Antibiotic Resistance Profile in Co-Infecting Bacteria Isolated from Sheep Displaying Orf Lesions

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Bulletin UASVM Veterinary Medicine 77(2)/2020

Print ISSN 1843-5270; Electronic ISSN 1843-5378

doi:10.15835/buasvmcn-vm:2020.0025

Abstract

Antibiotic resistance in veterinary medicine poses lately increasing risks in therapeutic success and threatens the life of patients with various bacterial infections. Environmental bacteria are very likely to develop on the initial lesions induced by orf virus in sheep, worsening the development and prognosis of the disease. The research aimed at verifying the hypothesis that the persistence of the bacteria and thus, increased severity of the orf lesions is residing in the high antibiotic resistance of the co-infecting bacteria. The antibiotics used were gentamicine, cefuroxime, amoxicilline clavulanic acid, enrofloxacin, ciprofloxacin, penicillin and thrimetoprim. Multi antibiotic resistance indices were calculated for each strain. Based on the MAR index, at least two of the investigated environmental bacteria strains could further complicate orf lesions and delay the positive effects of the antibiotic therapy.

Keywords: co-infecting bacteria, antibiotic multiresistance, orf, sheep

Introduction

Orf virus belongs to the genus *Parapoxvirus*, family *Poxviridae* and it is causative agent of ovine and caprine pustulo vesicular dermatitis also known as contagious ecthyma or soremouth. Usually evolution of the lesions in orf is from the stage of erythema to macula, papules, vesicles, pustules and crusts, which are located mostly around the mouth, on the nipples of lactating sheep or around the hooves (Abbas *et al.*, 2019). The formed crusts contain a high number of viable viruses, which in a dry environment, can remain infectious enough to maintain the evolution of the disease (Constable *et al.*, 2017). Severe evolutions can be found in flocks that haven't made contact with the virus, in which tendencies of confluence and proliferation of the lesions are higher and do not heal spontaneously. Evolution of orf is more severe in suckling lambs

due to oral lesions, the animals dying of hunger and thirst. At the first evolution in herds, morbidity can reach 100%, while mortality is usually less than 1%. However, mortality may increase by 20% ~ 50% as a result of secondary bacterial infection, stress, immunosuppression or in concomitant evolution with other diseases and may exceed 90% in the case of malignant orf (Hosamani *et al.*, 2009). Frequent suprainfections occur with bacteria such as *Staphylococcus* and *Streptococcus* (Chi *et al.*, 2017) but also with other bacteria (Venkatesan *et al.*, 2018). Scarification or microabrasions in the epidermis are a condition for the onset of the disease. Clinically, the evolution is more severe in lambs causing proliferation of lesions around the mucocutaneous junction of the mouth. Overall, clinical presentation of the disease is characterized by the formation of crusts and proliferation

of the epidermis causing painful lesions on the vulva, udder and nipples in sheep, while in lambs the lesions affect the mouth, nostrils and lips and extend to the esophagus, in some cases disease is progressing to the hooves (Onyango *et al.*, 2014). Secondary bacterial infections involve *Staphylococcus aureus* and *Arcanobacterium pyogenes*, *Streptococcus spp.*, *Pseudomonas aeruginosa*, bacteria that can create severe complications of the disease due to the phenomenon of antibiotic resistance often encountered in orf evolution. Resistance to antibiotics is a problem of great concern in animals as well as in humans. The widespread and unwise use of antibiotics can lead to their use in the treatment of resistant bacteriosis. These resistant bacteria cannot only become predominant species in a population, but they can also transfer their genetic characteristics to other bacterial species, making them resistant to antibiotics (Quinn *et al.*, 2016). Resistance to an antibacterial agent often leads to cross-resistance to other agents in the same class. The transport of several resistance genes by plasmids and transposable elements often allows bacteria to become resistant to a number of drugs of different classes (Quinn *et al.*, 2016). Bacteria have developed several mechanisms to avoid the action of commonly prescribed antibiotics, some types of resistance appearing all over the planet, so that the effectiveness of many drugs and even classes of drugs decreases (Kone-man *et al.*, 2017). The alarming increase in antibiotic resistance is one of the main threats in modern medicine.

Materials and methods

For bacteriological examination, perilesional exudate was collected using sterile swabs soaked in sterile saline solution and stored in sterile sterile containers, then was transported refrigerated to the animal and food safety laboratory in Satu Mare to be processed for detection of bacterial flora. The transport was carried out avoiding thermal or other shocks, in Amies transport media. Transport media do not contain a rich substrate for the multiplication of bacteria, but they can provide the necessary support to survive the during transport and handles of preparing bacteriological investigations. Samples have been carefully collected avoiding contamination with other infectious agents, preventing desiccation or long term exposure to air. Sample processing was carried out

