

Effect of Sialylation of *Histophilus somni* Lipooligosaccharide on
Virulence and Resistance to Host Defenses

Rajiv Balyan

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Dr. Thomas J. Inzana, Chair

Dr. Yasuhiro Suzuki

Dr. Stephen B. Melville

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by

Rajiv Balyan

Chairman: Professor Thomas J. Inzana, Ph.D.

Department of Biomedical Sciences and Pathobiology

(Abstract)

Incorporation of N-acetyl neuraminic acid (NANA), or sialic acid, onto lipooligosaccharide (LOS) enhances the virulence of several bacterial species. In the present study, we assessed the effect of sialylation of *Histophilus somni* LOS on complement-mediated killing, binding of complement factor H (which converts C₃b to inactive C₃b (iC₃b) and inhibit the alternative complement pathway) to the bacteria, complement activation by the LOS, and phagocytosis and killing of the bacteria by bovine polymorphonuclear leukocytes (PMN). Killing of *H. somni* by alternative complement pathway was measured by incubation of sialylated or non-sialylated *H. somni* with antibody-free precolostral calf serum (PCS) followed by viable plate count. A complement dose-dependent response to killing of non-sialylated *H. somni* by PCS was observed. However, sialylated *H. somni* were significantly ($P = 0.001$) more resistant to killing at any of the concentrations of PCS used.

Sialylated *H. somni* LOS activated ($P = 0.025$) and consumed ($P = 0.001$) less complement than non-sialylated LOS, as determined by reduction in hemolysis of opsonized sheep red blood cells or rabbit red blood cells, and by western blotting of C₃ activation products. Sialylated *H. somni* bound more factor H than non-sialylated

bacteria (determined by enzyme-linked immunosorbent assay) ($P = 0.004$), supporting the deficiencies observed in complement activation and consumption by sialylated LOS. Sialylation of *H. somni* inhibited both PMN phagocytosis of ^3H -thymidine-labelled bacteria ($P = 0.004$) and intracellular killing of the bacteria ($P = 0.0001$), compared to non-sialylated bacteria. Therefore, sialylation of the LOS results in enhanced binding of complement factor H to the bacteria, resulting in diminished complement activation, resistance to complement-mediated lysis, and PMN phagocytosis and killing.

Dedication

For my parents, Vimla and Mahipal Singh, you are my inspiration.

For my wife, Ruchi, this would not have been possible with out your love and support!

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List of Abbreviations

Abs	absorbance
BHI	brain heart infusion broth
CBA	Columbia Blood Agar
CFU	Colony Forming Unit
ChoP	Phosphorylcholine
CM	Carboxy-methyl
CMP-NANA	Cytidine monophosphate-N-acetyl neuraminic acid
CNS	central nervous system
CTT	Columbia broth with 0.1% Trizma base and 0.01% TMP
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
fH	Complement factor H
GPS	Guinea pig serum
HBSS	Hank's Balanced Salt Solution
HBSS-G	HBSS containing 0.1% gelatin
IgBP	Immunoglobulin Binding Protein
kDa	Kilodalton
KDO	3-deoxy-D-manno-2-octulosonic acid
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAb	Monoclonal Antibody
MASP	MBL-associated serine proteases
MBL	Mannan-binding lectin
MW	molecular weight
N	number of samples
NANA	N-acetylneuraminic acid
NHS	Normal human serum
OMP	Outer Membrane Antibody
ORFs	open reading frames

PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PCM	PBS supplemented with CaCl ₂ and MgCl ₂
PCS	Pre-colostral calf serum
PEG	Poly-ethylene glycol
PMN	Polymorphonuclear leucocytes
RRBC	Rabbit red blood cells
SD	standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRBC	Sheep red blood cells
TBS	Tris-buffered saline
TLR	Toll-like receptor
TME	Thrombotic Meningoencephalomyelitis

Chapter 1

Introduction

Histophilus somni (formerly *Haemophilus somnus*) is a non-spore forming, non-capsulated, non-motile, capnophilic, facultatively anaerobic gram-negative coccobacillus in the family Pasteurellaceae. *H. somni* is a host-specific commensal and an opportunistic pathogen of cattle (Harris and Janzen, 1989; Humphrey and Stephens, 1983) and sheep (Lees et al., 1994; Ward et al., 1995). Commensal isolates are relatively avirulent and found predominantly in the urogenital system (Corstvet et al., 1973). Pathogenic isolates cause multi-systemic infections in bovines.

In 1956 infectious embolic meningoencephalitis (IEM) was observed for the first time in cattle in Colorado (Brown et al., 1956). The symptoms were vasculitis, encephalitis, meningitis and production of infarcts in the parenchyma of the brain. At that time the etiology of the infectious agent could not be determined. In feedlot cattle in California a similar disease was reported in 1960, and a bacterial agent was isolated. Based on biochemical, serological, morphological, and cultural characteristics a ‘*Haemophilus*-like organism’ was described as the etiologic agent by Kennedy *et al* (Kennedy et al., 1960). This disease of central nervous system (CNS) of cattle, which is now recognized as being caused by *H. somni* was later termed thrombotic meningoencephalitis (TME) as the lesions were not the result of emboli to the affected tissues (Stephens et al., 1981). Thrombosis and severe vasculitis are prominent features of this disease. By 1965 TME had become a major neurological disease of cattle (Little and Sorensen, 1969), but the number of incidences of TME

later declined. However, other *H. somni* infections have increased, such as abortion, myocarditis, septicemia, polyarthritis, bovine respiratory disease complex (shipping fever), arthritis, orchitis, endometritis, mastitis, vaginitis, myelitis, otitis, conjunctivitis, laryngitis, and retinal hemorrhage (Corbeil et al., 1986; Harris and Janzen, 1989; Humphrey and Stephens, 1983; Kitching and Bishop, 1994; Stephens et al., 1981).

Microbiology and epidemiology

Histophilus somni was initially placed under the genus *Haemophilus* and was known as *Haemophilus somnus*. Unlike other members of the genus *Haemophilus*, *H. somni* does not require nicotinamide adenine dinucleotide (V factor) or hemin (X factor). For growth of *H. somni*, enriched media such as Columbia blood agar supplemented with 5-10% sheep or bovine blood and incubated under 5-20% CO₂ at 37°C is required (Humphrey and Stephens, 1983; Kitching and Bishop, 1994; Merino and Biberstein, 1982). For growth in broth, CO₂ incubation is not required, but the addition of thiamine monophosphate or thiamine pyrophosphate is required in the absence of fresh serum. *H. somni* forms round, convex, gray-yellow colored colonies on blood agar after 48 h of growth. There is weak or no β-haemolysis on blood agar. More hemolysis is observed after longer incubation, particularly at 4°C. *H. somni* is oxidase and indole-positive, catalase and urease-negative, and is capable of fermenting D-glucose to produce acid without gas (Kilian and Biberstein, 1984).

Outbreaks of *H. somni* infection have been reported from Canada, Mid and western parts of the USA, Argentina, Australia, New Zealand, Europe, Egypt, Russia,

and South Africa (Kitching and Bishop, 1994). *H. somni* is widely prevalent, affects a variety of organ systems, and is thus considered an important pathogen of economic interest to the cattle industry.

Virulence Factors

Virulence factors of *H. somni* include phase variation of lipooligosaccharide,(LOS) incorporation of sialic acid and phosphorylcholine onto the LOS, resistance to complement-mediated killing (Corbeil et al., 1995), apoptosis of bovine endothelial cells (Sylte et al., 2001), inhibition of the oxidative burst by bovine macrophages, binding of the Fc receptors of host immunoglobulins by outer membrane proteins (OMP) (Widders et al., 1988; Yarnall et al., 1988a; Yarnall et al., 1988b), activation of Toll-like receptors (TLRs) by LOS (Howard, 2005), biofilm formation et al. 2007) and exopolysaccharide production (Inzana and Corbeil, 2004). A thorough understanding of the virulence factors of a pathogen aid in designing better approaches to diagnosis and treatment.

Lipooligosaccharide

Lipooligosaccharide (LOS) is a component of the outer membrane of gram-negative bacteria that consists of lipid and carbohydrate components and has endotoxic activity. The LOS is analogous with the lipopolysaccharide (LPS) present in enteric gram-negative bacteria, but differs from LPS by a lack of O-side chains. The presence of O antigen on LPS makes it more hydrophilic and suitable to thrive in the gastrointestinal tract. Similarly, the absence of O antigen in LOS makes the

component less hydrophilic and more suitable to thrive in respiratory and reproductive tracts (Preston et al., 1996). The structure of LOS can be divided into three components: lipid A, outer core, and inner core. The lipid A mediates endotoxic shock and it is conserved in many bacterial genera. Hexose is the most prominent component of LOS, exhibiting maximum variation across strains and is responsible for the heterogeneity of LOS. The LOS has been shown to be important in binding of the bacteria to host cells (Vaara and Nikaido, 1984). The LOS is recognized by cells of the immune system and stimulates the immune response against the bacteria (Erridge et al., 2002). The LOS of *H. somni* is similar to the LOS of *H. influenzae* and *Neisseria* species in size and level of heterogeneity. The lipid moiety of endotoxins is responsible for inducing mediators of inflammation and causing tissue damage (Morrison and Ulevitch, 1978). *H. somni* LOS is suspected to contribute to the inflammatory lesions produced in diseases such as pneumonia, necrotizing placentitis, suppurative vasculitis, thrombosis, and broncholithiosis (Inzana et al., 1988). *H. somni* and culture supernatant LOS has been found to cause apoptosis of endothelial cells (Sylte et al., 2001; Sylte et al., 2003).

Complement System

The complement system is an important part of the innate and humoral immune system in mammals. The complement plays a crucial role in resistance to microbial infection by enhancing phagocytosis, inflammation, vascular permeability, and lysis of the invading microbial cells. The bactericidal activity of serum is due to the heat-stable component and the heat labile component. Ehrlich used the term

complement for the heat-labile component in 1899 (Coligan et al., 1994). The complement system consists of about 30 glycoproteins found in the serum of mammals as precursor enzymes, regulatory proteins, enzyme cofactors, and effector molecules (Mayer, 1984). Twelve of these proteins are directly involved in complement pathways. Complement proteins are also known as complement factors and make up ~5% of the serum globulin fraction. The biosynthesis of complement factors primarily takes place in hepatocytes of the liver. Other cells like monocytes, macrophages, and epithelial cells of the gastrointestinal tract also produce small amounts of complement proteins. There are three pathways of complement activation; the classical pathway, the alternative pathway, and the recently discovered lectin pathway (Sell, 2001b; Verschoor and Carroll, 2004).

The complement pathways differ in regard to the types of molecules they recognize, the substance of non-self or foreign origin, initiation of complement activation and formation of the C₃ convertase enzyme. Complement factor C₃ is the critical component of both classical and alternative pathways, and deposition of C₃b on the microbial membrane initiates formation of the membrane attack complex C5b-9, which is the end-result of all 3 pathways. After complement activation, the biologically active peptides C₅a and C₃a produce a number of proinflammatory effects, such as chemotaxis of leukocytes, degranulation of phagocytic cells, mast cells, and basophils, smooth muscle contraction, and increase of vascular permeability (Kohl, 2001; Sell, 2001b; Verschoor and Carroll, 2004).

Classical Complement Pathway

The classical pathway is initiated by the binding of antibody, whose Fc region has been altered due to the binding of antigen. The IgG subclasses IgG1, IgG3 and particularly IgM are the predominant antibodies that activate complement. The binding of the antigen-antibody complex with the C1-complex (which consists of one molecule C1q and two molecules C1r and C1s) produce conformational changes in the C1q molecule, which then activates the C1q and C1r molecules. The C1r molecules cleave C₄ and C₂, resulting in the formation of C₃ convertase (C4b2a). C₃ convertase cleaves C₃, resulting in deposition of C₃b on the microbial surface. C₃b binds with C4b2a and forms C₅ convertase (C4b2a3b). The C₅ convertase cleaves C₅ into C₅a and C₅b. C₅b binds to the cell surface and binds to the complement components C₆, C₇, C₈ and C₉ to form the membrane attack complex (MAC), which forms a pore in the cell membrane and causes cell lysis. The complement fragments C₃a, C₄a and C₅a have anaphylotoxin and chemotactic activity. These fragments increase inflammation, attract neutrophils, release histamine from mast cells, and increase vascular permeability (Gadjeva et al., 2001; Goldman and Prabhakar, 1996; Sell, 2001b; Verschoor and Carroll, 2004).

Alternative Complement Pathway

The alternative complement pathway is activated by microbial antigens such as yeasts, bacterial endotoxin, plant polysaccharides (Inulin), fungi, viruses, certain mammalian cells or IgA antibody, in the absence of specific antibody. This pathway recognizes foreign or non-self antigens and responds to them. The terminal

components C₃, and C₅ to C₉ are common to both the alternative and classical pathways. In addition, complement factors D, B, P (properdin) are unique to the alternative pathway. The complement cascade is initiated by the presence of antigen or by spontaneous activation of complement factor C₃. The coupling of factor B to activated C₃ leads to formation of C₃ convertase, which in turn cleaves C₃ into C_{3a} and C_{3b}. C_{3a} is released as an anaphylotoxin, while C_{3b} is deposited on an antigen to opsonize the antigen or initiate the formation of MAC. The alternative pathway is important in the early stages of infection, when antigen-antibody-mediated complement activation is not functional. The alternative complement pathway enhances complement action by amplifying the activity of classical and MBL pathways. The addition of EGTA to guinea pig serum binds calcium and inhibits the classical complement pathway. Surface determinants of rabbit erythrocytes (RRBC) activate the alternative complement pathway due to the comparative deficiency of the sialic acid on the surface of rabbit erythrocytes compared with erythrocytes of other species such as sheep and guinea pig, which do not activate the alternative complement pathway (Czop et al., 1978; Kazatchkine et al., 1979; Platts-Mills and Ishizaka, 1974). These properties of the alternative complement pathway have helped in designing protocols for studies involving only the alternative pathway such as activation of the alternative complement pathway by RRBC in absence of antibody against RRBC (Sell, 2001b; Verschoor and Carroll, 2004).

