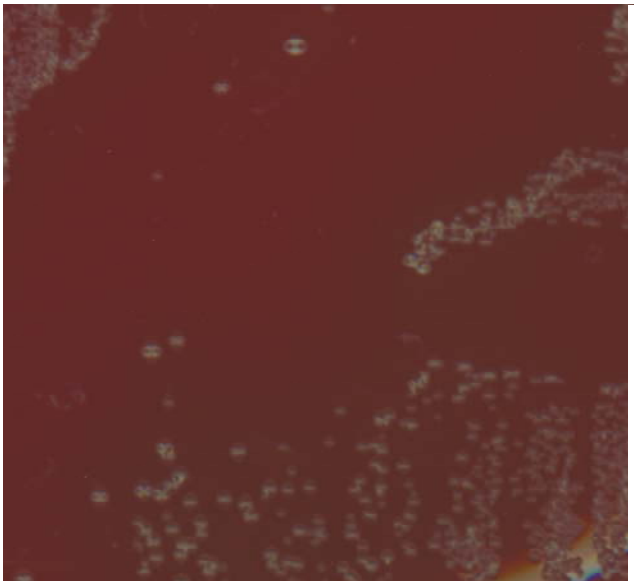

Fig. 1.01: *H. somni* strain 2336 colonies appear as circular, grayish, moist, and glistening structures with smooth edges on Columbia blood agar supplemented with 5% sheep blood. A greenish discoloration of the background is visible at the lower half, indicating active hemolysis. The plate was incubated at 37^0C in 5% CO_2 for 48 hours.

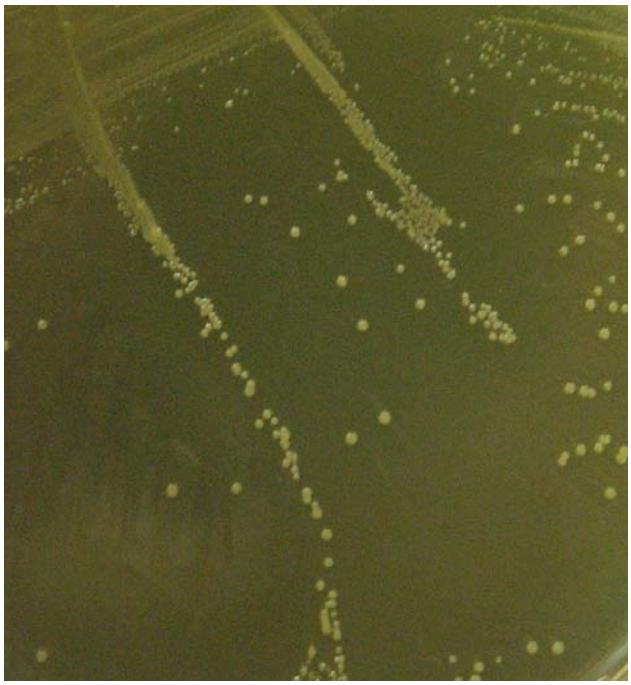

Fig. 1.02: *H. somni* strain 2336 colonies appear as circular, light-yellow colored, moist, and glistening structures with smooth edges on BHI agar supplemented with 0.1% TMP. The plate was incubated at 37^0C in 5% CO_2 for 96 hours.

Fig. 1.03: *H. somni* strain 2336 colonies scraped from blood agar plates using a cotton swab show the characteristic yellow color.

Fig. 1.04: *H. somni* strain 2336 cultured on Columbia blood agar supplemented with 5% sheep blood. A single colony has been magnified 100X to highlight the surrounding zone of hemolysis.

Fig. 1.05: *H. somni* strain 2336 cultured on Columbia blood agar supplemented with 5% sheep blood. An intersection of two colonies has been magnified 100X to visualize the surrounding zone of hemolysis and the embedded sheep red blood cells.

Fig. 1.06: *H. somni* strain 2336 cultured on BHI agar supplemented with 0.1% TMP. An intersection of two colonies has been magnified 100X to visualize the surrounding zone of 'clearing', which may be due to enzymatic activity.

Classification

The taxonomy of bovine isolates of *Haemophilus somnus* and the ovine isolates of *Histophilus ovis* and *Haemophilus agni* had continually been an issue of disagreement since their first discovery. However, recent phylogenetic analyses, utilizing the 16s ribosomal DNA (rDNA) and RNA polymerase B (*rpoB*) gene sequences, have shown that these bacteria differ significantly from *Haemophilus influenzae*, the type species of the genus *Haemophilus* (Angen et al., 2003). These analyses, like previous ones (Walker et al., 1985; Lees et al., 1994), also revealed that *Histophilus ovis* and *Haemophilus agni* are genetically and phenotypically similar to *Haemophilus somnus*. Based on these analyses, the genus '*Histophilus*' containing the sole species "*Histophilus somni*" was proposed within the family *Pasteurellaceae* and encompasses bacteria previously described as *Haemophilus somnus*, *Histophilus ovis,* and *Haemophilus agni* (Angen et al., 2003). Based on their ability to acquire ovine and/or bovine transferrin-bound iron (Ward et al., 2006), it may be suggested that the bovine and ovine *H. somni* isolates represent two very closely related organisms that evolved from a common ancestor in the near past.

Strains of *H. somni* that do not possess the typical features described previously have been reported (Garcia-Delgado et al., 1977; Corbeil et al., 1985). Some strains of *H. somni* show micro-heterogeneity in their morphology, biochemistry, as well as serology (Canto and Biberstein, 1982; Stephens et al., 1983; Stephens et al., 1987). Therefore, a universally applicable typing system to classify clinical isolates of *H. somni* is not yet available. Although biotyping, ribotyping (Fussing and Wegener, 1993), and other categorization schemes (Appuhamy et al., 1998; Tegtmeier et al., 2000) for *H. somni* have been reported, they have not been widely used. Epidemiological characterization of *H. somni* is thus ambiguous for want of a reliable typing scheme, antigenic or otherwise (Howard et al., 2000).

Geographic distribution

While *H. somni* is a constituent of the normal flora of cattle and sheep, the prevalence of *H. somni* as a disease agent appears to be common in those circumstances where husbandry practices are very intensive. Although the majority of *H. somni* reports have been from Western Canada, the Midwestern U.S.A., parts of Western Europe and Scandinavia, there have also been records of *H. somni* disease incidences in Argentina (Descarga et al., 2002), Australia (Stephens et al., 1986), Bulgaria (Buchvarova, 1985), Czech Republic (Svastova, 1988), Egypt (Ismail, 1991), Japan (Ishikawa et al., 1984), New Zealand (Thompson et al., 1987), and South Africa (Last et. al., 2001). Therefore a particular geographic location or climatic condition is probably not associated with disease episodes of *H. somni*. Differences in the predisposition of *Bos indicus* (zebu) and *Bos taurus* cattle to colonization and/or infection by *H. somni* probably exist, but have not been systematically examined thus far. Studies on the occurrence of *H. somni* in other species of the domesticated *Bovidae* are rare. Occurrences of *H. somni* in farmed North American bison have been reported (Ward et al., 1999; Dyer, 2001). However, it is unknown if *H. somni* occurs in bison and other large ruminants in the wild.

Within the continental U.S.A., reports of *H. somni* appear to be limited to those states with a higher density of cattle population and/or feedlot enterprises. Based on the 2002 census by the United States Department of Agriculture, the distribution of cattle in the 48 states is shown in figs. 1.07 and 1.08 (obtained from http://www.nass.usda.gov/research/atlas02/).

Fig. 1.07: Dairy cattle population

Fig. 1.08: Beef cattle population

Clinical diseases

H. somni exhibits considerable versatility as a commensal by colonizing disparate sites in the host and as a pathogen by causing different diseases in those sites. Excluding the digestive system, diseases of several organs of cattle and sheep have been caused by *H. somni* in male and female, young and old, and feedlot and dairy cattle (Bisgaard, 1993; Corbeil et al., 1995). Because of the wide-ranging implications of septicemia due to *H. somni* infection within a given host, the terms 'haemophilosis' and "*Haemophilus somnus* complex" are sometimes used in the literature to collectively describe diseases due to *H. somni* (Pennell and Renshaw, 1977; Harris and Janzen, 1989; Inzana, 1999).

One of the most frequently reported manifestations of disease due to *H. somni* was thrombotic meningoencephalitis (TME), and several of the initial reports of *H. somni* in the U.S.A. and Canada came from cases of TME in numerous cattle herds (Stephens et al., 1981). However, the incidence of bovine TME has been sporadic in recent years. Either independently or in association with *Mannheimia haemolytica* and *Pasteurella multocida*, *H. somni* also causes bovine shipping fever pneumonia (Inzana, 1999), and bovine pneumonia appears to be the most commonly reported *H. somni* disease in the recent past. However, illnesses such as abortion (Chladek et al., 1975), infertility (Kwiecien and Little, 1991), arthritis (Pritchard et al., 1979), myocarditis (Wessels and Wessels, 2005), and mastitis (Hazlett et al., 1985) can be caused by *H. somni* with varying degrees of frequency and severity in cattle (Fig. 1.09). Similar disease conditions associated with strains of *H. somni* have been described in sheep. As in other members of the *Pasteurellaceae*, relatively less pathogenic and/or avirulent variants of *H. somni* have also been isolated from cattle, most frequently from the mucosal surfaces of the reproductive tract (Widders et al., 1989a).

