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Research Article

Biofilm Formation Ability in Streptococci Causing Acute Otitis Media in Children and Their Treatment with Lantibiotic Gallidermin

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Abstract

Background: Acute otitis media in children is one of the most frequently occurring infections. Increased resistance of the causative agents to antibiotics leads to problems in treating this infection. Many causative bacteria can form biofilm, so the aim of this study was to test susceptibility to gallidermin of biofilm-forming streptococci isolated from otitis media.

Methods: Quantitative methods were used to analyze biofilm-forming ability and susceptibility to bacteriocins. Antibiotics susceptibility was checked using agar diffusion method.

Results: *Streptococcus pneumoniae* Spn 60, *Streptococcus pyogenes* Sp 114, Sp 115 and Sp 117 (4 out of 17) were assessed as highly biofilm-forming ($A_{570} \geq 1.0$); seven strains showed low-grade biofilm formation ($0.1 \leq A_{570} < 1.0$) and seven strains showed biofilm-forming ability less than 0.1 ($A_{570} < 0.1$), meaning they were negative following the biofilm assessment protocol. Among 10 *S. pneumoniae*, five strains did not form biofilm. In seven strains of *S. pyogenes*, three were highly biofilm-forming, three were low-grade biofilm-forming and one strain was non-biofilm-forming. *S. pyogenes* with low enzyme production showed high biofilm formation. Those strains with the highest enzyme production showed low-grade biofilm formation or they did not produce biofilm. Biofilm-forming streptococci were susceptible to gallidermin with the highest inhibition activity 51 200 AU/mL.

Discussion: Gallidermin treatment against streptococci causing otitis media has never been tested previously; results achieved in this study indicate the possibility of using gallidermin for this purpose.

Keywords: Susceptibility; Biofilm; Gallidermin; Otitis media; Streptococci

Introduction

Otitis Media (OM) in children is one of the most frequently occurring infections [1]. It is often diagnosed in children up to two years of age, and it is probably associated with the character of their microbiota, anatomy of the upper respiratory tract and immunological response in children of that age [2]. The most commonly-detected causes of OM in children are reported to be *Moraxella catharalis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. Moreover, representatives of the species *Streptococcus pyogenes* were detected in those cases [2]. *S. pyogenes* has been classified in the A group of streptococci in the family *Streptococcacae* and the phylum *Firmicutes*. Streptococci of that A group and/or *S. pneumoniae* are known to cause invasive or non-invasive infections [2]. *S. pneumoniae* is also from the phylum *Firmicutes*, family *Streptococcacae* and genus *Streptococcus*, but it does not belong in any special antigen group. Regarding the background of infection it is important to know if the causative agent has the ability to form biofilm. Any type of microbiota, including the spoilage and pathogenic kinds, could form and play role in different infections. Biofilm is an aggregation of microorganisms attached to and growing on the surface of bacteria [3]. The role of biofilms in chronic otitis media was first documented by Rayner et al. [4], Akyldiz et al. [5] even indicated chronic otitis media in the middle ear mucosa as biofilm-related disease. Some enzymes which can serve as additional markers in the diagnosis of various disorders (e. g. cancer) could also contribute to OM in the character of causative agents/strains. The standard treatment of OM involves administration of oral antibiotics. However, there is a problem with increased resistance of the causative bacteria to antibiotics. Zielnik-Jurkiewicz and Bielicka [6] performed a prospective study of 157 children with acute OM aged from 6 months to 7 years, admitted due to unsuccessful oral antibiotic treatment. They identified *S. pneumoniae* as the most frequently-isolated pathogen from the middle ear in children with acute OM treatment failure, and established that the majority of strains were antibiotic-resistant. This problem with increasing resistance necessitates searching for new approaches to eliminate/treat these resistant bacteria agents. One possibility in this regard lies with bacteriocins. Bacteriocins are antimicrobial substances of proteinaceous character which have the ability to inhibit growth of more or less related bacteria. Among the bacteriocins, a group of enterocins has been separated and classified [7,8]. They are mostly produced by representatives of *Enterococcus faecium* species [9,10], but enterocins produced by strains of the species *E. mundtii*, *E. durans* or *E. hirae* have also been reported [7]. Some of these enterocins have been purified into

