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Regular paper

Synergistic hemolysins of coagulase-negative staphylococci (CoNS)*

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A total of 104 coagulase negative staphylococci, belonging to *S. capitis, S. hominis, S. haemolyticus* and *S. warneri,* originating from the collection of the Department of Pharmaceutical Microbiology (ZMF), Medical University of Lodz, Poland, were tested for their synergistic hemolytic activity. 83% of strains produced δ-hemolysin, however, the percentage of positive strains of *S. haemolyticus, S. warneri, S. capitis* and *S. hominis* was different — 98%, 78%, 75% and 68%, respectively. Highly pure hemolysins were obtained from culture supernatants by protein precipitation with ammonium sulphate (0–70% of saturation) and extraction by using a mixture of organic solvents. The purity and molecular mass of hemolysins was determined by TRIS/Tricine PAGE. All CoNS hemolysins were small peptides with a molar mass of about 3.5 kDa; they possessed cytotoxic activity against the line of human foreskin fibroblasts ATCC Hs27 and lysed red cells from different mammalian species, however, the highest activity was observed when guinea pig, dog and human red blood cells were used. The cytotoxic effect on fibroblasts occurred within 30 minutes. The *S. cohnii* ssp. *urealyticus* strain was used as a control. The antimicrobial activity was examined using hemolysins of *S. capitis, S. hominis, S. cohnii* ssp. *cohnii* and *S. cohnii* ssp. *urealyticus*. Hemolysins of the two *S. cohnii* subspecies did not demonstrate antimicrobial activity. Cytolysins of *S. capitis* and *S. hominis* had a very narrow spectrum of action; out of 37 examined strains, the growth of only *Micrococcus luteus, Corynebacterium diphtheriae* and *Pasteurella multocida* was inhibited.

Key words: coagulase-negative staphylococci, CoNS, synergistic hemolysin, antimicrobial activity, cytotoxicity

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INTRODUCTION

Hemolysins are important factors of bacterial virulence. Coagulase-positive staphylococci (*S. aureus*) produce four hemolysins: α, β, γ and δ, which are well-known and extensively characterized, including the mechanism of their action and cytotoxicity (Wiseman, 1975; Bohach *et al*., 1997; Dinges *et al*., 2000; Foster, 2002). These hemolysins are also referred to as toxins or lysins. They all belong to the family of pore forming toxins (Verdon *et al.,* 2009b). Only particular species of coagulase-negative staphylococci (CoNS) produce α and β hemolysins, while γ hemolysin is not produced. The α-toxin was detected in *S. epidermidis, S. haemolyticus* and *S. saprophyticus* by Molnar *et al.* (1994), and β-toxin was detected in *S. schleiferi* by Hebert (1990). In the group of CoNS, δ-hemolysin is produced by the majority of species: *S. capitis, S. chromogenes, S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. simulans, S. warneri*, *S. xylosus* and others. A CAMP test is used to detect the δ-hemolysin. This method is also referred to as a test of the synergistic hemolysis and is applied routinely in clinical bacteriology for the identification of *Streptococcus agalactiae* or *Listeria monocytogenes* (Bhakdi, 1985; Hebert & Hancock, 1985; Hebert *et al*., 1988a, b; Hebert, 1990). The δ-hemolysin action against erythrocytes depends on its concentration. At low concentrations, this hemolysin forms pores in the lipid bilayer after interacting with a cell's membrane. At higher concentrations, δ-hemolysin acts as a surfactant which causes a detergent-like solubilization of the erythrocyte membrane. δ-hemolysin is able to lyse not only erythrocytes, but also other mammalian cells, as well as subcellular structures, such as membrane-bound organelles (Verdon *et al.,* 2009b).

The first δ-hemolysin of CoNS studied in detail was the hemolysin produced by *S. epidermidis*. It was isolated from the faeces of infants with necrotizing enterocolitis (NEC) (Scheifele *et al.,* 1987; Scheifele & Bjornson, 1988; McKevitt *et al*., 1990). Its two other producers are *S. haemolyticus* (Frenette *et al.,* 1984, Watson *et al.,* 1988) and *S. lugdunensis* (Donvito *et al.,* 1997). δ-Hemolysin synthesized by these species has also been characterized at a molecular level.

The production of this hemolysin was also described by Różalska & Szewczyk (2008) who examined a large collection of *S. cohnii*, isolated from hospital and non-hospital environments, patients and medical staff (Różalska *et al.,* 1993; Szewczyk *et al.,* 2000). It was found that 91% of *S. cohnii* ssp. *cohnii* strains and 74.5% of *S. cohnii* ssp. *urealyticus* strains produced δ-hemolysin. The activity of δ-hemolysin was possessed by three peptides – H1, H2 and H3. The peptide sequences of both *S. cohnii* subspecies were identical. Two of them – H1 and H3

