The Ovine Cathelicidin SMAP29 Kills Ovine Respiratory Pathogens In Vitro and in an Ovine Model of Pulmonary Infection

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Abstract
Cathelicidins are antimicrobial peptides from sheep (SMAP29 and SMAP34), rabbits (CAP11 and CAP18), rodents (CRAMP), and humans (FALL39, LL37, and h/CAP18). In a broth microdilution assay against nine ovine pathogens, SMAP29, SMAP34, mouse CRAMP, CAP18, CAP18\textsubscript{31}, CAP18\textsubscript{28}, CAP18\textsubscript{22}, and CAP18\textsubscript{21a} were the most active, with MICs as low as 0.6 μg/ml. Other cathelicidins were less active. In lambs with pneumonia, 0.5 mg of SMAP29 reduced the concentration of bacteria in both bronchoalveolar lavage fluid and consolidated pulmonary tissues. Hence, the antimicrobial activity of SMAP29 suggests that it has applications in the treatment of respiratory tract infections.

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Cathelicidins are antimicrobial peptides from sheep (SMAP29 and SMAP34), rabbits (CAP11 and CAP18), rodents (CRAMP), and humans (FALL39, LL37, and h/CAP18). In a broth microdilution assay against nine ovine pathogens, SMAP29, SMAP34, mouse CRAMP, CAP18, CAP1831, CAP1828, CAP1822, and CAP1821a, were the most active, with MICs as low as 0.6 μg/ml. Other cathelicidins were less active. In lambs with pneumonia, 0.5 mg of SMAP29 reduced the concentration of bacteria in both bronchoalveolar lavage fluid and consolidated pulmonary tissues. Hence, the antimicrobial activity of SMAP29 suggests that it has applications in the treatment of respiratory tract infections.

Innate host defenses in the respiratory tract of sheep are currently being examined as models for airway bacterial clearance (2), cystic fibrosis (2, 3, 10–13), and Pseudomonas aeruginosa pneumonia (6, 7). SMAP29, a cathelicidin from sheep, is also being considered as a therapeutic agent against microbial infections (5, 9), including P. aeruginosa associated with chronic respiratory inflammation in cystic fibrosis patients (14). Although SMAP29 is highly effective against P. aeruginosa PA01 and other common gram-positive and gram-negative organisms (14), its efficacy against a panel of pathogens originating from the same host species is unknown. Therefore, a broth microdilution assay was used to obtain both MICs and minimum bactericidal concentrations (MBCs) of SMAP29 against a panel of ovine pathogens (15, 18). SMAP29, mouse CRAMP, rat CRAMP, FALL39, FF21, CAP18, and truncated congeners of CAP18 were included for comparison and synthesized as previously described (14). Stock solutions of peptides were diluted in 0.4% bovine serum albumin containing 0.02% acetic acid (0.16 to 80.00 μg/ml) and added to polypropylene microtiter plates (Sigma, St. Louis, Mo.); 10 mM sodium phosphate buffer (pH 7.2) with 140 mM NaCl (PBS) was added to control wells. Mueller-Hinton broth containing a 10^3-CFU/ml concentration of Manheimia haemolytica serovar 1 (strain 82-25), 2, or 6; Pasteurella multocida serovar 4; Salmonella enterica subspp. arizonae; ovine Pasteurella multocida serovar 3A (strain P-2062); Klebsiella pneumoniae ATCC 10031; Corynebacterium pseudotuberculosis ATCC 19410; Staphylococcus aureus; or P. aeruginosa PA01 was added. Mueller-Hinton broth was added to wells containing PBS and used as the plate blank. After 24 and 48 h at 37°C, the optical density of bacterial growth was determined (Spectromax Microplate Reader; Molecular Devices Corp., Sunnyvale, Calif.). The MIC (e.g., the lowest concentration of peptide that reduced growth by more than 50% compared to control wells) and the MBC (e.g., the lowest concentration of peptide that prevented well growth) were determined.

Peptides were active against PA01, included as a susceptible control, and these results (Tables 1 and 2) were similar to those previously reported for other assays (14). Wide ranges of activity against gram-negative bacteria were seen with mouse CRAMP (MIC, 3.3 to 15.0 μg/ml), rat CRAMP (MIC, 5.0 to 20.0 μg/ml), FALL39 (MIC, 2.1 to ≥20.0 μg/ml), FF21 (MIC, 3.3 to ≥20.0 μg/ml), and SMAP34 (MIC, 5.0 to 11.7 μg/ml) (Table 1). MBCs were comparable to the corresponding MICs. SMAP29 (MIC, 0.6 to 2.5 μg/ml) and CAP18 (MIC, 1.3 to 10.0 μg/ml) had the highest activities. The activity of CAP18 congeners also varied, and CAP1831, CAP1828, CAP1822, and CAP1821a were very active against gram-negative bacteria, but the others were not (Table 2). All peptides were less active against C. pseudotuberculosis and S. aureus (MIC, 10.0 to ≥20.0 μg/ml).