Depending on the organ system involved and the host immune status, the disease pattern may either be acute and fatal or chronic and sublethal (Inzana, 1999). Morbidity rates vary across cattle herds with either very few or several animals affected at a time and the intermingling of cattle from disparate sources tends to precipitate disease onset (Radostits et al., 2000). The clinical presentation of *H. somni* systemic infection varies from sudden death to pyrexia, anorexia, hyperesthesia, ataxia, stiffness, blindness, dullness, hypersalivation, cough, and cachexia (Inzana, 1999; Radostits et al., 2000).

To confirm the etiologic role of *H. somni*, some of the afore-mentioned disease manifestations have been reproduced in cattle (Widders et al., 1986; Jackson et al., 1987; Silva and Little, 1990) and with limited success in some laboratory animals (Nivard et al., 1982; Dewey and Little, 1984; Inzana and Todd, 1992) using pathogenic isolates of *H. somni*. The suitability of new laboratory species such as *Drosophila melanogaster* and *Caenorhabditis elegans* for *in vivo* investigation of *H. somni* is yet to be explored.

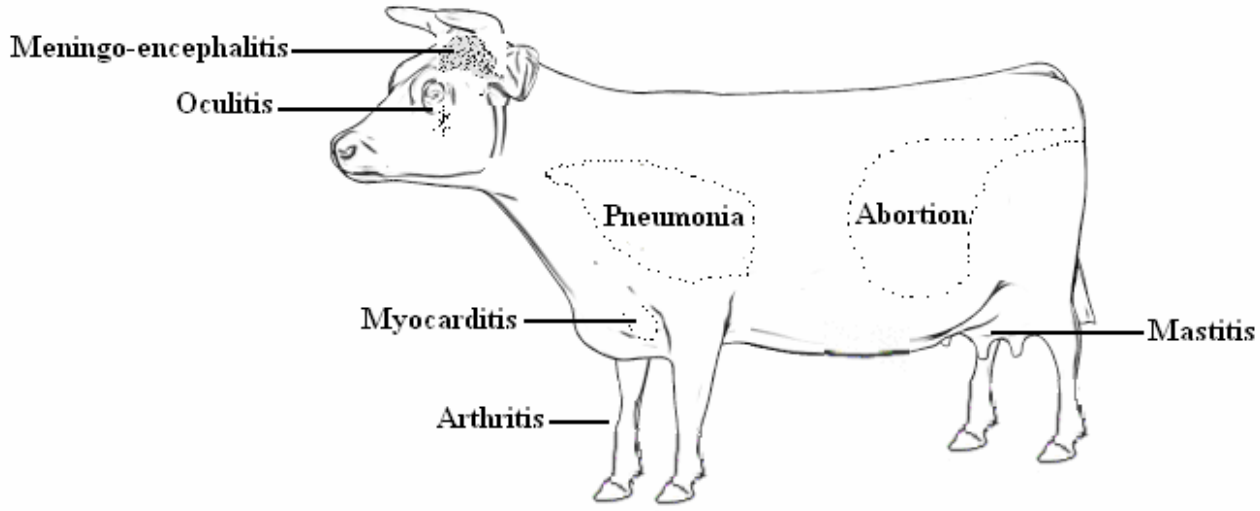

Fig. 1.09: Diseases caused by *H. somni*

(Picture courtesy of Rusinkiewicz, DeCarlo, Finkelstein, and Santella, http://www.cs.princeton.edu/gfx/proj/sugcon/models/)

Virulence attributes

A successful bacterial pathogen often uses a variety of virulence factors to survive and perpetuate within the most suitable host (Finlay and Falkow, 1997). Although the subset of virulence attributes essential for pathogenesis is unknown in the case of several bacteria, it is plausible that a pathogen alters its ability to express the minimum virulence subset in relation to adversities it encounters within and outside the host (Finlay and Falkow, 1997). An understanding of the versatility that bacteria such as *H. somni* exhibit while they transform from harmless commensals to debilitating pathogens is of fundamental importance.

Like most commensal pathogens, *H. somni* is probably not a pathogen by choice, but by chance. In the absence of factors that favor disease, such as the stress of transportation, concurrent viral infection, overcrowding, pregnancy, lactation, and/or harsh weather, which can all compromise immunity, *H. somni* leads a relatively noninvasive existence as a commensal of the mucosal surfaces of respiratory and reproductive tracts of cattle. Some of the virulence factors that may enable *H. somni* to cause diseases include lipooligosaccharide (LOS) phase variation and decoration of the LOS with sialic acid and/or phosphoryl choline, inhibition of the oxidative burst by bovine macrophages, apoptosis of bovine endothelial cells, synthesis of proteins capable of binding host immunoglobulin Fc receptors, and the production of an exopolysaccharide (Inzana and Corbeil, 2004). Furthermore, some mechanisms used by *H. somni* to escape the host immune system have been shown to be similar to those of *H. influenzae, Neisseria meningitidis*, and *Neisseria gonorrhoeae* (Howard et al., 2000). This indicates that these bacteria have retained certain common phenotypes, despite years of independent evolution. Nevertheless, it is possible that *H. somni* possesses several other as yet unknown and uncharacterized virulence mechanisms that it uses to escape host immune components and cause diseases.

Phase variation of lipooligosaccahride

Phase variation of genes encoding cell surface components involved in interaction with the host is a well characterized feature among bacterial pathogens. Phase variation of *H. somni* LOS is a modification in the structure of its oligosaccharide components. This type of variation also occurs in the LOS of several pathogenic bacteria related to *H. somni*. Although other mechanisms of phase variation exist, particularly in genes involved in virulence (Salaun et al., 2003), phase variation of genes involved in LOS biosynthesis appears to occur by slipped-strand mispairing (SSM) between the template and the nascent strands during semi-conservative DNA replication (Kahler and Stephens, 1998). Regions within (or in the promoters of) genes containing tracts of recurring 1-6 bp DNA, called variable number of tandem repeats (VNTRs), are highly vulnerable to SSM (van Belkum et al., 1998). The molecular switch function of SSM, which turns 'on/off ' the associated genes, may occur at the level of transcription or at the level of translation based on whether the VNTRs are within the promoter region or downstream of the start codon, respectively (Henderson et al., 1999). Phase variation of surface components because of SSM within genes involved in their biosynthesis has been well characterized in *H. influenzae*, *N. meningitidis*, *N. gonorrhoeae, Campylobacter jejuni*, and *Helicobacter pylori* (Kahler and Stephens, 1998; Salaun et al., 2003).

In *H. somni*, the LOS outer core oligosaccharides of strains isolated from sick animals have been shown to undergo a reversible, random, structural and antigenic phase variation at a comparatively higher rate than the LOS outer core oligosaccharides of preputial strains isolated from apparently healthy bulls (Inzana et al., 1992; Inzana et al., 1997). Monoclonal antibodies raised against epitopes of *N. gonorrhoeae* and *H. influenzae* LOS also react with *H. somni* LOS in a phase variable manner, confirming phase variation-based antigenic heterogeneity (Howard et al., 2000).

The association between the VNTRs in *H. somni* and LOS phase variation has been experimentally established. Proteins of various lengths would be predicted based on the number of 5'-(CAAT)-3' repeats in the *lob1* gene involved in LOS biosynthesis and the relative position of the stop codon. However, *H. somni* preputial isolate 129Pt has a non-phase varying LOS, and it has been shown that the *lob1* gene in this strain is truncated. Furthermore, *H. somni* strain 129Pt expressing *in trans* the functional *lob1* gene from a phase varying strain consisting of 5'-$(CAAT)_{33}$-3'repeats was able to phase vary its LOS after *in vitro* passage (McQuiston et al., 2000). Nonetheless, a correlation was not established between the reactivity of LOS epitopes with monoclonal antibodies, the SDS-PAGE profile of the extracted LOS, and the number of 5'-(CAAT)-3' repeats in *lob1*, indicating that *H. somni* LOS phase variation may be under the control of multiple genes (McQuiston et al., 2000).