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homogenoity, e.g. Enterocin A [11], Enterocin B, Enterocins L50A, L50B, Enterocin P, [12-14], Enterocin M [15], Enterocin C [16], Enterocin I [17], and Enterocin X [18]. But more enterocins exist with the same characteristics as the already purified/sequenced enterocins [10,15,19]. Enterocins are mostly small, thermo-stable peptides with a broad antimicrobial spectrum [7]. Inhibition effects of different enterocins against *S. pyogenes* and *S. pneumoniae* isolated from OM were shown in our previous study [20]. Nine enterocins were used there, produced by *E. faecium* strains of animal, environmental and rabbit meat origin. The most effective was reported as being dipeptide Enterocin A/P produced by the environmental strain *E. faecium* EK13=CCM 7419, which inhibited growth in three out of eight *S. pneumoniae* (inhibition activity 100-400 AU/mL). Growth in four out of eight *S. pyogenes* strains was also inhibited with activity 200 AU/mL-3200 AU/mL. However, in the range of bacteriocins, there is a special group consisting of lantibiotic bacteriocins, e.g. gallidermin. This is a polypeptide-antimicrobial substance originally produced by *Staphylococcus gallinarum* TU 3928. It contains amino acids such as lanthionine, β-methyllanthionine and/or α, β-didehydroamino acids which can form intramolecular thioether bridges [21]. Gallidermin is known for its predominant inhibition activity against Gram-positive microbiota, the group which streptococci belong in. The inhibitory effect of gallidermin against Gram-positive, faecal staphylococci isolated from roe and red deer was reported in our recent study [22]. Based on that information, streptococci involving those biofilm-forming strains isolated from children with OM were tested for their susceptibility to gallidermin. This will contribute to basic research but it will also indicate further opportunities for gallidermin application. To our knowledge, gallidermin has not previously been tested against acute otitis media agents.

Materials and Methods

Strains targeting, hemolysis, enzymatic activity

The target streptococci [18], originally isolated from samples obtained from children with acute otitis media, then identified and stored in the collection of our colleagues, Dr. Hupková and Dr. Bukovský at Comenius University in Bratislava (Slovakia), were kindly supplied to our laboratory for further analyses. Ten strains of the species *S. pneumoniae* and seven strains of the species *S. pyogenes* were analyzed. They were maintained on BH agar (Difco, USA) enriched with 5% defibrinated sheep blood and cultivated in an incubator (in an atmosphere with CO_2) at 37°C for 24 h [20]. These streptococci were mostly susceptible to clinically-used antibiotics, except for oxacillin resistance in eight strains. The strain Sp 114 was resistant to four antibiotics involving erythromycin. Six strains also showed resistance to rifampicin, and resistance to gentamicin was found in one strain (Sp 112; unpublished data).

Hemolysis testing was used to establish a basic characteristic of the isolated strains. Streptococci were inoculated onto BH agar (Difco, USA) enriched with 5% defibrinated sheep blood and cultivated in the incubator in an atmosphere with CO_2 at 37°C for 24 h. Typical for *S. pyogenes* is β-hemolysis, and α-hemolysis for *S. pneumoniae*. Presence/absence of clear zones around the bacterial colonies was read as α- or β-hemolysis respectively. Negative strains registered γ-hemolysis [23].