on the same day as the day of collection. The purpose of the microbiological determinations was to highlight the suprainfecting flora, which may complicate the subsequent evolution of contagious ecthyma. For microbiological investigations samples have been collected from forty sheep of different ages, sexes and with different lesion scores from five herds. The microbiological methods used were the classical ones. First direct smear examination was performed, then the smear has been stained by Gram method and methods for evidentiating spores. After this step, the culture media were inoculated and incubated according to the information provided by the bacterioscopic examination. Then, at the expiration of the incubation times, the colonies that grew were examined and, if necessary, the inoculation was performed on selective and special media, in order to establish a safe diagnosis and to study the characteristics of the isolated bacteria. Methods for studying biochemical characteristics such as catalase testing, manitol fermentation or nitrate reduction were also used for diagnosis. Where there was a possibility, the API galleries were also used, the results being read on the miniApi device. The Kirby Bauer method was used to assess antibiotic susceptibility. The Kirby-Bauer test, also called the disc diffusion test, is a valuable standard tool for assessing the effect of antibiotics on microorganisms. The test uses microcomprimates impregnated with antimicrobial substances, which are placed on a plate that is inoculated so that a film is formed on the entire surface of the plate. If bacteria is sensitive to the action of an antimicrobial agent, a clear area will appear around the disc where growth has been inhibited. Thick-layer agar slows lateral diffusion and thus produces smaller areas than standard 4 mm agar plates (LeBoffe *et al.*, 2010).

Performing antibiogram is important because it may indicate the treatment protocol in case of secondary infections occur. For testing we used microcomprimates that can be found in commercial use stored in cartridges. Antibiotics were: gentamicin (10µg), amoxicillin with clavulanic acid (20 / 10µg), cefuroxime (30µg), enrofloxacin (10µg), ciprofloxacin (5µg), penicillin (10µg) and trimethoprim (5µg), the interpretation of the results being performed according to CLSI 2015. We tested the sensitivity of the 50 strains of bacteria isolated to the action of 7 antibiotics. The standardized method developed criteria

for interpreting the zones of inhibition, by consulting which the level of sensitivity of various antimicrobial substances of the tested strains can be established. These interpretation criteria were established by correlating the zones of inhibition with the minimum inhibitory concentration. Their elaboration was based on the study of a large number of bacterial germs, including those that possess known resistance mechanisms for certain classes of antimicrobial agents and in correlation with the pharmacokinetics of antimicrobial substances used in therapy, as well as their clinical efficiency (CLSI, 2015). Depending on the diameter of the inhibition zone, the tested bacterial strain can be interpreted as "sensitive", "moderately sensitive" (or intermediate) or "resistant" to the action of anti-infectious substances. The „sensitive“ category assumes that the strains tested are inhibited by usual concentrations of the antimicrobial agent when used in the usual dose. The „moderately sensitive“ or „intermediate“ category includes situations in which the minimum inhibitory concentration of the antibiotic reaches a certain blood and tissue level, but the body's response rate may be lower than those in the „sensitive“ category. Substances included in the „moderately sensitive“ category may be used in the treatment of localized infections, where the antibiotic is physiologically concentrated (eg quinolones and β -lactams in the urine) or in situations where it is possible to use higher than usual doses (this is the case for β -lactams). The „resistant“ category assumes that the tested strain is not inhibited by the usual systemic concentration reached by the antimicrobial administered in the usual dosage or that the tested strain possesses specific

resistance mechanisms (eg β -lactamases); In such cases the therapy is ineffective. The isolates were transferred to a tube containing 5 ml of nutrient broth. The mixture was incubated at 37°C until a visible turbidity of 0.56 was found on the MacFarland scale. The isolates were strained on Mueller-Hinton agar containing 5% defibrinated blood. Antibiotic plates were incubated for 18 to 24 hours at 37°C and growth inhibition diameters were measured in millimeters.

In addition, we calculated the antibiotic resistance index (MAR). This index and how the value was obtained was first achieved by Krumperman in 1983. The value of the index is calculated by reporting the number of antibiotics to which a bacterial strain was resistant or had an intermediate reaction to the total number of antibiotics tested. The values obtained are subunitary, a value greater than 0.2 indicating multiple antibiotic resistance.

For statistical determinations the GraphPad Prism 8 application has been used, correlation tests, One Way Anova and descriptive statistics elements.

Results and discussions

Following bacterioscopic and bacteriological investigations, bacteria from species *Staphylococcus aureus* (30 strains), *Corynebacterium pseudotuberculosis* (6 strains), *Bacillus Cereus* (7 strains) and *Pseudomonas aeruginosa* (9 strains) were identified. More than half of the samples were suprainfected with *S.aureus* followed by *P.aeruginosa*, probably because of the characteristics of these bacteria that can grow in various environmental conditions, making them ubiquitous (figure 1).

Figure 1. Graphical representation of percentage of each isolated bacteria

From the 40 superinfected samples in a number of 12 samples, two bacteria were isolated simultaneously (figure 2). This can make the decision to use an antibiotic difficult because a bacteria may be sensitive to a particular antibiotic and the one it is associated with may not be sensitive to the same antibiotic.

In the case of secondary infection with *Staphylococcus Aureus*, we tested the sensitivity of the 30 staphylococcal strains to the action of 7 antibiotics (table 1). In 29 cases, the isolated *Staphylococcus* strains were found to be resistant to the action of penicillin, the area of inhibition being less than 28 mm, which is explained by the presence of β -lactamase, which is carried by a plasmid and it is activated in the presence of penicillin or other β -lactam antibiotics (Koneman *et al.*, 2017). Koneman *et al.*, 2017, mention about the existence of a multidrug resistance of *S.aureus*, to the action of fluoroquinolones, drugs considered very effective in curing *S.aureus* infections. An ionic pump called NorA appears to be responsible. This phenomenon of fluoroquinolone resistance was also observed in our case, in the case of testing the sensitivity of the detected strains to the action of ciprofloxacin and enrofloxacin. It is also proven that the β -lactamase produced by *S.aureus* interferes with the bactericidal / bacteriostatic activity of antibiotics in the β -lactam category, in this sense these antibiotics are associated with substances that inhibit the activity of β -lactamases, such as clavulanic acid. According to these characteristics we tested the sensitivity to amoxicillin with clavulanic acid, the success rate being 76.67%, as in the case of ciprofloxacin.