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Abbreviations: API, Analytical Profile Index; ATCC, American Type Culture Collection; CAMP, Christie, Atkins, Munch-Peterson; CCM, Czech Collection of Microorganisms; CoNS, coagulase negative staphylococci; FBS, fetal bovine serum; HD_{50} specific hemolytic activity; HU, hemolytic unit; IL, interleukin; IMDM, Iscove's Modified Dulbecco's Medium; MIC, minimal inhibition concentration; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; NCTC, National Collection of Type Culture; NEC, necrotizing enterocolitis; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PSM, phenol soluble modulin; SDS, sodium dodecyl sulfate; ShlA, Serratia marcescens hemolysin A; TNF, tumor necrosis factor; Tricine, *N*-[Tris(hydroxymethyl)methyl]glycine; TRIS, Tris(hydroxymethyl)aminomethane

– had a high amino acid sequence similarity to three hemolysins of *S. lugdunensis* and a relatively low similarity to three peptides of *S. haemolyticus*. There was no sequence similarity to the peptides produced by *S. aureus* and *S. epidermidis*. Peptide H2 of *S. cohnii* did not have amino acid sequence similarity to any other known hemolysins (Mak *et al.,* 2008).

Another synergistic hemolysin included in this work was studied in 2005 by a group of researchers from the University of Poitiers, in France, and the University of Würzburg, in Germany. It was isolated from a strain of *Staphylococcus warneri* RK. This strain produced two bacteriocins: the first inhibited the growth of Gram-positive cocci of the *Pediococcus* genus, and the second — Gram-negative rods from the genus *Legionella*. The second product was named warnericin RK (Hechard *et al.,* 2005). In further studies, researchers found that warnericin RK exhibited a synergistic hemolysin activity, and the strain of *S. warneri* RK produced other peptides expressing this activity, called δ-lysine I and II. The amino acid sequences of these three peptides were established. There was a high sequence similarity of lysine I and II, but not warnericin RK, with the δ-hemolysin of *S. aureus* (Fitton *et al.,* 1980), and delta-like hemolysin produced by *S. epidermidis* (McKevitt *et al.*, 1990). Cytolysine I and II showed a high similarity, and therefore further experiments were performed with hemolysin I. This peptide, like warnericin RK, also inhibited the growth of *Legionella* (Verdon *et al.,* 2008, 2009a). In 2011 Marchand *et al.* studied peptides possessing the anti-*Legionella* and synergistic hemolysin activity, which were produced by other species of *Staphylococcus*. The collection of strains used in this study included 15 species representatives. It was found that the culture supernatants of nine of them contained these peptides. Peptides produced by 5 species – *S. aureus, S. epidermidis*, *S. haemolyticus, S. lugdunensis* and *S. warneri* – were analyzed. They were purified and separated by chromatography. The authors of this paper were not able to get sufficient quantities of peptides from *S. cohnii*. Therefore, they obtained a synthetic analog of peptide H2U, one of the three peptides produced by these bacteria. The hemolytic and anti-*Legionella* activities were studied. The establishment of two groups of peptides was proposed according to their mode of action on the *Legionella* cells and their hemolytic activity. The first is a group of peptides that have a high hemolytic ability and bactericidal action. Warnericin RK belongs to this group. Peptides of the second group are bacteriostatic and have poor hemolytic properties. This class of peptides is represented by PSMα (Phenol-soluble modulin-alfa) from *S. epidermidis*. Both types of hemolysins are good candidates to be used as a specific anti-*Legionella* drug. The H2U peptide from *S. cohnii* was classified in this group. Peptides produced by *S. capitis* and *S. hominis* were not analyzed in this research.

Recent investigations aimed at understanding the relationship between the structure of peptides, representatives of both groups – warnericin RK and PSMα from *S. epidermidis*, and the mechanism of their antibacterial and hemolytic activity (Marchand *et al.,* 2015).

The aim of the present study was to supplement the knowledge of the synergistic hemolysins of *S. warneri*, *S. haemolyticus* or *S. cohnii*, which had been characterized earlier, and of those produced by *S. capitis* and *S. hominis* that have not been characterized so far.

Table 1. Synergistic hemolysin exhibited by coagulase-negative staphylococci (CoNS)

Species	Number of strains (% positive)
S. capitis	12 (75.0)
S. haemolyticus	45 (97.8)
S. hominis	38 (68.4)
S. warneri	9 (77.8)
Total	104 (82.7)

MATERIALS AND METHODS

Screening test for the detection of synergistic hemolysin producers. One hundred and four strains, originating from the collection of the Department of Pharmaceutical Microbiology (ZMF), Medical University of Lodz, and belonging to *S. capitis, S. hominis, S. haemolyticus* and *S. warneri* species, were examined. The synergistic hemolysis tests with *Staphylococcus aureus* ATCC 25923, as a producer of β-hemolysin, and *Streptococcus agalactiae,* as a positive control, were applied. The total lysis in the area of β-hemolysin around the line of inoculation indicated the presence of a synergistic hemolysin. The intensity of hemolysis was evaluated.

