Survey and Dynamics of bacteria involved in secondary infections in sheep affected by orf virus

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Abstract

In recent decades, secondary infection bacteria quickly increased their pathogenicity in both human and veterinary patients, due to their continuously increasing antibiotic-resistance. When such bacteria intervene in infections as orf in sheep, they can determine an important impact either in the sanitary or in the economic aspect of the breeding sector. Considering the relatively high spread and economic impact of orf in Romanian sheep, this research aimed to estimate the complexity of the secondary infections in diseased sheep in connection with the severity of the lesions displayed. The research was conducted in five herds, on 3 to 96 month old sheep, with orf lesions, scored from mild to severe. The bacteria were isolated from the lesions and identified by cultivation on broth and agar plates plus identification by use of API galleries. The severity of the lesions did not seem to be influenced by the number of bacterial species isolated, but by the herd environment and individual pathogenicity of the isolates.

Keywords: secondary infection bacteria, dynamics, orf, sheep

Introduction

Parapoxviruses are epitheliotropic viruses that affect a wide range of domestic and wild animals, even humans (Hosamani *et al.*, 2007).

We can say that this virus is an opportunistic pathogen because it is often associated with other viral infections such as peste des petits ruminants or smallpox virus (Saravanan *et al.*, 2007). Infections with this virus develop mainly local infections such as epidermitis that progresses rapidly through the stages of erythema, papule, vesicle, pustule with crusts characteristic to *Parapoxvirus* infections (Hosamani *et al.*, 2009). Contagious ecthyma is a zoonosis affecting humans too, especially people in contact with infected animals, the clinical signs consisting of acute skin lesions, malaise and lymphadenopathy (Groves *et*

al., 1991). In immunodeficient patients have been reported severe cases (Ara *et al.*, 2008). Among the complications following orf infection can be mentioned bacterial mastitis which can even lead to gangrenous mastitis. Also complications such as secondary necrobacillosis are possible, leading to lameness and economical losses (<http://www.msdsvetmanual.com/integumentary-system/contagious-ecthyma/overview-of-contagious-ecthyma>). Necrobacillosis is a serious infectious disease caused by an anaerobic bacteria *Fusobacterium Necrophorum* associated or not with *Corynebacterium Pyogenes*, and produces primary interdigital lesions, the deformation of the hooves then affecting even the joints of the animal that can no longer feed properly (<http://.edu/cgi/viewcontent.cgi?article=1994> &

context = iowastate_veterinarian). In rare cases, digestive cases or hepatic necrobacillosis or even transmission to the fetus can occur, producing dermatitis or suppurative bronchopneumonia in the fetus (Handeland *et al.* 2007). Other secondary infections may be with *Staphylococcus Aureus* and *Arcanobacterium pyogenes* (Housawi *et al.*, 2000) or infections with papillomatosis virus, smallpox virus or fungi (Wilson *et al.* 2002). The treatment of orf depends on the severity of the infection and healing can last from a few weeks to 3-6 months in complicated cases with secondary infections (Demiraslan *et al.* 2017). There is no specific treatment, but just topic disinfection of the lesions (Bora *et al.* 2015).

Materials and methods

The research was conducted in five herds, on 3 to 96 month old sheep, with orf lesions, scored from mild (1) to severe (3). The bacteria were isolated from the lesions and identified by cultivation on liquid and solid media plus identification by use of API galleries with related software. The biological material was collected from 5 flocks of sheep from Sântău, Romania. The samples have been collected from animals belonging to Țurcana, Merinos and Ile de France breeds, mostly young both males and females. In the case studied, the lesions appeared on the lips, nostrils, and nipple, with a tendency to expand on the mucosa in some cases. To perform bacteriological procedures exsudate has been collected from the lesions with sterile tampons soaked in sterile physiological solution and stored in sterile recipients. Biological materials have been transported refrigerated to the animal and food health laboratory Satu Mare for the analyses, in order to detect the bacterial flora involved in secondary infections. The transport was carried out avoiding thermal or other shocks, in Amies transport medium. Transport medium do not contain a rich substrate for the multiplication of bacteria, but they can provide the necessary support to survive during transportation and manipulations that precede bacteriological determinations. Ninety-six samples were collected carefully avoiding contamination with other infectious agents, carrying attention on preventing their desiccation or prolonged exposure to air, processing being carried out on the same day of the collection.

In order to perform statistical determinations, the severity of clinical signs has been shown in lesion scores: 1-mild, 2-moderate, 3-severe. Also for the sex of the animals we used scores 1-for males and 2 for females. To detect secondary infection flora we used the classic bacterioscopic and bacteriological methods. Depending on the bacteria observed when examining direct smears, we used the Gram staining method to highlight the gram negative and gram positive bacteria. In addition, we used the method with malachite green, to highlight the sporogenesis and staining of the capsule with Congo Red. The results of the bacterioscopic examination provide information to guide us further to perform bacteriological examination and biochemical tests that will accurately identify the existing flora. Depending on the results of the bacterioscopic examination, we used simple, selective, special media to examine the cultural and biochemical characteristics of the isolated bacteria. Additionally, API galleries have been used, the results of which were read in the MiniApi device.

For the statistical determinations we used the Graphpad Prism 8 application, in order to observe the evolution of the obtained values.

Results

A number of 96 samples were collected in order to reveal secondary infections, of which 40 turned out to carry bacteria as follows in table no.1.

