

STUDY ON THE ANTIBIOTIC RESISTANCE OF STRAINS OF STAPHYLOCOCCUS AUREUS ISOLATED FROM ANIMALS WITH CONTAGIOUS ECTHYMA

STUDIU ASUPRA FENOMENULUI DE ANTIBIOTICOREZISTENȚĂ A TULPINILOR DE STAPHYLOCOCCUS AUREUS IZOLATE DE LA ANIMALE CU ECTIMĂ CONTAGIOASĂ

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ABSTRACT | REZUMAT

Contagious ecthyma is a viral infectious disease that produces pustular vesicular rashes on the skin and mucosa, affecting a wide range of species, goats, sheep, wild ruminants, even carnivores and human.

In this paper, it were investigated the strains of *Staphylococcus aureus*, isolated from a contagious ecthyma outbreak, from the lesions caused by this virus in 5 flocks of sheep in 3 hotbeds of the disease. Attention was paid to the exact establishment of the type of isolated *Staphylococcus*, and the assessment of the antibiotic sensitivity by performing the antibiogram (CLSI, 2012). The results of the antibiogram showed an increased sensitivity to the action of Gentamicin (96,6%) and of quinolones (ciprofloxacin 86,6%, enrofloxacin 83,3%), even of trimethoprim (80%). *Staphylococcus aureus* is a ubiquitous germ, the cases of antibiotic resistance encountered due to the unusual use of antibiotics are frequently recorded in this germ. The increase of the penicillin resistance phenomenon, widely used in the past, has led to new generations of antibiotics in this group (semi-synthetic penicillin). Based on the sensitivity of the strains of *Staphylococcus aureus* isolated in 7 types of antibiotics, it was computed the MAR index (multiple antibiotic resistance index), in order to establish the risk degree represented by these types of strains for animals and humans. The values of the MAR index were between 0,14 and 0,57, in 63,3% of the cases the value exceeding the limit of 0,2, showing the possibility of the occurrence of the multiple antibiotic resistance. Moreover, the values over than 0,2 also show the fact that antibiotics are frequently used in the outbreaks of the disease investigated.

Keywords: contagious ecthyma, superinfection, *Staphylococcus*, antibiotic resistance, MAR index

Ectima contagioasă este o boală infecțioasă virală, care produce erupții veziculo-pustuloase, pe tegumente și mucoase, afectând o gamă variată de specii, caprine, ovine, rumegătoare sălbatice, chiar și carnivorele și omul.

În lucrarea de față, s-au investigat tulpinile de *Staphylococcus aureus*, izolate din 3 focare de ectimă contagioasă, din leziunile provocate de acest virus la 5 turme de ovine. S-a insistat asupra stabilirii exacte a tipului de stafilococ și a evaluării sensibilității la antibiotice prin efectuarea antibiogramei. Rezultatele antibiogramelor au arătat o sensibilitate crescută la acțiunea gentamicinei (96,6%) și a quinolonelor (ciprofloxacină 86,6% și enrofloxacină 83,3 %), chiar și a trimetoprimului (80%). *Staphylococcus aureus* este un germen ubicvitar, cazurile de antibioretistență întâlnite din cauza utilizării nejudicioase a antibioticelor fiind frecvent înregistrate la acest germ. Accentuarea fenomenului rezistență la penicilină, utilizată pe larg în trecut a dus la obținerea unor noi generații de antibiotice din această grupă (peniciline semisintetice).

Pe baza sensibilității tulpinilor de *Staphylococcus aureus* izolate la 7 tipuri de antibiotice, s-a recurs la calcularea indicelui MAR (multiple antibiotic resistance index), pentru stabilirea gradului de risc reprezentat de aceste tulpini pentru animale și om. Valorile obținute ale indicelui MAR au fost între 0,14 și 0,57, în 63,3% din cazuri valoarea depășind limita de 0,2 ceea ce denotă posibilitatea apariției fenomenului de rezistență multiplă la acțiunea antibioticelor.

Totodată, valorile peste 0,2 indică și faptul că în focarele de boală investigate se utilizează frecvent antibiotice.