Microorganisms for determining the antimicrobial action. Thirty seven species of Gram-positive and Gram-negative bacteria belonging to the genera: *Brevibacterium, Corynebacterium, Enterococcus, Micrococcus, Staphylococcus, Citrobacter, Enterobacter, Escherichia, Klebsiella, Morganella, Pasteurella, Pseudomonas* and *Proteus,* were used for testing the antimicrobial activity of hemolysins (Table 2). In the study, two groups of strains were used. The first one consisted of 13 reference strains of bacteria from ATCC, NCTC and CCM collection of microorganisms. The second group consisted of 24 strains of bacteria from the collection of the Department of Pharmaceutical Microbiology (ZMF), Medical University of Lodz, Poland. Most of these strains were isolated from clinical specimens. These strains were identified by the Analytical Profile Index (API) System (BioMérieux).

Bacterial strains used in the examination of cytotoxic and hemolytic activity. The following strains were applied in these experiments: *S. capitis* ZMF D25 (an isolate from a nose of a newborn baby), *S. haemolyticus* ZMF 3013 (an isolate of discharge from the penis of a man), *S. hominis* ZMF 3016 and *S. warneri* ZMF 2009 (both isolates from the nose of an adult man), and *S. cohnii* ssp. *urealyticus* ZMF 535, which was used as a control (an isolate from the hospital environment). Hemolysins isolated from *S. cohnii* ssp.*cohnii* ZMF 77 (an isolate from the skin of a newborn baby), *S. cohnii* ssp. *urealyticus* ZMF 535, *S. capitis* ZMF D25 and *S. hominis* ZMF 3016 were used to examine the inhibition of microbial growth.

Bacterial culture and hemolysin isolation. The strains were grown on a dialyzed brain heart infusion medium (bioMerieux). The hemolysins were obtained from a culture supernatant according to the method developed by Różalska & Szewczyk (2008). The proteins were precipitated with the ammonium sulfate (0–70% of saturation) and lyophilized, then extracted by vigorous shaking with a mixture of chloroform and methanol (2:1 v/v). After centrifugation, the organic solvents were evaporated at the temperature of 37°C.

Polyacrylamide gel electrophoresis. The homogeneity of these samples and molecular mass of hemolysins under reducing conditions were estimated using the TRIS/Tricine/SDS/PAGE method (Schager & Jagow, 1987). Electrophoresis was carried out in the MiniPro-

Table 2. The antimicrobial activity (MIC) of the CoNS synergistic hemolysins for the selected Gram–positive and Gram–negative bacteria

Strains	MIC (HU per mL medium)		
	S. cohnii both ssp.	*S. capitis*	*S. hominis*
Brevibacterium sp. ZMF	–	–	–
Corynebacterium diphtheriae t. gravis ZMF	–	62.5	–
Corynebacterium diphtheriae t. mitis ZMF	–	–	–
Corynebacterium pseudodiphtheriticum ZMF	–	–	–
Enterococcus faecalis ZMF E5	–	–	–
Enterococcus faecalis ZMF E6	–	–	–
Micrococcus luteus ATCC 4698	–	–	–
Micrococcus luteus ZMF 2012	–	37.5	62.5
Staphylococcus aureus ATCC 25923	–	–	–
Staphylococcus aureus ATCC 6538	–	–	–
Staphylococcus aureus MM3	–	–	–
Staphylococcus capitis ZMF 3000	–	–	–
Staphylococcus chromogenes ZMF 2008	–	–	–
Staphylococcus epidermidis ZMF 3019	–	–	–
Staphylococcus haemolyticus ZMF 3001	–	–	–
Staphylococcus haemolyticus ZMF3014	–	–	–
Staphylococcus intermedius ZMF 3033	–	–	–
Staphylococcus lugdunensis ZMF 3018	–	–	–
Staphylococcus saprophyticus CCM 883	–	–	–
Staphylococcus xylosus ZMF 1115	–	–	–
Citrobacter freundii ZMF P5	–	–	–
Enterobacter cloaceae ZMF P3	–	–	–
Escherichia coli ATCC 10538	–	–	–
Escherichia coli ATCC 25922	–	–	–
Escherichia coli ATCC 25942	–	–	–
Escherichia coli ATCC 35218	–	–	–
Escherichia coli ATCC 8739	–	–	–
Klebsiella oxytoca ZMF P26	–	–	–
Klebsiella pneumoniae ssp.*pneumoniae* ZMF P14	–	–	–
Morganella morganii ZMF P27	–	–	–
Pasteurella multocida ZMF P16	–	87.5	–
Proteus mirabilis ZMF P28	–	–	–
Pseudomonas aeruginosa ATCC 15442	–	–	–
Pseudomonas aeruginosa ATCC 27853	–	–	–
Pseudomonas aeruginosa ATCC 6749	–	–	–
Pseudomonas aeruginosa NTCC 10490	–	–	–
Pseudomonas aeruginosa ZMF P8	–	–	–

tean 3 apparatus (BioRad). Polypeptide SDS-PAGE Molecular Weight Standards (BioRad) were used.