In CAP18, truncations of the N terminus did not adversely affect the antimicrobial activity of congeners with a complete C terminus (e.g., CAP1831 and CAP1828), and truncations of the C terminus did not adversely affect the antimicrobial activity of congeners with a complete N terminus (e.g., CAP1831a). However, a severe truncation of the N terminus (e.g., CAP1828) or simultaneous truncations at both termini (e.g., CAP1821b, CAP1819, CAP1817, CAP1815, CAP1813a, and CAP1813b) decreased antimicrobial activity. Unfortunately, no central region with all of the antimicrobial activity could be identified. Instead, congeners needed to be at least 21 or more residues long to be effective. For PA01, this number could drop to a minimum of 18 residues (e.g., CAP1812a). Whether this size is related to the amphiphatic structure of the peptide needed to form pores or otherwise disrupt the cytoplasmic membrane is not known.

* Corresponding author. Mailing address: Respiratory Diseases of Livestock Research Unit, National Animal Disease Center, 2300 Dayton Ave., P.O. Box 70, Ames, IA 50010. Phone: (515) 663-7534. Fax: (515) 663-7588. E-mail: kbrogden@nadc.ars.usda.gov.
### TABLE 1. Antimicrobial activities of synthetic cationic peptides against *P. aeruginosa* PAO1 and ovine pathogens

<table>
<thead>
<tr>
<th>Organism</th>
<th>mCRAMP MIC&lt;sup&gt;a&lt;/sup&gt; (μg/ml)</th>
<th>rCRAMP MIC</th>
<th>SMAP29 MBC&lt;sup&gt;b&lt;/sup&gt; (μg/ml)</th>
<th>SMAP34 MBC</th>
<th>FALL39 MBC</th>
<th>FF21 MBC</th>
<th>CAP18 MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>3.3 ± 0.7</td>
<td>6.7 ± 1.4</td>
<td>10.0 ± 0.0</td>
<td>20.0 ± 0.0</td>
<td>0.8 ± 0.2</td>
<td>8.0 ± 0.2</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td><em>M. haemolytica</em> serovar 1</td>
<td>15.0 ± 2.9</td>
<td>13.3 ± 2.7</td>
<td>10.0 ± 0.0</td>
<td>8.3 ± 1.4</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>7.5 ± 1.5</td>
</tr>
<tr>
<td><em>M. haemolytica</em> serovar 2</td>
<td>8.3 ± 1.4</td>
<td>8.3 ± 1.4</td>
<td>8.3 ± 1.4</td>
<td>8.3 ± 1.4</td>
<td>0.8 ± 0.2</td>
<td>3.8 ± 2.6</td>
<td>5.0 ± 0.0</td>
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<tr>
<td><em>M. haemolytica</em> serovar 6</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>10.0 ± 0.0</td>
<td>10.0 ± 0.0</td>
<td>1.3 ± 0.0</td>
<td>1.3 ± 0.0</td>
<td>5.8 ± 1.8</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>3.3 ± 0.7</td>
<td>5.0 ± 2.0</td>
<td>16.7 ± 2.7</td>
<td>20.0 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>6.7 ± 1.4</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>3.3 ± 0.7</td>
<td>5.0 ± 2.0</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>2.5 ± 0.0</td>
<td>3.3 ± 0.7</td>
<td>10.0 ± 0.0</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>arizonae</em></td>
<td>7.5 ± 1.5</td>
<td>20.0 ± 0.0</td>
<td>20.0 ± 0.0</td>
<td>&gt;20.0</td>
<td>0.8 ± 0.2</td>
<td>1.5 ± 0.5</td>
<td>11.7 ± 3.6</td>
</tr>
<tr>
<td><em>P. tychovia</em> serovar 4</td>
<td>10.0 ± 0.0</td>
<td>10.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>2.5 ± 0.0</td>
<td>3.3 ± 0.7</td>
<td>10.0 ± 0.0</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td><em>S. enterica</em> pseudotuberculosis</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td><em>P. trehalosi</em></td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td><em>C. pseudotuberculosis</em></td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC, the lowest concentration of peptide that reduces growth by more than 50% compared to control wells. Results are means ± standard errors of the means (three replications).

<sup>b</sup> The MBC, determined by culturing 150 μl from each of the first three wells showing no visible growth (50 μl per spot) on Trypticase soy agar containing 5% defibrinated sheep blood and incubating the plates overnight at 37°C, was the lowest concentration of peptide that prevents any growth. Results are means ± standard errors of the means (three replications).

