ANTIBACTERIAL ACTIVITY OF Rhynocoris marginatus (FAB.) AND Catamirus brevipennis (SERVILE) (HEMIPTERA: REDUVIIDAE) VENOMS AGAINST HUMAN PATHOGENS


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ABSTRACT: The reduviid predators Rhynocoris marginatus (Fab.) and Catamirus brevipennis (Servile) use their venoms to paralyze their preys. We detected the antibacterial activity of R. marginatus and C. brevipennis venoms against seven Gram-negative and four Gram-positive bacteria by using the disc diffusion method. Rhynocoris marginatus venom exhibited antibacterial activity against four Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhimurium) and one Gram-positive (Streptococcus pyogenes). Catamirus brevipennis venom showed antibacterial activity against six Gram-negative (Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, and Salmonella typhimurium) and three Gram-positive (Bacillus subtilis, Staphylococcus aureus, and Bacillus sphaericus) bacteria. Both C. brevipennis (90.91%) and R. marginatus (45.45%) venoms were more effective against Gram-negative bacteria (80% and 70% for R. marginatus and C. brevipennis, respectively). The venoms of both reduviid predators are composed of low molecular weight proteins (7-33 kD).

KEY WORDS: antibacterial activity, reduviid saliva, protein content, peptides.

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INTRODUCTION

Reduviids have been recognized as important natural enemies against several field crop pests, especially hemipteran and lepidopteran pests (23-27). *Rhynocoris marginatus* (Fab) and *Catamirus brevipennis* (Servile) are polyphagous, multivoltine, entomophagous and crepuscular bugs. The vast majority of venomous arthropods are predators and feed exclusively on living prey which can be other arthropods, mainly insects (32). Fabre (9) demonstrated that the insect oral secretion could be paralyzant. Reduviid predators immobilize their prey by injecting venomous saliva into them (1, 13).

The large number of antibacterial molecules that are used as therapeutic agents target the bacterial metabolic machinery. The salivary venom of reduviids consists of digestive enzymes, which immobilize the prey and help in its external digestion (3, 5). In literature, there are reports on the biochemistry of venoms from reduviids such as *Peirates affinis* Serville (6), *Platymeris rhadamanthus* [Gerst] (7), *Acanthaspis pedestris* [Stal] (19), *Haematorrhophus nigroviolaceus* [Reuter] (31), *Holotrichius innesi* [Horrvath], *Peirates turpis* [Walker], *Agriosphodrus dohni* [Stal] and *Isyndus obscurus* [Dallas] (10), but none from *C. brevipennis* and *R. marginatus*.

Venom from predators including reduviids has been a great source of novel peptides with a notable potential for agricultural and medicinal use. The toxic saliva of predatory assassin bugs contains a complex mixture of small and large peptides for diverse uses such as immobilizing and defending against competitors and predators. Most research on venoms has focused on the identification, isolation and purification of proteins and peptides with pharmacological activity like neurotoxins (8, 20, 22) and antimicrobial peptides, whose presence in the venom has been suggested to be a defense mechanism against infections arisen during ingestion of the prey (29, 30).

The prey immune system not only plays a major role in dealing with large foreign bodies, but also eliminates microorganisms from its hemocoel. For the predator, it may be advantageous to prey using venom with antimicrobial factors, which could help to protect immunocompromised preys from opportunistic pathogens. This strategy could contribute to the survival of predators. Broad-spectrum antimicrobial activity has been reported for venom or its peptides purified from spiders such as *Cupiennius salei* (12), *Vespa crabro* (15), *Anoplius samarischenis* (14), and *Protopolybia exigua* [Saussure] (18). Antibacterial activity has been widely reported
in venoms from different snakes including *Pseudechis australis* (28). The presence of these antimicrobial peptides in venom has been suggested to be a defense mechanism against infections that may arise with the ingestion of prey (30). As far as we are aware, antibacterial activity in reduviid venom has not been reported. Thus, this study was undertaken to verify the role of antimicrobial activity of two reduviids venoms on selected human pathogens.

**MATERIALS AND METHODS**

**Reduviid collection and rearing**

The reduviid *Rhynocoris marginatus* and *Catamirus brevipennis* were collected from Sivanthipatti agroecosystems (77° 47’ E and 8° 30’ N), Tirunelveli District, Tamil Nadu, India. They were reared on fifth instar *Spodoptera litura* (Fab.) larvae kept in plastic containers (7X6) under laboratory condition (28±2°C, 70%-80% RH, and 11 dark : 13 light).

**Venom collection**

Venom was milked using a capillary tube by simply pressing the insects abdomen with the hands. The venom collected from both male and female was stored at 4°C for further study.