Additional evidence for the involvement of VNTRs in *H. somni* LOS phase variation has been obtained by mutational analyses. A mutation in the *lob2A* gene, which is involved in LOS biosynthesis in *H. somni*, not only caused significant alterations in the LOS profile compared to the wildtype, but also resulted in a reduced rate of phase variation (Wu et al., 2000). In addition, normal bovine serum had greater bactericidal activity for the *lob2A* knock-out mutant, and this strain was not as virulent as the parent in a mouse model of *H. somni* septicemia. Furthermore, a LOS profile similar to that of the *lob2A* mutant was observed in a *H. somni* wildtype strain that had 5'-$(GA)_{19}$-3' VNTRs within its *lob2A.* This observation indicates that *lob2A* undergoes phase variation *in vivo* and that the gene can be turned 'on' or 'off' based on the number of VNTRs (Wu et al., 2000). Several VNTRs in other genes of *H. somni* have also been identified and characterized using traditional approaches like Polymerase Chain Reaction (PCR) and genomic hybridization (Inzana and Corbeil, 2004; Elswaifi, 2006).

Phosphorylcholination of lipooligosaccharide

Among bacterial commensals and pathogens associated with mucosal surfaces, the ability to attach phosphorylcholine **(**ChoP) to cell surface components and remove it by alteration of genes involved in ChoP biosynthesis is known to facilitate their attachment and survival within the host (Table 1.01).

Table 1.01: Expression of ChoP in pathogenic bacteria

Bacterial Species	Molecules anchoring ChoP	Functional implication
H. influenzae	Lipooligosaccharide (Weiser et al., 1998a)	Enhances colonization of the infant rat upper respiratory tract epithelium
Actinobacillus actinomycetemcomitans	Lipooligosaccharide (Schenkein et al., 2000)	Enhances invasion of human vascular endothelial cells
N. gonorrhoeae	Pili and Lipooligosaccharide (Serino and Virji, 2000)	Enhances adherence to and invasion of human epithelial cells
Pseudomonas aeruginosa	43 kD membrane protein (Weiser et al., 1998b)	Possible role in adherence to biotic and abiotic surfaces
Streptococcus pneumoniae	Lipoteichoic and teichoic acid (Cundell et al., 1995)	Enhances invasion of human endothelial and epithelial cells

It has been demonstrated that the ChoP moiety of *H. influenzae* is capable of phase variation because of the 5'-$(CAAT)_n$-3' VNTRs in the *lic1A* gene, which encodes a ChoP kinase (Weiser et al., 1998a). In the infant rat model, the phase variable expression of ChoP in *H. influenzae* provides for efficient colonization of the epithelium in the upper respiratory tract (Weiser et al., 1998a). Swords et al. (2000) have shown by *in vitro* studies that the receptor on human epithelial cells for platelet activating factor mediates adherence to *H. influenzae* ChoP. Paradoxically, *H. influenzae* ChoP binding to the mammalian C-reactive protein has also been shown to activate complement-mediated bactericidal activity (Weiser et al., 1998a). In the light of these observations, it is possible that ChoP expression in the initial stages of infection serves to augment bacterial adherence to the upper respiratory tract, and phase variable loss of ChoP facilitates protection against host defenses.

In *H. somni*, ChoP is anchored to the primary glucose residue in the outer core of the LOS. The presence of ChoP at this site may camouflage the adjoining sugar residues (Cox et al., 1998). In a study using a monoclonal antibody specific to the ChoP of *H. influenzae* (biotype *aegyptius*) in an ELISA, 44 *H. somni* strains showed remarkable interstrain variation in the accessibility of their ChoP (Howard et al., 2000). A colony blot analysis of a phase variable strain of *H. somni* using the same monoclonal antibody established the presence of intrastrain variation in ChoP expression. A consistently ChoP-positive clonal variant of this strain presented a curtailed LOS profile when analyzed by SDS-PAGE. Electrospray mass spectrometry revealed that the ChoP-positive clonal variant also had a truncated outer core oligosaccharide (Howard et al., 2000). This increased reactivity of the ChoP-positive clonal variant compared to the non-reactive clone is proposed to be due to greater accessibility of ChoP because of a reduction in steric interference by sugars in the outer core (Howard et al., 2000).

In vivo studies have provided insights into the role of ChoP in the adherence of *H. somni* to the bovine respiratory tract. Naive calves inoculated via the respiratory route with a ChoP-deficient (>95% of cells were ChoP-negative in the population) variant of a pathogenic *H. somni* strain gave rise to ChoP-reactive (>35% cells were ChoP-positive in the population) bacteria in the upper respiratory tract 24 hours post-inoculation. A similar challenge with a predominantly ChoP-positive *H. somni* strain maintained the ChoP-positive state (Elswaifi, 2006). Thus it is possible that the phase variable expression of ChoP on the LOS enhances colonization of the bovine respiratory tract by *H. somni*. Furthermore, *H. somni* strain 129Pt has a non-phase varying LOS profile and it is possible that *H. somni* strains from the urogenetial tract have a different molecular mechanism of expression and regulation of the LOS phenotype (Howard et al., 2000; Elswaifi, 2006).

Sialylation of lipooligosaccharide

In some bacterial species, sialylation results from the addition of sialic acid residues to cell surface glycoforms, and this process is catalyzed by one or more sialyltransferases. Sialylation may play an important role in pathogenesis because sialic acid, which is also a nonimmunogenic component of mammalian tissues, may interfere with antibody binding to sialylated epitopes, thus rendering bacteria resistant to serum (Vimr and Lichtensteiger 2002). Sialylation of LOS has been observed in several bacteria including the human pathogens *N. gonorrhoeae* (Smith et al., 1995), *H. influenzae* (Vimr et al., 2000), and *Haemophilus ducreyi* (Schilling et al., 2001). The latter two species can metabolize *N*-acetyl neuraminic acid (NeuAc) and have been shown to adorn their LOS with sialic acid residues to enhance immune evasion and survival (Vimr and Lichtensteiger, 2002).

A similar mechanism of utilization of NeuAc has also been observed among pathogenic strains of *H. somni*. While some pathogenic strains of *H. somni* are able to partially sialylate their LOS without extraneous sources of NeuAc, supplementation of the culture medium with NeuAc is required for LOS sialylation in other pathogenic strains (Inzana et al., 2002). Sialylation has been shown to interfere with the binding of LOS-specific monoclonal antibodies to *H. somni* LOS, as determined by ELISA and Western blotting. Furthermore, sialylation results in increased resistance of *H. somni* to the complement-mediated bactericidal action of normal serum and anti-LOS serum, compared to strains with nonsialylated LOS (Inzana et al., 2002). Whereas NeuAc supplementation enhanced sialylation in several pathogenic *H. somni* strains, isolates from the bovine prepuce, including *H. somni* strain 129Pt, were not able to sialylate their LOS (Inzana et al., 2002). In the bovine host, *H. somni* may therefore have a sialylated LOS phenotype for enhanced resistance to host defenses.

Apoptosis of bovine endothelial cells

Several *in vitro* studies have confirmed that bacterial lipopolysaccharide (LPS) can mediate apoptosis in bovine endothelial cells (Frey and Finlay, 1998). Vasculitis is the hallmark of pathological changes associated with *H. somni* infection and this bacterium has been shown to be cytotoxic to bovine endothelial cells (BECs) *in vitro* (Thompson and Little, 1981). These results suggest that *H. somni* infection may trigger endothelial cell apoptosis *in vivo*. Sylte et al. (2001) used cytochemical analyses to study the mechanisms underlying this process and reported that both pathogenic as well as preputial isolates of *H. somni* were equally capable of apoptosis induction, and that the ultrastructural changes observed were typical of apoptosis. Sylte et al. (2001) also found no difference in apoptosis when *H. somni* cells were washed off the BEC culture after incubation, demonstrating that biochemical components of *H. somni* are sufficient for apoptosis. Culture filtrates as well as heat-killed *H. somni* strains were shown to induce significant BEC apoptosis; heat inactivation of *H. somni* culture filtrates reduced, but did not eliminate, apoptosis (Sylte et al., 2001).

H. somni LOS is heat stable and has been found to trigger apoptosis of BECs in a time and dose-dependent fashion. Induction of BEC apoptosis by LOS was not inhibited by the addition of polymixin B (Sylte et al., 2001). This type of apoptotic signaling has been shown to be mediated by caspase-3 in a caspase-8-dependent pathway (Sylte et. al., 2003). The LOS of *H. somni* has also been found to elicit reactive oxygen intermediates (ROIs) during caspase-3-mediated apoptosis of BECs, and membrane-permeable, but not membrane-impermeable, ROI scavengers were shown to inhibit this function (Sylte et al., 2004). Furthermore, it has been shown that *H. somni* LOS can induce reactive nitrogen intermediates (RNIs) in BECs, and that RNI inhibitors can reduce *H. somni* LOS-mediated BEC apoptosis (Sylte et al., 2004).

