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Virulence Factors of Nonsporing Anaerobes; A revisit to explore their diagnostic & therapeutic potential

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Abstract: Infections with nonsporing anaerobes are common and often pose diagnostic and therapeutic challenges. There is an increasing trend of resistance among anaerobes against the commonly used antibiotics. Lot of development has occurred in terms of molecular and biochemical basis for pathogenecity and virulence factors among nonsporing anaerobes. Therefore, there is a need for development of newer, quicker and cost effective diagnostic, therapeutic and prophylactic strategies against such infections. A comprehensive review on the newer developments in this field is presented here, in order to provide basis for the future research avenues.

Key words: Virulence factors, Nonsporing anaerobes, capsule, fimbriae, protease, collagenase, gingipains.

Introduction :

Anaerobes are the most predominant components of the normal human skin and mucous membrane flora hence are the common cause of bacterial infections of endogenous origin. These infections contribute significantly to morbidity and mortality prompting an early diagnosis and treatment.[1] The fastidious nature of nonsporing anaerobes make them difficult to isolate from infectious sites and are often overlooked.[2] Despite the advances in the field of anaerobic bacteriology, we encounter diagnostic and therapeutic challenges in day to day practice. Antibiotic resistance is on rise among anaerobes.[3, 4, 5] Untreatable bacterial infections constitute a dark but valid threat. The ability of anaerobes to produce enzymes inactivating antibiotics has become well established and is an important factor in the persistence of an infection even during therapy. There is a situationally emergent need for expanding newer therapeutic and immunological strategies against an invading pathogen and for such venture, it is necessary to understand molecular mechanisms of pathogenesis and virulence factors of nonsporing anaerobes.[6, 7, 8].There is a recent insurgence of better molecular techniques which have facilitated extensive studies on molecular mechanisms of pathogenesis of anaerobic bacteria resulting in significant contributions towards pathogenecity, virulence factors, mechanisms of resistance and host defenses. Especially, the role of capsule in the pathogenesis anaerobic infections has been extensively studied.[9, 10, 11] Antibody to capsular polysaccharide of *B.fragilis* can be induced in animals and such immunization confers significant protection against subsequent abscess development from *B.fragilis* strains.[12] There is emerging evidence on the role of hydrolytic and proteolytic enzymes like hyaluronidase, chondratinsulfatase, gelatinase, collagenase, fibrinolysin, lecithinase, caseinase and lipase secreted by some anaerobic bacteria in infectious process.[13-20]

There is a need for review and reflection on the newer developments in the field of anerobic bacteriology and hence, an attempt is made here to describe and summarize the current state of knowledge about the pathogenecity and virulence factors of nonsporing anaerobes so that future research may be directed towards the development of newer and rapid diagnostic modalities, discovery of effective therapeutic and prophylactic measures to attack nonsporing anaerobes.[21,22]

Anaerobes behave both as friend and foe!

Of the more than 10^{14} cells of which make the human body, only about 10% are mammalian. The remaining cells are the microorganisms that make the commensal microbiota of the host. human colon, where oxygen tension is low, contains large populations of anaerobic bacteria, exceeding 10^{11} organisms/g of colon content. The bacteria generally maintain a complex and generally beneficial relationship with the host when retained in the gut, but when they escape this environment they can cause significant pathology, including bacteremia and abscess formation in multiple body sites contributing to significant morbidity and mortality. The similar scenario "commensal gone bad" is explained for *E.fecalis* [2, 23] *B. fragilis*, an important anerobic pathogen, is known to behave as both "friend and foe" since its capsular polysaccharide is not only important in the development of host immune system, but also

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responsible for abscess formation. Acquiring genes that favor the "new" resident (e.g., genes that code for improved adhesion, new nutrition pathways, antibiotic resistance, and inhibition of host defenses) will give these organisms an edge in establishing a niche for themselves. Indeed, some bacteria may not even need to acquire new genes. Organisms such as *Bacteroides* with such a large genome bank at their disposal may simply need to turn on certain genes (such as those involving new nutrition pathways, efflux pumps to rid the cell of toxic substrates, or new surface epitopes) to change from friendly commensal to dangerous threat. [24]