**Bacterial cultures**

*Escherichia coli* (NCIM – National Collection of Industrial Microorganisms, 2068), *Pseudomonas aeruginosa* (NCIM 2862), *Salmonella typhimurium* (NCIM 2501), *Klebsiella pneumoniae* (NCIM 2098), *Proteus mirabilis* (NCIM 3011), *Bacillus subtilis* (NCIM 2063), *Staphylococcus aureus* (NCIM 2079), *Bacillus sphaericus* (NCIM 3019), *Proteus vulgaris* (NCIM 2001), *Enterobacter aerogenes* (NCIM 2003), and *Streptococcus pyogenes* (NCIM 2943) were obtained from the Biochemical Division of National Chemical Laboratory, Pune, Maharashtra, India. The strains were routinely cultured and maintained in nutrient agar.

**Antibacterial bioassay**

The venom antibacterial activity was tested against the eleven pathogenic bacteria mentioned above using the disc diffusion method (2). Inocula were prepared from
exponential-phase culture in nutrient broth. Nutrient agar plates were prepared and inocula were seeded by the cotton swab method. Venoms were applied to sterile disc (Hi-Media, Mumbai), dried and placed on the seeded agar plates; samples were run in duplicate. As positive control we used standard antibiotic discs (10 μg/ml each): penicillin for *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus sphaericus*, *Streptococcus pyogenes*; amikacin for *Proteus mirabilis*, *Klebsiella pneumoniae*; carbenicillin for *Proteus vulgaris*; cephalexin for *Enterobacter aerogenus*; methicillin for *Staphylococcus aureus*; and ampicillin for *Salmonella typhimurium*. Phosphate buffered saline (PBS, 10 μg/ml) was used as negative control. After 24 hours of incubation at 37°C, the diameter of inhibition zones (IZ) surrounding the disc formed by diffusion of the venom was measured. From the results, the activity index (AI) was calculated using the following formula: AI = venom IZ (mm) / standard IZ (mm).

**Protein concentration**

Protein content in the venom samples was estimated by using bovine serum albumin as standard at 610 nm (17).

**Gel electrophoresis**

SDS-PAGE was performed using 12%-acryl amide gel at 120V and 20mA (16). Venom samples were dissolved in 20μl of double distilled water plus 5μl of sample buffer (0.001% mercaptoethanol and 75% of 0.313 M Tris-HCl : 10% glycerol) and 0.001% bromophenol blue (pH 6.8). They were boiled for two minutes, shaken in vortex for 30 seconds and applied to the gel. After electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue R250 solution (containing 50% methanol and 12% acetic acid) and destained with 30% methanol and 10% acetic acid to reveal proteins. To estimate molecular weights, we used standard molecular weight markers, namely Genei SDS 70L kit (PMW-B standard, Genei Pvt. Ltd., Bangalore, India).
Statistical analysis
The activity index (AI) of the reduviid venoms was converted to correlation coefficient using the Statistica software. Protein content was subjected to Duncan’s Multiple Range Test (DMRT); values were considered significant at 5% level.

RESULTS
The sensitivity of 11 bacterial strains against the two reduviid predators Rhynocoris marginatus and Catamirus brevipennis venoms was assessed. Both venoms showed antibacterial activity against the 11 bacterial strains tested. Moderate sensitivity percentage was observed with Gram-negative organisms (70%) followed by the Gram-positive (30%) against C. brevipennis venom. The highest sensitivity was recorded for Gram-negative (80%) followed by Gram-positive microorganisms (20%) against R. marginatus venom.

Table 1 shows the antibacterial spectrum of adult R. marginatus and C. brevipennis venoms in terms of inhibition zone (IZ) and activity index (AI). Phosphate buffered saline (PBS) had no inhibitory effect on the bacteria tested. Reduviid venom generally inhibits the growth of Gram-negative as well as Gram-positive bacteria. Catamirus brevipennis venom showed antibacterial activity against all the tested bacteria, except for Streptococcus pyogens. Its AI was the highest against Proteus mirabilis (0.52) followed by Proteus vulgaris (0.49) and Enterobacter aerogenes (0.33). Rhynocoris marginatus venom showed maximum AI against Escherichia coli (0.56), followed by Pseudomonas aeruginosa (0.44), Proteus vulgaris (0.36), and Salmonella typhimurium (0.32). However, no inhibitory effect was recorded against Proteus mirabilis, Bacillus subtilis, Klebsiella pneumoniae, Enterobacter aerogenes, Staphylococcus aureus, and Bacillus sphaericus. Comparatively, C. brevipennis venom had broad-spectrum and higher antibacterial activity than R. marginatus venom (Table 1). The activity indexes of C. brevipennis and R. marginatus venoms were negatively correlated (-0.18, p=0.615453).