To gentamicin 29 out of 30 isolated strains proved to be sensitive. Sensitivity to cefuroxime was also tested, finding that 27 strains were sensitive to its action. In the case of clavulanic acid with amoxicillin, from the 30 isolated strains, 23 were found to be sensitive to its activity. Enrofloxacin was shown to be effective in 24 strains, while ciprofloxacin was effective in 26 strains. 23 strains proved to be sensitive to the action of trimethoprim. One of the isolated *S.aureus* has been sensitive to penicillin (figure 3).

Resistance to trimethoprim of *S.aureus*, may be due to a mutation in the *dhfr* gene (Koneman *et al.*, 2017), and in our case there is a resistance to this antibiotic, in proportion of 26.6%.

The procentual representation of sensitivity to the 7 types of antibiotics is shown in the figure 4.

Analyzing the graphs above the percentage of sensitivity was good, except for penicillin. The best results were obtained when testing gentamicin, of the 30 strains only one was resistant, although the literature described the aminoglycoside resistance of *Staphylococcus aureus* (Quinn *et al.*, 2011). Meanwhile, trimethoprim and sulfamides in general are considered antibiotics with low efficacy in treating staphylococcal infections (Moon *et al.*, 2015, Quinn *et al.*, 2011). Expressed as a percentage, the test result on the action of antimicrobials indicates that except for penicillin, all the other 6 antibiotics tested proved to be effective in the event of a complication of the contagious ecthyma, the results being over 70%. Even though 6 of the 7 antibiotics tested showed an efficiency of over 70% when calculating MAR indexes has been proven the existence of the phenomenon of multidrug resistance, a fact often

Figure 2. Percentage representation of superinfected samples with two bacteria compared to those infected with a single bacteria

encountered in case of *S.aureus* (Moon *et al.*, 2015). In our case we identified 21 strains with a MAR index value higher than 0.2 (figure 5). Intermediate or moderate sensitivity reactions have been considered resistant.

Values obtained from descriptive statistics, in case of the diameters of the zones of inhibition (see table 2) returned results that show a higher stability of the averages of the diameters of inhibition for gentamicin and cefuroxime and therefore

Table 1. Diameters obtained at antibiotic susceptibility testing and MAR indices obtained for *S.aureus*

Sample no.	Genta.	Cef.	Amoxiclav	Enro.	Cipro.	Pen	Trim	MAR index
1	17S	25S	18R	23S	23S	21R	12R	0,43
3	19S	18R	17R	22S	22S	23R	18S	0,43
4	18S	23S	23S	25S	25S	20R	17S	0,14
6	17S	26S	25S	22S	26S	19R	12R	0,29
9	19S	21S	26S	25S	25S	21R	20S	0,14
12	20S	23S	14R	25S	25S	21R	21S	0,29
15	19S	23S	21S	24S	16R	20R	17S	0,29
16	20S	23S	22S	21S	27S	22R	20S	0,14
21	19S	25S	24S	14R	14R	16R	9R	0,57
35	18S	21S	14R	19S	26S	10R	20S	0,29
42	18S	21S	24S	14R	14R	21R	21S	0,43
45	20S	22S	27S	17I	13R	31S	11R	0,43
49	19S	24S	23S	14R	14R	25R	18S	0,43
52	19S	23S	15R	21S	25S	12R	19S	0,29
56	18S	25S	25S	20S	27S	20R	18S	0,14
66	20S	21S	25S	17R	17R	21R	18S	0,43
71	21S	27S	25S	20S	25S	22R	17S	0,14
73	19S	23S	21S	22S	22S	18R	17S	0,14
75	13R	11R	21S	25S	25S	17R	18S	0,43
76	20S	22S	21S	25S	25S	20R	18S	0,14
77	21S	25S	23S	16R	16R	19R	20S	0,43
78	18S	13R	26S	21S	27S	18R	21S	0,29
79	19S	18R	17R	23S	24S	19R	20S	0,43
80	20S	22S	17R	23S	23S	23R	19S	0,29
82	19S	21S	23S	20S	25S	21R	9R	0,29
85	19S	22S	21S	21S	23S	23R	10R	0,29
87	18S	22S	25S	22S	22S	23R	9R	0,29
88	20S	21S	26S	23S	25S	23R	13R	0,29
91	18S	25S	23S	21S	23S	26R	19S	0,14
93	18S	25S	24S	24S	24S	21R	19S	0,14

Note: Genta.-gentamicin, Cef.-cefuroxime, Amoxiclav-Amoxicillin+clavulanic acid, Enro-enrofloxacin, Cipro.-ciprofloxacin, Pen.-penicillin, Trim.-Trimethoprim