Cuvinte cheie: ectimă contagioasă, suprainfecție, stafilococ, antibioretistență, indice MAR

Contagious ecthyma is a viral disease that affects predominantly domestic ruminants, wild animals but also pets (9). It is an infectious disease with acute evo-

lution, produced by an epitheliotropic virus, clinically characterized by bladder rash, with predominantly buccal, podal, genital, mammary and ocular localizations (5, 9). The infection can also occur in humans, especially in people who handle diseased animals, manifesting in the form of hand or face rashes (7). The causal agent of this disease is a epitheliotrope virus, which contains DNA and is included in the *Poxviridae* family,

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the *Parapoxvirus* genre, known as "orf virus" or "contagious ecthyma virus", with sizes of 158 x 252 nm and morphology similar to the vaccine virus (9). The virus can also be found in the body of seemingly healthy animals, these being called carriers. In the infected crusts, it can withstand even 12 years and is resistant to glycerol and ether action (16).

The most serious complication of this disease is represented by secondary infections, which may lead to the worsening and extension of the disease's evolution, the result being even the death of the animal. In case of secondary infections, under the crust, the purulent collections can appear (2). Among the bacteria populating the lesions of contagious ecthyma are *Staphylococci*, *Streptococci*, *Escherichia coli*, *Fusobacterium necrophorum*, *Bacillus cereus*, *Corynebacterium spp.* Of the 28 species of the *Staphylococcus* genre, the most frequent species involved in the sheep pathology is *Staphylococcus aureus* causing a wide variety of predominantly suppurative disorders (14).

Antibiotic resistance testing has special indications especially for germs known to belong to microbial species that exhibit resistance to common antibiotics (1). The resistance mechanisms include the production of enzymes that inactivate the anti-inflammatory agent, target destruction, or blockage of the mode of action. An example is represented by the Gram-positive bacteria (*Staphylococcus aureus*) that produce intracellular β -lactamases - penicillinases - that degrade the β -lactam rings of penicillins (12). In the case of Gram-negative bacteria, the situation changes, besides the wide range of β -lactamases, to the acquiring of antibiotic resistance also contributes to the low permeability of the bacterial capsule or few penicillin receptors on the surface of the bacterial cell. Another situation is the presence of static methicillin staphylococci that lead to their remarkable resistance to all cephalosporin groups. Gram-negative bacteria can develop plasmid-mediated resistance to both penicillins and cephalosporins as well as to nitroimidazole antibiotics (8). Sulfamides and trimethoprim have lost their effectiveness in recent years due to the possibility of increased production of paraaminobenzoic acid but also by increasing the enzyme production that mediates the production of dihydrofolic acid, which decreases the affinity for this group of antibiotics. In order to prevent such phenomena, it was started the combined used of antibiotics, even if the associated antibiotic has no remarkable effects on the incriminated infectious agents, the combination triggers mechanisms that increase the curative effect (12).

As a consequence of the unproductive use of anti-infectives, the phenomenon of antibiotic resistance in the world of microorganisms has spread to alarming levels, thus becoming a major concern of medicine worldwide, concretized in the epidemiological monitoring of microbial antibiotic resistance (14). For these reasons, it was necessary to check the behavior of anti-infectives in all pathogenic microbial agents isolated from various sources (clinical human or animal cases, food, etc.). This study assessed the sensitivity to different antibiotics of *Staphylococcus aureus* strains isolated in contagious ecthyma outbreaks in Santău, Sudurău and Chereușa localities in 2013.

MATERIALS AND METHOD

The materials used are represented by crusts from suspicious lesions of bacterial superinfection. The crusts were harvested from 40 sheep from 5 flocks of sheep from Santău commune, from 3 outbreaks of disease. In 30 of the 40 samples it was isolated the *Staphylococcus aureus* as superinfection agent, i.e. 30 strains. The samples were harvested using sterile swabs imbedded in sterile physiological saline and stored in sterile containers containing sterile Amies transport medium(13) and transported, avoiding thermal shock or any other shock at LSVSA Satu Mare, where the determinations necessary for the elaboration of this study were made. In the first stage, the isolation of bacterial strains was performed and the usual culture mediums usually indicated by thioglycolate broth and selective Agar-agar were seeded. In the liquid medium, uniform turbidity was found, with a homogeneous deposit, and the solid medium was observed to have large, smooth, dense and creamy colonies (about 8 mm in size). To demonstrate coagulation activity, the coagulation test in the tube was performed. The morphological characteristics of the isolated bacteria were evidenced by the Gram staining on the smears, presenting them in the form of gram-positive coils placed in clusters. The biochemical characteristics of the strains isolated on API ID 32 Staphylococcus galleries were also tested, the results being read by using the miniAPI device.