Production of enzymes (serving as disease markers) was tested using the commercial API-ZYM system (BioMérioux, Marcy l'Etoile, France). The enzymes presented in Table 2 were evaluated following the manufacturer`s recommendation: 1, alkaline phosphatase; 2, esterase (C4); 3, esterase lipase (C8); 4, lipase (C14); 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, α-chymotrypsin; 10, acid phosphatase; 11, naphthol-AS-BI-phosphohydrolase; 12, α-galactosidase; 13, β-galactosidase; 14, β-glucuronidase; 15, α-glucosidase; 16, β-glucosidase; 17, N-acetylglucosaminidase; 18, α-mannosidase and 19, α-fucosidase. Inocula (65 µL) of McFarland standard one suspensions were pipetted into each well of the kit. Enzyme activities were evaluated after 4 h of incubation at 37°C and after addition of Zym A and Zym B reagents. Color intensity values from 0 to 5 and their relevant values in nanomoles (nmoL) were assigned for each reaction according to the color chart supplied with the kit.

Biofilm testing

Biofilm formation in streptococci was tested using the quantitative plate assay described by Chaieb et al. [24] and Slížová et al. [25]. One colony of the tested strain grown on BH agar overnight at 37°C (Difco, USA) was transferred into 5 mL Ringer solution (pH 7.0, 0.75% w/v) to obtain a suspension corresponding to 1×10^8 CFU/mL. A 100 µL aliquot from that dilution was transferred into 10 mL of BH infusion/broth (BHI, Difco, USA). Then the dilution was inoculated in 200 µL volumes into polystyrene microtiter plate wells (Greiner ELISA 12 Well Strips, 350 µL, flat bottom, Frickenhausen GmbH, Germany) and incubated for 24 h at 37°C. The biofilm formed in the microtiter plate wells was washed twice with 200 µL deionized water and dried at 25°C for 40 min. The remaining attached bacteria were stained for 30 min at 25°C with 200 µL 0.1 % (m/v) crystal violet in deionized water. The dye solution was aspirated away, and the wells were washed twice with 200 µL deionized water. After the water removal, the plate was dried for 30 min at 25°C, the dye bound to the adherent biofilm was extracted with 200 µL 95% ethanol and stirred. A 150 µL aliquot was transferred from each well into a new microplate well for absorbance (A) measurement at 570 nm using a Synergy TM4 Multi Mode Microplate reader (Biotek, USA). Each strain and condition was tested in two independent tests with 12 replicates. Sterile BHI was included in each analysis as negative control. *Streptococcus equi* subsp. *zooepidemicus* CCM 7316 was used as positive control (kindly provided by Dr. Eva Styková, University of Veterinary Medicine and Pharmacy, Košice, Slovakia). Biofilm formation was classified as highly -positive (A570 ≥ 1.0), low-grade positive (0.1 ≤ A570 <1.0) or negative (A570<0.1) according to Chaieb et al. [24] and Slížová et al. [25].

Susceptibility to gallidermin of streptococcal strains

Gallidermin used for testing was pure substance (Enzo Life Sci. Corporation USA, MW2069.4). Based on previous testing results, it was used at concentration 0.5 mg/mL in 2 µL doses. Susceptibility of the streptococcal strains to gallidermin was tested using the agar spot method [26]. Briefly, BH infusion/broth supplemented with 1.5 % agar (BHIA, Difco, USA) was used for the bottom layer, and volume 4 mL 0.7 % BHIA enriched with 200 µL of overnight culture of indicator strain (absorbance measured at 600 nm-A600, with optical density of strains up to 0.8) was used for the overlay. Dilutions of gallidermin in phosphate buffer (pH 6.5, ratio 1:1) were prepared and dropped on the surface of the overlaid agar containing each indicator (streptococcal) strain. The plates were incubated at 37°C for 24 h in an atmosphere containing 5% CO_2. Clear inhibition zones around dilutions of gallidermin were checked. Inhibition activity was expressed in arbitrary units per mL (AU/mL); this means the reciprocal of the highest two-fold dilution of gallidermin demonstrating complete

growth inhibition of the indicator (streptococcal) strain. All tests were performed twice. Positive control was the principal indicator strain *Enterococcus avium* EA5 (our isolate from piglets); its growth was inhibited by activity 25 600 AU/mL.