Toll-like receptor activation

Mammalian Toll-like receptors (TLRs) have generated significant interest recently as these cell membrane receptor molecules are components of the innate immune system (Akira et al., 2001; Beutler, 2003). Several TLRs have been identified in mammals and two TLRs, TLR-2 and TLR-4, have been implicated in binding LPS from different bacteria (Hajishengallis et al., 2003). Upon stimulation of the cell surface LPS-signaling complex, which consists of the TLRs, a composite cascade of events involving the NF-κB transcription factors and MAP kinases is initiated, which brings about apoptosis and/or inflammation (Akira et al., 2001; Bannerman and Goldblum, 2003). In professional antigen presenting cells that express TLRs, such as dendritic cells (DCs), LPS enhances the expression of MHC molecules as well as induces pro-inflammatory cytokines like TNFα. These matured DCs then recruit naïve T cells to generate adaptive immunity (Barton and Medzhitov, 2002). Murine systems have also been shown to exhibit endotoxin tolerance by altering the profile of the TLR-transduced signals and/or by way of reduced expression of the TLRs, in order to moderate unwarranted immunoinflammatory responses. Such responses have tissue damaging potential that may result in sepsis and/or endotoxic shock (Hajishengallis et al., 2003).

Although *H. somni* LOS has been implicated in apoptosis of BECs, the receptor that transduces the death signal is not yet known. It is possible that this signaling also occurs through one or more bovine TLRs, as similar mechanisms have been found in endothelial cells of other species (Bannerman and Goldblum, 2003). Furthermore, LOS from *H. somni* has been shown *in vitro* to signal through the murine TLR-4 system (Howard, 2005). Since among bovine pathogens *H. somni* is known to phase vary its LOS, it is an ideal candidate to investigate the bovine TLRs involved and the effects of phase variation, if any.

Resistance to intracellular killing

Molecular oxygen and reactive oxygen intermediates (ROIs) play a crucial role in host innate and adaptive immunity against microbial pathogens (Beaman and Beaman, 1984). The oxidative burst of phagocytic cells, such as polymorphonuclear cells (PMNs), is central to innate immunity against invading pathogens (Elsbach and Weiss, 1985). However, several pathogenic bacteria have evolved mechanisms to subvert these host functions and survive in the very cells designed to kill them. A characteristic histopathological finding in *H. somni* infections is the abundance of bacteria and phagocytic cells (macrophages and PMNs) in the diseased organs (Stephens et al., 1981). Several studies have shed light on the role of PMNs in counteracting *H. somni*. Czuprynski and Hamilton (1985) demonstrated that opsonization with either immune or normal bovine serum enhances the internalization of *H. somni* by PMNs and that this response was not affected by complement. In the same study, although bovine PMNs were shown to internalize *H. somni*, the bacteria remained viable inside the cells. This indicates that PMNs may be ineffective in killing *H. somni*.

It has been suggested that the intracellular survival of *H. somni* may be due in part to inhibition or reduction of the production of ROIs, like superoxide anion, indicating inhibition of bovine PMN and macrophage oxidative burst by *H. somni* (Czuprynski and Hamilton, 1985; Howard et al., 2004). However, other studies have shown that bovine serum, complement, and PMNs are bactericidal for *H. somni* (Pennell and Renshaw, 1977; Simonson and Maheswaran, 1982). Corbeil et al. (1985) and Widders et al. (1989a) demonstrated variable serum sensitivity among *H. somni* isolates from different bovine organs. The disparities between these studies may have been due to differences in the strains tested, the source and concentrations of the sera used, and the experimental methodologies.

Live cells, heat-killed whole cells, or supernatant from heat-killed cells of *H. somni* have been described to drastically reduce the phagocytic ability of bovine PMNs as determined by a reduction in protein iodination and *Staphylococcus aureus* internalization, whereas these same components did not interfere with the nitroblue tetrazolium reducing activity of PMNs (Hubbard et al., 1986). The fractions of *H. somni* that inhibited *S. aureus* ingestion and protein iodination were reported to be of 300,000 and 10,000 Da, respectively (Hubbard et al., 1986).

Since *H. somni* does not reduce 3% H_2O_2 *in vitro*, it has been categorized as catalase-negative for diagnostic testing. Nonetheless, viable bacteria, and not heat- or formalin-killed, or sonicated *H. somni*, eliminated H_2O_2 from solution in a time, dose, and carbon source-dependent pattern (Sample and Czuprynski, 1991). In addition, a heat-killed suspension of *H. somni* was observed to induce H_2O_2 production by bovine PMNs *in vitro*, whereas this response was inhibited by viable *H. somni* (Sample and Czuprynski, 1991).

Techniques such as flow cytometry, which facilitate detection of the degree of H_2O_2 production as well as phagocytosis by PMNs, have also been used in conjunction with traditional chemiluminescence assays to evaluate the effect of *H. somni* on PMN activities *in vitro* (Pfeifer et al., 1992). These studies indicate that phagocytosis by PMNs from *H. somni* infected calves was diminished compared to PMNs from naïve calves (Pfeifer et al., 1992), and substantiate previous observations that *H. somni* is capable of interfering with bovine PMN functions. Yang et al. (1998) observed that opsonized *H. somni* cells were capable of inducing bovine PMN apoptosis *in vitro* and that formalin or heat treatment of *H. somni* reduced this outcome. Both serum-resistant and serum-sensitive strains of *H. somni* were capable of effecting these morphological changes. Whereas culture supernatants of *H. somni* have been shown to induce BEC apoptosis, (Sylte et al., 2001), culture supernatants did not trigger apoptosis in PMNs (Yang et al., 1998).

Although studies by Hubbard et al. (1986) identified some *H. somni* components that inhibit PMN functions, the mechanisms of action of these components remain unknown. It has also not been determined if these components are synthesized by *H. somni* or are bound to the cells from components of the growth medium. Nevertheless, based on the studies of Sample and Czuprynski (1991) as well as Pfeifer et al. (1992), induction of H_2O_2 production in bovine PMNs by heat-killed *H. somni* and inhibition of oxidative burst within these cells by viable *H. somni* are important features that need to be explored further.

Lederer et al. (1987) reported that *H. somni* survives in bovine alveolar macrophages and blood monocytes, whereas the same cells effectively killed *Escherichia coli* under analogous experimental conditions. Gomis et al. (1997a) have used flow cytometry to study the phagocytic functions of alveolar macrophages and blood monocytes from calves challenged with *H. somni*. The addition of exponential phase cultures of *H. somni* inhibited the phagocytosis of killed, opsonized *S. aureus* by alveolar macrophages, indicating that *H. somni* can diminish the phagocytic capacity of these cells. *H. somni* is also capable of reducing luminol-dependent chemiluminescence function of bovine alveolar macrophages and blood monocytes, and can survive within these cells in the presence of active nitric oxide production (Gomis et al., 1997b). However, stimulation of monocytes containing *H. somni* with LPS from *E. coli* and various cytokines, which are known to enhance phagocytic function, reduced the numbers of *H. somni* within these cells (Gomis et al., 1998).

Furthermore, pathogenic strains of *H. somni* have been found to significantly impede, in a dose- and time-dependent manner, the production of superoxide anion by bovine alveolar as well as mammary macrophages and PMNs (Howard et al., 2004). Culture supernatants from the same strains did not inhibit superoxide anion production, demonstrating that viable *H. somni* is

required. Addition of cytochalasin B did not reduce superoxide anion inhibition by *H. somni*, indicating that cell contact with, but not phagocytosis of, *H. somni* is essential (Howard et al., 2004). In contrast, superoxide anion inhibition by *H. somni* preputial strains was absent or less pronounced (Howard et al., 2004). These results support previous observations that inhibition of the oxidative burst by *H. somni* may contribute to intracellular survival and pathogenesis.

It is interesting to note here that *H. somni* LOS has been shown to generate ROIs in BECs (Sylte et al., 2004), but *H. somni* inhibits ROIs in bovine PMNs and macrophages (Howard, 2005). Although it is not known if these responses are specific to *H. somni*, it is possible that whole cells of *H. somni* and its LOS components interact disparately with different bovine cell types *in vivo* and *in vitro*.

Several isolates of *H. somni* from the respiratory tract have been reported to produce histamine on blood agar plates as well as in broth cultures. Variation in growth conditions, particularly CO_2 percentage, were shown to influence histamine synthesis (Ruby et al., 2002). Although bacterial histamine has not directly been implicated in intracellular pathogenesis, exogenous addition of interleukin-18 or histamine has been shown to reduce the numbers of *Mycobacterium bovis* BCG cells in histidine decarboxylase knock-out murine bone marrow macrophages *in vitro* (Megyeri et al., 2006). However, the genetic basis of histamine biosynthesis in *H. somni* remains unknown.

Induction of bovine platelet aggregation

Bovine platelets have been shown to be activated by *H. somni* and its LOS *in vitro* (Kuckleburg et al., 2005). Induction of bovine platelet aggregation by *H. somni in vitro* has also been shown to be influenced by ChoP expression (Kuckleburg et al., 2007). It is possible that these activated platelets contribute to formation of thrombi *in vivo* and pathogenesis of TME.