Based on the tests performed, it was found that the isolated bacterium was *Staphylococcus aureus* in 30 of the 40 harvested samples. To the resistance to antibiotics, we used the Kirby-Bauer method, based on the presence or absence of an inhibition area whose diameter depends on the sensitivity of the tested bacterial strain (13). For the testing we used micro-ta-

blents that are available on the market in cartridges, such as: gentamicin (10µg), amoxicillin with clavulanic acid (20µg), cefuroxime (30µg), enrofloxacin (10 µg), ciprofloxacin 10µg) and trimethoprim (5µg), interpreting the results according to CLSI 2012.

RESULTS AND DISCUSSIONS

In 30 of the total 40 samples harvested, there were isolated germs of the species *Staphylococcus aureus* according to the morphological features, cultures and biochemistry. Thus, after examination of the Gram stained smears (Fig. 1), in each sample the characteristics of gram-positive cocci, unfolded in clusters, were observed.

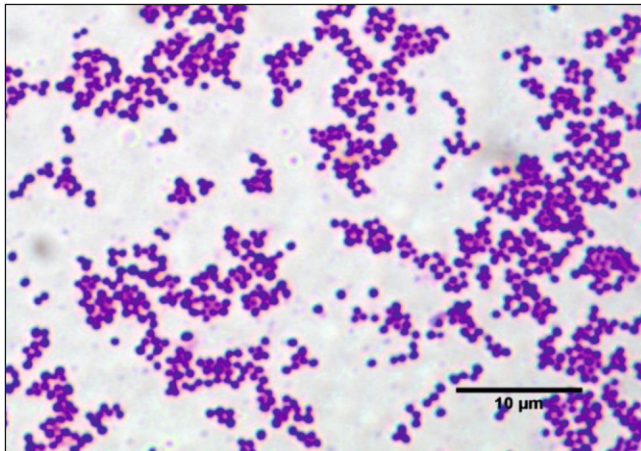


Fig. 1. *Staphylococcus aureus*, Gram staining 1000x

After the standard broth sowing according to the above method, moderate turbidity with a slightly homogeneous deposit was observed (Fig. 2).



Fig. 2. Standard broth cultivation, comparatively with a sterile control sample

In the passage on non-selective solid medium - blood agar, large, smooth, creamy colonies with large hemolysis zones were observed and on the selective medium (Baird Parker), there were seen some glossy black colonies, with white border, surrounded by a halo (Fig. 3).

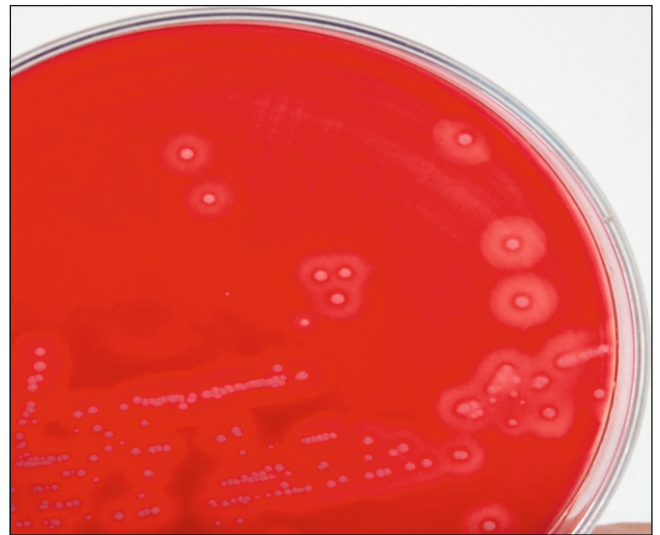


Fig. 3. *Staphylococcus aureus* colonies on selective Baird Parker medium

The coagulase activity, given by staphylocoagulase, was also highlighted, which is an essential criterion in the identification of *Staphylococcus aureus*. We tested the sensitivity of the 30 strains of *Staphylococcus* Genus at the action of 7 antibiotics (Fig. 4).