Pathogenesis of anaerobic infections

Pathogenecity is defined as the capacity of a microorganism to produce disease. The microorganism needs to *enter* the host, *multiply* on or in the host tissues, *resist* or *not stimulate* the host defenses, and damage host. Destruction of tissues happens as a response to very intricate host-parasite interactions. Historically, pathogenesis research has focused on the identification and characterization of virulence factors. The first evidence that microorganisms might live under anaerobic conditions was provided by Antonie van Leeuwenhoek, who noted that some 'animalcules' were able to live and move about in the absence of air.[25] Hippocrates provided a clear clinical description of tetanus in the fourth century B.C. The first study of a patient with an anaerobic infection was apparently done by E. Levy in Germany, and a report of the study was published in 1891 [23] Recently, genomic and proteomic analyses have vastly added to our understanding of the pathogenesis in Bacteroides species like (i) complex systems to sense and adapt to nutrient availability, (ii) multiple pump systems to expel toxic substances, and (iii) the ability to influence the host immune system so that it controls other (competing) pathogens.[2] Generally, there are three broad qualities of pathogenic bacteria by the means of which they cause disease namely, the ability of *adherence* and initial multiplication, ability to *invade* tissues and the ability to *overcome host defense mechanisms.*

Virulence factors of nonsporing anaerobes

Virulence is defined as the relative capacity of a microbe to cause damage in a host and virulence factors are those molecules, or components of a microbe, that have a damaging effect on host cells.[27] Virulence factors of nonsporing anaerobes have been extensively studied. Capsules surrounding some anaerobic bacteria probably interfere with phagocytosis and act as a barrier against penetration by antimicrobial agents.[12, 28] It is found that, the Bacteroides, Prevotella, and Porphyromonas species are capable of expressing adhesion factors (e.g., capsular polysaccharide, fimbriae and hemagglutinin), tissue-damaging exoenzymes (e.g., proteases, collagenase, hyaluronidase, fibrinolysin, gelatinase, elastase, and chondoitin sulfatase) and antiphagocytic factors (e.g., capsule, short-chain fatty acids, and immunoglobulin A [IgA], IgM, and IgG proteases)[29,30] Other enzymes secreted by nonsporing anaerobes are proteases, lipases, hyaluronidase, chondroitin sulfatase and neuraminidase which might play a role in infection by causing tissue cell destruction. In addition, ß-lactamase is known to inactivate antibiotics like penicillins & cephalosporins. [12, 28]

The virulence factors can be classified on the structural bases as Cellular & Extracellular or functionally as those helping the bacteria in ***adherence, invasiveness or overcoming host defense mechanisms.*** (Table.1) Important virulence factors which have been extensively studied are described below.

Table 1.

Capsule: Capsules were among the first known bacterial virulence determinants. The *B. fragilis* capsule was first analyzed with a prototype strain. Two distinct high-molecular-weight polysaccharides (PS-A and PS-B) that are co-expressed were described, which are known to mediate specific or nonspecific attachment [31,32] a third capsular polysaccharide (PS-C) was also found, and the biosynthetic loci involved were cloned and sequenced. [33, 34] Capsule can be readily demonstrated in light microscope by negative staining with India ink. Ruthenium red stained electron micrographs reveal that,

Table 1. Functional classification of virulence Factors of Nonsporing anaerobes

VIRULENCE FACTORS	
Helping in Bacterial Adhesion	Capsule, Fimbriae, Haemagglutinin, Lectin
Resisting Oxygen Toxicity.	Superoxide Dismutase, Catalase
Helping in Antiphagocytic activity.	Capsule, IgA, IgM, IgG proteases, Lipopolysaccharide, Volatile fatty acids
Favoring tissue destruction	Phospholipase C, Hemolysins, Proteases, Collagenase, Fibrinolysin, Neuraminidase, Heparinase, Chondroitin sulfatase, Glucuronidase, N-Acetylglucosaminidase, Volatile fatty acids, Hyaluronidase, Leucocidin, Lecithinase,

even within an individual strain of *B. fragilis* clinical isolates, one might observe a large capsule, a small capsule, and noncapsulate variants. Thus expression of the different capsular types is inheritable. [35, 36, 37] *B.fragilis* capsule stimulates a T cell-mediated response [38] which is intended to wall off the infection and protect the host from dissemination, but in fact, formation of an abscess protects the Bacteroides and neighboring bacteria from exposure to high concentrations of antibiotics and further attack from the immune mechanisms. The capsule promotes abscess production,[30, 39] acts as antiphagocytic,[40] adhesin [41, 42] and inhibits macrophage migration.[40] The pathogenicity of Bacteroides, and Fusobacterium, was demonstrated by their ability to induce subcutaneous abscesses in mice. Encapsulated starins were found to be more virulent as compared to the non-encapsulated isolates.[43] Brook I et al, reported the presence of piliated Bacteroides spp. mostly *B.fragilis* and *B.melaninogenicus,* 83% from blood, 78% from abscesses. On the contrary, only 10% of encapsulated strains were isolated from the faeces or pharynx of healthy persons ($P < 0.001$) [44]