Mannan-binding lectin (MBL) pathway

This pathway is also known as ‘the lectin pathway’ and is mediated by the binding of MBL to carbohydrates. MBL is a protein of the family ‘collectin’. This protein binds to carbohydrate structures on microbial surfaces (bacteria, yeast, viruses, and parasitic protozoa) and activates the MBL-associated serine proteases, MASP-1, MASP-2, and MASP-3. Activation of MASPs proteins leads to conversion of C₄ and C₂ into C_{4b} and C_{2a}. The C_{4b} and C_{2a} complement components bind together to form C₃-convertase, which is also a component of the classical pathway. The MBL pathway is also activated by Ficolins, which are homologous to MBL and function via MASP in a similar way. Ficolins play a significant role in immunity of non-vertebrates, who do not have an adaptive immune system (Goldman and Prabhakar, 1996). The MBL-like and MASP-like proteins are found in protochordates, indicating that the MBL pathway is an older mechanism of host defense in terms of evolution and originated before the classical and alternative pathways (Gadjeva et al., 2001; Sell, 2001b; Verschoor and Carroll, 2004).

Complement Factor H

Factor H (fH) is a negative regulator of the alternative pathway of complement activation. It is a soluble glycoprotein of 158 kDa present in plasma (Mhatre and Aston, 1987). Uncontrolled activation and proliferation of the complement cascade may cause detrimental effects on host tissues. Regulatory molecules are required to check the complement activity. Factor H regulates the complement pathway by acting as a cofactor in factor I-mediated cleavage of C_{3b} into

its inactive form iC_3b (Pangburn et al., 1977), and inactivating the C_3 convertase by dissociating Bb from the C_3Bb complex (Pangburn and Muller-Eberhard, 1978; Pangburn et al., 1977; Weiler et al., 1976). By inactivation of C_3 convertase, factor H controls the deposition of C_3b onto the bacterial surface. The binding of fH onto the bacterial surface also results in decreased deposition of C_4 , a key factor in the activation of the classical complement pathway (Gill et al., 1996; McQuillen et al., 1999), and mannan-binding lectin, a component of lectin complement pathway (Gulati et al., 2002). Factor H has affinity for polyanions like chondroitin sulphate A, heparin, carrageenan (types III and IV), and sialic acid (Meri and Pangburn, 1994). Binding of factor H to sialylated LOS is highly specific. Factor H has been demonstrated to bind only with sialylated LOS containing lacto-*N*-neotetraose structures (Gulati et al., 2005). Other proteins like glycoproteins 120 and 41 of the AIDS virus HIV-1 (Stoiber et al., 1995) and Yad A from *Yersinia enterocolitica* (China et al., 1993) enhance the binding of factor H and increases the virulence of the pathogen in a similar fashion as sialic acid. Factor H-mediated inhibition of complement activation has also been demonstrated to be responsible for the anti-phagocytic ability of M protein of streptococci (Fischetti et al., 1995; Horstmann et al., 1988).

Polymorphonuclear Leucocytes

Polymorphonuclear leucocytes (PMNs) constitute the predominant leucocyte population in blood. These cells are also known as ‘granulocytes’ and include neutrophils (contain neutrophilic granules), eosinophils (contain eosinophilic

granules), and basophils (contain basophilic granules). PMNs are the first cells of the host defense system to reach the site of infection and have the ability to protect the host from bacterial and fungal infections (Malech and Nauseef, 1997). Complement factor C₅a, chemotactic factors released by bacteria, and host molecules released from the site of infection attract PMNs to the pathogen (Sell, 2001a). PMNs release cytokines, interleukins, and TNF- α , all of which produce inflammation (Kuijpers and Roos, 2004). For successful phagocytosis, opsonization of bacteria by antibody and complement is required. However, it has been shown that for the phagocytosis of *H. somni* by PMNs, complement opsonization is not important (Czuprynski and Hamilton, 1985; Lederer et al., 1987). PMNs bind the Fc portion of antibodies that opsonize the bacteria, engulf the bacteria in an intracellular phagosome, release their granular contents into the phagosome, and kill the bacteria by toxic radicals and hydrolytic enzymes. PMNs may also get ‘congested’ due to ‘overeating’ of the bacteria during a severe infection, resulting in death of the PMNs and pus formation (Kuijpers and Roos, 2004; Sell, 2001a). Phagocytosis and elimination of bacteria by PMNs is a major function of the cellular arm of the innate immune system (Kuijpers and Roos, 2004; Sell, 2001a)

Sialylation

Sialic acid [also known as N-acetylneuraminic acid (NANA)], is a nine carbon keto sugar with the structure 2-keto-3-deoxy-5-acetamido-*glycero-D-galactonanulosonic acid*, and is found both in eukaryotic and prokaryotic systems (Kenne and Lindberg, 1983; Vimr, 1994). Sialic acid is found both in free form and

complexed to other molecules. Sialic acid has many functions, including mediating cell-cell and cell-molecule interaction (Vimr et al., 2004). Sialic acid is very important during early developmental stages of complex metazoans. Homozygous mice with mutations in sialic acid synthesis have been observed to die during early embryonic stages (Schwarzkopf et al., 2002). Binding of sialic acid to the terminal glucose on a cell surface masks that antigen against the action of the immune system. The presence of sialic acid on trophoblasts protects embryos from maternal antibodies. However, it becomes harmful to the host when sialic acid is on cancer cells or on a pathogen (Schauer, 1985).

It has been observed that gonococci (*Neisseria gonorrhoeae*) immediately after isolation are resistant to killing by normal human serum and rabbit hyperimmune serum. However, this resistance is lost upon subculture (Ward et al., 1970). A low-molecular weight dialyzable substance was later discovered that makes the *N. gonorrhoeae* resistant to killing by normal human serum (NHS). This substance was also found in human serum (Martin et al., 1981; Veale et al., 1981), guinea pig serum (Nairn et al., 1988; Patel et al., 1984b; Tan et al., 1986), human erythrocytes (Nairn et al., 1988; Parsons et al., 1988; Patel et al., 1984a; Patel et al., 1984b), and human leukocytes (Patel et al., 1988). This substance was later determined to be 5'-cytidinemonophospho-*N*-acetylneuraminic acid (CMP-NANA) (Nairn et al., 1988; Parsons et al., 1989; Parsons et al., 1988).

Growth of gonococci in media containing CMP-NANA results in the incorporation of sialic acid onto the terminal galactose at the Gal β 1 \rightarrow 4GlcNac residue of the LOS (Griffiss et al., 1988; Mandrell et al., 1990; Parsons et al., 1988).

The presence of CMP-NANA increased the molecular weight of the LOS of gonococci without affecting the structure of the outer membrane proteins of the bacteria (Patel et al., 1988).

Apicella et al. observed that the LOS of gonococci grown on media containing fresh blood is sialylated in a similar fashion to that of bacteria grown in media containing CMP-NANA. Under both types of growth conditions, antibody binding to the sialylated epitope is blocked, and inhibition of antibody binding is reversed after removal of sialic acid (Apicella et al., 1990).

Sialylation of bacteria occurs by incorporation of sialic acid into the LOS, cell membrane, fimbriae (van Ham et al., 1995) and/or the capsule (Kenne and Lindberg, 1983). The LOS is sialylated in *H. influenzae*, and *N. gonorrhoeae*, while capsular polysaccharide containing NANA is produced in *Escherichia coli* and *N. meningitides* (Kenne, 1983). The LOS of *Neisseria* spp. is sialylated exogenously by the transfer of NANA onto the terminal galactose of lacto-*N*-neo-tetraose of *N. gonorrhoeae* LOS by α -2,3-sialyltransferase (Gao et al., 2000).

H. influenzae, *H. ducreyi* and *H. somni* have a sialylsynthetase enzyme, which can endogenously synthesize CMP-NANA using sialic acid present in blood or body secretions of the host (Howard, 2005; Inzana et al., 2002; Vimr and Lichtensteiger, 2002). Sialic acid on pathogenic bacteria has been shown to mask specific antigens and protect the pathogen from the innate immune system of the host (Vimr et al., 2004).

Antibody-mediated opsonophagocytosis and killing of bacteria is influenced by the quantity of complement deposited on the bacterial surface during the initiation

of the complement cascade. The alternative complement pathway plays a predominant role in the generation and deposition of activated complement on bacteria prior to the presence of specific antibody, but also when complement is activated via the classical pathway (Wetzler et al., 1992).

Sialylation of the lacto-neo-tetraose moiety of *N. gonorrhoeae* LOS also results in increased binding of factor H, a regulator of the alternative complement pathway (Ram et al., 1998). The phenomenon of binding of higher amounts of factor H by sialic acid on LOS explains the increased resistance of sialylated *N. gonorrhoeae* to complement-mediated killing and opsonophagocytosis (Wetzler et al., 1992). In *N. meningitidis* available carbon source affects the biosynthesis of sialic acid and which in turn have been demonstrated to affect complement mediated killing and virulence (Exley et al., 2005).

The LOS of *H. somni* is sialylated by a mechanism similar to that of other *Haemophilus* species (Howard, 2005). However, *H. somni* abortion isolate 649, as well as preputial isolates 129Pt and 1P, cannot sialylate their LOS (Inzana et al., 2002). Three genes that may be involved in LOS sialylation have been found in *H. somni* pathogenic strain 2336 and two of the three genes are in strain 738 (Howard, 2005). The corresponding genes have been reported to be absent or interrupted by an insertion sequence in *H. somni* preputial isolate 129Pt, based on analysis of the complete genome sequence (Howard, 2005).

H. somni can synthesize CMP-NANA from free sialic acid in growth media by a sialyl synthetase and presumably transfer it to LOS following catabolism, as for other *Haemophilus* species. *H. somni* strain 738 has a sialyltransferase with a high

affinity for the terminal Gal β in the Gal β -(1-3)-GlcNac structure of lacto-*N*-neo-tetraose moiety of LOS. The affinity of the sialyltransferase for strain 738 is greater than the affinity of same enzyme for the terminal Gal β in the Gal β -(1-4)-GlcNac epitope in strain 2336 (Inzana et al., 2002). The sialylation of *N. meningitidis* LOS in vivo is affected by the conformation of the acceptor molecule and other structures on the LOS, such as phosphorylcholine (choP) or phosphoethanolamine (Wakarchuk et al., 2001). Sialylation of the bacterial cell surface has been reported to inhibit the binding of antibody and enhance bacterial resistance to complement-mediated killing (Estabrook et al., 1997; Moran, 1996; Moran et al., 1996; Vimr and Lichtensteiger, 2002; Vimr et al., 2004)

H. somni strain 2336 can incorporate NANA into its LOS when grown on CBA containing sheep blood (Howard, 2005; Inzana et al., 2002). Sialic acid on *H. somni* LOS can be determined by reactivity with the lectin *Limax flavus* (garden slug), which specifically binds to sialic acid, or by the Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of sialylated compared to non-sialylated LOS (Howard, 2005; Inzana et al., 2002). Sialylation of LOS has been proposed to be a virulence mechanism in many bacterial species by increasing bacterial resistance to host immunity (Nairn et al., 1988; Parsons et al., 1988). Sialylation can also reduce the binding of complement, enhance the binding of factor H, and make bacteria less susceptible to neutrophil-mediated killing (Estabrook et al., 1992; Rest and Frangipane, 1992). Sialylation also prevents the binding of the mannan-binding lectin, activator of lectin complement pathway (Gulati et al., 2002).

Sialylation has been demonstrated to decrease signaling through Toll-like receptor 4 (TLR4) by *H. somni* (Howard, 2005). Sialylation of *H. influenzae* LOS has been shown to influence biofilm formation in vitro, and enhance bacterial survival in the middle ear of male Mongolian gerbils and the lungs of male Sprague-Dawley rats (Swords et al., 2004). LOS sialylation can also inhibit the binding of antibody to nearby epitopes [e.g., steric interference with binding of monoclonal antibody MAb-5F5.9 to the ChoP epitope on LOS (Howard et al., 2000)].

A better understanding of the role of sialic acid on *H. somni* LOS in virulence may facilitate devising improved vaccines and diagnostic tests. In view of this, the current study examines the effect of sialylation of *H. somni* LOS on:

- (a). binding of complement factor H and C₃ onto bacteria
- (b). complement-mediated killing of bacteria
- (c). Phagocytosis and killing of bacteria by PMNs
- (d). Complement consumption and activation

Chapter 2

Materials and Methods

Bacterial strains and growth conditions

Four strains of *H. somni* (kindly provided by Dr. Lynette Corbeil, University of California, San Diego) were used in this study (Table 2.1). *Haemophilus influenzae* type b strain Eagon (kindly provided by Dr. Porter Anderson, University of Rochester Medical Center) is a capsulated strain. *Actinobacillus pleuropneumoniae* strain J45-100 is a nonencapsulated and nontypeable mutant of J45 (Ward et al., 1998). *Escherichia coli* strain DH5 α (Invitrogen Life Technologies, Carlsbed, CA) is an attenuated strain.

Table 2.1: Bacterial strains used in this study:

Bacterial strain	Source or characterization	Reference
<i>H. somni</i> strain 2336	Pneumonic lung isolate	(Corbeil et al., 1985)
<i>H. somni</i> strain 738	Clonal isolate of 2336	(Gogolewski et al., 1987)
<i>H. somni</i> strain 129Pt	Preputial isolate	(Corbeil et al., 1985)
<i>A. pleuropneumoniae</i> strain J45-100	Nonencapsulated mutant of strain J45	(Ward and Inzana, 1997)
<i>H. influenzae</i> type b strain Eag	Capsulated strain from meningitis	(Anderson et al., 1972)
<i>E. coli</i> strain DH5 α	Common laboratory strain	(Brest et al., 2004)

Strains of *H. somni* from frozen stocks were streaked onto Columbia blood

agar (CBA) plates containing 5% sheep blood and incubated overnight at 37°C in 5% CO₂. For growth in broth, *H. somni* colonies were inoculated into brain heart infusion (BHI) broth containing 0.1% Trizma base and 0.01% thiamine monophosphate (TMP) (BHI-TT) and incubated at 37°C with shaking (200 RPM). Optical density was measured using a Klett-Summerson meter. A Klett absorbance of 150 units was found to correspond to 10⁹ colony forming units (CFU)/ml as determined by serial dilution and viable plate count. *E. coli* was cultured on LB plates and incubated overnight at 37°C. *A. pleuropneumoniae* was streaked onto BHI agar plates containing 5 µg/ml nicotinamide adenine dinucleotide (NAD; Sigma-Aldrich Corp., St Louis, MO) and cultured in BHI broth containing 5 µg/ml NAD at 37°C with shaking (200 RPM).