Graphical representation of antibiotic sensitivity and resistance of *S. aureus* **Figure 3.** Graphic representation of the number of *S. aureus* strains sensitive and resistant to the tested antibiotics **Figure 4.** Graphical representation of *S. aureus* strains showing sensibility to tested antibiotics **Figure 5.** Graphical representation of the phenomenon of multidrug resistance, according to calculated MAR indices, of *S. aureus* strains

a higher success rate in their possible use, the small values of the standard deviations obtained associated with the coefficients of variation of 8 and 16% respectively ensuring that we can obtain a favorable response to their action. Although enrofloxacin, ciprofloxacin, amoxicillin and trimethoprim showed high averages and success rates, statistically analyzed results in measuring inhibition diameters oscillate between values indicating sensitivity and resistance, as well as coefficients of variability have higher values which results in larger oscillations of these diameters. Regarding the MAR indices, the average obtained was 0.3 ± 0.13 , which indicates that the tendency of isolated *S.aureus* strains is to be resistant to 2,3 or even 4 antibiotics.

In the case of isolates from which *C. pseudotuberculosis* was isolated, the same procedures and antibiotics were used to test susceptibility to antimicrobials as to *S.aureus*. These bacteria produce caseous lymphadenitis, a chronic disease of small ruminants, characterized by the formation of abscesses in the superficial and deep lymph nodes, but can affect more internal organs such as the lungs. The animal loses weight progressively due to anorexia, and later, usually within a few months, dies due to toxemia and piemia (Zarraga *et al.*, 2005).

Like other bacteria, *C. pseudotuberculosis* has developed resistance to certain groups of antibiotics, especially to the group of aminoglycosides (Koneman *et al.*, 2017), proven in our case. Of

Table 2. Descriptive statistics on the diameters of the zones of inhibition and MAR indices in the case of *S.aureus*

Parameters	Genta.	Cef.	Amoxiclav	Enro.	Cipro.	Pen.	Trim.	MAR index
Minimum	13	11	14	14	13	10	9	0,14
Maximum	21	27	27	25	27	31	21	0,57
Mean	19	22	22	21	22	21	17	0,3
Std. Deviation	1,5	3,4	3,7	3,4	4,4	3,9	4	0,13
Std. Error of Mean	0,27	0,63	0,68	0,61	0,81	0,71	0,72	0,023
Lower 95% CI of mean	18	21	20	20	21	19	15	0,25
Upper 95% CI of mean	19	23	23	22	24	22	18	0,34
Coefficient of variation	8,0%	16%	17%	16%	20%	19%	24%	42%

Note: Genta.-gentamicin, Cef.-cefuroxime, Amoxiclav-Amoxicillin+clavulanic acid, Enro-enrofloxacin, Cipro.-ciprofloxacin, Pen.-penicillin, Trim.-Trimethoprim

Table 3. MAR indexes and inhibition diameters obtained when testing antibiotic susceptibility in *C.pseudotuberculosis* isolates

Sample no.	Genta.	Cef.	Amoxiclav	Enro.	Cipro.	Pen	Trim	MAR index
29	15I	25S	28S	7R	11R	30S	30S	0,29
45	18S	22S	27S	17I	13R	31S	28S	0,14
55	20S	22S	28S	9R	9R	30S	27S	0,29
73	19S	23S	21S	22S	22S	18R	17S	0,14
84	5R	21S	19S	10R	16I	32S	20S	0,29
96	6R	16I	20S	9R	14R	31S	20S	0,43

Note: Genta.-gentamicin, Cef.-cefuroxime, Amoxiclav-Amoxicillin+clavulanic acid, Enro-enrofloxacin, Cipro.-ciprofloxacin, Pen.-penicillin, Trim.-Trimethoprim

the 6 isolated strains, only 3 responded favorably to gentamicin testing, the efficiency being 50%. There was a strain to which gentamicin testing returned an intermediate sensitivity result, but not being a clearly positive response, it fell into the “resistant” category (Figure 6).

According to research by Algammal *et al.* (2016), *C. pseudotuberculosis* had an increased resistance to penicillin and an increased susceptibility to the action of fluoroquinolones. In our case the sensitivity percentage to penicillin was 83.33% and to ciprofloxacin and enrofloxacin of only 16.67% (Figure 7).

Unlike in *S.aureus*, we also tested antibiotics with 100% efficiency, amoxicillin with clavulanic acid and trimethoprim in case of *C.pseudotuberculosis*. But gentamicin showed a small amount of 50% sensitivity and penicillin unlike in *S.aureus* infection showed a very high percentage of sensitivity.

Antibiotic resistance was also observed in *C. pseudotuberculosis*, to a lesser extent than in *S.aureus* (see figure 8). However, in the case of *C. pseudotuberculosis*, multidrug resistance to 2 antibiotics predominated, the values of MAR indices being lower. In general, abscesses in caseous lymphadenitis are refractory to antibiotic therapy due to the thick capsule possessed by *C.pseudotuberculosis*. However, in sheep and goats, long-term systemic antibiotics (4-6 weeks) combined can be effective in treating internal abscesses and decrease the chance of recurrence of external abscesses.