Immunoglobulin- and transferrin-binding proteins

Several studies have identified a variety of protein components of *H. somni* that may be involved in virulence and induction of protective immunity (Corbeil et al., 1995). A group of proteins known as immunoglobulin-binding proteins (IgBPs) are conspicuous in *H. somni*. Two IgBPs of *H. somni* have been well characterized and shown to bind the bovine immunolgobulins (Widders et al., 1988; Yarnall et al., 1988). Bastida-Corcuera et al. (1999a) demonstrated that the Fc region of bovine IgG2b adheres to IgBPs of *H. somni*. These proteins could act in concert *in vivo* to block binding of specific antibodies to *H. somni*. The enhanced serum resistance of pathogenic isolates of *H. somni* has been attributed, in part, to the actions of these IgBPs (Corbeil et al., 1995; Corbeil et al., 1997a). Sanders et al. (2003) have shown by mutational analysis that these proteins are among the virulence determinants of *H. somni*. A mutant strain expressing truncated IgBPs was found to be less virulent than the wildtype strain in a mouse model of septicemia and adhered poorly to BECs (Sanders et al., 2003). However, the molecular mechanism of action of these proteins in affording serum resistance is unknown.

Bacterial transferrin-binding proteins (Tbps) facilitate iron acquisition from host transferrin. Tbps may be important for *H. somni* survival *in vivo* since free iron is not available in the bovine host, and these proteins could also confer host specificity upon *H. somni* (Corbeil et al., 1995). The loss of Tbps has been found to compromise *in vivo* growth and survival in other bacteria (Cornelissen and Sparling, 1994), but *H. somni* mutants lacking Tbps have not been reported. Won and Griffith (1993) proposed a hemolytic role for a 31 kDa protein from *H. somni* since heterologous expression of this protein in a strain of *E. coli* induced lysis of bovine red blood cells *in vitro*. The role of this protein in virulence *in vivo* is unknown and it is possible that this protein is partially responsible for *H. somni* hemolytic activity *in vitro*.

Host immune response

Since *H. somni* is not a true intracellular pathogen, humoral immunity is thought to be adequate in affording protection against systemic diseases. Numerous studies have characterized this aspect of host immunity against *H. somni*. Gogolewski et al. (1987) reported that calves challenged with opsonized *H. somni* were protected against pneumonia, whereas calves challenged with *H. somni* incubated with preimmune serum developed pneumonia, indicating the importance of serum in protection against *H. somni*. In a similar calf challenge experiment using bacteria preincubated with immune or nonimmune serum, Gogolewski et al. (1988) also reported that antiserum against the 40 kDa outer membrane protein (OMP), but not antiserum against the 78 kDa OMP of *H. somni*, protected calves from *H. somni* pneumonia.

Furthermore, cattle challenged with *H. somni*, or vaccinated with killed whole cells of *H. somni*, were found to have persistently high serum IgG2 antibody titers (Widders et al., 1986: Widders et al., 1989b), indicating the importance of this immunoglobulin isotype in protection against *H. somni*. Corbeil et al. (1997b) also compared the protective ability of different immunoglobulin isotypes specific to the 40 kDa OMP of *H. somni*, and showed the importance of IgG2a in bovine resistance against *H. somni* pneumonia.

Butt et al. (1993) reported that intramuscular immunization of approximately 12 month old Holstein calves on day 1 and day 35 with 10 mg of 270 kDa OMP of *H. somni* in Freund's incomplete adjuvant resulted in systemic induction of 270 kDa OMP-specific IgG1, IgG2, and IgM antibodies approximately 30 days after the second immunization. However, no 270 kDa OMP-specific IgA antibodies were detected in either serum or uterine secretions following immunization (Butt et al., 1993). In view of this, the relative roles of IgA and IgG subclasses in systemic and local protection against *H. somni* need to be investigated further.

A variation in the allelic expression of IgG2 allotypes has also been reported among cattle of different age groups. This variation might partially explain the relative susceptibilities of calves and cows to pathogenesis by *H. somni* (Corbeil et al., 1997b). A bovine IgG2b allotype has been found to activate the classical complement pathway twice as effectively as IgG2a (Bastida-Corcuera et al., 1999b), indicating the differential roles of IgG2 subclasses in immunity and pathogenesis during *H. somni* infection. In other studies, mice immunized with *H. somni* lipid A conjugated to protein made antibodies against lipid A, but were not protected against challenge with an abortion isolate of *H. somni* (Inzana and Todd, 1992). Therefore, it is plausible that an assortment of *H. somni* lipoprotein and LOS antigens are crucial in generating active immunity upon natural infection or vaccination, as are the particular isotypes and allotypes of host immunoglobulins.

H. somni has been shown to persist for 6-10 weeks in the lungs of challenged calves, despite the presence of systemic as well as local *H. somni*-specific antibodies (Gogolewski et al., 1989). This indicates that humoral immune responses may not be adequate in clearing *H. somni* infections. However, these calves were shown to clear a second *H. somni* challenge within three days (Gogolewski et al., 1989). Therefore, it is possible that the induction of cell-mediated immune responses, in addition to IgG2- and IgA-mediated humoral immune responses, is important in affording complete protection against *H. somni*, particularly when the bacteria reside in bovine PMNs and other cells. However, the current understanding of cell-mediated immune responses against *H. somni* is limited.

In summary, previous research indicates that challenged or vaccinated calves have moderate levels of resistance against subsequent *H. somni* infection, and that vaccination could be used to protect cattle against certain manifestations of disease due to *H. somni*.

Vaccination

A variety of concoctions of bacterial cultures have been used in the field for prophylaxis against *H. somni*, in hopes of reducing infection and/or disease severity (Yancey, 1993). Although the usefulness of bacterins against the spectrum of *H. somni* diseases in cattle of different age groups has been unpredictable, the decrease in cases of TME in the recent past has been attributed in part to such preventive measures (Harris and Janzen, 1989; Schuh and Harland, 1991).

Some examples of commercial vaccine formulations available to protect cattle against diseases induced by *H. somni* are shown in table 1.02. However, these vaccine formulations may not be as efficacious against pneumonia and myocarditis as they are against other forms of diseases. In view of this, live-attenuated *H. somni* vaccines will need to be developed and tested.

Table 1.02: Examples of commercial *H. somni* vaccines available in different countries

Trademark	Manufacturer	Contents
SOMNU SHIELD™	Novartis Animal Health US, Inc. Larchwood, IA	*H. somni* bacterin
SOMUBAC®	Pfizer Animal Health New York, NY	*H. somni* bacterin
TRIANGLE® 4 + HS	Fort Dodge Animal Health Division Of Wyeth Fort Dodge, IA	*H. somni* bacterin in addition to four other killed viral agents
POLY-BAC® B3	Texas Vet Lab San Angelo, TX	*H. somni, M. haemolytica* A1, *and P. multocida* A3 bacterin-toxoid
SOMNUGEN™	Boehringer Ingelheim (Canada) Ltd. Burlington, ON	*H. somni* bacterin
BOVINE HAEMOPHILUS INACTIVATED VACCINE®	Kyoto Biken Laboratories,Inc. Kyoto, Japan	Formalin-inactivated whole cell suspension of *H. somni*

Conclusions

From the review in this chapter, it is clear that *H. somni* can cause a range of diseases with varying severity, when opportunities arise. Apart from factors such as age, sex, husbandry practices, and stress that influence host immunity, unidentified and as yet uncharacterized *H. somni* determinants may be important in disease induction.

The stark phenotypic contrast between pathogenic and commensal isolates is one of the most striking features of *H. somni*. Previous studies utilizing these two types of strains have shed some light on the genetic differences that correspond to the phenotypic dissimilarities. It is possible that these two types of *H. somni* strains originated from a common ancestor and evolved independently to colonize different sites within the bovine host.

The virulence potential of *H. somni* has only been partially understood in the pre-genomic era using traditional genetic, biochemical, and immunologic approaches. Several studies have shown that the LOS of *H. somni* is a complex molecule with many functions, including providing structural integrity to the cellular architecture and mediating endotoxic shock through its lipid A. It is also known that multiple components in the LOS of *H. somni* can undergo phase variation. The clinical implication of LOS antigenic variation is that it may help *H. somni* to evade the humoral immune responses and adapt to different sites within the host. This also suggests that *H. somni* LOS components may be inapt diagnostic and vaccine candidates.

The ability of *H. somni* to survive in monocytes, macrophages, and PMNs, as well as induce apoptosis in BECs has been relatively well characterized. It appears that *H. somni* utilizes mechanisms specific to each of the above cell types during different stages of infection and dissemination. As a commensal of the mucosal surfaces, *H. somni* may cause damage to

epithelial cell layers of the respiratory and reproductive tracts before colonizing the endothelial cell layers of blood vessels to cause vasculitis. However, our understanding of this phase of pathogenesis is still nascent. The roles of *H. somni* OMPs, IgBPs, LOS, and other components in virulence will thus need to be examined further for a comprehensive understanding of pathogenesis.