***In vitro* sialylation of *H. somni* LOS**

To sialylate *H. somni* LOS in vitro, strain 738 was grown in BHI-TT broth containing 1.5 mg/ml N-acetylneuraminic acid (NANA; Toronto Research Chemicals, Toronto, Canada). For larger volumes, a 25 ml starter culture was inoculated to 55 klett units, cultured to 10⁹ CFU/ml, and added to 225 ml of fresh BHI-TT medium and growth was continued to mid-log phase. Desialylation of *H. somni* LOS was performed by incubating bacteria grown to mid-log phase with neuraminidase (Type II from *Vibrio cholerae*; 0.0145 Units/ml; Sigma-Aldrich Corp., St Louis, MO) for one h at 37°C with shaking.

LOS preparation

H. somni LOS was prepared as reported previously (Inzana et al., 1988). Briefly, pelleted bacterial cells were digested with egg white lysozyme, incubated with DNase and RNase for 10 minutes at 37°C, followed by 60°C for ten minutes, and continued at 60°C incubation with proteinase K for one h. The bacterial suspension was further extracted with 45% phenol at 68°C and the aqueous phase and the phenol were separated by centrifugation at 10,000 rpm for 15 minutes. The top aqueous phase was dialyzed extensively against distilled water. The insoluble material was removed by centrifugation at 5,000 x g for 15 minutes. The supernatant was centrifuged at 105,000 x g for 16 h to sediment the LOS. The LOS pellet was suspended in high performance liquid chromatography (HPLC) grade water and lyophilized. LPS from *H. influenzae* type b was extracted by Dr. Michael D. Howard.

Bactericidal assay

Sialylated or non-sialylated *H. somni* cultures were grown to 10^9 CFU/ml with shaking (200 RPM) at 37°C, and diluted to 10^5 CFU/ml in PCM (phosphate buffered saline, pH 7.4 containing 0.5 mM MgCl₂ and 0.15 mM CaCl₂). A 20 µl bacterial suspension was added to serial dilutions of antibody-free pre-colostral calf serum (PCS), and 10 µl was plated immediately (T₀) onto CBA plates and after 60 minutes of incubation (T₆₀) at 37°C (Inzana et al., 1988). *A. pleuropneumoniae* strain J45-100, which lacks capsule and is serum sensitive (Ward and Inzana, 1997), was used as a positive control for killing by PCS, heat-inactivated PCS was used in negative control assays. The percent survival of bacteria was expressed as $T_{60}/T_0 \times 100$. For desialylation, sialylated bacteria were treated with neuraminidase as described for

LOS.

Purification of bovine complement factor H

Complement factor H was semi-purified from bovine serum as described by Mhatre and Auston (Mhatre and Aston, 1987). The bovine serum was prepared by clotting of blood at room temperature for 30 minutes and centrifugation at 1500 x g for 30 minutes at 4°C. Selective precipitation of serum components was accomplished by addition of 500 ml of 15% polyethylene glycol 4000 (PEG; Sigma Chemical Co., St. Louis, MO) in 0.1M phosphate buffer, pH 7.4 containing 0.015M EDTA, 0.15M NaCl and 0.03 M epsilon amino-caproic acid (Sigma Chemical Co., St. Louis, MO). The reaction mixture was centrifuged at 1900 x g for 10 minutes at 4°C to remove precipitate. Thirty three percent PEG in phosphate buffer was gradually added to the supernatant to increase the PEG level from 5% to 12%. The centrifugation was repeated and the precipitate was washed with 12% PEG. The precipitate was dissolved in 0.005M phosphate buffer, pH 7.4, containing 0.005 M EDTA, 0.01 M EACA and 0.2 M NaCl and loaded onto a DEAE-sepharose (Sigma-Aldrich, Inc., St. Louis, MO) column (2.5 x 30 cm; Bio-Rad Laboratories, Hercules, CA) equilibrated with 0.005 M phosphate buffer. After extensive washing with three volumes of equilibrating buffer, 500 ml of a 0 to 0.3 M NaCl gradient was passed through the column. Fractions were pooled and protein content was measured at 280 nm. The biological activity of factor H was determined by performing a hemolytic assay with rabbit RBC (RRBC) and fresh bovine serum as described below. Fractions containing factor H were pooled and concentrated using a amicon ultrafiltration cell

(Amicon Corp., Danvers, MA) with a Diaflo YM-30 membrane (Amicon Inc., Beverly, MA). Pooled fractions were dialyzed against 0.02 M Tris-acetate buffer, pH 6.8 containing 0.005 M EDTA and 0.02 M NaCl. The dialyzed product was loaded onto a 2.5 x 30-cm CM-sepharose (Sigma-Aldrich, Inc., St. Louis, MO) column equilibrated with 0.02 M Tris-acetate buffer. The column was washed with three volumes of equilibrating buffer, followed by a 500 ml salt gradient from 0 to 0.3 M NaCl. The fractions containing factor H activity were pooled and concentrated as described above. The concentrated product was loaded onto a 2.5 x 100-cm sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column for gel filtration. Factor H activity was determined by hemolytic assay and the sample was subjected to electrophoresis on a SDS-PAGE gel to confirm the presence of factor H. Fractions exhibiting factor H activity by inhibiting the lysis of RRBC by bovine serum were stored at 4°C.

Hemolytic assay to assess the activity of bovine factor H

Bovine factor H inhibits the lysis of rabbit RBC (RRBC) by bovine serum. To monitor factor H activity during purification, procedure by Mhatre and Aston was used (Mhatre and Aston, 1987), with modification as below. Bovine serum and RRBC were diluted in barbital buffered saline (pH 7.2) containing 4 mM MgCl₂, 0.1% gelatin, and 15 mM ethylene glycol bis-amino tetraacetate (EGTA; Sigma Chemical Co., St. Louis, MO). Twenty µl of 4% RRBC (v:v) and 20 µl of semi-purified bovine factor H were added to 160 µl of bovine serum (1:36 dilution) and incubated at 37°C for 30 minutes. The reaction was stopped by addition of 3.5 ml ice-

cold PBS-EDTA. The samples were harvested by centrifugation at $800 \times g$ for 5 minutes to pellet unlysed RRBC, and the absorbance of the supernatant was measured at 412 nm. The activity of bovine factor H was expressed as the inhibition of lysis compared with the sample without semi-purified bovine factor H.

Dot blot assay

Bovine serum (5 μ l) was deposited on a nitrocellulose membrane and allowed to dry for 30 minutes. Unbound sites were blocked by incubating with 5% skimmed milk in TBS-Tween (100 mM Tris-HCl, 0.85% NaCl, Ph 7.3 with 0.05% Tween-20) at room temperature with shaking. Primary antibody (sheep anti-human factor H; The Binding Sites, UK) at a dilution of 1: 1000 was added and the membrane was incubated at room temperature for one h. The membrane was washed three times with TBS-Tween, incubated with a 1:4000 dilution of peroxidase conjugated rabbit anti-sheep IgG (Jackson Immunoresearch, West Grove, PA), and incubated at room temperature for one h. The membrane was washed again three times with TBS-Tween and the band was visualized by addition of the TMB substrate; the reaction was stopped by addition of 50 ml of distilled water.

ELISA for assessing binding of factor H, C₃ and iC₃b

Binding of complement factor H and inactive C3b (iC₃b) to sialylated and non-sialylated *H. somni* strain 738 was assessed by ELISA. Sialylated or non-sialylated *H. somni* 738 was grown to mid log phase and 100 μ l of culture was suspended in HBSS (Hanks' balanced salt solution; Sigma Chemical Co., St. Louis, MO) and incubated with 100 μ l of bovine serum for one h at 37 °C to activate and

initiate the complement cascade. Bacterial cells were centrifuged at 3200 x g for 5 minutes, resuspended in 600 μ l of carbonate buffer (pH 9.5), and 100 μ l of this mixture was added to wells of a 96-well Immulon 4 HBX micro-titer plate (Thermo Fisher Scientific, Waltham, MA) and incubated for one h at 37 °C. Purified human factor H (Calbiochem, San Diego, CA) was used as a positive control. The plate was washed three times with PBS-Tween. To block nonspecific binding of other proteins, 100 μ l of 2% dry skim milk in PBS (blocking buffer) was added per well and the plates incubated for one h at 37 °C. The plates were washed 3 times with PBS-Tween, primary antibody (100 μ l of sheep anti-human factor H; The Binding Site, UK) diluted 1: 1000 in blocking buffer was added at 100 μ l/well, and the plate incubated overnight at 4°C. The plate was washed as above, secondary antibody (peroxidase conjugated rabbit anti-sheep IgG (H+L); Jackson ImmunoResearch, West Grove, PA), diluted 1:5000 in blocking buffer was added at 100 μ l/well and incubated at 37°C for one h. The plate was washed again and 100 μ l/well of peroxide solution (TMB Substrate Kit; Pierce Biotechnology, Inc., Rockford, IL) was added. The reaction was stopped by addition of 1 M sulphuric acid (100 μ l/well). Binding of factor H and iC₃b was measured at 450 nm using a ELISA plate reader (Molecular Devices, Sunnyvale, CA).

To quantitate the binding of iC₃b with sialylated or non-sialylated *H. somni* strain 738, the same procedure was used except murine monoclonal anti-human antibody to iC₃b (Quidel Corporation, San Diego, CA; 1:2000 dilution) and peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA; 1:4000 dilution) were used. To quantitate binding of C₃ to sialylated or non-

sialylated *H. somni* strain 738 and sialylated or non-sialylated *H. somni* strain 738 LOS, goat anti-C₃ (Fitzgerald Industries International Inc., Concord, MA; diluted at 1:2000) and peroxidase-labeled rabbit anti-goat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD; diluted at 1:4000) were used. To quantitate the amount of anti-bacterial antibody present in bovine serum for opsonization by PMNs, bovine serum diluted 1:10 in blocking buffer and peroxidase conjugated rabbit anti-bovine IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:4000 in blocking buffer were used.

Complement Consumption Assay

The LOS from sialylated or non-sialylated *H. somni* strain 738 and *H. influenzae* type b were suspended in distilled water at 2.5 mg/ml. From this stock solution between 10 and 70 µg of LOS were added to 100 µl of 15% guinea pig serum (GPS) (Cedarlane labs, Burlington, NC) in veronal buffered saline (VBS; Cambrex Bio Science, Walkersville, MD) and incubated at 37°C for 30 minutes. The sheep RBC (SRBC) were opsonized by incubation of SRBC with rabbit anti-sheep RBC antibody (Rockland Immunochemicals Inc., Gilbertsville, PA; 1:200 titer) for 60 minutes at 37°C. The LOS-GPS suspension was diluted to 325 µl using VBS and one hundred µl of the mixture was added to 1.3 ml of opsonized SRBC (10⁹ SRBC/ml) for 30 minutes at 37°C. The number of SRBC was optimized using a previously described protocol (Coligan et al., 1994). The cells were sedimented by centrifugation at 800 × g for 5 minutes, and the OD of the supernatant was measured at 546 nm. The amount of serum complement producing 50% hemolysis of the SRBC was considered

equivalent to 100 hemolytic units of complement. Complement consumption was expressed as the decrease in hemolytic units compared with a sample lacking LOS (Galanos and Luderitz, 1976; Inzana et al., 1987).

To evaluate complement consumption by the alternative pathway, a 2.5% suspension of unopsonized rabbit RBC (RRBC) was prepared in VBS containing 7 mM MgCl₂ and 10 mM EGTA. The number of RRBC for this experiment was optimized using a previously described protocol (Coligan et al., 1994). LOS concentrations of 30, 50, 70 and 100 µg were suspended in 20 µl of distilled water, added to 50 µl guinea pig serum containing 7 mM MgCl₂ and 10 mM EGTA, and incubated for 30 minutes at 37°C. Following incubation, 1.25×10^5 RRBC in VBS were added, and the mixture incubated for 1 h at 37°C. Unlysed RRBC were sedimented by centrifugation at $800 \times g$ for 5 minutes, and the OD of the supernatant was determined at 412 nm. One hundred hemolytic units of complement was considered the amount of serum required to cause 50% hemolysis of the RRBC. Complement consumption was expressed as the decrease in hemolytic units compared with the control sample lacking LOS (Riches and Stanworth, 1980).

Western Blotting

Sialylated or non-sialylated *H. somni* 738 LOS was incubated with fresh guinea pig serum (0.2µg/ µl) for 30 minutes at 37°C. Ten µl of this suspension was added to 10 µl of 2x SDS-PAGE loading buffer, boiled for 5 minutes, and the samples loaded onto 4-12% NuPAGE Bis-Tris pre-cast gels (Invitrogen, Carlsbad, CA). Following electrophoresis at 200 V for 40-60 minutes (Ram et al., 1998) the

proteins were transferred to a nitrocellulose membrane (Whatman Inc., Sanford, ME), which was blocked with 5% skim milk in tris buffered saline (TBS; 8.5% M NaCl, 0.1 M Tris, pH 7.3) containing 0.05% Tween 20 (Sigma-Aldrich, Inc., St. Louis, MO; blocking buffer) overnight at 4° C. After washing three times with TBS for ten minutes each, goat anti-C₃ (Fitzgerald Industries International Inc., Concord, MA; 1:2000 in blocking buffer) was added and the incubation continued at room temperature for 2 h with gentle shaking. The nitrocellulose membrane was washed 3 times with TBS, peroxidase-labeled rabbit anti-goat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:4000 in blocking buffer) was added and the incubation continued at room temperature for one h, followed by washing the membrane 3 times with TBS. The bands on the membrane were developed with 25 ml of TMB membrane peroxidase substrate (KPL, Gaithersburg, MD) and reaction was stopped by addition of 50 ml of distilled water (Schneider et al., 2006). Quantitative measurement of activated complement C₃ was performed by scanning densitometry of the bands using a Gel logic 200 imaging system (Kodak Scientific Imaging Systems, New Haven, CT).