Hemolytic and cytotoxicity assays. The geometric dilutions of hemolysins ranging from 1:2 to 1:256, in a final volume of 100 µL in phosphate-buffered saline pH 7.2 (PBS) in 96-well V-bottom plates, were made. 50 µL of 3% suspension in PBS of human, sheep, dog, rabbit and guinea pig erythrocytes were added to each hemolysin dilution. The 100% of hemolysis was achieved in distilled H_2O. After incubation at 37°C for 30 min, the plates were centrifuged at 750×*g*, at room temperature for 10 min (Centrifuge *MPW 341)*. The supernatants were transferred into 96-well flat-bottom plates, and the absorbance of each well was measured at 550 nm by a plate reader (Multiscan Ex Labsystem). One hemolytic unit (HU) causes 50% lysis of a 3% suspension of

tested red blood cells in phosphate buffered saline, pH 7.2, after 30 min at 37°C. The specific hemolytic activity (HD_{50}) of the tested hemolysins was expressed as HU per mg of protein.

ATCC Hs27 cells (normal dermal human fibroblasts from the foreskin), which were used in the cytotoxicity assay, were grown and maintained in Iscove's Modified Dulbecco's Medium (IMDM; PAA Laboratories) supplemented with 5% fetal bovine serum (FBS; PAA Laboratories), 100 U/mL penicillin and 100 μg/mL streptomycin sulfate (Polfa) at 37°C in a humid atmosphere containing 5% CO_2. Twenty hours before experimental use, the Hs27 cells were subcultured in 48-well flat-bottom plastic culture plates (Falcon) at a density of 2×10^4 cells/well. 500 μL samples of serial dilutions of hemolysins in a culture medium without serum were added to each well. A negative control (untreated cells) was included. The plates were incubated for 18 hours and then the cytotoxic effect was examined. The viability of the hemolysin treated cells was determined using the tetrazolium blue (MTT; Sigma) method (Mossman, 1983). Cytotoxicity was determined as a relative percentage of survival, with the equation: % survival = absorbance of treated cells ×100/ absorbance of untreated cells. The cytotoxic activity was expressed as LC_{50}, i.e. the concentration of a hemolysin that caused the death of half of the Hs27cells. The hemolysins at the LC_{50} value were used to determine the time-course of cytotoxic action. The plates were incubated for 0.5, 1, 2, 3, 6 and 24 hours, and then they were examined for cytotoxic effects by the MTT test. In addition, a hemolysin treated monolayer of tested cells was fixed with 2% formalin in PBS, stained with 0.13% crystal violet (Merck) in 5% etanol-2% formalin-PBS and observed under the light microscope NICON ECLIPSE TE 2000-S for analyzing the cytotoxic effect.

Determination of antimicrobial activity. The hemolysins were dissolved in 70% aqueous solution of acetone and they were added in the amounts of 100, 75, 50, 25 HU/mL to molten and subsequently cooled to 56°C agar medium (Mueller Hinton II, Emapol). After vigorous mixing, they were quickly poured into Petri dishes. Standardized suspensions of the microorganism (2 μL) were placed on the agar surface. After incubation, the intensity of growth was assessed. Microbial growth was controlled on agar with or without acetone. Acetone did not have an influence on the microbial growth. The tests were replicated three times with hemolysins obtained from various cycles of experiments. The Minimal Inhibition Concentration (MIC) of hemolysin, expressed as HU/mL, which completely inhibits growth of the bacteria, was determined.

Protein concentrations were determined according to the Bradford's method using a plate reader (Multiscan Ex Labsystem). Bovine serum albumin (Sigma) was used as a standard (Bradford, 1976).

RESULTS AND DISCUSSION

Staphylococci, including CoNS, are able to produce numerous hemolytic peptides with various activities. *S. epidermidis* is an example of CoNS that secrets such peptides, termed phenol-soluble modulins (PSM). It consists of PSMα, PSMβ and PSMγ peptides; the latter is identical to the δ-toxin. This toxin is an important virulence factor of *S. epidermidis*. It shows inflammatory properties, which are involved with the symptoms of neonatal necrotizing enterocolitis (NEC). PMSs activate macrophages and induce cytokine release (TNF-α, IL-1β, IL-6) (Otto, 2004). It was also found that PSMs of *S. epidermidis* are highly produced in biofilms, as compared to the planktonic growth of bacteria. These peptides, due to the action typical for surfactants, play a crucial role in detachment of the biofilm structure and dissemination of bacteria (Cheung *et al.,* 2014). The synergistic hemolysins of other CoNS species are less characterized and that is why they were the objects of analysis in this paper, which is a continuation of the research conducted at the Department of Pharmaceutical Microbiology of the Medical University of Lodz (Różalska & Szewczyk, 2008; Mak *et al.,* 2008). Hemolysins of two subspecies: *Staphylococcus cohnii – S. cohnii* ssp. *cohnii* and *S. cohnii* ssp. *urealyticus,* are well-known and have been characterized in detail. A fast, simple and reproducible method for the preparation of hemolysins from the crude lyophilized proteins developed by Różalska, allowed for conducting research that requires sufficient amount of sample. This method is a modification of the procedure described by Heatley (1971), where δ-hemolysin was obtained from the culture supernatant in the aqueous environment. The preparations obtained in this way can be stored in a dry environment at a temperature of 4°C for a long period of time (several years) without the loss of activity. On the other hand, a strong decrease in the hemolysin activity was observed when preparations were frozen and refrozen in an aquatic environment. In addition, these hemolysins were fully soluble in 70% aqueous acetone, a volatile organic solvent which can be easily removed (Różalska, data not shown). This method was also successfully used in studies on bacteriocin produced by the strain of *S. aureus* CH-91 (Wladyka *et al.,* 2013).

