STAPHYLOCOCCUS WARNERI CLINICAL ISOLATE SUSCEPTIBILITY TO ANTIBIOTICS AND ITS MODIFICATION BY EMOXYPINE

*N.A. Bobrova, G.A. Loban, E.M. Vazhnichaya, M.O. Faustova, M.M. Ananieva
UKRAINIAN MEDICAL STOMATOLOGICAL ACADEMY, POLTAVA, UKRAINE

Background. S. warneri is a common commensal organism, but it can cause serious infections. One of the ways to increase a susceptibility of this microorganism to antibiotics is their combining with adjuvant remedies.

Objectives. The aim of the research is to study the susceptibility of S. warneri clinical isolate to antibiotics and its modification by emoxypine - 2-ethyl-6-methyl-3-hydroxypyridine hydrochloride.

Methods. Samples of purulent wound exudation were obtained from a patient with infective complications after a bone fracture and osteosynthesis by metal plates. Susceptibility of S. warneri clinical isolate to antibiotics and their combinations with emoxypine (1000 μg/disk) was studied by a standard disc diffusion technique. In the case of microbial resistance, the susceptibility of the investigated isolate to such antibiotic was determined by a serial broth dilutions method without or with emoxypine and evaluated according to the minimum inhibitory concentration (MIC).

Results. By the disk diffusion method, S. warneri isolate was susceptible to all tested antibiotics, except chloramphenicol. The growth inhibition zones also were formed around disks containing emoxypine, but the susceptibility of S. warneri to this agent was low. Applying of emoxypine on the disks with antibiotics resulted in the increase of S. warneri growth inhibition in all cases, except using the amikacin, gentamicin, and fusidic acid. The most significant changes were evidenced for a composition chloramphenicol / emoxypine. Using chloramphenicol alone in the liquid medium, the MIC was over a cut-off point. Adding of emoxypine (209 μg/ml) decreased the MIC of the antibiotic and restored the susceptibility S. warneri to chloramphenicol.

Conclusions. The susceptibility of S. warneri clinical isolate to antibiotics can be increased by combining with emoxypine, which among other overcomes the resistance of the studied microorganism to chloramphenicol.

KEY WORDS: S. warneri, susceptibility, antibiotics, 2-ethyl-6-methyl-3-hydroxypyridine (emoxypine).

Introduction
Coagulase-negative staphylococci (CoNS) were taken as insignificant contaminant earlier, but now they are regarded as a cause of many multidrug resistant infections. The significant change in the patients profile, that is an increased number of premature newborns, elderly patients, chronically ill patients, and immunocompromised patients along with greater use of indwelling or implanted foreign bodies has made CoNS a predominant nosocomial pathogen [1]. S. warneri is a catalase-positive, oxidase-negative, and CoNS, and is a common commensal organism found as part of the skin flora in humans and animals [2]. This pathogen can cause abortion in domestic animals and humans [3]. In humans, it causes discitis, urinary tract infections, and meningitis [4, 5, 6]. Orthopedic infections due to S. warneri also have been reported [7, 8]. Over the last two decades, similarly to other CoNS species, S. warneri has been reported as a new emerging pathogen, capable of causing serious infections usually in association with the presence of implant materials [9, 10]. Very often S. warneri strains isolated from clinical material are resistant to one-two antibiotics or even all tested antimicrobials (to 18% of clinical isolates) [11].

One of the ways to increase a susceptibility of the microorganism to antibiotics is their use in a combination with adjuvant remedies. Synthetic antioxidant 2-ethyl-6-methyl-3-hydroxyypyridine hydrochloride (emoxypine) is used in clinic, has its own antimicrobial activity and is able to enhance microbial susceptibility to antibiotics in the experiments with etalon strains of microorganisms [12, 13, 14]. For implementation of these experimental data into the anti-infective therapy, it is interesting to evaluate the influence of 2-ethyl-6-methyl-3-hydroxyypyridine hydrochloride on the clinical isolate of S. warneri as a representative of CoNS.