### TABLE 2. Antimicrobial activities of truncated congeners of CAP18 against *P. aeruginosa* PAO1 and ovine pathogens

<table>
<thead>
<tr>
<th>Organism</th>
<th>CAP18&lt;sub&gt;S3&lt;/sub&gt; MIC&lt;sup&gt;a&lt;/sup&gt; (μg/ml)</th>
<th>CAP18&lt;sub&gt;S5&lt;/sub&gt; MBC&lt;sup&gt;b&lt;/sup&gt; (μg/ml)</th>
<th>CAP18&lt;sub&gt;S7&lt;/sub&gt; MBC</th>
<th>CAP18&lt;sub&gt;S9&lt;/sub&gt; MBC</th>
<th>CAP18&lt;sub&gt;S11&lt;/sub&gt; MBC</th>
<th>CAP18&lt;sub&gt;S13&lt;/sub&gt; MBC</th>
<th>CAP18&lt;sub&gt;S15&lt;/sub&gt; MBC</th>
<th>CAP18&lt;sub&gt;S17&lt;/sub&gt; MBC</th>
<th>CAP18&lt;sub&gt;S19&lt;/sub&gt; MBC</th>
<th>CAP18&lt;sub&gt;S21&lt;/sub&gt; MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>1.3 ± 0.0</td>
<td>3.3 ± 0.7</td>
<td>4.2 ± 0.7</td>
<td>10.0 ± 0.0</td>
<td>&gt;20.0</td>
<td>13.0 ± 0.0</td>
<td>1.7 ± 0.3</td>
<td>1.3 ± 0.0</td>
<td>1.3 ± 0.0</td>
<td>20.0 ± 0.0</td>
</tr>
<tr>
<td><em>M. haemolytica</em> serovar 1</td>
<td>10.0 ± 0.0</td>
<td>8.3 ± 1.4</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>11.7 ± 3.6</td>
<td>10.0 ± 0.0</td>
<td>25.0 ± 0.0</td>
<td>25.0 ± 0.0</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td><em>M. haemolytica</em> serovar 2</td>
<td>9.2 ± 0.9</td>
<td>11.3 ± 5.1</td>
<td>40.0 ± 2.5</td>
<td>58.0 ± 1.8</td>
<td>7.5 ± 2.0</td>
<td>8.3 ± 1.4</td>
<td>29.0 ± 0.9</td>
<td>42.0 ± 0.7</td>
<td>42.0 ± 0.7</td>
<td>13.3 ± 2.7</td>
</tr>
<tr>
<td><em>M. haemolytica</em> serovar 6</td>
<td>13.3 ± 2.7</td>
<td>13.3 ± 2.7</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>13.3 ± 2.7</td>
<td>16.7 ± 2.7</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td><em>P. tychovia</em> serovar 4</td>
<td>4.2 ± 0.7</td>
<td>4.2 ± 0.7</td>
<td>&gt;15.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>15.0 ± 0.5</td>
<td>7.5 ± 2.0</td>
<td>7.5 ± 2.0</td>
<td>20.0 ± 0.0</td>
<td>&gt;13.3 ± 2.7</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. <em>arizonae</em></td>
<td>3.3 ± 0.7</td>
<td>13.3 ± 2.7</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>16.7 ± 2.7</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>2.5 ± 0.0</td>
<td>3.3 ± 0.7</td>
<td>3.3 ± 0.7</td>
<td>10.0 ± 0.0</td>
<td>13.3 ± 2.7</td>
<td>&gt;20.0</td>
<td>58.0 ± 1.8</td>
<td>12.5 ± 4.3</td>
<td>&gt;10.0 ± 0.0</td>
<td>&gt;16.7 ± 2.7</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>13.0 ± 0.0</td>
<td>13.0 ± 0.0</td>
<td>6.0 ± 0.0</td>
<td>1.7 ± 0.3</td>
<td>58.0 ± 1.8</td>
<td>25.0 ± 0.0</td>
<td>13.3 ± 2.7</td>
<td>20.0 ± 0.0</td>
<td>10.8 ± 4.1</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td><em>C. pseudotuberculosis</em></td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
<td>&gt;20.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC, the lowest concentration of peptide that reduces growth by more than 50% compared to control wells. Results are means ± standard errors of the means (three replications).