A search for the δ-hemolysin in other CoNS species: *S. capitis*, *S. haemolyticus*, *S. hominis* and *S. warneri* from the ZMF collection was conducted. It was found that the production of this hemolysin was widespread among the strains in this collection (83% of the strains were positive), which is in accordance with the literature data (Hebert & Hancock, 1985; Hebert *et al*., 1988a, b). Table 1 shows the percentage of positive strains for each species. Based on the intensity of synergistic hemolysis, the strains representing different species were selected for further studies. Classification of these strains was confirmed by phenotyping, which was carried out with the API Staph ID 32 system in accordance with the recommendations of Heikens *et al.* (2005).

Figure 1 illustrates the test of synergistic hemolysis with the obtained hemolysins. As a positive control, both subspecies of *S. cohnii* and the extract of δ-toxin were used. The obtained preparations of hemolysins showed very high purity, as presented in Fig. 2. Electrophore-

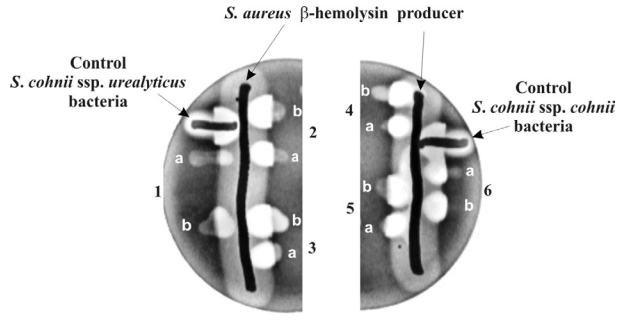

Figure 1. Test of synergistic hemolysis (CAMP).
Extracts of hemolysins (a – 20 μg and b – 40 μg) – *S. capitis* (1), *S. hominis* (2), *S. warneri* (3), *S. c.urealyticus* (4), *S. haemolyticus* (5) and *S. c.cohnii* (6).

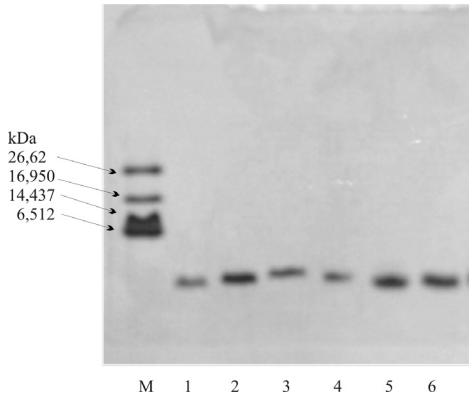

Figure 2. Polyacrylamide gel electrophoresis under reducing conditions of CoNS synergistic hemolysins in the Tris-Tricine system (15.5% peptide gel).
Extracts of hemolysin (20 µg): 1 – *S. capitis,* 2 – *S. warneri,* 3 – *S. haemolyticus,* 4 – *S. hominis* and 5 – *S. cohnii* ssp.*urealyticus* and 6 – *S. cohnii* ssp. *cohnii,* M – marker; the sizes of the molecular size marker, in kDa, are shown on the left side of the figure. Gel stained with Coomassie Blue G-250.

sis of all of hemolysins in 15.5% polyacrylamide gel revealed only single peptide bands, located at a position similar to that of the two *S. cohnii* subspecies hemolysin, whose molecular weight had been previously established. The size of these hemolysins is approximately 3.5 kDa (Różalska & Szewczyk, 2008).

The analysed hemolysins showed different activity toward the tested red cells of sheep, rabbit, dog, guinea pig and human (Fig. 3). The *S. cohnii* ssp. *urealyticus* hemolysin was used as a positive control. The obtained HD_{50} values highly differed from 35 HU/mg of protein, for the hemolysin produced by *S. haemolyticus* and tested with rabbit erythrocytes, to 761 HU/mg of the protein for the hemolysin from *S. capitis* or *S. warneri* and tested with guinea pig red cells. The most active hemolysins against all tested erythrocytes are peptides from *S. capitis*, whereas the lowest activity was shown by a hemolysin from *S. hominis*. As expected, erythrocytes of various species exhibited different sensitivity to the studied hemolysins – the most sensitive were guinea pig, dog, rabbit and human red cells, the most resistant were the sheep red blood cells. This may have resulted from various mechanisms of action or specific physicochemical properties of the studied hemolysins. The hemolysin used as a control was the most active against the erythrocytes of dog and guinea pig. Similar results had been obtained in previous studies (Różalska, data not published).