It is not surprising that cattle naturally infected with *H. somni*, or vaccinated using commercial *H. somni* bacterins, mount an immune response and are partially protected against subsequent infection. However, this immunity may not be long-lasting and the host immune responses may also exacerbate pathogenesis. Perhaps the balance between host immune responses and the interaction of *H. somni* components with those responses determine the outcome of an infection, disease process, and/or manifestation of chronic illness.

With the unreliable efficacy of commercial vaccines, *H. somni* continues to be an important pathogen of cattle. It is estimated that the cattle industry in North America loses $1 billion per annum due to bovine respiratory disease, whose etiology includes *H. somni*, *P. multocida*, *M. haemolytica*, and a host of other bacteria as well as viruses (NCE, 2002/2003). Therefore, it is imperative to continue to understand the fundamental biological aspects of *H. somni*. This understanding is expected to facilitate the development of new and improved diagnostic tests and vaccines.

In view of the above considerations, this dissertation presents comparative data on the chromosomes (chapter II), plasmids (chapter III), biofilms (chapter IV), and restriction-modification systems (chapter V) of *H. somni*. Particular emphasis is placed on whole genome comparison and understanding the evolution of strains of *H. somni* in the context of other members of the *Pasteurellaceae*.

References

Appuhamy S, Low JC, Coote JG, Parton R (1998). PCR methods and plasmid profile analysis for characterisation of *Histophilus ovis* strains. *Journal of Medical Microbiology* **47**: 987-992.

Angen O, Ahrens P, Kuhnert P, Christensen H, Mutters R (2003). Proposal of *Histophilus somni* gen. nov., sp. nov. for the three species *incertae sedis 'Haemophilus somnus'*, '*Haemophilus agni*' and '*Histophilus ovis*'. *International Journal of Systematic and Evolutionary Microbiology* **53**: 1449-1456.

Asmussen MD and Baugh CL (1981). Thiamine pyrophosphate (cocarboxylase) as a growth factor for *Haemophilus somnus*. *Journal of Clinical Microbiology* **14**: 178-183.

Akira S, Takeda K and Kaisho T (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunology* **2**: 675-680.

Bannerman DD and Goldblum SE (2003). Mechanisms of bacterial lipopolysaccharide-induced endothelial apoptosis. *American Journal of Physiology Lung Cellular and Molecular Physiology* **284**: L899-914.

Barton GM and Medzhitov R (2002). Control of adaptive immune responses by Toll-like receptors. *Current Opinion in Immunology* **14**: 380–383.

Bastida-Corcuera FD, Nielsen KH, Corbeil LB (1999a). Binding of bovine IgG2a and IgG2b allotypes to protein A, protein G, and *Haemophilus somnus* IgBPs. *Veterinary Immunology and Immunopathology* **71**:143-149.

Bastida-Corcuera FD, Butler JE, Yahiro S, Corbeil LB (1999b). Differential complement activation by bovine IgG2 allotypes. *Veterinary Immunology and Immunopathology* **71**: 115-123.

Beaman L and Beaman BL (1984). The role of oxygen and its derivatives in microbial pathogenesis and host defense. *Annual Review of Microbiology* **38**: 27-48.

Beutler B (2003). Innate immune responses to microbial poisons: discovery and function of the Toll-like receptors. *Annual Review of Pharmacology and Toxicology* **43**: 609–628.

Bisgaard M (1993). Ecology and significance of *Pasteurellaceae* in animals. *Zentralblatt fur Bakteriologie* **279**: 7-26.

Buchvarova Ia (1985). [Isolation of *Haemophilus somnus* from cattle (article in Bulgarian)]. *Veterinarno Meditsinski Nauki* **22**: 15-21.

Butt BM, Besser TE, Senger PL, Widders PR (1993). Specific antibody to *Haemophilus somnus* in the bovine uterus following intramuscular immunization. *Infection and Immunity* **61**: 2558-2562.

Canto GJ and Biberstein EL (1982). Serological diversity in *Haemophilus somnus*. *Journal of Clinical Microbiology* **15**: 1009-1015.

Chladek DW (1975). Bovine abortion associated with *Haemophilus somnus*. *American Journal of Veterinary Research* **36**: 1041.

Corbeil LB, Blau K, Prieur DJ, Ward ACS (1985). Serum susceptibility of *Haemophilus somnus* from bovine clinical cases and carriers. *Journal of Clinical Microbiology* **22**: 192–198.

Corbeil LB, Gogolewski RP, Stephens LR, Inzana TJ (1995). *Haemophilus somnus*: antigen analysis and immune responses. In: Donachie W, Lainson FA, Hodgson JC (Editors). *Haemophilus, Actinobacillus, and Pasteurella.* New York, NY: Plenum Press, pp. 63-73.

Corbeil LB, Bastida-Corcuera FD, Beveridge TJ (1997a). *Haemophilus somnus* immunoglobulin binding proteins and surface fibrils. *Infection and Immunity* **65**: 4250-4257.

Corbeil LB, Gogolewski RP, Kacskovics I, Nielsen KH, Corbeil RR, Morrill JL, Greenwood R, Butler JE (1997b). Bovine IgG2a antibodies to *Haemophilus somnus* and allotype expression. *Canadian Journal of Veterinary Research* **61**: 207-213.

Cornelissen CN and Sparling PF (1994). Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. *Molecular Microbiology* **14**: 843-850.

Cox AD, Howard MD, Brisson JR, van der Zwan M, Thibault P, Perry MB, Inzana TJ (1998). Structural analysis of the phase-variable lipooligosaccharide from *Haemophilus somnus* strain 738. *European Journal of Biochemistry* **253**: 507-516.

Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI (1995). *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature* **43**: 435–438.

Czuprynski CJ and Hamilton HL (1985). Bovine neutrophils ingest but do not kill *Haemophilus somnus*. *Infection and Immunity* **50**: 431–436.

Descarga CO, Piscitelli HG, Zielinski GC, Cipolla AL (2002). Thromboembolic meningoencephalitis due to *Haemophilus somnus* in feedlot cattle in Argentina. *Veterinary Record* **150**: 817.

Dewey KJ and Little PB (1984). The pathogenicity of *Haemophilus somnus* in various laboratory animal species. *Canadian Journal of Comparative Medicine* **48**: 27-29.

Dyer NW (2001). *Haemophilus* somnus bronchopneumonia in American bison *(Bison bison).* *Journal of Veterinary Diagnostic Investigation* **13**: 419-421.

Elswaifi S (2006). The molecular characterization of phosphorylcholine (ChoP) on *Histophilus somni* lipooligosaccharide: contribution of ChoP to bacterial virulence and pathogenesis. *PhD dissertation*, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Elsbach P and Weiss J (1985). Oxygen-dependent and oxygen-independent mechanisms of microbicidal activity of neutrophils. *Immunology Letters* **11**: 159-163.

Frey EA and Finlay BB (1998). Lipopolysaccharide induces apoptosis in a bovine endothelial cell line via a soluble CD14 dependent pathway. *Microbial Pathogenesis* **24**: 101–109.

Finlay BB and Falkow S (1997). Common themes in microbial pathogenicity revisited. *Microbiology and Molecular Biology Reviews* **61**: 136-169.

Fussing V and Wegener HC (1993). Characterization of bovine *Haemophilus somnus* by biotyping, plasmid profiling, REA-patterns and ribotyping. *Zentralblatt fur Bakteriologie* **279**: 60-74.

Garcia-Delgado GA, Little PB, Barnum DA (1977). A comparison of various *Haemophilus somnus* strains. *Canadian Journal of Comparative Medicine* **41**: 380-388.

Gogolewski RP, Kania SA, Inzana TJ, Widders PR, Liggitt HD, Corbeil LB (1987). Protective ability and specificity of convalescent serum from calves with *Haemophilus somnus* pneumonia. *Infection and Immunity* **55**: 1403-1411.

Gogolewski RP, Kania SA, Liggitt HD, Corbeil LB (1988). Protective ability of antibodies against 78- and 40-kilodalton outer membrane antigens of *Haemophilus somnus*. *Infection and Immunity* **56**: 2307-2316.

Gogolewski RP, Schaefer DC, Wasson SK, Corbeil RR, Corbeil LB (1989). Pulmonary persistence of *Haemophilus somnus* in the presence of specific antibody. *Journal of Clinical Microbiology* **27**: 1767–1774.

Gomis SM, Godson DL, Beskorwayne T, Wobeser GA, Potter AA (1997a). Modulation of phagocytic function of bovine mononuclear phagocytes by *Haemophilus somnus.* *Microbial Pathogenesis* **22**: 13–21.