The aim of the research is to study the susceptibility of S. warneri clinical isolate to anti-
biotics and its modification by 2-ethyl-6-methyl-3-hydroxypridine hydrochloride (emoxypine).

**Methods**

Some samples of purulent wound exudation were obtained from a 57-year old female patient with infective complications developed on the 7th day after the bone fracture and osteosynthesis by metal plates without subsequent antibiotic therapy. It was used for microbiological assessments and diagnosis. The patient has given an informed consent for sampling of wound exudation and the use of these samples for investigation. After collection, the sample was transferred to a microbiological laboratory in less than 1 hour. Bacteriological culture was collected in sterile ball vials. Gram staining was made on the first day. Cultivation was carried out in thioglycolic medium manufactured by the Mechnikov Ilya Institute of Vaccines and Serums. 1.5% of agar “Dyfko”, 5% of blood and 0.5-1% of yeast hydrolysate was added to promote growth of microorganisms, followed by isolation of pure cultures of facultative anaerobic bacteria in presence of 5-10% CO₂. The medium was observed daily for microbial growth. Final identification was performed by the automatic bacteriological analyzer Vitrek 2 Staphylococcus cards (Biomérieux®, France). The research was performed in the Medical Laboratory BRight-Bio (License AB No. 526132, dated February 4, 2010) and partly in the Research Laboratory of the Department of Microbiology, Virology and Immunology of Ukrainian Medical Stomatological Academy. The experiments were approved by the Commission on Bioethics of Ukrainian Medical Stomatological Academy.

Susceptibility of the tested isolate to antibiotics was determined using disk diffusion method [15]. The following disks with antibiotics were used: penicillin G (10 μg), oxacillin (30 μg), ampicillin (30 μg), cefoxitin (30 μg), norfloxacin (10 μg) erythromycin (15 μg), tetracycline (30 μg), amikacin (30 μg), gentamicin (10 μg), co-trimoxazole (1.25/23.75 μg), chloramphenicol (30 μg), and fusidic acid (10 μg). All the antibiotic disks were procured by HiMedia Laboratories Pvt Ltd., India. Substance of such antibiotic (chloramphenicol) was produced by the Biopharma Company (Ukraine). Mother solutions contained 312 μg/ml of the antibiotic (0.031% solution), 10000 μg/ml of emoxypine (1% solution), or 312 μg of the antibiotic and 10000 μg of the potential adjuvant per 1 ml. Twofold serial dilutions of the antibiotic, adjuvant and their combination were prepared and dispensed into the laboratory tubes. A growth control tube and a sterility (uninoculated) tube also were prepared. The inoculum was prepared using the direct colony suspension method. An 18-hour old culture of *S. warneri* was grown on blood agar. For inoculation microbial suspension equivalent to 1.0 by McFarland Equivalence Standards, diluted 1/100 in saline, was used, and then the concentration of microorganisms in this suspension was 3 x 10⁷ CFU/cm³. Laboratory tubes with macrodilutions were incubated for 24 hours at 37 °C. The amount of growth in the tubes containing the antimicrobial agent was compared with the amount of growth in the growth control tubes. The results were registered visually. The susceptibility of the tested microorganism was evaluated according to the minimum inhibitory concentration (MIC).

Every determination was repeated five times and digital data was processed by the computer programs Statistica for Windows 6.0. The average mean (M) and its standard error (m, SE) were calculated. The lines of variants were checked for normal values. Since in all the cases there was a normal distribution of data, the difference between the groups was evaluated by the Student’s t-test.