Fig. 4. Mueller Hinton agar, *Staphylococcus aureus*, antibiogram

The standardized method has presented criteria for the interpretation of inhibition areas, through which it is possible to determine the level of sensitivity of the

Table 1

The result of testing the action of the 7 antibiotic types on isolated *Staphylococcus aureus* strains, calculated by measuring the inhibition zone

Antibiotic Sample no.	Genta-micin	Cefu-roxim	Amoxi-cillin with clavulanic acid	Enro-floxacin	Cipro-floxacin	Peni-cillin	Trime-thoprim
1	19S	18R	17R	22S	22S	23R	18S
2	18S	23S	23S	25S	25S	20R	17S
3	17S	26S	25S	22S	26S	19R	12R
4	19S	21S	26S	25S	25S	21R	20S
5	20S	23S	14R	25S	25S	21R	21S
6	20S	23S	22S	21S	27S	22R	20S
8	19S	25S	24S	14R	14R	16R	9R
10	18S	21S	14R	19S	26S	10R	20S
11	18S	21S	24S	14R	14R	21R	21S
12	19S	24S	23S	14R	14R	25R	18S
13	19S	23S	15R	21S	25S	12R	19S
14	18S	25S	25S	20S	27S	20R	18S
15	20S	21S	25S	17R	17R	21R	18S
17	21S	27S	25S	20S	25S	22R	17S
18	21S	25S	23S	16R	16R	19R	20S
19	18S	13R	26S	21S	27S	18R	21S
20	19S	18R	17R	23S	24S	19R	20S
21	20S	22S	17R	23S	23S	23R	19S
22	19S	21S	23S	20S	25S	21R	9R
23	19S	22S	21S	21S	23S	23R	10R
25	18S	22S	25S	22S	22S	23R	9R
27	20S	21S	26S	23S	25S	23R	13R
28	20S	26S	25S	20S	25S	26R	20S
29	18S	25S	23S	21S	23S	26R	19S
30	18S	25S	24S	24S	24S	21R	19S
33	19S	23S	21S	24S	16R	20R	17S
34	19S	23S	21S	22S	22S	18R	17S
37	13R	11R	21S	25S	25S	17R	18S
38	20S	22S	21S	25S	25S	20R	18S
39	17S	25S	18R	23S	23S	21R	12R

various antimicrobial substances of the tested strains. These interpretation criteria were established by correlating the inhibition zones with the minimal inhibitory concentration. Their design was based on the study of a large number of bacterial germs, including those with known resistance mechanisms for certain classes of antimicrobial agents and in correlation with the pharmacokinetics of the antimicrobial substances used in therapy, as well as their clinical effectiveness. Depending on the diameter of the inhibition zone, the tested bacterial strain may be interpreted as being sensitive, moderately sensitive or resistant to the ac-

tion of the anti-microbians. The "sensitive" category implies that the test strains are inhibited by the usual concentrations of the antimicrobial agent, when used in the usual dose. The „moderately sensitive" category includes situations in which the minimal inhibitory concentration of the antibiotic reaches a certain blood and tissue level, but the response rate of the body may be lower than in the "sensitive" category. Substances included in the "moderately sensitive" category can be used in the therapy of localized infections, where the antibiotic is physiologically concentrated (e.g. quinolone and β -lactam in urine) or where it is possible to

Table 2

Percentage representation of the sensitivity to each tested antibiotic

Tested antibiotic	Genta-micin	Cefu-roxim	Amoxi-cillin with clavulanic acid	Enro-floxacin	Cipro-floxacin	Trime-thoprim	Peni-cillin
Sensitivity percentage	96,6%	86,6%	76,6%	83,3%	86,6%	80%	0%
Resistance percentage	3,4%	13,4%	23,4%	16,7%	13,4%	20%	100%

use higher doses than usual (In the case of β -lactams). The "resistant" category means that the test strain is not inhibited by the usual systemic concentration achieved by the antimicrobial substance administered at the usual dosage or the test strain possesses specific resistance mechanisms (e.g. β -lactamases); in such cases, therapy is ineffective. In all cases, isolated *Staphylococcus* strains were found to be resistant to penicillin action, the inhibition zone being less than 28 mm, indicating the existence of β -lactamase. For the rest of the tested antibiotics the results were satisfactory, most of the isolated strains being susceptible to the action thereof. It was found that 29 of the 30 isolated strains proved to be sensitive to the action of gentamicin. It was also tested the sensitivity to cefuroxime, finding that 26 strains were sensitive to the activity thereof. In the case of amoxicillin with clavulanic acid, 23 of the 30 isolated strains proved to the sensitivity thereof. Enrofloxacin proved to be effective only for 25 strains, while ciprofloxacin was effective for 26 strains. 24 strains proved to be sensitive to trimethoprim. In Table 1 are represented the diameters of the inhibition areas for each tested strain and antibiotic.