To assess the cross-reactivity of bovine factor H with anti-human factor H, the previously described procedure was used except that the samples were bovine serum, human serum, PBS (negative control) and human factor H (Calbiochem, San Diego, CA; positive control). Sheep anti-human factor H (The Binding Site, UK) diluted 1:500 in blocking buffer and peroxidase conjugated rabbit anti-sheep IgG (H+L) (Jackson Immunoresearch, West Grove, PA) diluted 1:4000 in blocking buffer were used as primary and secondary antibodies. Human serum for this experiment was

obtained from pooled human serum for other experiments.

Isolation of bovine Polymorphonuclear Leukocyte (PMNs)

Bovine PMNs were isolated from venous blood as previously described (Howard et al., 2004; Roth and Kaeberle, 1981) with slight modifications. Briefly, blood was collected by jugular venipuncture from healthy adult cattle into a 50-ml centrifuge tube containing 2x ACD (acid-citrate-dextrose anticoagulant) and subjected to centrifugation at 1000 x g for 20 minutes at 25°C. The plasma, buffy coat and top layer RBCs were removed and the RBC pellet was washed in 1x HBSS at 1000 x g for 20 minutes at 25°C. The plasma, buffy coat, and top layer RBCs were removed and the remaining 5 ml of RBC pellet was lysed with 10 ml of ACK lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA, pH 7.3) for one minute at 0°C. Five ml of 2x HBSS and 20 ml of 1x HBSS were added to the lysate, followed by centrifugation at 480 x g for 10 minutes at 25°C. The supernatant was decanted and the pellet was again treated with ACK buffer as described above. The pellet was resuspended in 20 ml of 1x HBSS followed by centrifugation at 300 x g for 10 minutes at 25°C. The supernatant was discarded and the PMNs were suspended in 5 ml of RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10% heat-inactivated fetal calf serum, counted using a hemocytometer, and suspended to 10⁶ cells/ml. Cell viability was greater than 95% by trypan-blue exclusion assay.

Polymorphonuclear Leukocyte (PMN) Phagocytosis Assay

Sialylated or non-sialylated *H. somni* strain 738 and *E. coli* strain DH5 α were radiolabelled by growing the bacteria in BTT containing 10 μ Ci 3 H-thymidine/ml (specific activity 30 Ci/mMol) to 10^9 cfu/ml. The bacteria were washed in PBS three times by centrifugation at 6000 x g for 5 minutes each. After centrifugation the bacteria were resuspended in PBS and opsonized by incubation with 10% fresh bovine serum for 45 minutes at 37°C. Opsonized bacteria were washed three times in PBS, suspended in HBSS-G (HBSS containing 0.1% gelatin) and held on ice until incubation with PMNs (<1 hr). Opsonized bacteria (100 μ l) were added to 250 μ l of PMNs (2.6×10^6 cells/ml) at a final ratio of 100:1 respectively, in 1.5-ml microfuge tubes and incubated at 37°C for 45 minutes. After incubation extracellular bacteria were removed by adding the bacteria-PMN suspension to 7 ml of PBS, layering it onto 5 ml of Histopaque-1107 (Sigma Chemical Co., St. Louis, MO) in 15 ml-conical tube, and centrifuging the mixture at 400 x g for 30 minutes at room temperature with the break off.

After centrifugation, the supernatant was discarded, the bacterial-PMN suspension at the bottom was mixed well by pipetting, and 200 μ l of this suspension was added to 1 ml MicroscintTM 20 cocktail (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) for analysis in a liquid scintillation counter (Inzana et al., 1987). Bacterial uptake was determined by comparing the counts per minute (CPM) of washed PMNs to CPM of sample containing opsonized bacteria alone. Adherence of bacteria to the PMN surface was determined by incubation of bacteria with control PMNs treated with cytochalasin-D (Sigma Chemical Co., St. Louis, MO) at 5 μ g/ml

at 37°C for one h. The CPM of control PMNs was subtracted from washed PMNs to determine actual bacterial uptake. *E. coli* strain DH5 α was used as a positive control for phagocytosis.

PMN Bactericidal assay Procedure

Bacterial killing by PMNs was determined by a previously described method (Czuprynski and Hamilton, 1985; Czuprynski et al., 1984; Fischer and Rest, 1988). Sialylated or non-sialylated bacteria at midlog phase were washed three times in HBSS (by centrifugation at 6000g for 5 minutes) and resuspended in HBSSII buffer (HBSS containing 10 mM glucose, 0.1% gelatin, 1 mM CaCl₂ and 1 mM MgCl₂) at a concentration of 10⁶ cells/ml. Bovine PMNs were isolated from venous blood as previously described and adjusted to 2.5 x 10⁶ cells/ml in HBSSII buffer. The bactericidal mixtures contained the following: 450 μ l of bacterial suspension (4.5 x 10⁶ CFU/ml), 450 μ l of PMN suspension (3.75 x 10⁶ cells/ml), and 100 μ l fresh bovine serum. This mixture was incubated at 37°C for 120 minutes on a rotating shaker (10 rpm). Aliquots (50 μ l) were taken at 0, 30, 60, 90, and 120 minutes, serially diluted in sterile distilled water, and 10 μ l was streaked on CBA plates, which were incubated overnight at 37°C in 5% CO₂. The bactericidal activity of PMNs was determined by viable plate count with *E. coli* strain DH5 α used as a control for bactericidal activity of PMNs. Bacteria incubated with bovine serum without PMNs, bacteria incubated with PMNs without bovine serum, and bacteria incubated with bovine serum and PMNs on ice at 0°C were used as negative controls.

Chapter 3

Results

Effect of LOS sialylation on serum resistance

The effect of LOS sialylation on complement-mediated killing of *H. somni* by serum was determined by incubating different concentrations of pre-colostral calf serum (PCS), which lacks antibodies, with sialylated or non-sialylated *H. somni* strain 738 (Figure 3.1). A complement dose-dependent killing was observed with non-sialylated *H. somni*. However, there was significant ($P < 0.0001$) serum resistance of sialylated *H. somni* at all concentrations of PCS tested. Thus sialylation of LOS increases the resistance of *H. somni* against serum complement. *A. pleuropneumoniae* strain J45-100 was used as positive control, while non-sialylated *H. somni* strain 738 incubated with heat-inactivated PCS (30 minutes at 56°C) was used as negative control.

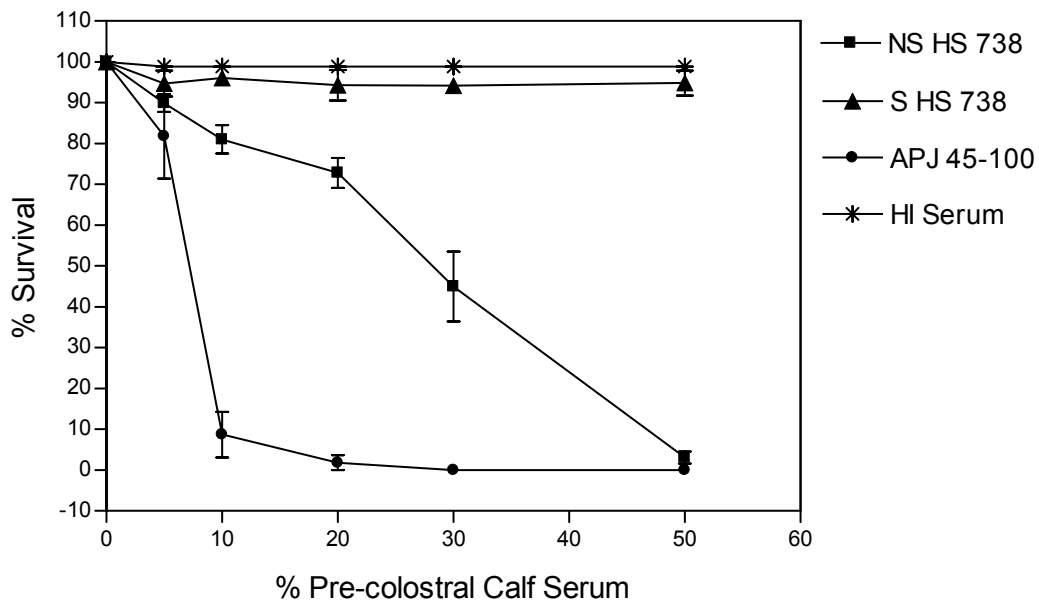


Figure 3.1: Effect of LOS sialylation on complement-mediated killing of bacteria.

Non-sialylated *H. somni* 738 (NS HS 738) or sialylated *H. somni* 738 (S HS 738) was incubated with 5, 10, 20, 30, and 50% pre-colostral calf serum for one hour at 37°C with shaking and the % survival of the bacteria was calculated. *A. pleuropneumoniae* strain J45-100 (APJ 5-100) was used as a positive control and non-sialylated *H. somni* 738 incubated with heat-inactivated PCS (HI Serum) (30 minutes at 56°C) was the negative control. Error bars represent standard deviation above and below the mean, where n = 3.

Effect of desialylation of sialylated LOS on serum resistance

To further confirm the role of sialylation of LOS in resistance to killing by the alternative complement pathway, sialylated and non-sialylated *H. somni* strain 738 grown to mid-log phase were treated with neuraminidase (0.0145 U/ml) for one h at 37°C with shaking. This treatment did not affect the survival of non-sialylated *H. somni*, whereas survival of sialylated *H. somni* was significantly ($P < 0.0001$) reduced (Figure 3.2). Therefore, specific removal of sialic acid increased the killing of sialylated bacteria. Treatment with higher doses (0.0210, 0.0290 and 0.0435 U/ml) of neuraminidase reduced the killing of both sialylated and non-sialylated *H. somni*, presumably due to interference of neuraminidase with complement activity (data not shown).

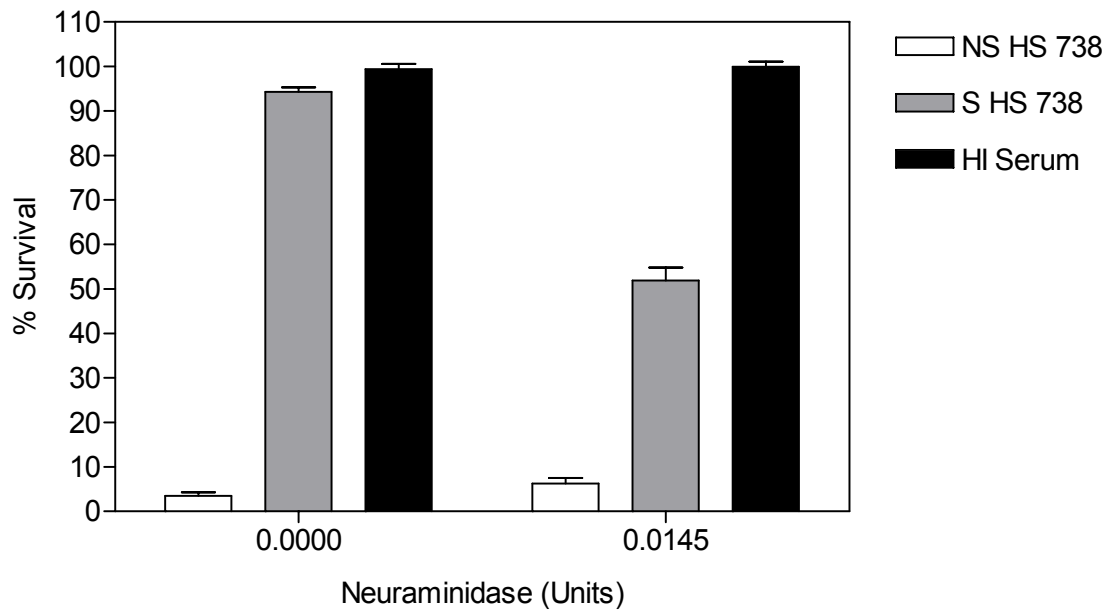


Figure 3.2: Effect of desialylation of sialylated *H. somni* on serum resistance.

Sialylated (S HS 738) and non-sialylated *H. somni* strain 738 (NS HS 738) was treated with or without neuraminidase and incubated with 50% PCS for one h at 37°C with shaking. The non-sialylated bacteria incubated with heat-inactivated PCS (HI Serum) was used as control. The percent survival was calculated by viable plate count.

Error bars represent standard deviation above and below the mean, where n = 3.

Purification of bovine complement factor H

Bovine complement factor H is a 158 KDa-glycoprotein present in plasma. It inhibits the activation of the alternative complement pathway. To purify complement factor H, bovine serum was precipitated and eluted through two ion-exchange chromatography columns (DEAE-sepharose anion exchange column and CM-sepharose cation exchange column), and then a Sephadex G-200 gel-filtration column as described in materials and methods. The protein profile of the eluted fractions was monitored by measuring the absorbance of individual fractions at 280 nm. The biological activity of factor H that was from each column was determined for each fraction by a hemolytic assay using bovine serum and rabbit RBC (RRBC). Factor H inhibited the activation of the alternative complement pathway in bovine serum and thus prevented the lysis of RRBC by bovine complement. The presence of factor H in the fractions eluted from each column was further confirmed by SDS-PAGE. However, factor H was still not purified after elution through these three columns, and biological activity was lost before further purification could be completed.

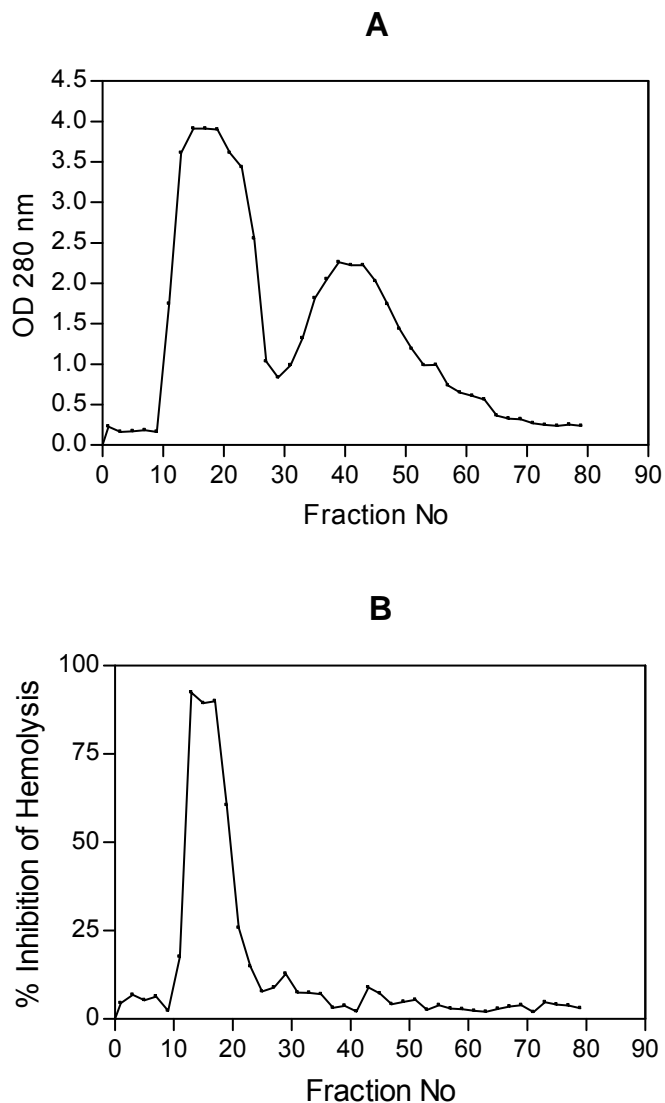


Figure 3.3: Purification of factor H by DEAE-sepharose anion exchange chromatography.