Results and Discussion

Altogether seventeen streptococcal strains were tested for enzyme production (ten *S. pneumoniae* and seven *S. pyogenes*) (Table 1). *S. pyogenes* strains showed β-hemolysis, and α-hemolysis was read for tested strains *S. pneumoniae*. According to the enzyme production test results, *S. pneumoniae* Spn 46, 49, 60 and *S. pyogenes* Sp 114, 115 and 117 did not produce any enzyme, or enzyme production in these strains was slight or low. Spn 40 showed no or slight enzyme production, except for alkalic and acidic phosphatase (20 nmoL, 40 nmoL). Spn 50 with no or slight enzyme activity produced 30 nmoL of β-galactosidase, and Spn 754 mostly did not produce enzymes; however, acidic phosphatase reached 30 nmoL. The highest enzyme production was assessed in strains Spn 57 (alkaline phosphatase, leucin arylamidase, naphthol-AS-BI-phosphohydrolase, 40 nmoL; acidic phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase and α-glucosidase, 30 nmoL), Spn 59 (acidic phosphatase and β-galactosidase, 40 nmoL and 30 nmoL for leucin arylamidase) and Spn 922 (esterase, lipase 30 nmoL, α-chymotrypsin, acidic phosphatase, naphthol-AS-BI-phosphohydrolase and β-galactosidase, 40 nmoL). *S. pyogenes* Sp 112 and Sp 113 showed low or no enzyme production, except for leucin arylamidase which was evaluated with 30 nmoL in these strains (Table 1). The absolute highest enzyme production was found in *S. pyogenes* Sp 111; leucin arylamidase, α-chymotrypsin, acidic phosphatase, naphthol-AS-BI-phosphohydrolase, α- glucosidase were high - 40 nmoL and 30 nmoL was measured for β-galactosidase. In general, the highest values of enzymes measured in the tested streptococci were found for leucin arylamidase, α-chymotrypsin, β-galactosidase, naphthol-AS-BI-phosphohydrolase, and acidic phosphatase. Alkaline phosphatase is an enzyme which plays a role in the liver and skeleton metabolism. In human blood it works as a marker for hepatitis or osteomalacia diagnosis. Esterase, esterase lipase, acidic phosphatase or naphthol-AS-BI-phosphohydrolase represent hydrolyses. In some streptococci high levels of undesirable enzymes were measured, which may indicate their harmful character.

S. pyogenes are known to produce complete (β)-hemolysis and *S. pneumoniae* incomplete (α)-hemolysis, as it found in the tested streptococci. Incomplete hemolysis means that cell membranes of the blood red cells are left intact [23].

Regarding biofilm formation, four strains (Spn 60, Sp 114, Sp 115 and Sp 117) out of 18 were evaluated as highly- positive (A570 ≥ 1.0), seven strains showed low-grade biofilm formation (0.1 ≤ A570 <1.0) and seven strains showed biofilm activity less than 0.1 (A570), meaning no biofilm formation, (Table 2). Among ten *S. pneumoniae*, while Spn 60 was highly-positive, five others showed absorbance (A570) less than 0.1; they were classified as non-biofilm-forming; and four strains did not form biofilm at all. In seven *S. pyogenes* strains, three were highly-positive, three were low-grade biofilm-forming and one strain did not form biofilm (Table 2). Among *S. pyogenes* strains, those with low enzyme production showed high biofilm formation (Table 2). On the other hand, those strains with the highest value of enzyme production were assessed with low-grade biofilm formation or they did not produce biofilm at all. Recently some studies have reported on enzyme-based biofilm induction strategies [27]. It appears that in the case of strains with high biofilm formation, this ability did not depend on enzyme production, and/or enzyme production was only slight. Liaqat et al. [28] also reported that proteolytic enzymes such as trypsin, chymotrypsin and proteinase can reduce biofilm formation in many pathogenic strains, and even increase their susceptibility to conventional antibiotics [29], so strains not producing those enzymes can show high biofilm formation ability. In our case for example, Sp111 with the highest enzyme production showed low-grade biofilm formation, and Sp114 with high biofilm formation did not produce any enzyme, which confirms the indicated and previously reported strategies. In general, those acute OM-causing agents producing proteolytic or other enzymes show some impact on their biofilm formation ability. However, all tested streptococci were susceptible to gallidermin with inhibition activity ranging from 800 up to 51 200 AU/mL (Table 2). Susceptibility to gallidermin with the highest inhibition activity was measured in *S. pneumoniae* Spn 57 (51 200 AU/mL), this strain did not form biofilm, but it showed high enzyme activity (Tables 1 and 2). The majority of strains was susceptible to gallidermin with activity 3 200 AU/mL.