Moreover, the tested hemolysins had a cytotoxic effect on Hs27 cells – human skin fibroblasts (Fig. 4). Interestingly, despite the different hemolytic activity, the hemolysin doses resulting in a reduction of cell metabolism by 50% were similar for all extracts, and ranged from 1 to 3 HU. However, the kinetics of cytotoxic activity of individual hemolysins slightly differed. The effect of *S. cohnii* ssp. *urealyticus* and *S. capitis* hemolysins on Hs27 cells was relatively rapid, as within 1 hour the metabolic activity was reduced to 50%. Other hemolysins caused a decrease to 56–58% of Hs27 cells' metabolic activity after 6 hours of incubation. The decrease in the cytotoxic activity of tested hemolysins was observed after approx. 2 hours of incubation. Interestingly, the Hs27 control cells not exposed to hemolysins showed a slight increase in the metabolic activity during 24 hours of incubation, except the time between 1 and 2 hours of incubation where the same metabolic activity level was noted (data not shown). A similar phenomenon was observed earlier during research on cytotoxicity of hemolysins produced by *S. cohnii* ssp. *urealyticus* and *S. cohnii* ssp. *cohnii* (Mak *et al.*, 2008). The decline in hemolysins' cytotoxic activity could have resulted from a temporary slowdown of the Hs27 cells metabolic activity. Also, the pore-forming toxins used at low doses could be inactivated by one of the possible ways: closing the pores, proteolysis, or the toxin shedding from the membrane. Hertle *et al.* (1999) noted that *Serratia marcescens* hemolysin (ShlA) present at sublytic doses, caused a reversible intracellular ATP reduction in the fibroblasts and HEp-2, HeLa, and Hec1B cells. In these cells, the restoration of the initial ATP concentration in a medium lacking ShlA probably resulted from the repair of ShlA pores and depended on the cellular protein synthesis. Additionally, the ability to restore the ATP level was related to the duration of the hemolysin action and decreased with time. *Further* studies need to be undertaken to understand the mechanism of interaction between the tested CoNS δ-hemolysins and Hs27 cells.

The cytotoxic effect on the Hs27 cells caused by the tested peptides was also observed under the microscope after 6 hours of incubation (Fig. 5). The cells showed

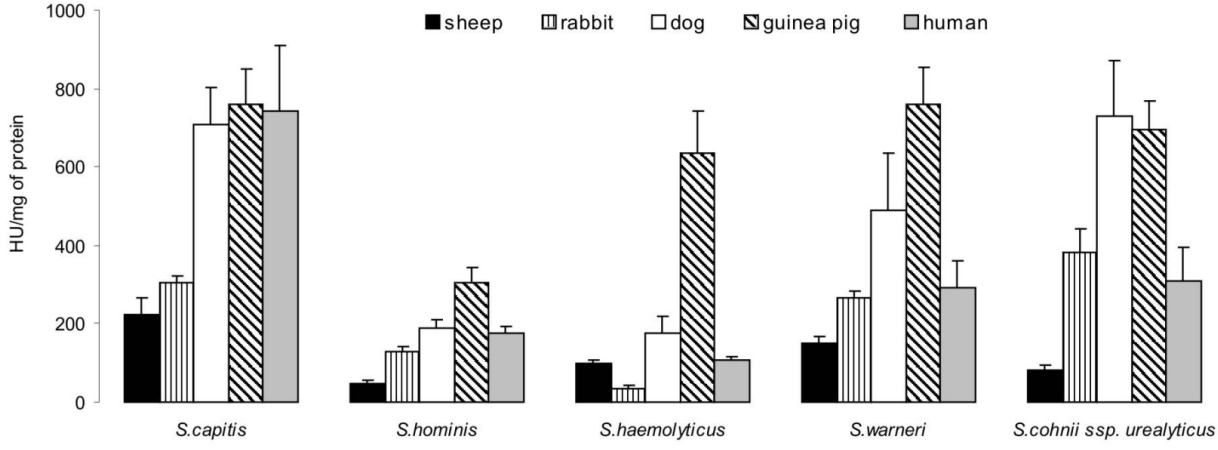

Figure 3. Hemolytic activity of synergistic hemolysins produced by the studied *Staphylococcus* species.
The hemolytic activity was expressed as HD_{50}, i.e. the concentration of hemolysin (HU/mg of protein) that caused 50% of hemolysis.