A: Protein profile of bovine plasma protein eluted from DEAE-sepharose following a salt gradient. The absorbance of each fraction was measured at 280 nm.

B: Hemolytic assay of bovine plasma protein eluted from DEAE-sepharose. The presence of factor H in eluted fractions was determined by hemolytic assay with bovine serum and RRBC.

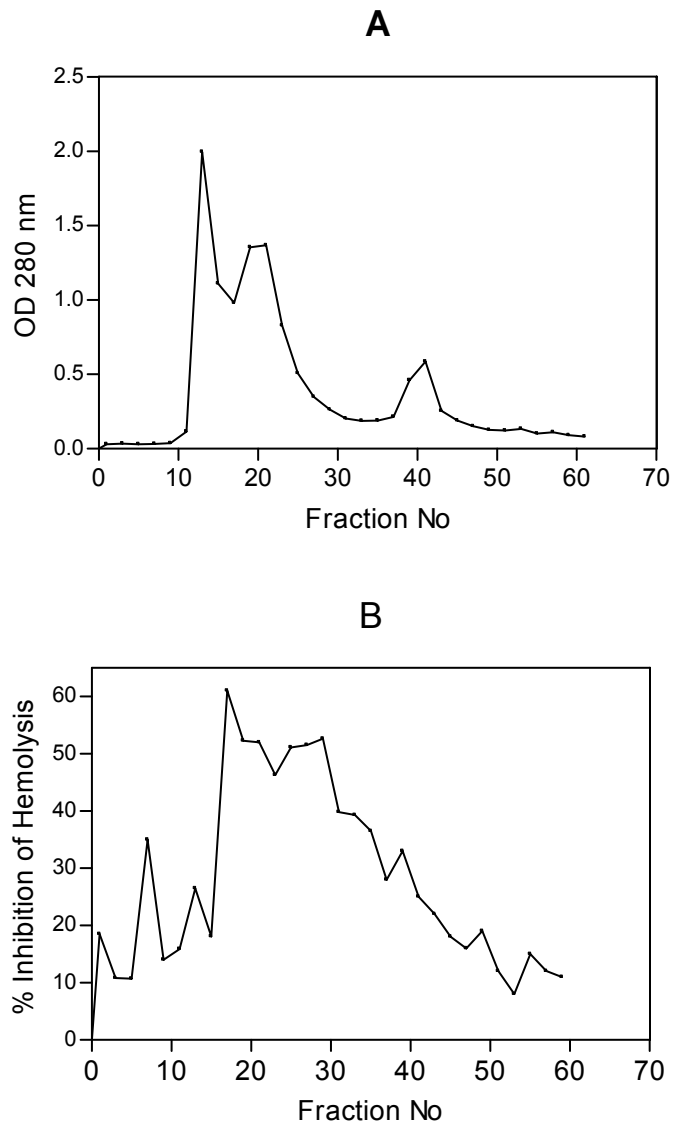


Figure 3.4: Purification of factor H by CM-sepharose cation exchange chromatography.

A: Protein profile of fractions eluted from CM-sepharose following a salt gradient. The absorbance of each fraction was measured at 280 nm.

B: Hemolytic assay of bovine factor H containing fractions eluted from CM-sepharose. The presence of factor H in eluted fractions was determined by hemolytic assay with bovine serum and RRBC.

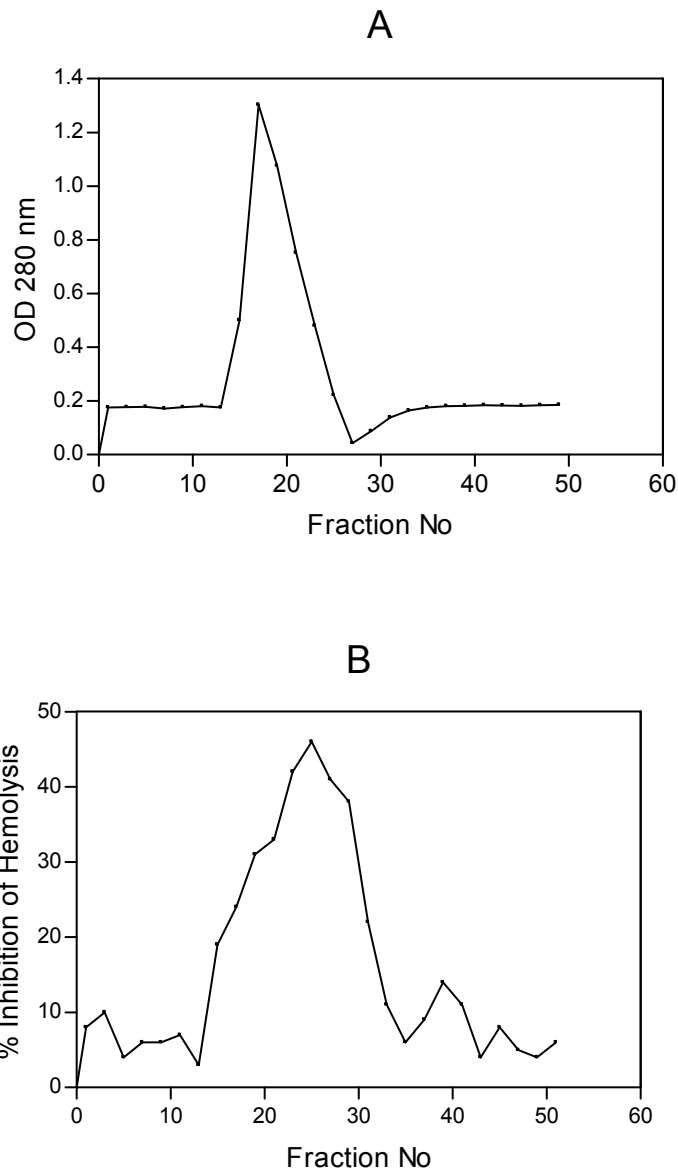


Figure 3.5: Purification of factor H by fractionation on Sephadex G-200 gel-filtration chromatography.

A: Protein profile of fractions eluted from Sephadex G-200. The absorbance of each fraction was measured at 280 nm.

B: Hemolytic assay of bovine factor H containing fractions eluted from Sephadex G-200. The presence of factor H in eluted fractions was determined by hemolytic assay with bovine serum and RRBC.

Cross-reactivity of bovine factor H with anti-human factor H antibody

Complement factor H is conserved among different mammalian species. Human factor H is 61% identical (76% similar) across the entire length of the protein to bovine factor H, based on the genomic blast search of human and bovine factor H (Personal communication, Dr. Shivakumara Siddaramappa). Considering the close relationship between human factor H and bovine factor H proteins, cross-reactivity of a commercially available anti-human factor H antibody with partially purified bovine factor H was investigated using Western blotting and dot blot assays. Bovine factor H did cross-react, at least weakly, with sheep anti-human factor H antibody (Figures 3.6 and 3.7).

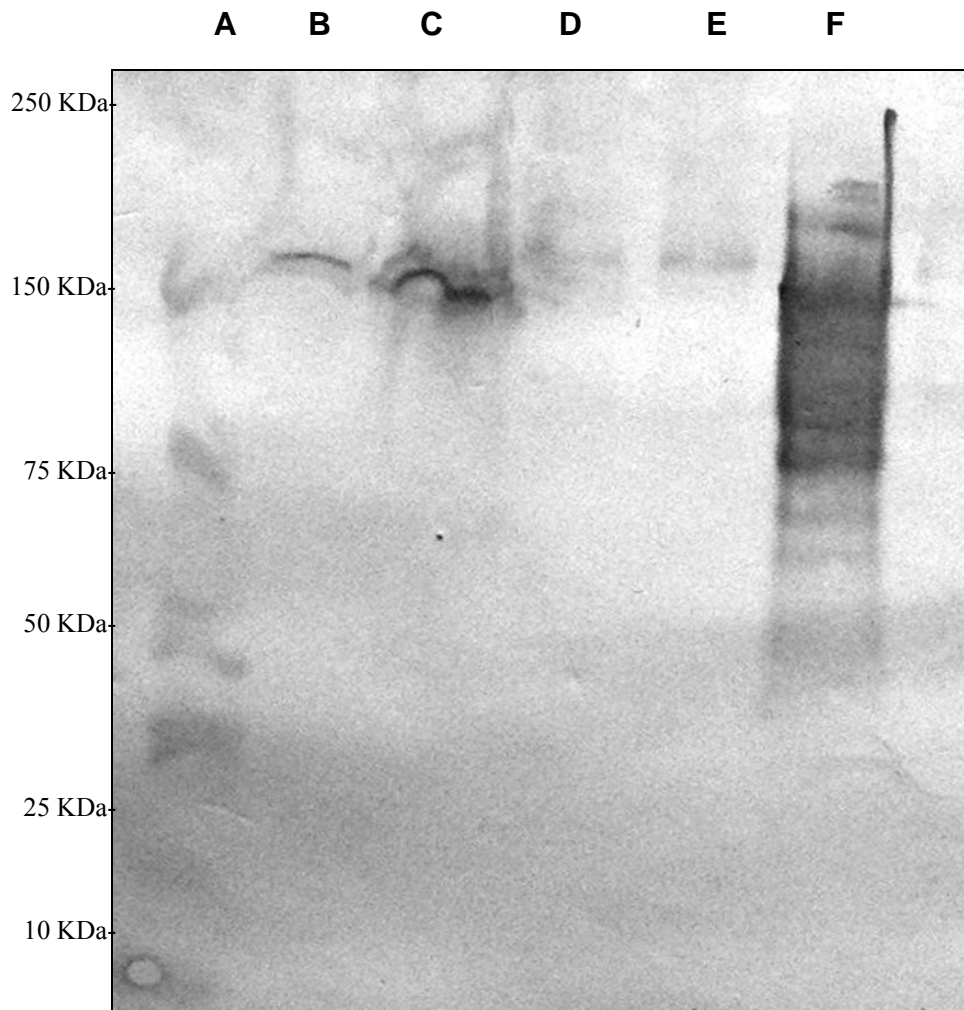


Figure 3.6: Western blot showing cross-reactivity of bovine factor H with anti-human factor H antibody. Serum samples were digested with loading buffer and subjected to electrophoresis on a 4-12% NuPAGE Bis-Tris gel followed by Western blotting. Blots were probed with sheep anti-human factor H. Lane A: Pre-stained protein standard (Bio-Rad Laboratories, Hercules, CA); lane B: bovine serum; lane C: human serum; lane D: PBS (negative control); lane E: blank; lane F: human factor H (positive control).

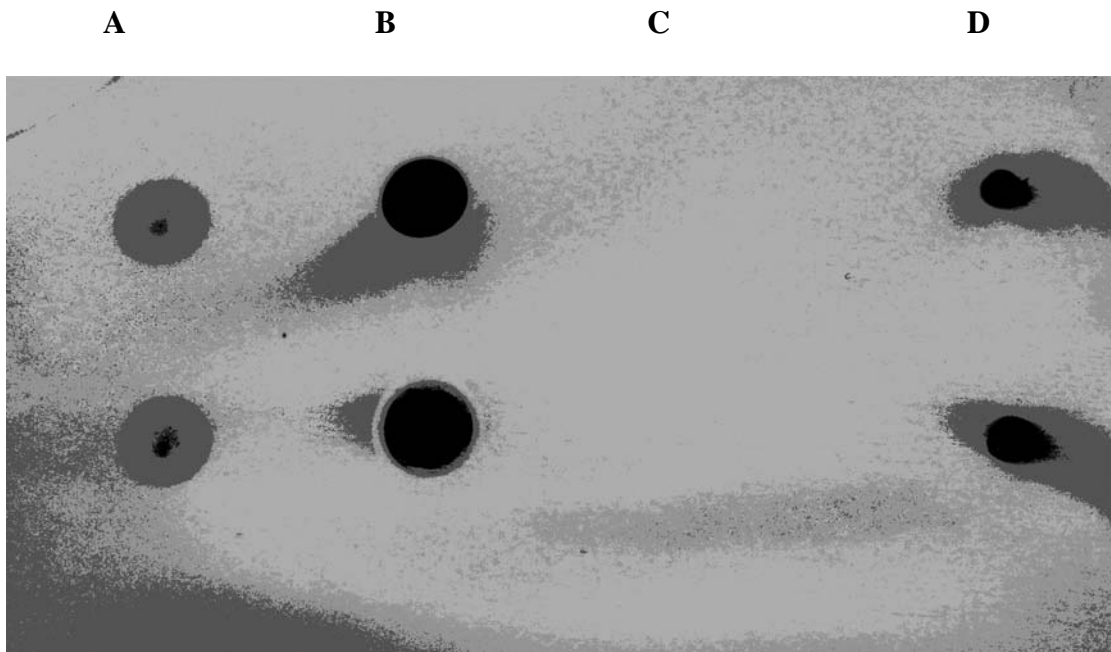


Figure 3.7: DOT blot showing weak cross-reactivity of bovine factor H with anti-human factor H antibody. A: Bovine serum; B- Human serum; C- PBS (Negative control); D- Human factor H (Positive control).