As shown in Table 4, in the case of *C. pseudotuberculosis* statistical analyzes confirm the success rates of antibiotics without any doubt. The average values for gentamicin ciprofloxacin and enrofloxacin fall into the category of resistance to these antibiotics, and the results obtained when using them can give highly variable results,

Figure 6. Graphic representation of susceptibility and resistance of *C.pseudotuberculosis* strains

Figure 7. Percentual representation of resistance and susceptibility of *C.pseudotuberculosis* strains

indicated by standard deviations and coefficients of variation greater than 20% (see table no.4). The mean values obtained for MAR indices indicated a tendency to fit resistance to two types of antibiotics tested, 0.26 ± 0.11 . However, the standard deviation extends this trend and if we analyze the coefficients of variability, there is a tendency to fall within the resistance to 1, 2 or even 3 antibiotics, depending on the strain of *C. pseudotuberculosis* isolated.

Bacillus cereus, is a bacteria that is found anywhere in the environment due to the resistance conferred by the existence of spores, however in animals it rarely causes complications. It is involved in abortions and mastitis in cattle, in sheep not having a pathological significance clinical signs in sheep from which isolated this bacterium being alarming only in the case of association with other opportunistic bacteria (McVey *et al.*, 2013). However, the literature describes the existence of a resistance to the action of penicillins (which also represents a differential diagnostic criteria from

B.anthraxis) and antibiotics from the β -lactam group (McVey *et al.*, 2017) but also to the action of aminoglycosides, due to enzymes ANT type (Koneman *et al.*, 2017).

Contrary to the findings made by McVey *et al.*, 2013, gentamicin sensitivity of the 7 isolated *B.cereus* strains was 100%, but the penicillin resistance was 100%. This is a feature of *B.cereus* that represents the differential diagnostic value of *B.anthraxis*. There is no evidence of resistance to amoxicillin, probably due to its association with clavulanic acid, which potentiates its effectiveness. Enrofloxacin sensitivity was 100% and also cefuroxime sensitivity showed a high percentage. Moderate sensitivity has been considered resistance, because moderately sensitive strains can show uncertain reactions to treatment with such antibiotics.

Overall, with the exception of penicillin, the positive results were greater than 50% for all antibiotics tested (Figure 10).

Figure 8. Representation of multiresistance to antibiotics tested on *C.pseudotuberculosis*

Table 4. Descriptive statistics of inhibition diameters and MAR indexes

Parameters	Genta.	Cef.	Amoxiclav	Enro.	Cipro.	Pen.	Trim.	MAR Index
Minimum	5	16	19	7	9	18	17	0,14
Maximum	20	25	28	22	22	32	30	0,43
Mean	14	22	24	12	14	29	24	0,26
Std. Deviation	6,7	3	4,3	5,9	4,5	5,3	5,3	0,11
Std. Error of Mean	2,7	1,2	1,7	2,4	1,9	2,2	2,2	0,045
Lower 95% CI of mean	6,8	18	19	6,2	9,4	23	18	0,15
Upper 95% CI of mean	21	25	28	18	19	34	29	0,38
Coefficient of variation	48%	14%	18%	47%	32%	18%	22%	42%

Table 5. Inhibition diameters and MAR index in *B.cereus* susceptibility testing

Saple no.	Genta.	Cef.	Amoxiclav	Enro.	Cipro.	Pen.	Trim	MAR index
4	18S	23S	23S	25S	25S	20R	17S	0,14
6	17S	26S	25S	22S	26S	19R	12R	0,29
12	20S	23S	14R	25S	25S	21R	21S	0,29
15	19S	23S	21S	24S	16R	20R	17S	0,29
33	26S	11R	5R	24S	23S	3R	15I	0,43
71	21S	27S	25S	20S	25S	22R	17S	0,14
74	29S	7R	2R	27S	27S	4R	16I	0,43

Note: Genta.-gentamicin, Cef.-cefuroxime, Amoxiclav-Amoxicillin+clavulanic acid, Enro-enrofloxacin, Cipro.-ciprofloxacin, Pen.-penicillin, Trim.-Trimethoprim

Figure 9. Graphical representation of the numbers of *B.cereus* tested "sensitive" or "resistant" to antibiotics **Figure 10.** Procentual representation of the numbers of *B.cereus* tested "sensitive" or "resistant" to antibiotics

Even if the result of testing two antibiotics was 100% successful in calculating MAR indices, only in the case of two strains was the existence of resistance to a single antibiotic found. The rest of the strains being multi-resistant, their proportion being 71.43%. Gentamicine and enrofloxacin showed a high efficiency in treating *B.cereus* (figure 11).

The average value of the MAR index was 0.29, which confirms the fact that the isolated *B.cereus* strains are also multi-resistant. *Bacillus cereus* is the second most common bacterial agent responsible for foodborne outbreaks. In hospital infections have also been reported, of interest in human medicine, the symptoms being sepsis, endophthalmitis, pneumonia, endocarditis, meningitis and encephalitis, especially in individuals with immune problems and newborns (Glasset

et al., 2018). Very rarely there are cases of fulminant infections similar to anthrax and affecting healthy people (Mc.Vey *et al.*, 2013). Being a bacteria spread in all environments, the risk factors are represented by intravenous injections, surgeries or traumatic wounds, intravascular catheters. Avoiding such infections requires strict adherence to sterilization and disinfection protocols.