**Results**

Gram positive cocci were found in the samples of purulent exudation at the first stage of bacteriological investigation. They were identified as *S. warneri* by automatic microbiological analysis.
Routine disk diffusion method has demonstrated that this clinical isolate is susceptible to majority of antibiotics (Table 1). Zones of growth inhibition around the disks with all antimicrobials, except chloramphenicol, ranged between 24-32 mm that was characteristic to the susceptible strain of CoNS [16]. At the same time, a diameter of growth inhibition zone around a disk with chloramphenicol was less than 18 mm, a cut-off value of susceptibility that testified the resistance to this antibiotic. The growth inhibition zones also were formed around disks containing emoxypine, but their diameters confirmed low susceptibility of S. warneri culture to this synthetic antioxidant. For all antibiotics, except chloramphenicol, zones of growth inhibition were more than the same for emoxypine with a credibility from t=11.18 p<0.001 to t=6.47 p<0.005. Diameter of the test culture growth inhibition around disks with chloramphenicol did not differ from this parameter around disks with emoxypine.

Applying of emoxypine on the standard disks with antibiotics resulted in the increase of growth inhibition zones of S. warneri test culture as compared to appropriate antibiotic in all cases, except the use of amikacin, gentamicin, and fusidic acid (Table 1). Diameter of the growth inhibition zone for a combination of emoxypine with benzylpenicillin was greater by 9.2 mm (t=4.03, p<0.02), oxacillin – by 9.6 mm (t=7.0, p<0.005), ampicillin – by 6.8 mm (t=2.92, p<0.05), cefoxitin – by 7.0 mm (t=4.43, p<0.02), norfloxacin – by 5.2 mm (t=2.83, p<0.05), erythromycin – by 4.5 mm (t=2.95, p<0.05), tetracycline – by 6.8 mm (t=3.03, p<0.05), co-trimoxazole – by 4.2 mm (t=3.88, p<0.02), chloramphenicol – by 15.4 mm (t=11.32, p<0.001), rifampicin – by 5.5 mm (t=2.78, p<0.05) compare to antibiotic itself. The most significant changes of growth inhibition were observed for a composition of chloramphenicol/emoxypine. Distinguishes between the growth inhibition of the S. warneri isolate caused by a combination of amikacin, gentamicin, and fusidic acid with an adjuvant were not significant as compared to the control administered with the standard antibiotic. At the same time, all zones of growth inhibition by combinations of antibiotics with emoxypine were more than the same of the adjuvant drug alone (a credibility from t=22.03, p<0.001, to t=8.10, p<0.002).

Chloramphenicol was only one antibiotic from the tested antimicrobials for which S. warneri isolate was resistant in cases of the disk diffusion method and for which effect of emoxypine was the most as compared to other compositions. That is why it also was investigated by a serial broth dilutions method. Results of MIC determination are represented in Fig.1.

MIC was over the cut-off point for CoNS susceptibility, when chloramphenicol was used

| Antimicrobial agent | Diameter of growth inhibition zone, mm |
|---------------------|----------------------------------------|
| Emoxypine (1000 μg) | 10.6 ±1.1                               |
| Benzylpenicillin (10 μg) | 27.4±2.0*                             |
| Oxacillin (30 μg)    | 28.8±1.2*                              |
| Ampicillin (30 μg)   | 26.6±2.0*                              |
| Cefoxitine (30 μg)   | 26.8±1.5*                              |
| Norfloxacin (10 μg)  | 20.8±1.6*                              |
| Erythromycin (15 μg) | 23.5±1.3*                              |
| Tetracycline (30 μg) | 23.2±1.2*                              |
| Amikacin (30 μg)     | 25.0±1.8*                              |
| Gentamicin (10 μg)   | 25.4±1.0*                              |
| Co-trimoxazole (1.25/23.75 μg) | 19.8±0.9* |
| Chloramphenicol (30μg) | 7.8±0.8*                           |
| Fusidic acid (10 μg) | 25.2±1.0*                              |
| Rifampicin (5 μg)    | 28.0±1.4*                              |

| Antibiotic or adjuvant (emoxypine) | Combination of antibiotic and adjuvant (emoxypine) |
|------------------------------------|-----------------------------------------------|
| 36±1.1*                            | 38±0.6*                                       |
| 28±1.2*                            | 33±1.2*                                       |
| 33±1.5*                            | 33±0.5*                                       |
| 26±0.9*                            | 28±0.8*                                       |
| 30±1.9*                            | 28±1.6*                                       |
| 27±1.3*                            | 24±0.6*                                       |
| 23±1.1*                            | 29±1.2*                                       |
| 33±1.9*                            |                                               |