Binding of bovine complement factor H to sialylated or non-sialylated *H. somni* strain 738

Binding of complement factor H to sialylated or non-sialylated *H. somni* strain 738 was determined by whole cell ELISA. Since bovine factor H cross-reacted with anti-human factor H antibody, human factor H was used as a standard. The ELISA was performed with different concentrations of human factor H as described in materials and methods, and a standard curve was plotted (Figure 3.8).

Sialylated or non-sialylated *H. somni* strain 738 was incubated with bovine serum to allow binding of factor H to the bacterial surface and activation of complement. Anti-human factor H antibody was used to assess the binding of bovine factor H onto the surface of *H. somni*. The binding of factor H to bacteria was quantified by ELISA and the amount of factor H bound was determined using the equation for the standard curve of factor H (Figure 3.9). Sialylated *H. somni* bound significantly more factor H compared to non-sialylated *H. somni* ($P = 0.0002$).

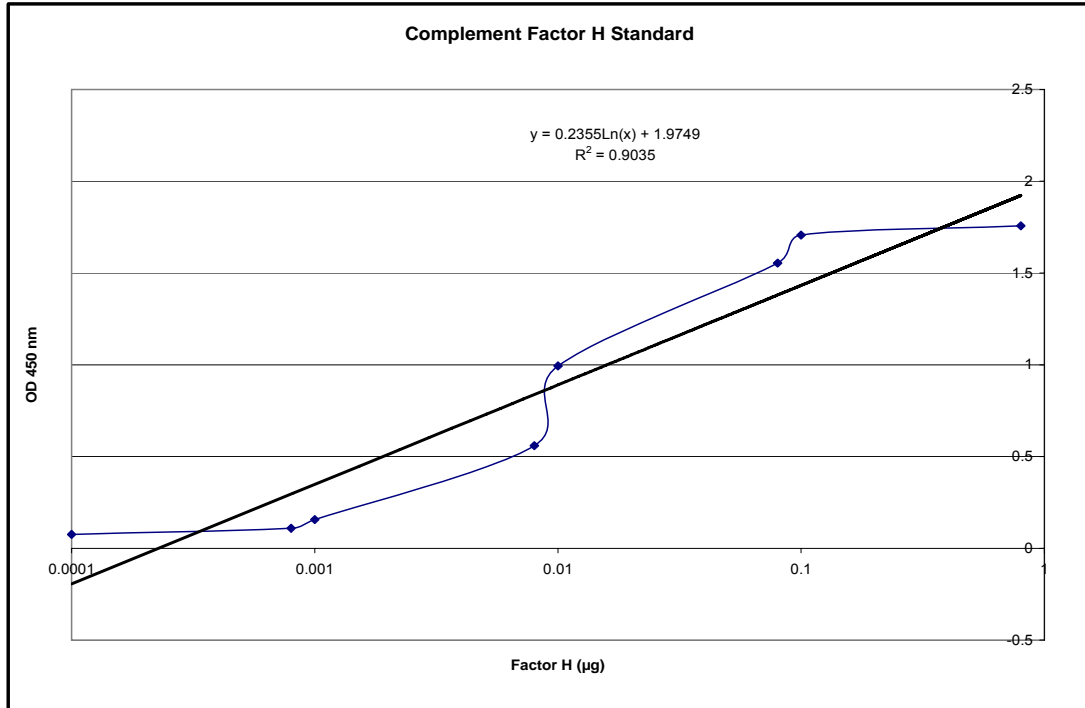


Figure 3.8: Best fit line of human complement factor H. Human factor H (0.0001 to 1.0 µg) was incubated with sheep antibody to human factor H and the absorbance values generated by ELISA. The best fit line was made by closest approximation of data points to the make the equation for a straight line and to compensate for the variability in data, where $n = 4$. This best fit line would help to predict the values which have not been displayed on the graph.

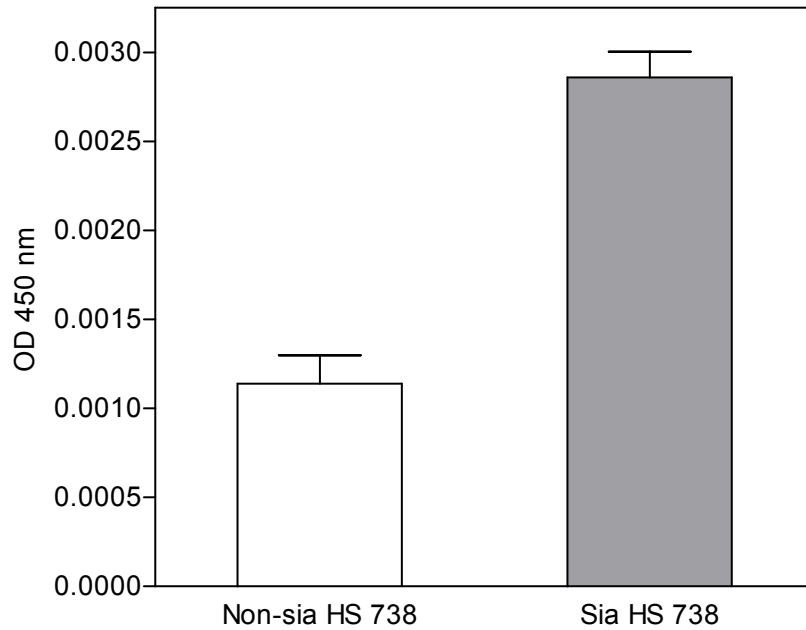


Figure 3.9: Quantification of binding of bovine factor H onto the surface of sialylated or non-sialylated *H. somni* strain 738 cells by whole-cell ELISA. After incubation of sialylated (Sia HS 738) or non-sialylated *H. somni* strain 738 (Non-sia HS 738) cells bacterial cells with bovine serum for 20 minutes, ELISA was performed as described in materials and methods, and the absorbance was measured at 450 nm. The amount of factor H bound was determined by the best fit line generated for human factor H. Error bars represent the standard deviation above and below the mean, where n = 5.

Deposition of complement factors C₃ and iC₃b on sialylated or non-sialylated *H. somni* strain 738

Deposition of complement factors C₃ and iC₃b on sialylated or non-sialylated *H. somni* 738 was quantified by whole cell ELISA. After incubation of bacterial cells with bovine serum, complement factor C₃ is converted into its conversion products C₃a and C₃b by C₃ convertase. C₃a is an anaphylotoxin, while C₃b is deposited on bacterial cell surface. Complement factor H converts C₃b to iC₃b. The non-sialylated *H. somni* 738 bound significantly more C₃ than sialylated *H. somni* ($P < 0.02$) (Figure 3.10). However, significantly more ($P = 0.0098$) iC₃b was present on sialylated *H. somni* than non-sialylated *H. somni* (Figure 3.11). This result corroborates the previous finding that sialylated *H. somni* binds more factor H, which mediates the conversion of C₃b to iC₃b by complement factor I, as compared with non-sialylated *H. somni*.

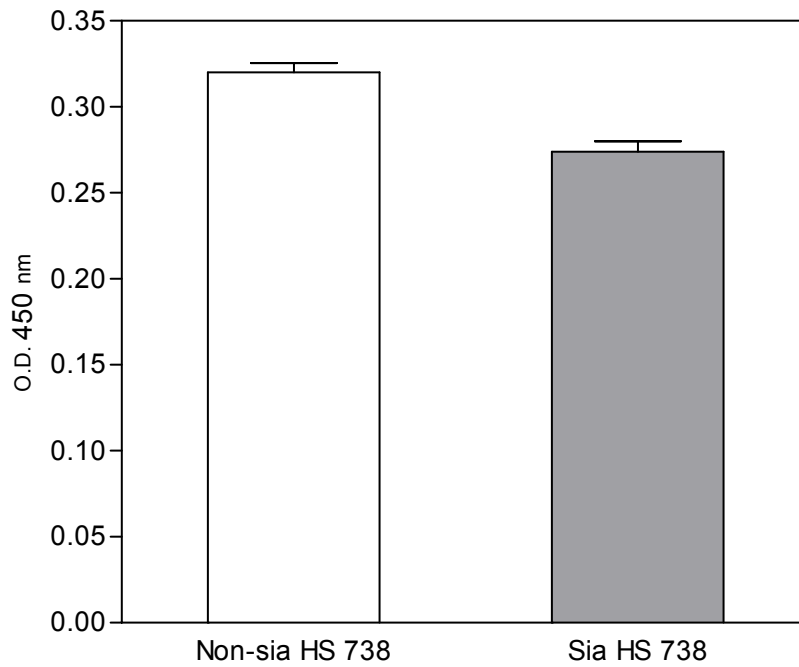


Figure 3.10: Quantification of C₃ binding to sialylated or non-sialylated *H. somni* 738 bacterial cell by ELISA. Sialylated (Sia HS 738) or non-sialylated (Non-sia HS 738) *H. somni* 738 bacterial cells were incubated with fresh bovine serum for 20 minutes to activate complement. ELISA was performed as described in materials and methods, and binding of C₃ was determined by measuring the absorbance at 450 nm. These results are representative of two separate experiments. Error bars represent standard deviation above and below the mean, where n = 4 for each experiment.

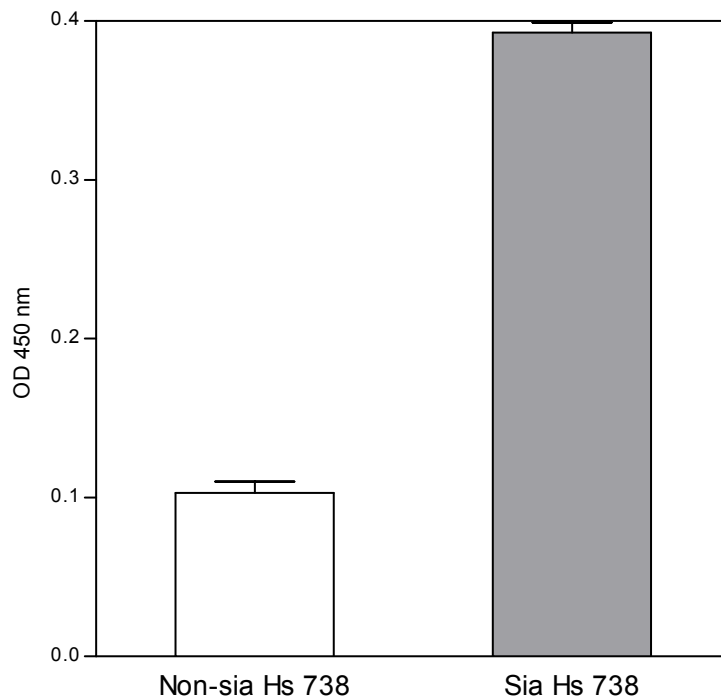


Figure 3.11: Quantification of iC_3b binding to sialylated or non-sialylated *H. somni* 738 bacterial cell by whole cell ELISA. Sialylated (Sia HS 738) or non-sialylated (Non-sia HS 738) *H. somni* 738 bacterial cells were grown to midlog phase and incubated with fresh bovine serum for 20 minutes to activate complement. The ELISA was performed as described in materials and methods, and binding of iC_3b was determined by measuring the absorbance at 450 nm. These results are representative of two experiments performed in triplicates. Error bars represent standard deviation above and below the mean.

Effect of LOS Sialylation on Complement Consumption

Bacterial LOS activates the alternative complement pathway in the absence of specific antibody, resulting in the formation of a membrane attack complex (MAC) and subsequent bacterial killing (Sell, 2001b; Verschoor and Carroll, 2004). Complement consumption by *H. somni* LOS was measured as described in materials and methods. The amount of complement producing 50% hemolysis (CH_{50}) was considered equivalent to 100 hemolytic units. The LOS of non-sialylated *H. somni* strain 738 consumed significantly more ($P < 0.0001$ at 70 μg LOS) complement compared to sialylated LOS. Complement consumption by the alternative pathway was measured using GPS chelated with MgCl_2 and EGTA. The classical pathway was blocked by removing Ca^{2+} present in the serum using EGTA. Non-sialylated LOS of *H. somni* strain 738 consumed significantly more ($P = 0.0004$ at 100 μg LOS level) guinea pig complement when compared to sialylated LOS (Figure 3.12). *H. influenzae* type b LPS, LOS without serum, veronal buffer, heat-inactivated serum, and total hemolysis by water were used as controls in the experiment. Earlier experiments were performed using GPS as a complement source. Similar results were obtained by repeating the experiment using bovine serum as a source of complement ($P = 0.0019$ at 70 μg LOS level). Complement consumption by non-chelated serum was higher when compared to complement consumption by EGTA- MgCl_2 chelated serum.

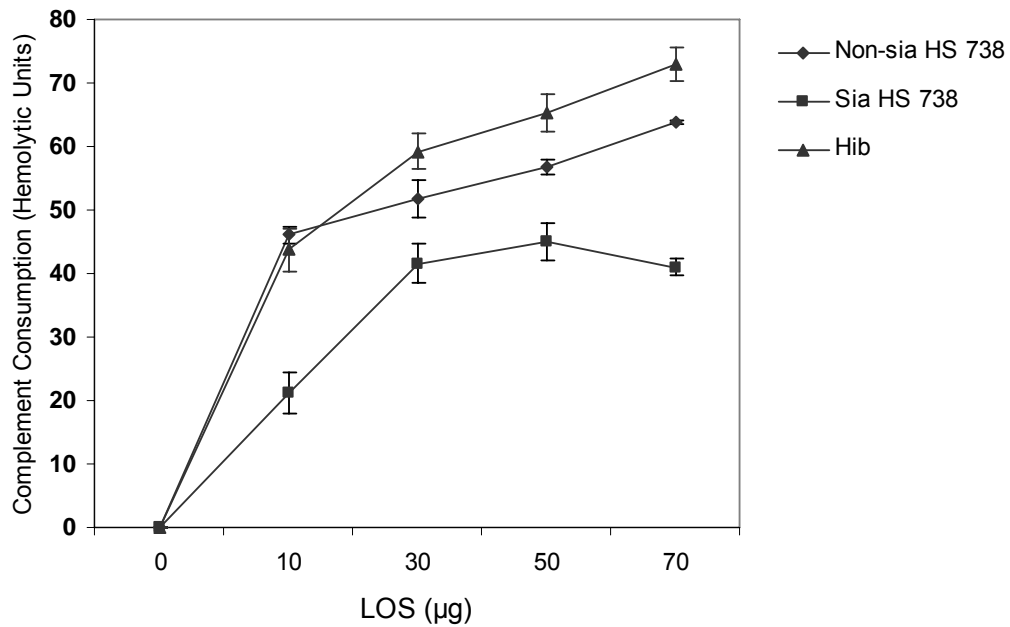


Figure 3.12: Complement consumption by LOS. Complement consumption was measured as described in materials and methods and is indicated by hemolytic units of complement consumed. Fresh guinea pig serum was used as the source of complement. Complement consumption was measured by inhibition of lysis of opsonized SRBC by complement. *H. somni* strain 738 non-sialylated LOS (Non-sia HS 738) consumed more complement compared to sialylated LOS (Sia HS 738). *H. influenzae* type b LPS (Hib), LOS without serum, buffer, heat-inactivated serum, and total hemolysis by H₂O were used as controls (Inzana et al., 1987). Error bars represent the standard deviation above and below the mean, where n = 3.