<sup>b</sup> The MBC, determined by culturing 150 μl from each of the first three wells showing no visible growth (50 μl per spot) on Trypticase soy agar containing 5% defibrinated sheep blood and incubating the plates overnight at 37°C, was the lowest concentration of peptide that prevents any growth. Results are means ± standard errors of the means (three replications).
10 ml of lung (pulmonary deposition site) in each animal. Two lambs in the dorsum of the caudal portion of the cranial lobe of the right lung of animals in group 1 and group 3 received an additional 10 ml of PBS-PS at the same deposition site. Lambs in group 2 received PBS-PS as a diluent to decrease atelectasis and facilitate pulmonary spreading (4, 17). At 24 h postinoculation, all lambs were euthanized, their lungs were evaluated grossly, and total lung involvement was calculated as previously described (1, 16). Bronchoalveolar lavage (BAL) fluids and consolidated lung tissue were collected for quantitative bacteriological culture, determination of total leukocyte counts, and histopathological examination. The extents of lymphocytic and/or neutrophilic infiltration, necrosis, and collapse were scored as previously described (1), with a maximum score of 4 for each category. The concentrations of M. haemolytica in pulmonary tissues and fluids were transformed [log_{10}(X + 1)], and differences among groups was assessed by one-way analyses of variance. When an analysis of variance resulted in a significant F-test statistic, indicating group differences, Duncan’s multiple-range test was used as the multiple comparison procedure for detecting pairwise differences among the treatment group means. The lungs of lambs in groups 1 and 2 were free of gross lesions and histopathology (Table 3). BAL fluids contained means of 0.4 × 10^6 and 0.9 × 10^6 leukocytes/ml, respectively. Bronchioles and surrounding alveoli contained minimal to mild infiltrates of lymphocytes in the bronchiolar wall, but there was no acute inflammatory response in the bronchioles or alveoli.

The lungs of lambs in group 3 had extensive lesions characterized by focal areas of consolidation with hemorrhage and necrosis. BAL fluids contained a mean of 3.3 × 10^6 leukocytes/ml. There were minimal multifocal lymphocytic peribronchial infiltrates, mild to suppurative bronchitis and/or pneumonia, and minimal to moderate necrosis, hemorrhage, and collapse. Four of five lambs had M. haemolytica in BAL fluid (2.3 log_{10} CFU/ml), and all five lambs had organisms in consolidated pulmonary tissues (5.1 log_{10} CFU/g) collected from the deposition site. (Table 3).

The lesions in the lungs of lambs in group 4 were not as severe, and BAL fluids contained a mean of 4.9 × 10^6 leukocytes/ml. Sections of bronchioles and surrounding alveoli showed moderate numbers of lymphocytes in the bronchiolar wall but no acute inflammatory response. Three of five lambs had M. haemolytica in the BAL fluid (0.9 log_{10} CFU/ml), and all of the lambs had less numbers of organisms in consolidated pulmonary tissues (3.7 log_{10} CFU/g) collected from the deposition site.

Previously, prevention or treatment of respiratory infections with cathelicidins has not been fruitful. For example, CAP18 mixed with P. aeruginosa prior to intratracheal instillation in mice significantly reduced pulmonary injury but did not reduce the number of bacteria that had been instilled, nor did it improve the survival of the infected mice (8). Interestingly, CAP18 alone induced edema, suggesting that it had some pulmonary toxicity (8). To eliminate these species differences within the model, we established a homologous ovine model of acute pneumonia using ovine SMAP29 against the ovine respiratory pathogen M. haemolytica. SMAP29 alone was well tolerated (group 2), and the 1.0-mg dose (twice the treatment dose in group 4) did not induce any significant gross pathology, histopathology, or inflammatory cell filtrates in BAL fluid. Also, lambs treated with only a single dose of SMAP29 had substantially lower gross pulmonary lesion scores, histopathological lesion scores, and concentrations of M. haemolytica in BAL fluids and consolidated pulmonary tissues than untreated lambs. Further studies will determine the optimal doses and intervals of SMAP29 therapy.

We thank Gwen Laird and Abby Lozano for technical assistance. This work was supported by the Cystic Fibrosis Foundation (BROGDE97Z, McCray 97ZO). P.B.M. is the recipient of a Career Development Award from the American Lung Association.

### REFERENCES


### TABLE 3. Protective effect of SMAP29 against M. haemolytica in an ovine model of acute pulmonary infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean (SE) % lung consolidation</th>
<th>Mean (SE) log_{10} (1 + CFU of M. haemolytica/ml of BAL fluid)</th>
<th>Mean (SE) log_{10} (1 + CFU of M. haemolytica/g of lung tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0 (0.0)</td>
<td>1.0 (0.0) bc</td>
<td>0.0 (0.0) b</td>
</tr>
<tr>
<td>2</td>
<td>0.0 (0.0)</td>
<td>1.3 (0.3) c</td>
<td>0.0 (0.0) b</td>
</tr>
<tr>
<td>3</td>
<td>8.8 (2.6)</td>
<td>6.1 (0.9) a</td>
<td>2.3 (0.8)</td>
</tr>
<tr>
<td>4</td>
<td>5.6 (2.3)</td>
<td>5.0 (1.3) ab</td>
<td>0.9 (0.4)</td>
</tr>
</tbody>
</table>

Means sharing the same letter(s) within the column are not significantly different from one another based on Duncan’s multiple-range test performed at the 0.05 level.