Notes: * – p<0.05 compare to growth inhibition zone for emoxypine; ** – p<0.05 compare growth inhibition zone for this antibiotic without emoxypine.
alone [16] (Fig. 1A). With adding of emoxypine MIC of the antibiotic decreased from average 52 μg/ml to 6.5 μg/ml (t=3.47, p<0.05) and the susceptibility of S. warneri clinical strain to chloramphenicol restored. At the same time, MIC of emoxypine alone was 416 μg/ml (average); it proved low susceptibility of the studied isolate to this agent (Fig. 1B). A concentration of emoxypine complied with MIC of a composition chloramphenicol/emoxypine was 209 μg/ml, that was 2 times less as compared to MIC of emoxypine (t=3.0, p<0.05).

**Discussion**

The investigated S. warneri clinical isolate was susceptible to the antibiotics of the penicillins, cephalosporins, fluoroquinolones, aminoglycosides, macrolides, tetracyclines, rifampin groups and steroids, which are recommended for screening of CoNS susceptibility to antimicrobial agents [16]. A high susceptibility of this isolate could be explained by the short history of the hospital treatment and the absence of previous antibiotic therapy in a patient. At the same time, the clinical isolate was resistant to chloramphenicol that was evidenced by a small growth inhibition in cases of disk diffusion method and high MIC in a serial broth dilutions method as compared to cut-off values susceptibility / resistance for CoNS [16]. This can be explained by the nature of chloramphenicol. As it is an ancient microbial metabolite, genetic elements conferring resistance against this drug are retained by and are frequently dispersed in microbial communities [17].

Response of the tested microorganism to emoxypine as a potential adjuvant of antimicrobials was similar to the results of our previous investigations [14, 18]. It was also consistent with other experimental results concerning etalon strains of gram negative rods [12, 13], as well as the retrospective computer prognosis of the biological activity for ethylmethylhydroxypyridine structure that showed a possible antimicrobial activity [19]. Emoxypine’s MIC in S. warneri test culture was less than this parameter of other CoNS (an etalon strain S. epidermidis ATCC 14990) [14]. There are no reference for susceptibility or resistance of bacteria to emoxypine, so we cannot conclude is clinical isolate susceptible to this agent or not, but it is possible to think that S. warneri isolates susceptibility to emoxypine is low and the use of this preparation as an adjuvant of antimicrobial therapy will be more perspective.

The mechanism of emoxypine antibacterial action is still unclear and the most likely it is due to modification of the bacterial cell membrane typical to antioxidants. Other mechanisms, e.g. protein synthesis inhibition, may also play a part in the antimicrobial action of this 3-hydroxypyridine derivative [19].

Combining of antibiotics with emoxypine led to an increase of growth inhibition zones of S. warneri test culture that proved enhance of the clinical isolate susceptibility to these agents. The effect observed was similar to the changes of susceptibility of the etalon strains of S. aureus and E. coli under the influence of combinations of antibiotics of different classes and relative

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**Fig. 1. Minimum inhibitory concentration of chloramphenicol (A) and emoxypine (B) alone and in a combination against S. warneri clinical isolate.**

*Note. * – p<0.05 as compared with antimicrobial or adjuvant themselves.*
preparation: 2-ethyl-6-methyl-3-hydroxypyridine succinate (mexidol) [14].