Gomis SM, Godson DL, Wobeser GA, Potter AA (1997b). Effect of *Haemophilus somnus* on nitric oxide production and chemiluminescence response of bovine blood monocytes and alveolar macrophages. *Microbial Pathogenesis* **23**: 327-333.

Gomis SM, Godson DL, Wobeser GA, Potter AA (1998). Intracellular survival of *Haemophilus somnus* in bovine blood monocytes and alveolar macrophages. *Microbial Pathogenesis* **25**: 227–235.

Hajishengallis G, Martin M, Schifferle RE, Genco RJ (2002). Counteracting interactions between lipopolysaccharide molecules with differential activation of toll-like receptors. *Infection and Immunity* **70**: 6658-6664.

Harris FW and Janzen ED (1989). The *Haemophilus somnus* disease complex (haemophilosis): a review. *Canadian Veterinary Journal* **30**: 816–822.

Hazlett MJ, Little PB, Barnum DA, Maxie MG, Leslie KE, Miller RB (1985). *Haemophilus somnus*: investigations of its potential role in bovine mastitis. *American Journal of Veterinary Research* **46**: 2229-2234.

Henderson IR, Owen P, Nataro JP (1999). Molecular switches-the ON and OFF of bacterial phase variation. *Molecular Microbiology* **33**: 919-932.

Howard MD, Cox AD, Weiser JN, Schurig GG, Inzana TJ (2000). Antigenic diversity of *Haemophilus* somnus lipooligosaccharide: phase-variable accessibility of the phosphorylcholine epitope. *Journal of Clinical Microbiology* **38**: 4412-4419.

Howard MD, Boone JH, Buechner-Maxwell V, Schurig GG, Inzana TJ (2004). Inhibition of bovine macrophage and polymorphonuclear leukocyte superoxide anion production by *Haemophilus somnus*. *Microbial Pathogenesis* **37**: 263-271.

Howard MD (2005). Investigation of *Haemophilus somnus* virulence factors: lipooligosaccharide sialylation and inhibition of superoxide anion production. *PhD dissertation*, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Hubbard RD, Kaeberle ML, Roth JA, Chiang YW (1986). *Haemophilus somnus*-induced interference with bovine neutrophil functions. *Veterinary Microbiology* **12**: 77-85.

Humphrey JD and Stephens L (1983). *Haemophilus somnus:* a review. *Veterinary Bulletin* **53:** 987–1004.

Inzana TJ and Corbeil LB (1987). Development of a defined medium for *Haemophilus somnus* isolated from cattle. *American Journal of Veterinary Research* **48**: 366-369.

Inzana TJ and Todd J (1992). Immune response of cattle to an *Haemophilus* somnus lipid A-protein conjugate vaccine and efficacy in a mouse abortion model. *American Journal of Veterinary Research* **53**: 175–179.

Inzana TJ, Gogolewski RP, Corbeil LB (1992). Phenotypic phase variation in *Haemophilus somnus* lipooligosaccharide during bovine pneumonia and after *in vitro* passage. *Infection and Immunity* **60**: 2943-2951.

Inzana TJ, Hensley J, McQuiston J, Lesse AJ, Campagnari AA, Boyle SM, Apicella MA (1997). Phase variation and conservation of lipooligosaccharide epitopes in *Haemophilus somnus.* *Infection and Immunity* **65**: 4675-4681.

Inzana TJ (1999). The *Haemophilus somnus* complex. In: Howard JL and Smith RA (Editors). *Current Veterinary Therapy: Food Animal Practice* (volume 4). Philadelphia, PA: W. B. Saunders Company, pp. 358–361.

Inzana TJ, Glindemann G, Cox AD, Wakarchuk W, Howard MD (2002). Incorporation of N-acetylneuraminic acid into *Haemophilus somnus* lipooligosaccharide (LOS): enhancement of resistance to serum and reduction of LOS antibody binding. *Infection and Immunity* **70**: 4870-4879.

Inzana TJ and Corbeil LB (2004). *Haemophilus*. In: Gyles CL, Prescott JF, Songer JG, Thoen CO (Editors). *Pathogenesis of Bacterial Infections of Animals* (third edition). Ames, IA: Blackwell Publishing, pp. 243–257.

Ishikawa Y, Tsukuda S, Nakajima Y, Ohshima K (1984). Atypical nervous lesion in *Haemophilus somnus* infection of cattle. *Cornell Veterinarian* **74**: 349-353.

Ismail M (1991). *Haemophilus somnus* as a bacterial cause of pneumonia in buffalo calves in Egypt. *Archives of Experimental Veterinary Medicine* **45**: 161-164.

Jackson JA, Andrews JJ, Hargis JW (1987). Experimental *Haemophilus somnus* pneumonia in calves. *Veterinary Pathology* **24**: 129–134.

Kahler CM and Stephens DS (1998). Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin). *Critical Review of Microbiology* **24**: 281-334.

Kilian M and Biberstein EL (1984). Genus II *Haemophilus*. In: Krieg NR (Editor). *Bergey's Manual of Systematic Bacteriology* (volume 1)**.** Baltimore, MD: Williams & Wilkins, pp. 558-569.

Kuckleburg CJ, Sylte MJ, Inzana TJ, Corbeil LB, Darien BJ, Czuprynski CJ (2005). Bovine platelets activated by *Haemophilus somnus* and its LOS induce apoptosis in bovine endothelial cells. *Microbial Pathogenesis* **38**: 23-32.

Kuckleburg CJ, Elswaifi SF, Inzana TJ, Czuprynski CJ (2007). Expression of phosphorylcholine by *Histophilus somni* induces bovine platelet aggregation. *Infection and Immunity* **75**: 1045-1049.

Kwiecien JM and Little PB (1991). *Haemophilus somnus* and reproductive disease in the cow: a review. *Canadian Veterinary Journal* **32**: 595–601.

Last RD, Macfarlane MD, Jarvis CJ (2001). Isolation of *Haemophilus somnus* from dairy cattle in kwaZulu-Natal. An emerging cause of 'dirty cow syndrome' and infertility? *Journal of the South African Veterinary Association* **72**: 95.

Lederer JA, Brown JF, Czuprynski CJ (1997). *"Haemophilus somnus",* a facultative intracellular pathogen of bovine mononuclear phagocytes. *Infection and Immunity* **55**: 381–387.

Lees VW, Yates WD, Corbeil LB (1994). Ovine *Haemophilus somnus*: experimental intracisternal infection and antigenic comparison with bovine *Haemophilus somnus*. *Canadian Journal of Veterinary Research* **58**: 202-210

McQuiston JH, McQuiston JR, Cox AD, Wu Y, Boyle SM, Inzana TJ (2000). Characterization of a DNA region containing 5'-$(CAAT)_n$-3' DNA sequences involved in lipooligosaccharide biosynthesis in *Haemophilus somnus. Microbial Pathogenesis* **28**: 301-312.

Megyeri K, Buzas K, Miczak A, Buzas E, Kovacs L, Seprenyi G, Falus A, Mandi Y (2006). The role of histamine in the intracellular survival of *Mycobacterium bovis* BCG. *Microbes and Infection* **8**: 1035-1044.

Merino M and Biberstein EL (1982). Growth requirements of *Haemophilus somnus*. *Journal of Clinical Microbiology* **16**: 798-802.

NCE (2002/2003). Investing in Prosperity, Achieving Results. *Annual Report of the Canadian Networks of Centres of Excellence*. pp 38-39 (available at http://www.nce.gc.ca/annualreport2002_2003/Eng/NCE_AR_02-03_e-Dec15.pdf).

Nivard JL, Ward GE, Stevens JB, Maheswaran SK (1982). Model infection of the chicken embryo with *Haemophilus somnus*. *American Journal of Veterinary Research* **43**: 1790-1792.

Pennell JR and Renshaw HW (1977). *Haemophilus somnus* complex: *in vitro* interactions of *Haemophilus somnus*, leukocytes, complement, and antiserums produced from vaccination of cattle with fractions of the organism. *American Journal of Veterinary Research* **38**: 759-769.

Pfeifer CG, Campos M, Beskorwayne T, Babiuk LA, Potter AA (1992). Effect of *Haemophilus somnus* on phagocytosis and hydrogen peroxide production by bovine polymorphonuclear leukocytes. *Microbial Pathogenesis* **13**: 191–202.

Pritchard DG, Shreeve J, Bradley R (1979). The experimental infection of calves with a British strain of *Haemophilus somnus. Research in Veterinary Science* **26**: 7-11.

Radostits OM, Gay CC, Blood DC, Hinchcliff KW (2000). Veterinary Medicine (ninth edition). London: W. B. Saunders Company, pp. 895-901.

Ruby KW, Griffith RW, Kaeberle ML (2002). Histamine production by *Haemophilus somnus*. *Comparative Immunology, Microbiology, and Infectious Diseases* **25**:13-20.