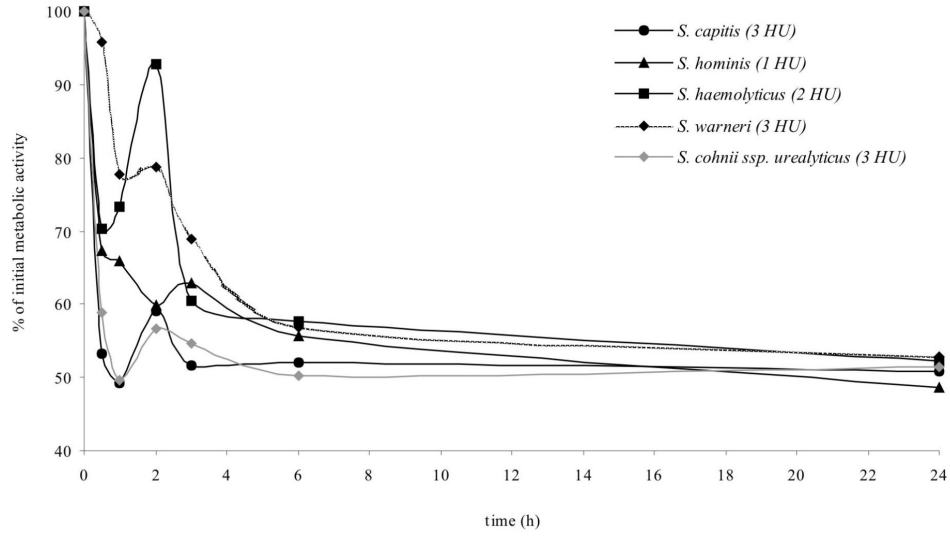

Figure 4. Time-course of cytotoxic action of the *Staphylococcus* species hemolysins on Hs27 cells, determined by using the MTT test. Hemolysins at the LC_{50} concentration were used — LC_{50} values are given in brackets on the chart. The percentage of initial metabolic activity represents an average of at least three experiments.

signs of cytotoxicity, such as a round shape, a damaged edge and a lower number of cells in the visual field, as compared to the unaffected control. Scheifele *et al.* (1987) also found that a δ-like toxin from CoNS isolated from neonates with necrotizing enterocolitis caused damage to the monolayer of the human foreskin. The toxic effect was also observed on murine fibroblasts and procine keraticoyte cells treated with culture supernatants of *Staphylococcus hyicus* strains producing a cytotoxin similar to δ-hemolysin of *S. aureus* (Allaker *et al.*, 1991). Moreover, purified δ-like toxin from *S. epidermidis* induced necrosis of mucus and hemorrhage in injected loops of the bowels of infant rats (Scheifele *et al.*, 1987). Other studies indicate that a chronic orofacial muscle pain (Butt *et al.*, 1998) or inflammation of bovine mammary glands (Watts & Owens, 1987) may be associated with δ-hemolysins produced by CoNS. All this data shows that δ-hemolysin is an important virulence factor of coagulase-negative staphylococci.

Determination of antimicrobial action of hemolysins complemented the study of their biological activity. Antimicrobial activity of the hemolysins of *S. haemolyticus* and *S. warneri* had been determined in other studies (Frenette *et al.,* 1984; Verdon *et al.,* 2008). Therefore, the research presented here focused on the investigation of the hemolysins of *S. capitis* and *S. hominis*. The antimicrobial activity of hemolysins produced by the two subspecies of *S. cohnii* was also tested since in the