Fimbriae: Fimbriae are the filamentous protein appendages found in variety of bacteria. The pioneering work of Duguid more than 30 years ago established the presence of fimbriae by agglutination of red blood cells from a range of species. Structurally they are made up of about 1000 protein subunits of mol.wt 17000 which form straight, tubular structure about 1 µm in length and 7-8 nm in diameter with a central hole.[45, 56, 47] Expression of fimbriae by bacteria is dependent on cultural conditions, and temperatures must be above 20^0C. Fimbriae appear to be a major adherence-mediating determinant of *P. gingivalis*. Investigations variously involving purified fimbriae, recombinant fimbrillin, isogenic mutants deficient in fimbriae production, and antibodies to fimbriae have revealed that the fimbriae per se mediate adherence to a variety of oral substrates and molecules. These include salivary molecules such as proline-rich proteins (PRPs), proline-rich glycoproteins, statherin, fibrinogen, fibronectin, and lactoferrin; oral epithelial cells; antecedent bacteria such as oral streptococci, and *A. naeslundii*.[48, 49] Pathogenic role of fimbriae in *P. gingivalis* infection has been documented in a number of studies. Immunization with purified fimbriae confers protection against periodontal destruction in a gnotobiotic rat model.[50] Moreover, insertional inactivation of the fimA gene, with concomitant loss of fimbria production, results in a phenotype significantly less able to cause periodontal bone loss in the gnotobiotic rat model.[51]

Hemagglutinins. Hemagglutinin proteins are established virulence factors for a number of bacterial species and *P. gingivalis* produces at least five hemagglutinating molecules. When expressed on the bacterial cell surface, hemagglutinins may promote colonization by mediating binding of bacteria to receptors (usually oligosaccharides) on human cells.The cloning of the first hemagglutinin gene (hagA) from *P. gingivalis* by Progulske-Fox et.al.[52] has led to major advances in our understanding of the genetic and functional complexities of the hemagglutination process. The hagA gene encodes a protein with a predicted molecular mass of 283.3 kDa (2,628 aa) and contains four contiguous direct 440- to 456-aa residue repeat blocks [53] Lepine et al.[54] constructed isogenic hag mutants of *P. gingivalis* 381 and examined their in-vitro adhesion phenotypes. Disruption of the hagA, hagB, or hagC gene in each case resulted in reduced hemagglutinating activity of cells, suggesting that all three genes were involved in determining the hemagglutination phenotype.[54] Emerging sequence data suggest that hemagglutinin-related sequences are not only present as independently expressed genes, e.g., hagA, but are also coexpressed with genes encoding proteolytic activities. It is proved that genetic and functional determinants of adhesion, hemagglutination, proteolysis, and fimbriation are inextricably linked. [55,56]

Superoxide Dismutase (SOD) and Catalase: Many pathogenic anaerobic species not only survive but are capable of growth at low oxygen tentions. *B.fragilis* for instance, may grow at oxygen tensions up to 8%. Protection from toxic effects of oxygen is provided in particular by the enzyme superoxide dismutase and catalase may play a role. [57] During initiation of an intra abdominal infection, oxygen tolerance is believed to allow the bacteria to survive in the oxygenated tissue of the abdominal cavity until *E.coli* and other synergistic organisms are able to reduce the redox potential at the site of infection. Additionally, this oxygen tolerance may help in surviving free radical production by the immune system PMNs. Superoxide dismutase is present in Bacteroides and Fusobacterium species in amounts varying from species to species and among strains within the same species.[58] Tally et al. have concluded that SOD in anaerobic bacteria is of clinical significance, after assaying twenty-two anaerobic bacteria isolated from infected sites and normal fecal flora for SOD, there was a correlation between the enzyme level and the oxygen tolerance, in that the aerotolerant organisms had SOD, whereas the extremely oxygen-sensitive isolates had low or undetectable enzyme. It is postulated that SOD may be a virulence factor that allows pathogenic anaerobes to survive in oxygenated tissues until the proper reduced conditions are established for their growth.[56, 59, 60] Catalase

that is released by the bacteria plays a role in protecting the bacteria from phagocytic effect. Bacteroides have been found to encode two major oxidative stress response genes, catalase,[61] superoxide dismutase,[62] as well as approximately 28 other oxygen-induced proteins.[63]