Total protein content of the tested reduviid venoms was analyzed using the Lowery method, being higher in the venoms from female (85.71 μg/ml) and male (26.6 μg/ml) C. brevipennis than in those from female (52.8 μg/ml) and male (22.9 μg/ml) R. marginatus; they were statistically significant at 5% level according to the DMRT. Protein profiles of venoms from male and female R. marginatus showed two different
molecular weight polypeptides. Between the two polypeptides, 7-kD polypeptides were common in both male and female venoms. The remaining band was different between sexes: 14 kD for male and 16 kD for female. Protein profile of male C. brevipennis venom also showed two polypeptides of different molecular weights and that of female C. brevipennis venom showed six polypeptides of different molecular weights. Two polypeptides (18 and 21 kD) were common for both sexes of C. brevipennis. However, 33-kD, 30-kD, 25-kD and 23-kD polypeptides were observed only in females.

Table 1: Inhibition zone (IZ; mm) and activity index (AI) of Rhynocoris marginatus and Catamirus brevipennis venoms against selected human pathogens.

<table>
<thead>
<tr>
<th>Microorganisms used</th>
<th>Standard</th>
<th>Rhynocoris marginatus venom</th>
<th>Catamirus brevipennis venom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IZ</td>
<td>AI</td>
<td>IZ</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>17</td>
<td>9.6</td>
<td>0.56</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>17</td>
<td>7.5</td>
<td>0.44</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>22</td>
<td>8.0</td>
<td>0.36</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
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<td>-</td>
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<tr>
<td>Staphylococcus aureus</td>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus sphaericus</td>
<td>17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>17</td>
<td>8.5</td>
<td>0.50</td>
</tr>
<tr>
<td>Salmonella typhimurim</td>
<td>23</td>
<td>7.5</td>
<td>0.33</td>
</tr>
</tbody>
</table>

- no activity was observed.

DISCUSSION

From the results, it is very clear that the antibacterial activity of the reduviid venoms differed according to the bacterial species used. These data confirm the general features of Gram-positive and Gram-negative antibacterial proteins, which can induce non-specific immunity in insects (4). Differences in the susceptibility of the tested bacteria can be attributed to the molecular characteristics of the antibacterial factors present in the reduviid venom. Insect antibacterial factors are fairly non-specific in nature. According to previous studies, insect hemolymph showed a broad-spectrum antibacterial action (2). The present study evidences that the peptides
present in *R. marginatus* and *C. brevipennis* venoms might be involved in their antibacterial activity. However, antibacterial activity of *C. brevipennis* was not detected against a Gram-positive bacterium (*Streptococcus pyogenes*). Similarly, antibacterial activity of *R. marginatus* was not detected against the Gram-positive bacteria *Bacillus subtilis*, *Staphylococcus aureus*, and *Bacillus sphaericus*. So, to detect antibacterial effect against both Gram-positive and Gram-negative bacteria, using the plate growth assay, a relatively large amount of proteins from *R. marginatus* and *C. brevipennis* venoms is required. Plate growth assay method is generally used to detect venom antibacterial activity. Both *R. marginatus* and *C. brevipennis* venoms are comprised of many proteins and the proportion of these proteins responsible for the antibacterial activity is not known. Hence this type of study is a prerequisite to isolate and identify the antibacterial proteins present in the reduviid venom. The antibacterial activity detected could be due to one or several peptides. Earlier findings reported that some or all of these activities might be due to the non-selective cytotoxic venom protein melittin, an abundant polypeptide in the venom of the honeybee *Apis mellifera* (11). Recently, it has been reported that the presence of protopolybia in *Protopolybia exigua* (Saussere) venom is responsible for its antibacterial activity (18).

Many of the characterized insect antimicrobial peptides inhibit both Gram-positive and Gram-negative bacteria (4). However, short-chain proline-rich antimicrobial peptides have been found to be active mainly against Gram-negative bacteria. Peptides with \( \sim 12 \) and/or \( \sim 13 \) residues exhibited potent broad-spectrum antibacterial activities. These include indolicidin, bacteriocins and tigerinins (21). Generally, the peptides bind to the lipopolysaccharides layer in the outer membrane of Gram-negative bacteria and disrupt its structure, which allows the entrance of peptide molecules to the inner membrane. The peptides insert into the inner membrane and break its permeability barrier, which results in cell death. The identified peptides have been used as drugs. To determine whether *R. marginatus* and *C. brevipennis* venoms inhibit exclusively Gram-negative bacteria, further studies are needed using a wider spectrum of Gram-positive and Gram-negative bacteria.
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