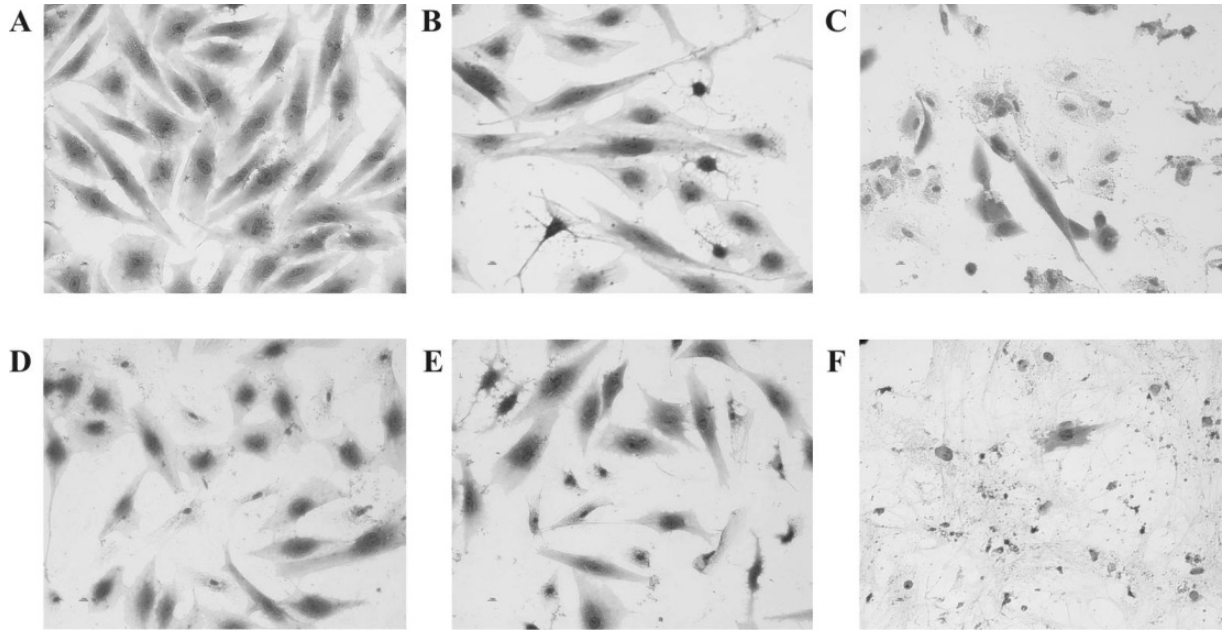

Figure 5. Cytotoxic effect of the tested *Staphylococcus* species' synergistic hemolysins on Hs27 cells.
(**A**) Cells after 6 hours of incubation in medium without a hemolysin; (**B–F**) Destruction of Hs27 cells by hemolysins from *S. capitis* (**B**), *S. hominis* (**C**), *S. haemolyticus* (**D**), *S. warneri* (**E**), *S. cohnii* ssp.*urealyticus* (**F**) as a positive control after 6 hours of incubation with peptides. The cells were stained with crystal violet and observed with the light microscope under 400x magnification.

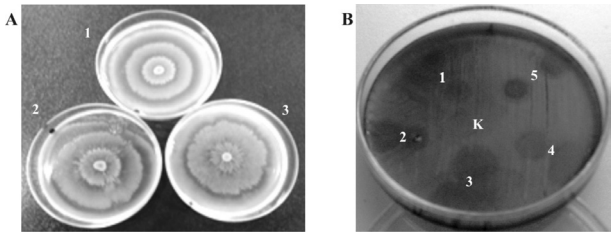

Figure 6. Influence of synergistic hemolysins produced by CoNS on other bacteria.
(**A**) No effect of *S. capitis* synergistic hemolysin on *P. mirabilis* ZMF P28 swarming growth (**1**) — medium supplemented with hemolysin (100 HU per mL) dissolved in 70% aqueous solution of acetone; controls: (**2**) — medium without hemolysin and (**3**) — medium with 70% acetone. (**B**) *Neisseria meningitidis* growth inhibition by δ-hemolysins of CoNS. Extracts of hemolysins (20 μL and 10 μL) — *S. capitis* (**1**), *S. hominis* (**2**), *S. c.cohnii* (**3**), *S. c.urealyticus* (**4**), *S. warneri* (**5**) and PBS as a control (**K**).

previous studies this feature had not been precisely defined. In this study, a collection of microorganisms was used, including references strains, bacteria isolated from pharynx, larynx and the postoperative wounds from patients with laryngeal cancer treated surgically (Różalska & Józefowicz-Korczyńska, 2001). Also, bacterial strains derived from the mucous membranes of the nasal vestibule of healthy people and from clinical specimens - blood, peritoneal fluid or surgical wounds were being investigated. One isolate, *Staphylococcus xylosus* ZMF 1115, came from a hospital environment. The hemolysin dilution method in solid medium was applied for testing the antimicrobial activity. This method was possible to use since staphylococcal synergistic hemolysins have a very high thermostability and the hemolysins were very easy to obtain in the amount sufficient for testing. It is also worth emphasizing that this method allowed for examining the inhibition of growth of several microorganisms at the same time. It was found that only three bacterial species, *Corynebacterium diphtheriae* type gravis, *Micrococcus luteus* ZMF2012 and *Pasteurella multocida* P16, did not grow in the presence of hemolysins. This growth was inhibited by hemolysin of *S. capitis,* while hemolysin obtained from *S. hominis* inhibited only Gram-positive cocci of the *Micrococcus luteus* ZMF2012 strain. It was observed that none of the synergistic hemolysins inhibited the swarming growth characteristic for *Proteus mirabilis* rods. Figure 6A shows an example of *P. mirabilis* growth in the presence of *S. capitis* hemolysin. To determine the Minimal Inhibitory Concentration (MIC), agar plates containing the hemolysin of *S. capitis* and *S. hominis* in the amounts of 100, 75, 50 and 25 HU/mL, were prepared. Table 2 summarizes the results of these experiments. As expected, the examined δ-hemolysins had a very narrow spectrum of antimicrobial activity. Similar results were presented by Kreger *et al.* (1971). The authors described δ-toxin produced by *S. aureus* which inhibited only the growth of three strains: *Micrococcus luteus*, *Streptococcus pyogenes* and *Bacillus megaterium* KM. The hemolysin isolated from the culture of *S. haemolyticus* inhibited only the growth of *Neisseria gonorrhoeae* and *Neisseria meningitidis* strains, and also of rods from the *Corynebacterium* genus, which were not classified to the species (Frenette *et al.,* 1984). Preliminary research showed that all hemolysins tested in the present study inhibit the *Neisseria meningitidis* growth (Fig. 6B). These studies should be continued with the use of other species of *Neisseria*. Recently, Marchand *et al.* (2011) also found that hemolysins of *S. haemolyticus* were active against Gram-negative rods of the *Legionella* genus. It would be interesting to analyze in the future the possible anti-*Legionella* activity of synergistic hemolysins isolated from the tested strains of *S. capitis, S. cohnii* and *S. hominis*. These issues require further investigation.

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