Lipopolysaccharide (LPS): LPS in *B. fragilis* has an unusual structure and is 10 to 1,000 times less toxic than that of *E. coli*.[64] The induction of endotoxin liberation on exposure to antibiotics was many times higher with *B. fragilis* than with the other species of the *B. fragilis* group, which may also help to explain why this species is particularly associated with clinical infections and higher mortality.[65] Bacteroides and Fusobacterium LPS activate complement by classical and mainly, the alternate pathways.[66] One of the biological effects of the activation is the generation of a split product, C5a, of fifth complement factor, which acts as a chemotactic factor for PMNs.[67] Brornson 1984 found that *B.fragilis* LPS activated Hageman factor, initiating the intrinsic pathway of coagulation. Thus it is possible that LPS may contribute to the local thrombophlebitis and subsequent septic embolization and metastatic abscess that occur in *B fragilis* infections.[68] Administration of *F. nucleatum* LPS into sterile root canals in monkeys (Macaca cynomolgus) produced inflammation and bone resorbtion.[69]

IgA, IgM and IgG proteases: Several virulent anaerobic species generate products that inhibit or destroy the humoral components of host's defenses. Many bacteria which colonize the mucous membranes produce an IgA protease which degrades secretory IgA. Immunoglobulin proteases elaborated by Bacteroides species are capable of dissolving and digesting tissue or cellular material.[70] Black pigmented species produce a range of proteolytic enzymes active against immunoglobulins and complement. Enzymes of Porphyromonas species (*P. gingivalis, P. asaccharolytica* and *P.endodontalis*) degrade IgA, IgM, IgG and plasma proteins such as C5 and the bacteriolytic component C3.[71] IgA proteases are also produced by *Prevotella melaninogenica* and *P.intermedia.*[72]

Short Chain Fatty Acids (SCFA): Various periodontal and root canal pathogens, such as the Bacteroides species, can produce significant amounts of short chain fatty acids (SCFA). The roles of SCFA in the pathogenesis of periodontal disease are still not fully understood. Jeng et al therefore investigated two main SCFA, butyrate and propionate, on the functional behavior of cultured human gingival fibroblasts (GF) such as cell growth, protein synthesis, cell adhesion capacity, and cell cycle progression. They found that Butyrate and propionate inhibited the growth of healthy and inflamed gingival fibroblasts in a dose dependent manner.[73] Succinate is an important short-chain fatty acid (SCFA) that is produced by both aerobic and anaerobic bacteria (e.g., *E. coli, K. pneumoniae,* Bacteroides spp., and Prevotella spp.); it accumulates in anaerobe-dominated infected sites [74] and inhibits the chemotaxis and phagocytosis of *E. coli* and *S. aureus.*[75, 76] Acetic butyric, succinic and propionic acids are produced by *P.gingivalis, B.fragilis and F.nucleatum* and High levels of succinic acid (>30 mmol) have been measured in clinical abscesses.[77]

Proteinases: The current status of over 15 years of work on AGNB proteinases is a somewhat confusing and still emerging functional studies are needed to resolve the complexities of proteinase production and regulation[77, 78] Of these two species, proteinases of *P.gingivalis* are the most intensively studied. During the last decade an impressive array of information has been accumulated with respect to the biochemical characterization of purified proteinases, structure of the genes encoding them, regulation of expression and the effects of these enzymes on host systems. In addition, studies on proteinase-deficient isogenic mutants have shed light on both their housekeeping functions and potential role(s) in the pathogenicity of periodontitis.[79] Among several proteinases produced by *P.gingivalis*, Arg- (Rgp) and Lys-gingipains (Kgp) are two individual cysteine proteinases which are clearly in the spotlight. Gingipains seem to be key players in subverting host defense systems with complement and neutrophils as the main target. Furthermore, the ability to interact with the cytokine networking systems has the potential to dysregulate the local inflammatory reaction.[56]

Collagenase: This enzyme catalyzes the degradation of collagen, a scleroprotein found in tendons, nails and hair. *P.melaninogenica* is one of the few bacteria that produce collagenase.[80] Cell extracts of *P .melaninogenica* strains with collagenolytic activity, when given with a live Fusobacterium species, produce more severe lesions in rabbits than does the organism or the extract given alone. Mayrand and Grenier studied collagenolytic activity of 12 species of oral bacteria, except for two species, all bacterial strains tested were capable of degrading at least one general protein substrate.[81] A number of substances used as adjuncts in periodontal therapy are also tested for their capacity to inhibit collagenase activity of *P. gingivalis.*[82]

Neuraminidase: It degrades neuraminic acid of mucosa (also called sialic acid), an intercellular cement of the epithelial cells. Neuraminidases are produced by many species of aerobic and anaerobic bacteria and can

contribute to their virulence [83, 84] Neuraminidase, the product of the *nanH* gene in *Bacteroides* species, cleaves mucin polysaccharides and enhances growth of the bacterium by generating available glucose.[85] This enzyme is found in many pathogenic bacteria and is generally considered a virulence factor and many strains of *B. fragilis* produce neuraminidase.[86-88] Neuraminidase can catalyze the removal of the sialic acid from host cell surfaces and from important immunoactive proteins such as IgG and some components of complement and may consequently disrupt important host functions. [89]

Regulation of Virulence gene Expression.