In the case of the resistance of S. warneri isolate to chloramphenicol, adding of emoxypine restored the susceptibility of the tested microorganism that was registered both in the disk diffusion and serial broth dilutions methods. It is established that resistance to chloramphenicol is mainly present due to the production of a specific inactivating chloramphenicol acetyl-transferase, reduced drug uptake or increased drug efflux [17, 20]. That is why it is reasonably that emoxypine effect is caused by its influence on all or some of the mentioned mechanisms, especially on the membrane permeability and efflux pumps because membrane modification is a fundamental property of 2-ethyl-6-methyl-3-hydroxypyridine structure [19].

Overcoming resistance of the clinical isolate S. warneri to chloramphenicol occurred with significant MIC of emoxypine in the medium, which is much higher than the maximum concentration of this substance or its salt with succinic acid in the blood plasma after the intravenous administration [21]. Therefore, despite the fact that the description of this phenomenon opens up new possibilities in controlling the susceptibility of CoNS, a matter of the ratio of the concentration of emoxypine in the in vitro experiments with a dose of this potential antimicrobial adjuvant in clinic is still unclear. The prospects of the research presented should cover the research of dose/concentration range of emoxypine adjuvant activity.

Conclusions

S. warneri isolate, susceptible to penicillin G, oxacillin, ampicillin, cefoxitin, norfloxacin, erythromycin, tetracycline, amikacin, gentamicin, cotrimoxazole, and fusidic acid, but resistant to chloramphenicol, was isolated from the clinical material. The susceptibility of this clinical isolate to all antibiotics listed, except amikacin, gentamicin, and fusidic acid, can be increased significantly when combined with 2-ethyl-6-methyl-3-hydroxypyridine hydrochloride (emoxypine), which, among other things, restores the susceptibility of the studied microorganism to conventional rates at its concentration of 209 µg/ml.

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Conflict of Interests

The authors declare no conflict of interest.

Author Contributions

Nellya O. Bobrova – methodology, investigation; Galina A. Loban – project administration, supervision; Elena M. Vazhnichaya – conceptualization, writing – review and editing; Mariia O. Faustova – formal analysis, writing the initial draft; Maiia M. Ananieva – investigation, visualization.

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містять емоксипін, але чутливість S. warneri до цього засобу була низькою. Нанесення емоксипіну на диски з антибіотиками призвело до посилення пригнічення росту S. warneri у всіх випадках, за винятком застосування амікацину, гентаміцину та фузидової кислоти. Найбільш виражені зміни спостерігалися для комбінації хлорамфеніколу / емоксипін. При використанні самого хлорамфеніколу у рідкому середовищі, МПК була вище точки відсічення. Додавання емоксипіну (209 мкг/мл) зменшило МПК антибіотика та відновило чутливість S. warneri до хлорамфеніколу.

Висновки. Чутливість клінічного ізоляту S.warneri до антибіотиків можна збільшити, поєднуючи їх з емоксипіном, який, крім іншого, здатний подолати резистентність досліджуваного мікроорганізму до хлорамфеніколу.

КЛЮЧОВІ СЛОВА: S. warneri; чутливість; антибіотики; 2-етил-6-метил-3-гідроксипіридин (емоксипін).

Відомості про авторів
Bobrova Неля Олександрівна – кандидат біологічних наук, викладач кафедри мікробіології, вірусології та імунології Української медичної стоматологічної академії, м. Полтава.

Лобань Галина Андріївна – доктор медичних наук, професор, завідувач кафедри мікробіології, вірусології та імунології Української медичної стоматологічної академії, м. Полтава.

Важнича Олена Митрофанівна – доктор медичних наук, професор кафедри експериментальної та клінічної фармакології Української медичної стоматологічної академії, м. Полтава.

Фаустова Марія Олексіївна – доктор медичних наук, доцент кафедри мікробіології, вірусології та імунології Української медичної стоматологічної академії, м. Полтава.

Ананьєва Майя Миколаївна – доктор медичних наук, доцент кафедри мікробіології, вірусології та імунології Української медичної стоматологічної академії, м. Полтава.
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