As already mentioned, gallidermin is a lantibiotic bacteriocinogenic substance, which can work like erythromycin or fusidin in clinical practice [30]. Because of its predominantly inhibitory effect against Gram-positive bacteria, its use against streptococci as causative agents of otitis media could be supposed. In addition to gallidermin, other lantibiotics are also involved in the group of lantibiotic bacteriocins, such as nisin, epidermin, Pep 5, and subtilin [31]. Among them, nisin is the best-studied lantibiotic. Besides nisin application in food [32], it has also been used against mastitis in cattle [33], against gingival inflammation in beagles [34], in broiler rabbits with significant increase in phagocytic activity and with antimicrobial effect [35], and recently, nisin use in cancer therapy has been indicated [36]. Gallidermin mode of action is very similar to that of nisin. Like nisin, gallidermin interacts with lipid I, II, III and IV; thus it inhibits not only murein biosynthesis but also wall teichoic acid biosynthesis [37,31]. After gallidermin application in broiler rabbits, phagocytic activity was increased and counts of Gram-positive and Gram-negative bacteria were reduced [37]. The pharmacological properties of gallidermin have led to extensive investigations to optimize its production process. Goetz et al. [31] reported that lantibiotics, in general offer various potential applications, e.g. as anti-infection regulators of immune systems, or as new lead structures for biotechnological studies and use. While the primary application of bacteriocins has always been in food preservation [32], increasing antibiotic resistance to conventional antibiotics presents new opportunities for the exploration of bacteriocins, including lantibiotics. Chikindas et al. [32] reported this era of bacteriocin research as exciting, as it will lead to new inventions and new applications through possibilities of new progressive sequencing techniques for studying bacteriocins.

In summary, *S. pneumoniae* and *S. pyogenes* isolated from children with acute otitis media, some strains with highly biofilm-forming ability and others producing damaging enzymes, were susceptible to gallidermin with the highest inhibition activity 51 200 AU/mL. On the other hand, fecal strains *E. hirae* EH52b and *E. faecium* EF42 (isolated from pheasants) for example, producing some of the same enzymes and having virulence factor genes, were susceptible to bacteriocin-enterocins [38]. Moreover, as also mentioned above, biofilm-forming fecal staphylococci from roe and red deer were

Table 1: Enzyme activity in streptococci isolated from acute otitis media (in nmoL).