Even if the results obtained from MAR indices and the diameters of the inhibition zones provide us with information about the susceptibility to a certain antimicrobial, it is not enough. It is useful to follow the evolution trend, because a good percentage of positive reaction to an antibiotic can hide some large variations in the diameters of the zones of inhibition, so its use can generate inconsistent results. Thus, in the case of gentamicin, ciprofloxacin and enrofloxacin, the

Figure 11. Graphical representation of multiresistant *B.cereus* related one antibiotic resistant strains

Table 6 Descriptive statistics for inhibitory diameters and MAR indexes for *B.cereus*

Parameters	Genta.	Cef.	Amoxiclav.	Enro.	Cipro.	Pen.	Trim.	MAR index
Minimum	17	7	2	20	16	3	12	0,14
Maximum	29	27	25	27	27	22	21	0,43
Mean	21	20	16	24	24	16	16	0,29
Std.Deviation	4,4	7,8	9,6	2,3	3,7	8,3	2,7	0,12
Std. Error of Mean	1,7	2,9	3,6	0,86	1,4	3,1	1	0,045
Lower 95% CI of mean	17	13	7,5	22	20	7,9	14	0,18
Upper 95% CI of mean	26	27	25	26	27	23	19	0,4
Coefficient of variation	21%	39%	59%	9,5%	15%	53%	16%	41%

Note: Genta.-gentamicin, Cef.-cefuroxime, Amoxiclav-Amoxicillin+clavulanic acid, Enro-enrofloxacin, Cipro.-ciprofloxacin, Pen.-penicillin, Trim.-Trimethoprim

mean values of the diameters of the inhibition zones of 21 and 24 mm respectively did not show large variations at a significance level of 95%. Small standard deviations and coefficients of variation that showed values below 25%, certainly indicated a tendency of the diameters to be very close to the average value (see table 6).

In the case of amoxicillin+clavulanic acid and trimethoprim, even if the sensitivity rates were above 50%, the mean values between the "sensitive" and "resistant" categories and the large standard deviations (especially in the case of amoxicillin with clavulanic acid) indicates large variations in the values of the diameters of the zones of inhibition. Even if in the case of cefuroxime the coefficient of variability indicates values that at a probability of 95% are close to the average value, this having the value of 16mm, indicates the sensitivity to this antibiotic is at the lower allowed limit and the use in therapy will have an uncertain success. The average values of the MAR indices showed oscillating evolutions between the resistance to 1, 2, 3 antibiotics.

Pseudomonas aeruginosa is the most common bacterium in nosocomial infections, being a bacterium that does not require complex conditions for survival. It is found everywhere in the environment and being an opportunistic bacterium creates complications when it meets favorable conditions to enter the body fluids. In our case, these conditions were met by the creation of breaches by orf virus. From the 40 superinfected samples, *Pseudomonas Aeruginosa*

was present in 9 samples. This bacterium causes problems in both human and veterinary medicine, the development of wound infection depends on the integrity and protective function of the skin. Secondary infections with this bacterium have a worldwide distribution varying only the strain causing the infection. In recent years, there has been an increase in the prevalence of Gram-negative organisms that tend to over-infect more frequently than *Staphylococcus aureus* (Anupurba *et al.*, 2006). Among the Gram-negative bacilli, *Pseudomonas aeruginosa* has been of particular interest, whose incidence in wound infection has increased compared to a ten-year study. What is worrying is that there are healthy organisms that carry this bacterium. *P. aeruginosa* can cause infections in almost any location in the host, although it does not cause clinical disease in the healthy host. *P. aeruginosa* contaminates areas of the body that have a small number of normal flora. Because *P. aeruginosa* is resistant to common antibiotics, it will replace the normal flora (Haenni *et al.*, 2015). Tissue destruction is due to the release of exotoxins and pyocyanin from the bacterial cell and the release of proinflammatory cytokines and reactive oxygen mediated by the host. *P. aeruginosa* is also isolated from animals that have no history of antimicrobial therapy. *Pseudomonas aeruginosa* is an opportunistic causing infections especially in patients with impaired immune systems or in people suffering from cystic fibrosis. It is also a cause of disease in both farm animals and pets, including otitis and urinary tract infections in dogs,

Table 7. Inhibition zone diameters and MAR indexes in *Paeruginosa*

Sample	Genta.	Cef.	Amoxiclav	Enro.	Cipro.	Pen.	Trim.	MAR index
1	17S	25S	18R	23S	23S	21R	12R	0,43
33	26S	11R	5R	24S	23S	3R	15I	0,57
35	18S	21S	14R	19S	26S	10R	20S	0,29
42	10R	21S	24S	22S	23S	21R	21S	0,29
56	15I	11R	9R	20S	27S	20R	18S	0,57
59	14I	7R	3R	25S	25S	12R	22S	0,57
81	15I	10R	9R	28S	24S	5R	25S	0,57
86	14I	10R	10R	28S	27S	7R	25S	0,57
94	15I	8R	11R	26S	25S	9R	23S	0,57

Note: Genta.-gentamicin, Cef.-cefuroxime, Amoiclav-Amoxicillin+clavulanic acid, Enro-enrofloxacin, Cipro.-ciprofloxacin, Pen.-penicillin, Trim.-Trimethoprim

mastitis in dairy cows, endometritis in horses, and hemorrhagic pneumonia in fur animals such as mink or foxes (Haenni *et al.*, 2015).