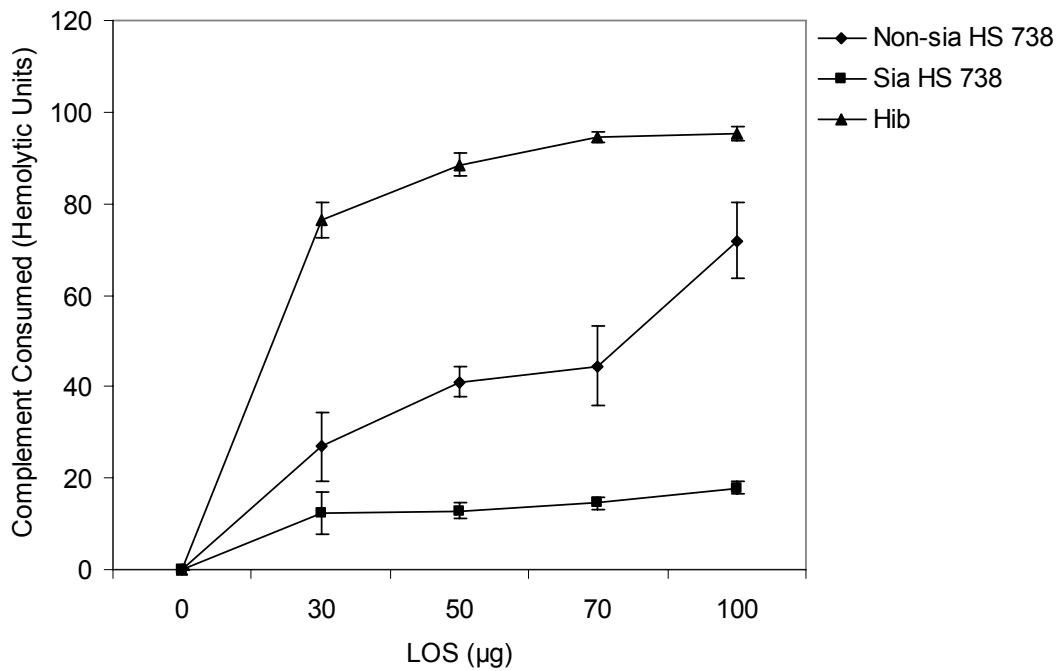


Figure 3.13: Complement consumption by bacterial LOS by the alternative complement pathway using guinea pig serum as a complement source.

Complement consumption via alternative pathway was measured as described in materials and methods and is indicated by hemolytic units of complement consumed. Fresh guinea pig serum chelated with 7 mM MgCl₂ and 10 mM EGTA was used as the source of complement. Complement consumption was measured by inhibition of lysis of RRBC by complement. *H. somni* strain 738 non-sialylated LOS (Non-sia HS 738) complement when compared to sialylated LOS (Sia HS 738). *H. influenzae* type b LPS (Hib), LOS without serum, buffer, heat inactivated serum and total hemolysis by H₂O were used as controls. Error bars represent the standard deviation above and below the mean, where n = 3.

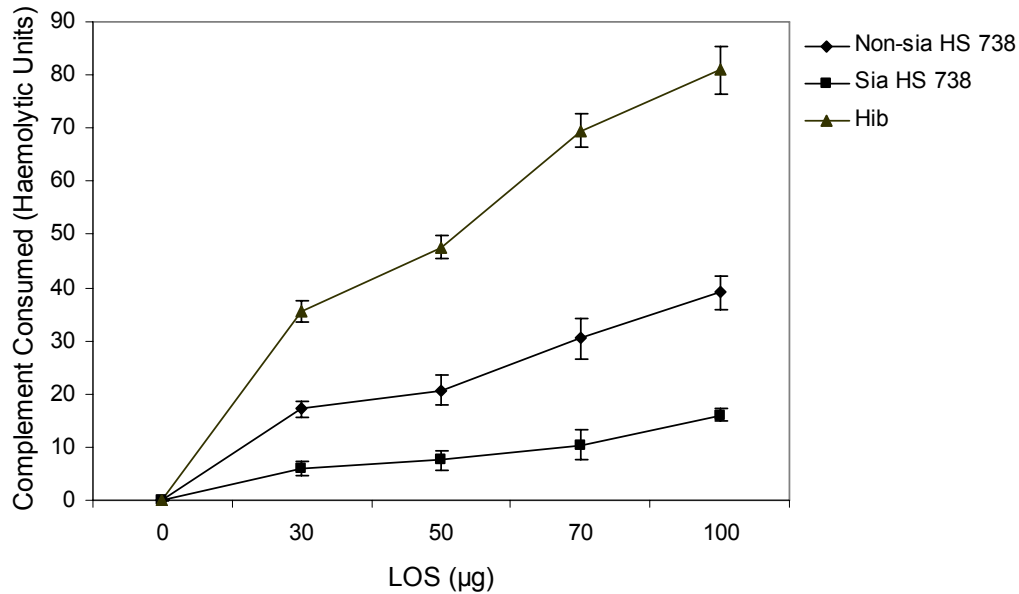


Figure 3.14: Complement consumption by bacterial LOS via the alternative pathway using bovine serum as the source of complement. Complement consumption via the alternative pathway was measured as described in materials and methods and is indicated by hemolytic units of complement consumed. Fresh bovine serum chelated with 7 mM MgCl₂ and 10 mM EGTA was used as a source of complement. Complement consumption was measured by inhibition of lysis of RRBC by complement. *H. somni* strain 738 non-sialylated LOS (Non-sia HS 738) consumed more complement when compared to sialylated LOS (Sia HS 738). *H. influenzae* type b LPS (Hib), LOS without serum, buffer, heat inactivated serum, and total hemolysis by H₂O were used as controls. Error bars represent the standard deviation above and below the mean, where n = 3.

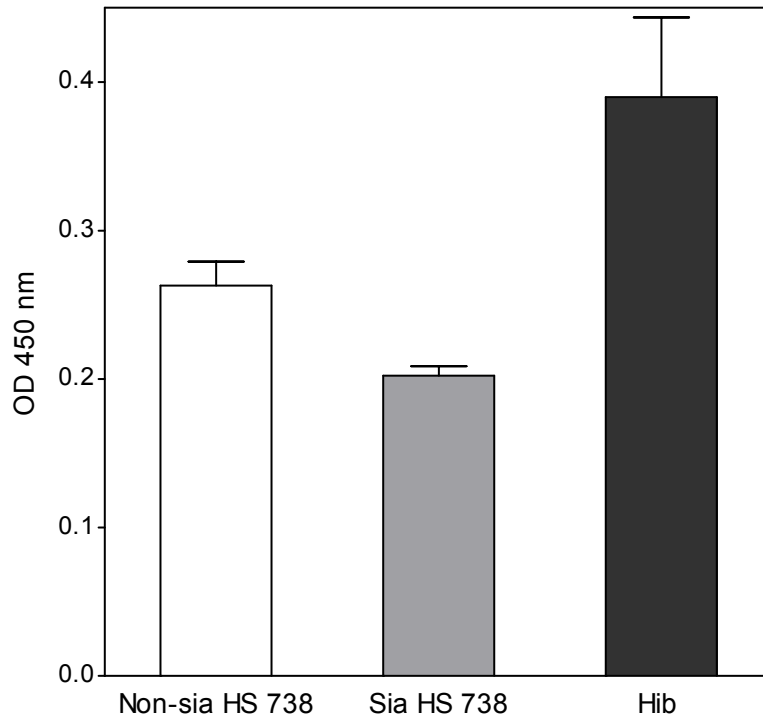


Figure 3.15: Quantification of C₃ binding to LOS by ELISA. Bacterial LOS was incubated with bovine serum for 30 minutes and binding of complement factor C₃ was determined by ELISA. *H. somni* strain 738 non-sialylated LOS (Non-sia 738) bound more complement factor C₃ when compared to sialylated LOS (Sia HS 738) ($P < 0.02$). *H. influenzae* type b LOS (Hib) was used as a control. Error bars represent the standard deviation above and below the mean, where n = 6.

Effect of Sialylation of LOS on Complement Activation

The effect of LOS sialylation on complement activation was further investigated by incubation of LOS with GPS for 30 minutes at 37°C and subjecting the activated complement to SDS-PAGE, followed by transfer to nitrocellulose membrane. After development of the membrane, the net intensity of the bands was measured and complement activation was determined by the percent degradation of C₃ to its end products. *H. somni* strain 738 non-sialylated LOS activated significantly more complement than sialylated LOS (Figure 3.16) ($P = 0.0043$). This result corroborates with findings of the complement consumption assay (Figures 3.12, 3.13 and 3.14). *H. influenzae* type b LPS was used as positive control, while GPS without LOS was used as negative control.

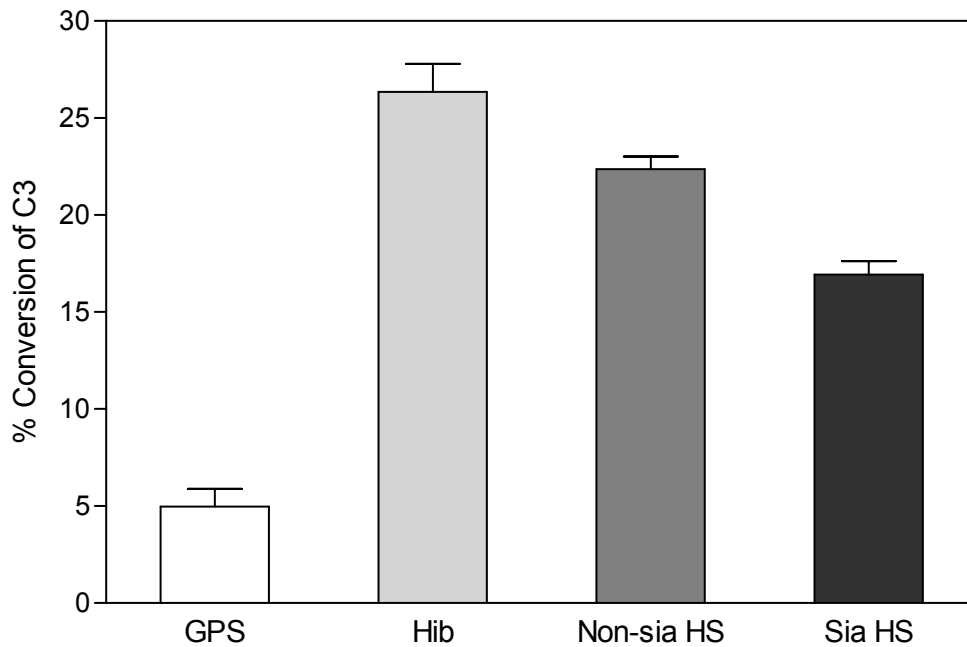


Figure 3.16: Complement activation by LOS. The percent conversion of C_3 to its degradation products was measured after incubation of sialylated (Sia HS) or non-sialylated *H. somni* LOS (Non-sia HS) with guinea pig serum. *H. influenzae* type b LOS (Hib) was a positive control, while guinea pig serum without LOS (GPS) was the negative control. Error bars represent the standard deviation above and below the mean, where $n = 3$ and $P = 0.0043$ between sialylated and non-sialylated *H. somni* 738 LOS.

Phagocytosis and killing of bacteria by Polymorphonuclear leukocytes

Polymorphonuclear leukocytes (PMNs) are an important component of the innate immune system along with complement. PMNs uptake and kill bacteria opsonized by antibody and complement. Sialylation of *H. somni* strain 738 LOS resulted in reduced phagocytosis and killing by PMNs (Figures 3.17 and 3.18). Less antibody in bovine serum bound to sialylated than to non-sialylated *H. somni* strain 738, as determined by ELISA ($P = 0.002$). Sialylated bacteria would be less effectively opsonized for phagocytosis (Figure 3.19).

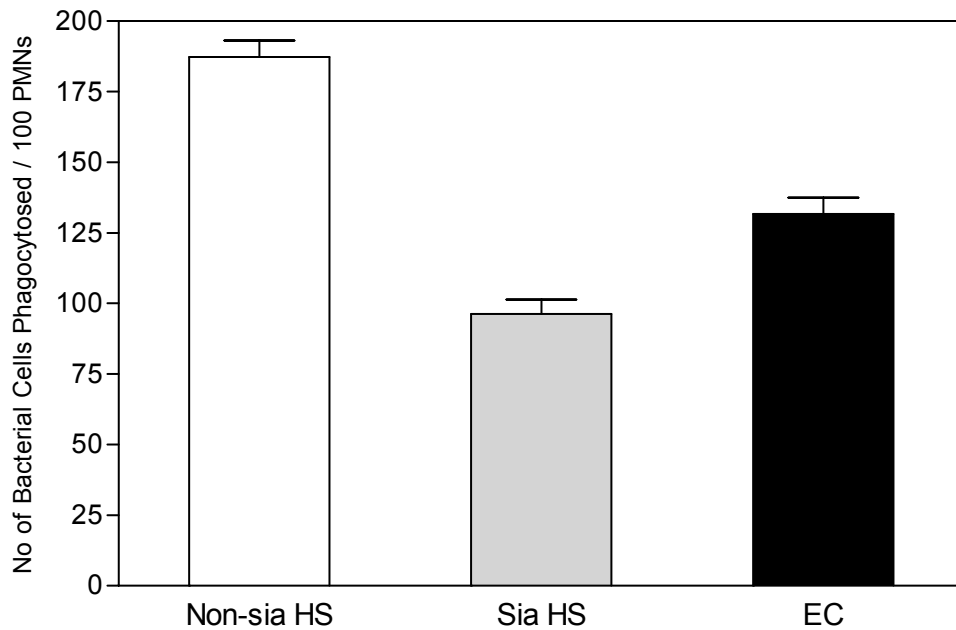


Figure 3.17: Effect of sialylation of LOS on PMN activity. Bacteria were radiolabelled, opsonized with 10% bovine serum and incubated with bovine PMNs as described in materials and methods. Extracellular bacteria were removed and the radioactivity of PMNs was measured to determine the internalization of bacteria. *E. coli* strain DH5 α (EC) was used as a positive control, while bacteria incubated with cytochalasin-D-treated PMNs were used as a negative control. Phagocytosis of sialylated *H. somni* strain 738 (Sia HS) by PMNs was significantly diminished when compared to non-sialylated *H. somni* (Non-sia HS) ($P < 0.05$). Error bars represent the standard deviation above and below the mean, where $n = 4$.