Sample AK and Czuprynski CJ (1991). Elimination of hydrogen peroxide by *Haemophilus somnus,* a catalase-negative pathogen of cattle. *Infection and Immunity* **59**: 2239–2244.

Salaun L, Snyder LA, Saunders NJ (2003). Adaptation by phase variation in pathogenic bacteria. *Advances in Applied Microbiology* **52**: 263-301.

Schilling B, Goon S, Samuels NM, Gaucher SP, Leary JA, Bertozzi CR, Gibson BW (2001). Biosynthesis of sialylated lipooligosaccharides in *Haemophilus ducreyi* is dependent on exogenous sialic acid and not mannosamine. Incorporation studies using N-acylmannosamine analogues, N-glycolylneuraminic acid, and ^{13}C-labeled N-acetylneuraminic acid. *Biochemistry* **40**: 12666–12677.

Schenkein HA, Barbour SE, Berry CR, Kipps B, Tew JG (2000). Invasion of human vascular endothelial cells by *Actinobacillus actinomycetemcomitans* via the receptor for platelet-activating factor. *Infection and Immunity* **68**: 5416-5419.

Schuh J and Harland R (1991). *Haemophilus somnus* myocarditis versus thrombotic meningoencephalitis in western Canada. *Canadian Veterinary Journal* **32**: 439.

Serino L and Virji M (2000). Phosphorylcholine decoration of lipopolysaccharide differentiates commensal Neisseriae from pathogenic strains: identification of licA-type genes in commensal Neisseriae. *Molecular Microbiology* **35**: 1550-1559.

Simonson RR and Maheswaran SK (1982). Host humoral factors in natural resistance to *Haemophilus somnus. American Journal of Veterinary Research* **43**: 1160-1164.

Silva SVPS and Little PB (1990). The protective effect of vaccination against experimental pneumonia in cattle with *Haemophilus somnus* outer membrane antigens and interference by lipopolysaccharides. *Canadian Journal of Veterinary Research* **54**: 326–330.

Smith H, Parsons NJ, Cole JA (1995). Sialylation of neisserial lipopolysaccharide: a major influence on pathogenicity. *Microbial Pathogenesis* **19**: 365-377.

Stephens L, Little RPB, Wilkie BN, D. Barnum A (1981). Infectious thromboembolic meningoencephalitis in cattle: a review. *Journal of American Vetetinary Medical Association* **178**: 378-384.

Stephens LR, Humphrey JD, Little PB, Barnum DA (1983). Morphological, biochemical, antigenic, and cytochemical relationships among *Haemophilus somnus, Haemophilus agni, Haemophilus haemoglobinophilus, Histophilus ovis, and Actinobacillus seminins. Journal of Clinical Microbiology* **17**: 728-737.

Stephens LR, Slee KJ, Poulton P, Larcombe M, Kosior E (1986). Investigation of purulent vaginal discharge in cows, with particular reference to *Haemophilus somnus*. *Australian Veterinary Journal* **63**: 182-185.

Stephens LR, Aukema R, Murray LJ (1987). Antigenic heterogeneity of *Haemophilus somnus*. *Australian Veterinary Journal* **64**: 113.

Svastova A (1988). [*Haemophilus somnus* as a cause of bronchopneumonia in calves (article in Czech)]. *Veterinary Medicine (Praha)* **33**: 193-200.

Swords WE, Buscher BA, Ver Steeg Ii K, Preston A, Nichols WA, Weiser JN, Gibson BW, Apicella MA (2000). Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Molecular Microbiology* **37**: 13-27.

Sylte MJ, Corbeil LB, Inzana TJ, Czuprynski CJ (2001). *Haemophilus somnus* induces apoptosis in bovine endothelial cells *in vitro*. *Infection and Immunity* **69**: 1650-1660.

Sylte MJ, Leite FP, Kuckleburg CJ, Inzana TJ, Czuprynski CJ (2003). Caspase activation during *Haemophilus somnus* lipooligosaccharide-mediated apoptosis of bovine endothelial cells. *Microbial Pathogenesis* **35**: 285-291.

Sylte MJ, Inzana TJ, Czuprynski CJ (2004). Reactive oxygen and nitrogen intermediates contribute to *Haemophilus somnus* lipooligosaccharide-mediated apoptosis of bovine endothelial cells. *Veterinary Immunolgy and Immunopathology* **97**: 207-217.

Tegtmeier C, Angen O, Ahrens P (2000). Comparison of bacterial cultivation, PCR, in situ hybridization and immunohistochemistry as tools for diagnosis of *Haemophilus somnus* pneumonia in cattle. *Veterinary Microbiology* **76**: 385-394.

Thompson KG and Little PB (1981). Effect of *Haemophilus somnus* on bovine endothelial cells in organ culture. *American Journal of Veterinary Research* **42**: 748–754.

Thompson KG, Vickers MC, Stevenson BJ, Davidson GW (1987). Thromboembolic meningocephalitis caused by *Haemophilus somnus* infection in a bull calf - a new disease in New Zealand. *New Zealand Veterinary Journal* **35**: 5-7.

van Belkum A, Scherer S, van Alphen L, Verbrugh H (1998). Short-sequence DNA repeats in prokaryotic genomes. *Microbiology and Molecular Biology Reviews* **62**: 275-293.

Vimr E, Lichtensteiger C, Steenbergen S (2000). Sialic acid metabolism's dual function in *Haemophilus influenzae*. *Molecular Microbiology* **36**: 1113–1123.

Vimr E and Lichtensteiger C (2002). To sialylate, or not to sialylate: that is the question. *Trends in Microbiology* **10**: 254-257.

Walker RL, Biberstein EL, Pritchett RF, Kirkham C (1985). Deoxyribonucleic acid relatedness among "*Haemophilus somnus*", "*Haemophilus agni*", "*Histophilus ovis*", "*Actinobacillus semnis*" and *Haemophilus influenzae*. *International Journal of Systemic Bacteriology* **35**: 46-49.

Ward AC, Dyer NW, Corbeil LB (1999). Characterization of putative *Haemophilus somnus* isolates from tonsils of American bison *(Bison bison). Canadian Journal of Veterinary Research* **63**: 166-169.

Ward AC, Weiser GC, Anderson BC, Cummings PJ, Arnold KF, Corbeil LB (2006). *Haemophilus somnus* (*Histophilus somni*) in bighorn sheep. *Canadian Journal of Veterinary Research* **70**: 34-42.

Weiser JN, N Pan, McGowan KL, Musher D, Martin A, Richards J (1998a). Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *Journal of Experimental Medicine* **187**: 631-640.

Weiser JN, Goldberg JB, Pan N, Wilson L, Virji M (1998b). The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in *Pseudomonas aeruginosa* and on pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Infection and Immunity* **66**: 4263-4267.

Wessels J and Wessels ME (2005). *Histophilus somni* myocarditis in a beef rearing calf in the United Kingdom. *Veterinary Record* **157**: 420-421.

Widders PR, Paisley LG, Gogolewski RP, Evermann JF, Smith JW, Corbeil LB (1986). Experimental abortion and the systemic immune response to *"Haemophilus somnus"* in cattle. *Infection and Immunity* **54**: 555–560.

Widders PR, Smith JW, Yarnall M, McGuire TC, Corbeil LB (1988). Non-immune immunoglobulin binding of *Haemophilus somnus. Journal of Medical Microbiology* **26**: 307–311.

Widders PR, Dorrance LA, Yarnall M, Corbeil LB (1989a). Immunoglobulin-binding activity among pathogenic and carrier isolates of *Haemophilus somnus*. *Infection and Immunity* **57**: 639-642.

Widders PR, Dowling SC, Gogolewski RP, Smith JW, Corbeil LB (1989b). Isotypic antibody responses in cattle infected with *Haemophilus somnus*. *Research in Veterinary Science* **46**: 212-217.

Won J and Griffith RW (1993). Cloning and sequencing of the gene encoding a 31 kilodalton antigen of *Haemophilus somnus*. *Infection and Immunity* **61**: 2813-2821.

Wu Y, McQuiston JH, Cox A, Pack TD, Inzana TJ (2000). Molecular cloning and mutagenesis of a DNA locus involved in lipooligosaccharide biosynthesis in *Haemophilus somnus.* *Infection and Immunity* **68**: 310-319.

Yancey RJ Jr (1993). Recent advances in bovine vaccine technology. *Journal of Dairy Science* **76**: 2418-2436.

Yang YF, Sylte MJ, Czuprynski CJ (1998). Apoptosis: a possible tactic of *Haemophilus somnus* for evasion of killing by bovine neutrophils? *Microbial Pathogenesis* **24**: 351–359.

Yarnall M, Widders PR, Corbeil LB (1988). Isolation and characterization of Fc receptors from *Haemophilus somnus*. *Scandinavian Journal of Immunology* **28**: 129–137.