Following the comparison of the results obtained, we found that in 19 of the 30 isolated strains, antibiotic resistance was multiple, these being resistant to 2, 3 or even 4 antibiotics tested, representing 63,33% of total number of isolated. 20% of the isolated strains were resistant to 3 of the antibiotics tested, 23,33 % in 2 of the antibiotics, 3,33 % in 4 of the antibiotics, 53,34% in one antibiotic. The best results were obtained when testing Gentamicin, of the 30 strains being only one resistant, although the literature described the resistance to aminoglycosides of the *Staphylococcus aureus* (12). Similarly, trimethoprim and, in generally, sulfamides are considered antibiotics with low efficacy in the treatment of staphylococcal infections but our results showed a sensitivity of 80 % (7, 12). Compared to the rest of the antimicro-

bials tested the amoxicillin-clavulanate combination were low as it is shown in Tables 1 and 2. Expressed as a percentage, the result of the antimicrobial test demonstrates that except for penicillin, all the other 6 antibiotics tested were found to be effective in the event of a contagious ecthyma complication, the results being higher than 70% (Tables 1 and 2).

Resistance to antibiotics is a problem representing a major concern in veterinary medicine and human medicine because many cases of staphylococcus have been found to be resistant to antibiotics. In this regard, after performing the antibiograms and the interpretation of the results thereof it was established the MAR index (multiple antibiotic resistance index) to determine whether or not the disease outbreaks of which they are isolated represent a major risk source.

The MAR index represents the ratio between the number of antibiotics to which the isolated *Staphylococcus* of each sample is resistant and the total number of antibiotics used (1, 7, 10). The index was computed for each *Staphylococcus* and the results obtained are presented in Table 3.

In 19 of the 30 isolated strains, the MAR index was higher than 0,2, which represents a percentage of 63,33%. This situation reflects that multiple antibiotic resistance occurred because at the values of 0,2 indicates a resistance to 2 antibiotics. According to Jorgensen et al. (6) and Obajuluwa et al. (11), the value of 0,2 is a high value for the MAR index. This, according to Adegoke and Mkize et al. (1), it means that there is a high risk of occurrence of the multiple antibiotic resistance phenomenon and that the isolated bacteria come from areas where anti-microbials are often and unjustifiably used. The highest possible value of MAR index is 1, that indicates a resistance to all the tested antibiotics, in our case we did not get such result, but the 8 samples with the value of 0,42 and the one with 0,57 represented a reason to concern.

Table 3
MAR index – multiple antibiotic resistance
for every sample

Sample no.	MAR Index
1	0,42
2	0,14
3	0,28
4	0,14
5	0,14
6	0,14
8	0,57
10	0,28
11	0,42
12	0,42
13	0,28
14	0,14
15	0,42
17	0,14
18	0,42
19	0,28
20	0,42
21	0,28
22	0,28
23	0,28
25	0,28
27	0,28
28	0,14
29	0,14
30	0,14
33	0,28
34	0,14
37	0,42
38	0,14
39	0,42

CONCLUSIONS

Most of the secondary infections occurred in the 3 outbreaks of contagious ecthyma are those with *Staphylococcus aureus*, this being the major cause of the complications occurred, such as the various dermatitis, or mammitis, gingivitis or palatinitis or other purulent processes. This is also due to the fact that *Staphylococci* are ubiquitous germs, even on the skin or mucous membranes (6, 14).

Given the frequency of secondary infections, it was required the isolation and identification of the causative agent of the complications to establish a correct treatment.

Most of the antibiotics tested proved to be efficient, therefore it was not difficult to choose the antibiotic required to establish the anti-infective treatment in the 3 outbreaks. Contrary to the information in literature (3, 12), high percentage of trimethoprim resistance was found although it is known that mecha-

nisms by which production of para-amino-benzoic acid increases and changes in the enzyme increases the dihydro-folate acid, decreases the affinity to trimethoprim.

The fact that the penicillin resistance was signaled in all the isolated strains show the fact that the activity β -lactamase of the isolated strains was present. Since penicillin is an affordable and widely used product in veterinary medicine, the result obtained could be expected.

Following the tests performed, it was found an increased sensitivity of the isolated *Staphylococcus* to the action of the products of the category of quinolones and gentamicin. In our case, the ability to inhibit the 30S ribosome is effective, as described in the literature (3, 11).

The high computed values of the MAR index in most of the cases show the fact that the possibility of the occurrence of resistance to the multiple antibiotic was high in the 3 outbreaks of disease and a risk for the contacts.

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