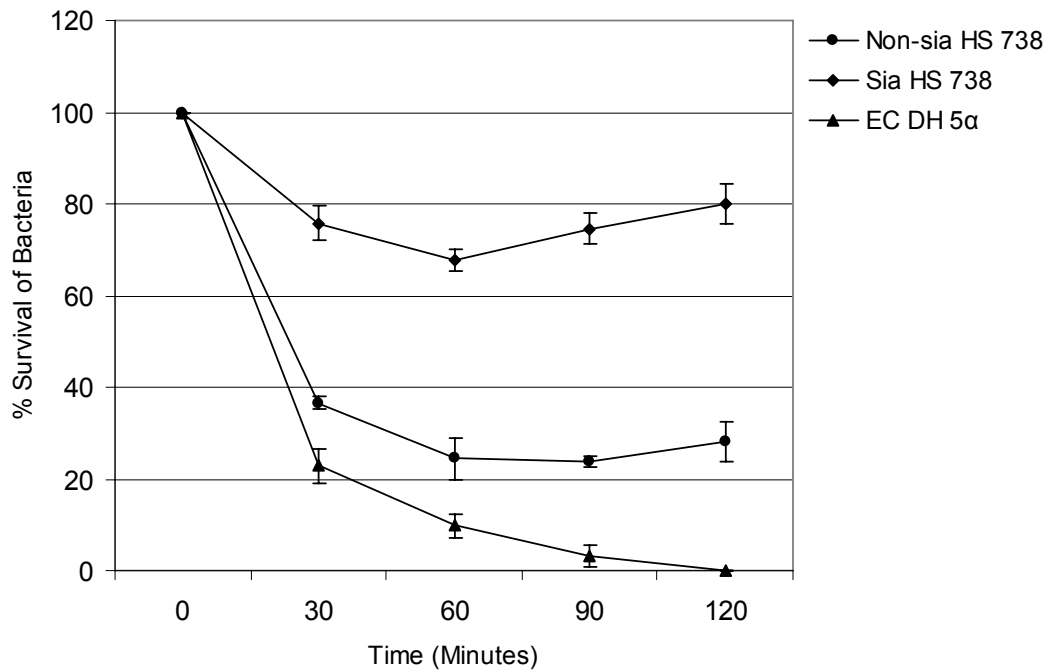


Figure 3.18: Effect of sialylation on bacterial killing by PMNs. Sialylated or non-sialylated *H. somni* strain 738 was incubated with bovine PMNs and bovine serum described in materials and methods. Killing of the bacteria was determined by viable plate count following PMN lysis. Sialylated *H. somni* (Sia HS) were more resistant the killing by PMNs as compared to non-sialylated *H. somni* (Non-sia HS) ($P = 0.0001$). *E. coli* strain DH5α (EC) was used as control for killing by PMNs. Bacteria incubated without PMNs, bacteria incubated with PMNs but without bovine serum, and bacteria incubated on ice were also used as controls. Error bars represent standard deviation above and below the mean, where $n = 3$.

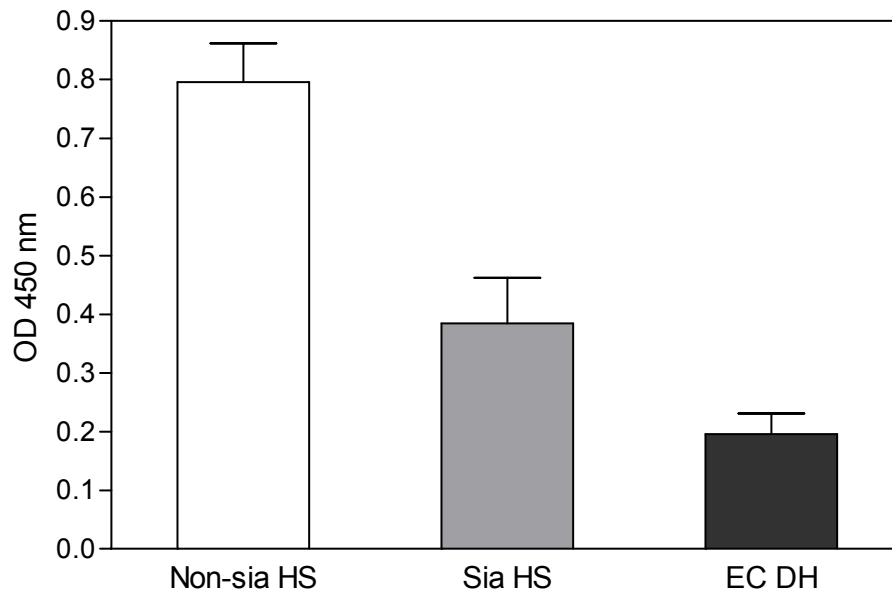


Figure 3.19: Binding of bovine serum antibodies to *H. somni* 738. Presence of bovine antibody bound to *H. somni* was quantified by ELISA using peroxidase-conjugated rabbit anti-bovine IgG as described in materials and methods. There was a significant difference between the amount of bovine antibody bound by non-sialylated (Non-sia HS) and sialylated *H. somni* (Sia HS) ($P = 0.002$). *E. coli* strain DH5 α (EC) was used as control. Error bars represent standard deviation above and below the mean, where $n = 6$.

Chapter 4

Discussion

To promote survival in the host, pathogenic bacteria develop mechanisms to overcome the effects of the host's immune system. Complement and polymorphonuclear leucocytes are important components of innate immunity. Bacterial sialylation affect many facets of the pathogenesis of *Neisseria gonorrhoeae* (Mandrell et al., 1990), *Neisseria meningitides* (Mandrell et al., 1991) and *Haemophilus influenzae* (Mandrell et al., 1992). This phenomenon has been found to be important for persistence of bacteria in the host, as it inhibits their killing by serum complement (Estabrook et al., 1997; Moran et al., 1996; Vimr et al., 2004), decreases complement activation by LOS (Vogel et al., 1997) , enhances the binding of complement factor H to the bacterial surface (Ram et al., 1998), and reduces the effectiveness of opsonization and killing by phagocytic cell (Kim et al., 1992).

Incorporation of sialic acid onto *Neisseria gonorrhoeae* by growing in medium containing CMP-NANA results in resistance to killing by normal human serum (Elkins et al., 1992; van Putten and Robertson, 1995) and decreased opsonophagocytosis upon incubation with human PMNs (Kim et al., 1992). About 91% killing occurs when bacteria are grown without CMP-NANA, whereas only 6% bacterial killing occurs when the bacteria are grown with CMP-NANA (Kim et al., 1992; Parsons et al., 1988). Similar results were observed after incubation of *H. influenzae* type b (Hood et al., 1999) and *N. meningitides* (Estabrook et al., 1997) with NHS. A dose-dependent increase in killing of non-sialylated *H. somni* from 10%

to 97% was observed after incubation with PCS (which lacks antibody). However, killing of the sialylated *H. somni* remained at about 6% even after increasing the serum concentration to 50%. A significant increase in killing of sialylated *H. somni* was observed after incubation of the bacteria with the neuraminidase to remove sialic acid, further confirming the role of sialic acid in resistance to killing by serum complement. The increased resistance to killing by serum after sialylation of the bacteria might be due to an ineffective penetration of the bacterial surface by the membrane attack complex (C₅b-C₉) (Joiner et al., 1983). The decreased amount of complement deposited on the sialylated bacteria has also been reported as a mechanism responsible for enhanced serum resistance (Elkins et al., 1992). In *N. meningitides*, resistance to complement-mediated killing is due to the masking of lacto-*N*-tetraose (LNnT) on the LOS by sialic acid (Estabrook et al., 1997).

Factor H is a regulator of alternative complement pathway and down-regulates activation of the complement cascade by dissociating the C₃b convertase. Therefore, less complement is deposited on the bacterial surface. Factor H also converts bound C₃b to iC₃b; thereby enhancing the virulence of the sialylated bacteria by inhibiting deposition of the complement cascade on the bacterial surface. In the current study, it was observed that the binding of factor H to *H. somni* is influenced by presence of sialic acid on bacterial LOS. Sialylated *H. somni* bound significantly more factor H, less total C₃ and more iC₃b compared to non-sialylated bacteria. These findings are in accordance with the findings of a previous study on the binding of factor H in NHS to *N. gonorrhoeae* (Ram et al., 1998). However, in *N. meningitides* binding of factor H is independent of sialic acid binding (Schneider et al., 2006). The differential binding

of factor H onto sialylated or non-sialylated LOS may be responsible for the difference in complement deposition. The reduced binding of complement to sialylated *H. somni* also appears to be responsible for resistance to complement-mediated killing.

Both *N. gonorrhoeae* and *N. meningitides* activates complement on the bacterial surface in the absence of sialic acid (Jarvis, 1994). However, in other studies *N. gonorrhoeae* strain F62 (wild type) consumed less C₃ compared with strain JB1 (sialyltransferase-deficient mutant) upon growth in medium containing sialic acid (Gill et al., 1996). The inhibition of bacterial killing and opsonophagocytosis upon sialylation of bacteria is due to hindrance in complement cascade rather than due to prevention of binding of the antibody (Wetzler et al., 1992). In the current study, it was found that the LOS of non-sialylated *H. somni* strain 738 activated and consumed significantly more complement compared to sialylated LOS. Similar findings were observed in experiments involving only an active alternative complement pathway. The inhibition of complement activation by incorporation of sialic acid onto the bacterial surface is well established (Fitzgerald, 1987; Jarvis and Vedros, 1987; Marques et al., 1992; Vogel et al., 1997). In this study and others the difference in complement activation and consumption by sialylated and non-sialylated *H. somni* LOS appeared to be due to increased binding of the factor H onto the sialylated bacteria. However, there was a marked difference in total complement consumption and complement consumption by the alternative complement pathway alone. This difference might be due to the consumption of some complement by the lectin pathway. Further research is required to determine the role of the lectin complement

pathway in complement consumption by the *H. somni* LOS.

Sialylation of *H. somni* has been observed to have a negative effect on phagocytosis and killing of the bacteria by PMNs. PMNs escalate the immune response and release many inflammatory mediators (Lederer et al., 1987). The bacteria are opsonized by deposition of specific antibody and complement factors onto the bacterial surface. Opsonization results in enhanced phagocytosis and it results in clearing of the bacteria from the circulation. Ingestion of *N. gonorrhoeae* strains VPI and MS11 by human epithelial cells was found to be inhibited by growth of the bacteria in medium containing CMP-NANA (Van Putten, 1993). The survival rate of *N. gonorrhoeae* strain F62 increased following incubation of human PMNs with bacteria grown in CMP-NANA (Kim et al., 1992; Rest and Frangipane, 1992; Wu and Jerse, 2006).

In the current study, it was observed that sialylation of LOS interferes with phagocytosis of the opsonized bacteria by PMNs. There was significantly less phagocytosis of sialylated compared to non-sialylated *H. somni*. Similarly, reduced PMN killing was observed for the sialylated *H. somni* compared with non-sialylated bacteria. The diminished phagocytosis of sialylated bacteria may be attributed to reduced binding of antibody and complement onto the surface of sialylated bacteria. The ineffective killing of sialylated bacteria by PMNs might be due to variation in deposition of C₃ or differential conversion of C₃b to iC₃b (Brown, 1991; McQuillen et al., 1991). However, results of the current study are in conflict with observations of Czuprynski et al., who reported that *H. somni* is not killed by PMNs (Czuprynski and Hamilton, 1985). In their experiment, bacteria were grown in media containing 10%

calf serum. There is a possibility that sialic acid present in serum sialylated the bacteria and the sialylated bacteria were resistant to killing by PMNs. It could be concluded from experiments that the sialylated LOS is antiphagocytic and it lowers the phagocytosis by depositing more factor H, which in turn activates reduced level of complement and there is reduced opsonization of bacteria. Sialylation inhibits the phagocytosis, thus producing reduced immune response and helping bacteria to survive better in host.

In summary, sialylation of *H. somni* LOS enhances the virulence of the sialylated bacteria by increasing the binding of factor H, decreasing the complement activation and consumption and thereby complement-mediated killing, and inhibiting phagocytosis and killing by PMNs. The enhanced virulence of sialylated bacteria may result from masking of the specific LOS epitopes that are potential targets of host immunity, by sialic acid or a change in interaction of sialylated epitopes with receptors on eukaryotic cells (Hood et al., 1999). Sialylation could also affect host-bacterial interaction by changing the bacterial surface charge (Hood et al., 1999), or by altering the surface determinants of the bacterial cell like outer membrane proteins (St Geme et al., 1993) or fimbriae (van Ham et al., 1995). A better understanding of the molecular mechanisms underlying sialylation of *H. somni* would enhance our understanding of bacterial pathogenesis and virulence.

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Vita

Rajiv Balyan was born in Muzaffarnagar, Uttar Pradesh, India. He attended school in Jhansi and Pathnamthitta, India. In January, 1998, he joined G B Pant University of Agriculture & Technology, Pantnagar, India and graduated with a Bachelor's of Veterinary Sciences and Animal Husbandry Degree in June, 2003. In January of 2004 he enrolled in the Master of Science program at the VA-MD Regional College of Veterinary Medicine, Department of Biomedical Sciences and Pathobiology. He studied under the supervision of Dr. Thomas J. Inzana and successfully defended his thesis in Summer 2007.