Although literature mentions phenomena of multiple drug resistance of *Paeruginosa* mostly in pets, in our case all isolated strains were multi-resistant to antibiotics (Figure 12).

The sensitivity to fluoroquinolones was 100%, all strains react positively to the testing of these antibiotics, a fact also observed by Anupurba *et al.*, 2006. The known resistance to aminoglycosides, given by enzymes that modify the structure of aminoglycosides, *Paeruginosa* was also shown in our case, 6 of the 9 isolated strains reacted unfavorably. Five of the strains considered resistant had an intermediate reaction but we counted them as resistant because the response to those antibiotics can be uncertain. Resistance to aminoglycosides due to the presence of β -lactamases (Koneman *et al.*, 2017) was evident in our case, even if we used the combination of amoxicillin with clavulanic acid, clavulanic acid being a substance that interferes with the activity of β -lactamases of this bacterium. Lately, the number of strains combining the presence of β -lactamases with the change in cell wall permeability to aminoglycosides has increased, especially in the case of bacteria that meet a favorable substrate for the synthesis of this cell wall (Koneman *et al.*, 2017). In our case this possibility exists considering the high resistance to amoxicillin and the refractory results observed when testing gentamicin. The presence of cations

(Ca, mg, Zn) and saccharides in the environment may increase antibiotic resistance.

Although it has been shown that some *Paeruginosa* strains are resistant to fluoroquinolones, in our case both enrofloxacin and ciprofloxacin testing proved to be 100% effective. Calculated MAR indices showed that all identified *Paeruginosa* strains were resistant to at least 2 antibiotics (Figure no.13). Most are resistant to 4 antibiotics. Even though the percentages of sensitivity to each individual antibiotic are over 77% in 3 of the antibiotics, the high values of MAR indices are worrying. Being a bacterium that possesses numerous defense mechanisms, resistance to the action of antibiotics and the wide variety of toxins released, before establishing a treatment protocol it is recommended the mandatory testing of susceptibility to antibiotics.

Even though the procentual sensitivity of *Paeruginosa* to gentamicin is only 33.33%, the mean values of the diameters of the inhibition zones were at the level that falls into the "sensitive" category, but the standard deviation of 4.4 places gentamicin at a oscillating level in terms of the effectiveness of a possible treatment. Given that *Paeruginosa* is a versatile bacterium and that it may develop gentamicin defense mechanisms, the use of gentamicin is not indicated in this case (see table no.8). Cefuroxime and amoxicillin with clavulanic acid showed mean values of the diameters of the inhibition zones at the lower level of sensitivity, so the low reactivity percentage is confirmed. The mean values of the diameters of

Figure 12. Graphic representation of drug resistance in *Paeruginosa*

Figure 13. Graphic representation of susceptibility and resistance percentage in *Paeruginosa*

Table 8. Descriptive statistics for inhibition zone diameters in *Paeruginosa*

Parameters	Genta.	Cef.	Amoxiclav.	Enro.	Cipro.	Pen.	Trim.	MAR index
Minimum	10	7	3	19	23	3	12	0,29
Maximum	26	25	24	28	27	21	25	0,57
Mean	16	14	11	24	25	12	20	0,49
Std. Deviation	4,4	6,6	6,5	3,2	1,6	7	4,4	0,12
Std. Error of Mean	1,5	2,2	2,2	1,1	0,55	2,3	1,5	0,041
Lower 95% CI of mean	13	8,7	6,5	21	24	6,6	17	0,4
Upper 95% CI of mean	19	19	16	26	26	17	24	0,59
Coefficient of variation	27%	48%	56%	13%	6,6%	58%	22%	25%

Note: Genta.-gentamicin, Cef.-cefuroxime, Amoxiclav-Amoxicillin+clavulanic acid, Enro-enrofloxacin, Cipro.-ciprofloxacin, Pen.-penicillin, Trim.-Trimethoprim

Figure 14. Numbers and percentage according multiresistance to antibiotics.

the inhibition zones when testing enrofloxacin and ciprofloxacin were stable, the variations at a significance level of 95% were very low and grouped around the mean with a variability index of 13 and 6.6%, respectively. The highest mean value of the MAR index was found to be in the case of *Paeruginosa* 0.49 ± 0.12 which indicates the trend of resistance to at least 3 antibiotics. At a significance level of 95% and a variability index of 25% we can say that the identified *Paeruginosa* strains tended to be resistant to 3-4 antibiotics.