Bacteria in the tissues, experience dramatic environmental changes as a consequence of host factors. In response to these kinds of dynamic processes, bacteria often regulate gene expression to maintain optimal phenotypic properties. Expression of virulence factors in a wide range of bacteria is tightly regulated in response to environmental cues. [90] Although the study of gene regulation in nonsporing anaerobes is in its infancy, it is likely that an organism with such fastidious requirements with regard to oxygen and iron availability will regulate gene expression accordingly. During establishment of an infection, pathogenic bacteria use carefully regulated pathways of conditional gene expression to transition from a free-living form to one that must adapt to the host milieu. This transition requires the regulated production of both extracellular and cell-surface molecules, often termed virulence factors. Although the mechanisms of pathogenesis among different bacteria vary, the principles of virulence are generally conserved. Bacterial virulence may therefore offer unique opportunities to inhibit the establishment of infection or alter its course as a method of antimicrobial chemotherapy.[91,92]

Future research avenues

Despite the availability of voluminous literature decribing the infections caused by AGNB, studies on host parasite interactions especially the host interaction with factors eg. lipopolysachharide, capsular or other surface layer polysachharide, fimbriae, toxic protein, various enzymes and other extra cellular factors are rather meager. Direct detection of encapsulated AGNB species from the clinical specimens, probably helps in reducing the time, effort, and the expenses involved in comprahensive anaerobic isolation and identification techniques, furthermore, opening up the avenues for the development of novel and rapid diagnostic techniques. Development of serological tests could complement laboratory diagnosis of anaerobic infections. Evaluation of the protective effects of various antibodies may be useful in the development of immunoprophylaxis. [93]

A preliminary study done to identify protease activity in otorrhea samples from patients with otitis media, which examined the ability of selective protease inhibitors to decrease protease activity a protease assay that used azocasein as the substrate to quantify protease activity, with and without addition of selective protease inhibitors. A statistically significant ($p<0.05$) decrease in protease activity was observed. This is the first study to quantify protease activity and inhibition by selective protease inhibitors in human otorrhea. Protease inhibitors effectively decreased protease activity in most cases and in addition to standard antibiotic therapy might prove beneficial in the treatment of otitis media.[94] In future, attemptss in this regard namely, sequencing of bacterial genomes may open new perspectives for identification of targets for treatment of infectious diseases. Garbom et al., have identified a set of novel virulence-associated genes (vag genes) by comparing the genome sequences of six human pathogens that are known to cause persistent or chronic infections in humans: *Y.pestis, N.gonorrhoeae* etc. Therefore, they propose that the corresponding vag gene products may constitute novel targets for antimicrobial therapy and that some vag mutants could serve as carrier strains for live vaccines. [95]

Gougen et al., considered potential of extracellular protease as targets for drug development.[96] Arg and Lys-gingipains of *P. gingivalis* are important virulence determinants in periodontal disease and may correspond to targets for immune or drug based treatment strategies.In the investigation carried out by Curtis et al., which aimed at determination of the enzymes representing the most promising molecular target for protease inhibitor-based therapy and to examine the effectiveness of the resultant compound in a murine virulence assay. The virulence of the mutants indicated that Kgp is a promising drug target. These data emphasize the conclusion that Kgp is an important factor for both nutrition and virulence of *P.gingivalis* and that, inhibitors of this enzyme may have therapeutic potential for the control of *P. gingivalis* infections. It was concluded that, protease inhibitors may be a potentially novel class of antimicrobial agents with relevance to the control of other bacterial pathogens. [97]

Conclusion

Existing data on extensive studies on biochemical and genetic analysis of the key virulence factors points to an essential, but highly complex roles of these factors in pathogenesis and provide scope further research. Some virulence factors, especially the capsule and proteinases can be potential targets for rapid diagnosis and treatment of anaerobic infections, possibly in the form of development of novel synthetic enzyme inhibitors.

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