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DETECTION OF EXTRA-CELLULAR ENZYMES OF ANAEROBIC GRAM-NEGATIVE BACTERIA FROM CLINICALLY DISEASED AND HEALTHY SITES

*JM Nagmoti, CS Patil, MB Nagmoti, MB Mutnal

Abstract

Anaerobic gram-negative bacteria (AGNB) produce enzymes that play a significant role in the development of disease. We tested 50 AGNB isolates, 25 each from clinically diseased and healthy human sites for in vitro production of caseinase, collagenase, etc. Majority of the isolates were Bacteroides fragilis and Porphyromonas gingivalis, which more commonly produced collagenase and haemolysin. Comparatively larger number of clinical AGNB produced collagenase ($P = 0.004$). No such difference was observed with other enzymes. Hence, collagenase is probably one of the key virulence markers of pathogenic AGNB, and the inhibitors targeting collagenases might help in the therapy of anaerobic infections.

Key words: Anaerobic gram-negative bacteria, extra-cellular enzymes, virulence factors

Infections due to anaerobes are common and associated with considerable morbidity and potential mortality. Anaerobic gram-negative bacteria (AGNB) belonging to the genera Bacteroides, Prevotella and Porphyromonas are most commonly encountered in clinical infections.[1,2] Clinical interests in these organisms are linked to the therapeutic problems usually encountered in treating mixed infections. Despite their clinical relevance, very little is known about the pathogenic mechanism of anaerobic infections.

Vast majority of anaerobic infections arise from the indigenous flora of the host. Although AGNB constitute a major portion of human microflora, very few exhibit marked pathogenic potential and are responsible for the majority of the infections.[3] There is some evidence that hydrolytic and proteolytic enzymes like chondroitinsulphatase, gelatinase, collagenase, caseinase and lipase secreted by AGNB play a role in the infectious process.[4] It has been observed that, the collagenases of P. gingivalis hallmark its presence at the infection sites.[4,5]

In the light of the above facts and in view of detecting key virulence factors of AGNB and shedding light on the diagnostic and possible therapeutic approaches by targeting the virulence factors of AGNB, the present study has made an attempt for the detection of cellular and extra-cellular virulence markers of AGNB isolates from both infected and healthy human sites.

Materials and Methods

A total of 50 AGNB isolates from clinically diseased (25 in number) and healthy (25 in number) sites were tested for the production of extra-cellular enzymes. (collagenase, caseinase, catalase, chondroitin sulfatase, gelatinase, haemolysin, hyaluronidase and lipase). Clinical isolates were from periodontitis orofacial and neck infections, brain, intra-abdominal abscesses and others. Commensal AGNB isolates were from healthy mucosal sites of oral cavity, upper respiratory tract, intestine and vagina. The isolation of AGNB was done by using ‘Internal gas generating system’ and their identification was done as per the standard techniques.[6,7]

Collagenase production was tested on an agar medium containing human collagen (Sigma C-9879); after inoculating the broth culture of the isolate and incubating anaerobically, the plates were stained with Coumassie brilliant blue. Collagenase (Sigma C-6885) served as a positive control.[8]

Caseinase producing AGNB on casein-containing medium produced zones of clearance beneath and around the areas of growth due to the digestion of casein. Catalase production was tested by the standard technique.[7] Hyaluronic acid (Sigma H-1876) and chondroitin sulphate (Sigma C-4134) containing plates were inoculated with the isolates and incubated anaerobically, the plates were stained with Coumassie brilliant blue. Collagenase (Sigma C-6885) served as a positive control.[8]

For gelatinase production test, the organisms were spot inoculated on 0.4% gelatin agar. After satisfactory growth, the cultures were flooded with mercuric chloride solution, which rapidly denatured and rendered opaque any
unhydrolyzed gelatin. Protease (Sigma P-5130) served as positive control.[11] Lipolytic organisms produced a restricted opacity and pearly layer on egg yolk medium. Purified lipase (Sigma L-2253) served as the positive control.[12]

Hemolysin production was tested by adding 50 µL of culture supernatant to the red blood cell suspension. The results were read after incubation for 18 h.[11] Statistical analysis of the data was done by using ‘Z’ test, with the level of significance set at a value of $P < 0.05$.