Strains	Enzymes																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Spn 40	30	20	20	0	10	5	10	0	0	40	10	10	20	5	5	10	5	0	0
Spn 46	20	10	5	0	0	0	0	0	0	20	20	0	5	0	5	5	0	0	0
Spn 49	10	20	10	0	0	0	0	0	0	20	10	0	0	5	10	5	0	0	0
Spn 50	0	20	20	10	20	10	10	5	5	5	5	0	30	0	0	0	0	0	0
Spn 51	20	10	10	5	20	10	10	5	10	30	30	10	30	30	20	10	0	0	0
Spn 57	40	5	5	5	40	5	30	5	5	30	40	30	30	30	30	5	5	5	5
Spn 59	10	10	10	5	30	10	10	10	20	40	20	20	40	5	5	20	5	5	5
Spn 60	5	20	5	5	5	5	0	0	5	5	0	0	0	0	0	0	0	0	0
Spn 754	20	5	10	0	0	0	0	0	0	30	10	0	5	0	5	0	0	0	0
Spn 922	10	20	10	10	30	20	10	5	40	40	40	10	40	0	30	20	20	10	10
Spn 111	5	10	20	5	40	20	20	5	40	40	40	5	30	5	40	10	5	5	5
Spn 112	20	10	10	5	30	20	20	0	10	20	20	20	20	10	20	20	0	0	0
Spn 113	30	20	10	5	30	10	10	10	5	20	20	10	20	20	20	10	10	10	10
Spn 114	0	10	10	0	0	0	5	0	0	5	5	0	0	5	5	0	0	0	0
Spn 115	5	20	10	0	0	0	0	0	0	10	10	0	0	10	10	0	0	0	0
Spn 116	0	20	20	5	30	20	20	0	10	10	10	10	30	0	2	30	5	0	0
Spn 117	20	10	10	5	20	5	10	5	5	20	20	10	20	20	20	20	0	0	0

1-alkaline phosphatase; 2-esterase (C4); 3-esterase, lipase (C8); 4-lipase (C14); 5-leucin arylamidase; 6-valin arylamidase; 7-cystine arylamidase; 8-trypsine, 9-α-chymotrypsin; 10-acidic phosphatase; 11- naphtol-AS-Bi-phosphohydrolase; 12- α-galactosidase; 13-β-galactosidase; 14- β-glucuronidase; 15- α-glucosidase; 16- β-glucosidase; 17-N-acetyl- β-glucosaminidase; 18- α-mannosidase; 19- α-fucosidase; SPn 58 was not tested.

Table 2: Susceptibility to gallidermin and biofilm formation ability in streptococci isolated from acute otitis media.

Strains	Plate assay (± SD)	Gallidermin (AU/mL)
Spn 40	0.056 (0.04)	nt
Spn 46	0.071 (0.04)	25600
Spn 49	0.319 (0.06)	6400
Spn 50	0.066 (0.04)	800
Spn 51	0.064 (0.04)	51200
Spn 57	0.524 (0.09)	12800
Spn 59	0.049 (0.04)	nt
Spn 60	1.386 (0.33)	3200
Spn 754	0.456 (0.19)	3200
Spn 922	0.156 (0.03)	3200
Spn 111	0.136 (0.03)	3200
Spn 112	0.135 (0.02)	3200
Spn 113	0.096 (0.04)	3200
Spn 114	1.946 (0.44)	3200
Spn 115	1.921 (0. 37)	3200
Spn 116	0.529 (0.09)	1600
Spn 117	1. 521 (0.28)	3200

S. pneumoniae Spn 50 was not treated with gallidermin. Nt-not tested. Biofilm-reading:highly-positive A_{570}>1.0; low-grade positive (0.1<A_{570}<1.0) or negative (A_{570}<0.1) meaning Absorbance A570 was less than 0.1 according to Chaieb et al. [24] and Slížová et al. [25].

susceptible to gallidermin [22].

Conclusion

This study is an original contribution to ongoing lantibiotic bacteriocin inhibition spectrum studies, as gallidermin treatment against streptococci isolated from children with acute otitis media has never been tested. The presented results indicate the possibility of using gallidermin for this purpose. Of course, clinical testing has to be undertaken and processed.

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Authors Contributions

Andrea Lauková: Idea, managing, strain targeting, results evaluating, summarizing, writing; Anna Kandričáková: Antimicrobial analysis testing, biofilm testing; Eva Bino: Biofilm testing.

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