As seen in the graphics above 25% of the strains of isolated bacteria were resistant to a

single antibiotic. Most bacteria are sensitive to at least two antibiotics. Predominant was multidrug resistance. More than half of the *S.aureus* strains were resistant to the action of at least two antibiotics. 38.46% of the isolated strains were sensitive to 3 or 4 antibiotics, which is a serious multidrug resistance phenomenon. In the case of *C. pseudotuberculosis*, the existence of a multidrug resistance to antibiotics was also found, as well as in the case of *B.cereus*. The highest resistance to antibiotics proved to be in the case of *Paeruginosa*, 66% of the isolated strains showed multidrug

Table 9. ONEWAY ANOVA for MAR indexes between bacterial species isolated

Bartlett's statistics for MAR indexes				0,149
P				0,9854
P summary				Ns
Dev.std. significantly different (P < 0.05)?				No
Tukeys multiple comparisons test	Mean differences	Confidence intervals between means	Significant?	Adjusted P value
Corynebacterium vs. Staphylococcus	-0,033	-0,1790 to 0,1130	No	0,9311
Bacillus vs. Staphylococcus	-0,00919	-0,1462 to 0,1279	No	0,998
Pseudomonas vs. Staphylococcus	0,1959	0,07179 to 0,3200	Yes	0,0006
Bacillus vs. Corynebacterium	0,02381	-0,1579 to 0,2055	No	0,9852
Pseudomonas vs. Corynebacterium	0,2289	0,05680 to 0,4010	Yes	0,0048
Pseudomonas vs. Bacillus	0,2051	0,04053 to 0,3696	Yes	0,0091
Test details	Mean 1	Mean 2	Differences between means	Standard error of differences
Corynebacterium vs. Staphylococcus	0,2633	0,2963	-0,033	0,05487
Bacillus vs. Staphylococcus	0,2871	0,2963	-0,00919	0,0515
Pseudomonas vs. Staphylococcus	0,4922	0,2963	0,1959	0,04663
Bacillus vs. Corynebacterium	0,2871	0,2633	0,02381	0,06826
Pseudomonas vs. Corynebacterium	0,4922	0,2633	0,2289	0,06466
Pseudomonas vs. Bacillus	0,4922	0,2871	0,2051	0,06183

resistance to 4 antibiotics. In *Paeruginosa* all 9 isolated strains were multidrug resistant.

Following the comparison of MAR indices for the 4 isolated bacteria, through the Anova Test - of multiple comparisons, it was found that the differences between the means of the MAR index values are not significant, in total, for the 4 types of bacteria. Comparing the averages between the 4 species of isolated bacteria, the biggest difference existed when comparing the MAR indices calculated for *Pseudomonas aeruginosa* with the other 3 bacteria.

The differences at a significance level $\alpha = 0.05$ are significant which means that the value of P was less than 0.05, when comparing the MAR indices for *Pseudomonas* with the MAR indices calculated for the other 3 bacteria. Thus, at a 95% probability, the difference between the mean MAR indices for *Pseudomonas* and the other isolated bacteria was large, which means that the multidrug resistance of *Paeruginosa* was much more obvious than in the other isolated bacteria. The averages of the MAR indices in *S.aureus*, *B.cereus*, *C.pseudotuberculosis* did not show large differences, the values being in the area of resistance to two or three antibiotics.

Conclusions

Based on the MAR indexes, at least two of the investigated environmental bacteria strains could further complicate orf lesions and delay the positive effects of antibiotic therapy. As mentioned in the literature, the lesions of the contagious ectima are often suprainfected with more or less dangerous bacteria, thus prolonging the healing time of the affected sheep (Venkatesan *et al.*, 2017). As expected and judging by its characteristics, *Staphylococcus Aureus* ranked first in terms of the number of secondary infections (Chi *et al.*, 2017), being a ubiquitous bacterium with a high capacity to adapt to environmental conditions. *Paeruginosa*, is also a very versatile and adaptable bacterium, surviving in the most surprising conditions (including distilled water), the capsule having a very resistant and complex structure and the metabolism adapts depending on the substrate on which it is fixed (Anupurba and col., 2006). However, secondary infections with serious consequences with *Pseudomonas* are less common in sheep than in humans, in the present case they were limited only to local infections that were more difficult to treat (Quinn

et al., 2017). The biggest challenge is the secondary infection with *Paeruginosa*, being resistant to at least two antibiotics. Antibiotic testing and the calculated MAR indices corroborated with the statistical calculations revealed that a good percentage of reaction to an antibiotic does not guarantee the success of the treatment with that certain antibiotic. Statistical investigations such as standard deviation and coefficient of variation revealed the existence of an instability in the response to the action of a particular antibiotic. Success rates for trimethoprim antibiotic therapy showed the highest stability because at a probability of $p = 0.05$ they tended to cluster around the mean. In the other 6 antibiotics, values of variability were obtained which had a high degree of dispersion compared to the average, the population values being in wide limits oscillating a lot between minimums and maximums. The groups of antibiotics from the group of β -lactams and penicillins proved to be ineffective, the responses being refractory, oscillating between favorable reaction percentages between over 90% and below 10%. These answers prove that in the case of many isolated bacteria the activity of β -lactamases and penicillinases was present, even if in case of amoxicillin we used a combination with clavulanic acid, which should have inhibited β -lactamase activity. With fluoroquinolones and aminoglycosides, there was a high variability of the response, the success of the treatment being ensured by the isolated strain. For cases of infections with two bacteria, a combination of various antibiotics will be tried to ensure the success of the treatment. Finally, we can conclude that antibiotic susceptibility testing and the calculation of MAR indices are not reliable information regarding the establishment of a treatment, so if a rebellious bacterium is found for additional treatment, it is advisable to test the incriminated strain, genetically. These genetic tests require a lot of time and high costs, the most convenient way to prevent multidrug resistance is the judicious use of antibiotics, strictly following a medical prescription.

Acknowledgments. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Founding has been supported from my personal resources.

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