**Results**

Of the 25 AGNB isolates from the clinical group, 10 were *B. fragilis*, 7 were *P. gingivalis*, 5 were *P. melaninogenica* and 3 were *Fusobacterium nucleatum*. Similar types and the number of isolates as mentioned above were obtained from commensal group as well. The AGNB were found to be highly variable with respect to the production of enzymes. Enzymes most often produced were collagenase (60%) followed by hemolysin (32%), Catalase (28%) and others by the clinical isolates, whereas commensal AGNB isolates less frequently produced the enzymes. In general, both clinical and commensal isolates of *P. gingivalis* and *B. fragilis* were more active in enzyme production (Tables 1 and 2). None of the AGNB produced all of the enzymes tested.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Clinical isolates (n = 25)</th>
<th>Commensal isolates (n = 25)</th>
<th>Z-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyaluronic acid</strong></td>
<td>12 (48)</td>
<td>6 (24)</td>
<td>1.77</td>
<td>0.768</td>
</tr>
<tr>
<td><strong>Chondroitin sulfatase</strong></td>
<td>7 (28)</td>
<td>3 (12)</td>
<td>1.41</td>
<td>0.158</td>
</tr>
<tr>
<td><strong>Catalase</strong></td>
<td>7 (28)</td>
<td>3 (12)</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Collagenase</strong></td>
<td>15 (60)</td>
<td>5 (20)</td>
<td>2.89</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Caseinase</strong></td>
<td>12 (48)</td>
<td>6 (24)</td>
<td>1.77</td>
<td>0.768</td>
</tr>
<tr>
<td><strong>Gelatinase</strong></td>
<td>12 (48)</td>
<td>6 (24)</td>
<td>1.77</td>
<td>0.768</td>
</tr>
<tr>
<td><strong>Hemolysin</strong></td>
<td>8 (32)</td>
<td>5 (20)</td>
<td>0.97</td>
<td>0.331</td>
</tr>
<tr>
<td><strong>Lipase</strong></td>
<td>7 (28)</td>
<td>3 (12)</td>
<td>1.41</td>
<td>0.158</td>
</tr>
</tbody>
</table>

Interestingly, the collagenase production by clinical isolates was significantly higher ($P = 0.004$) as compared to the commensal AGNB isolates (Table 3). On the other hand,

### Table 1: Enzymes produced by clinical anaerobic gram-negative bacterial isolates

<table>
<thead>
<tr>
<th>AGNB</th>
<th>Number of bacteria producing enzymes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAase</td>
</tr>
<tr>
<td>Bf (10)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Pg (7)</td>
<td>5 (71.4)</td>
</tr>
<tr>
<td>Pm (5)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Fn (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total 25</td>
<td>12 (48)</td>
</tr>
</tbody>
</table>


### Table 2: Enzymes produced by commensal anaerobic gram-negative bacterial isolates

<table>
<thead>
<tr>
<th>AGNB</th>
<th>Number of bacteria producing enzymes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAase</td>
</tr>
<tr>
<td>Bf (10)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Pg (7)</td>
<td>2 (28.5)</td>
</tr>
<tr>
<td>Pm (5)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Fn (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total 25</td>
<td>6 (24)</td>
</tr>
</tbody>
</table>


### Table 3: Comparison between enzymes produced by the clinical and commensal anaerobic gram-negative bacterial isolates

```
<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Clinical isolates (n = 25)</th>
<th>Commensal isolates (n = 25)</th>
<th>Z-value</th>
<th>P-value</th>
</tr>
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<tbody>
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<tr>
<td><strong>Lipase</strong></td>
<td>7 (28)</td>
<td>3 (12)</td>
<td>1.41</td>
<td>0.158</td>
</tr>
</tbody>
</table>
```
production of other enzymes did not differ significantly between clinical and commensal AGNB isolates ($P = >0.05$).

**Discussion**

*Porphyromonas gingivalis* and *B. fragilis* are the commonest AGNB isolated from human anaerobic infections and were also found to produce more number of enzymes tested and hence, the most virulent species among AGNB.

*Porphyromonas gingivalis* expressed the highest enzymic activity, followed by *B. fragilis* and others, as has been shown by many other studies.[3,12-15]

A positive relationship appears to exist between the production of collagenase enzyme by a given strain and virulence of that strain. All the collagenase-producing organisms invariably produced other enzymes as well, suggesting it to be a key virulence marker of AGNB. It has been shown that *P. gingivalis*, *B. fragilis* and *F. nucleatum* disrupt the epithelial cells and degrade a range of plasma proteins: albumin, fibrinogen, immunoglobulins, complement components, etc.[1,3,12]

Although some of the healthy AGNB isolates produced few enzymes, except for collagenase production there was no statistically significant difference in the production of other enzymes between these groups, which suggested that the commensal AGNB can exist in harmony with the host and the disease episodes ensuing from the shift in the ecological balance within the complex microenvironment.

It is concluded that AGNB produce a variety of hydrolytic and proteolytic enzymes, but that none of these enzymes are universally present in them. *Bacteroides fragilis* and *P. gingivalis* are the most virulent AGNB species. Collagenase is a key virulence marker of pathogenic AGNB and other enzymes being contributory for the pathogenesis of AGNB infections. Collagenase inhibitors might have a therapeutic role in AGNB infections. The study supports further investigations on qualitative and quantitative analyses of key enzymes, which help in the development of newer diagnostic tools.

**References**


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