Following bacteriological and biochemical examination, the presence of *Staphylococcus aureus*, *Corynebacterium pseudotuberculosis*, *Bacillus cereus* and *Pseudomonas aeruginosa* was detected (Table 1). Following the laboratory procedures, in 40 samples out of 96 were detected secondary infections (Figure 1). In 29 samples the presence of *S.aureus* has been demonstrated, while in 5 samples *C. pseudotuberculosis*, in 7 samples *B.cereus* and in 9 samples *P.aeruginosa*. Out of 40 samples showing bacterial presence, in 12 were isolated two bacteria at the same time.

Globally, the presence of *S.aureus* was the most common, followed by *P.aeruginosa* (Figure 2). Since these two bacteria are ubiquitous and very resistant in the environment, the percentage obtained is not surprising. Being both opportunistic bacteria causing infections, they can have easy access created by lesions determined by orf virus and multiply in these sites.

Table 1. Samples showing secondary bacterial infections

<i>Herd</i>	<i>Sample no.</i>	<i>Sex**</i>	<i>Age</i>	<i>Lesion score*</i>	<i>Isolated bacteria</i>
1	1	2	8	3	<i>S.aureus - Paeruginosa</i>
1	3	2	8	3	<i>S.aureus</i>
1	4	2	8	2	<i>S.aureus-B.cereus</i>
1	6	2	3	3	<i>S.aureus-B.cereus</i>
1	9	2	3	3	<i>S. aureus</i>
1	12	2	3	3	<i>S.aureus-B.cereus</i>
1	15	2	3	2	<i>S.aureus-B.cereus</i>
1	16	2	3	3	<i>S.aureus</i>
1	21	2	3	3	<i>S.aureus</i>
1	29	1	16	1	<i>C. pseudotuberculosis</i>
1	33	1	16	1	<i>B.cereus-Paeruginosa</i>
1	35	1	16	3	<i>S.aureus-Paeruginosa</i>
1	42	2	48	3	<i>S.aureusPaeruginosa</i>
1	45	2	48	3	<i>C.pseudotuberculosis-S.aureus</i>
2	49	2	5	3	<i>S.aureus</i>
2	52	2	5	3	<i>S.aureus</i>
2	55	2	5	3	<i>C.pseudotuberculosis</i>
2	56	2	5	3	<i>S.aureus-Paeruginosa</i>
2	59	2	120	3	<i>Paeruginosa</i>
2	66	2	4	3	<i>S.aureus</i>
3	71	1	4	2	<i>S.aureus-B.cereus</i>
3	73	2	4	3	<i>S.aureus-C.pseudotuberculosis</i>
3	74	2	48	1	<i>B.cereus</i>
3	75	2	48	3	<i>S.aureus</i>
3	76	2	48	2	<i>S.aureus</i>
4	77	1	4	3	<i>S.aureus</i>
4	78	1	4	1	<i>S.aureus</i>
4	79	2	4	3	<i>S.aureus</i>
4	80	2	3	3	<i>S.aureus</i>
4	81	2	3	3	<i>Paeruginosa</i>
4	82	2	3	3	<i>S.aureus</i>
4	84	2	96	3	<i>C.pseudotuberculosis</i>
4	85	2	60	1	<i>S.aureus</i>
4	86	2	60	3	<i>Paeruginosa</i>
5	87	2	96	2	<i>S.aureus</i>
5	88	2	72	3	<i>S.aureus</i>
5	91	1	4	3	<i>S.aureus</i>
5	93	2	4	3	<i>S. aureus</i>
5	94	2	4	3	<i>Paeruginosa</i>
5	96	1	4	2	<i>C.pseudotuberculosis</i>

Note* lesion scores that show severity of the symptoms, **Scores according to sex of the animals samples came from

Figure 1. Percentage of secondary infections detected in the analyzed samples

Figure 2. Percentage of the bacteria species detected in the samples with secondary infections

Figure 3. Percentage of secondary infections involving two bacterias

From the total of 40 samples with bacterial suprainfections in a number of 12 samples, the existence of two bacteria was found (Figure 3). The highest number of secondary infections involving two bacteria were in herd no.1 in proportion of 57.14% and in herd no.3 in proportion of 40%.

The ages of sheep with secondary infections ranged from 3 to 120 months, with an average of 23 months. However, the coefficient of variation and the standard deviation indicate a large variability of ages, at 95% significance level. The same trend was observed when calculating the average ages at each herd, the average ages were different from the global average, but the coefficients of variation and standard deviations indicated the same high variability of ages (Table 2). This explains that regardless of the age of the animals, secondary infections can occur if favorable conditions are met.

Regarding the distribution of secondary infections by sex in affected animals, lesion scores indi-

cated a trend of secondary infections in females in herds no. 1, 2, 3, 4 fact supported by the coefficients of variation, at a level of significance of 95%. The exception is represented by herd no. 5 where the occurrence of secondary infections was variable involving both males and females. In this case the coefficient of variation with a significance level of 95% showed a high variability of the sexes. The trend of secondary infections at global level involved mostly females in bacterial suprainfections (Table 3).

Secondary infections with *S.aureus* predominated in every herd, the percentages varying between 52.17% and 66.67%. In the case of *Paeruginosa* the percentages were different between the herds and there was one herd in which its presence was not detected.. *C. pseudotuberculosis* was present in all herds with the exception of herd no., while 1. *B.cereus* was isolated only in the herds 1 and 3 (Table 4).

Table 2. Age ditribution (in months) of the animals with secondary infections and statistic analysis

Descriptive statistics	Herd no.1	Herd no.2	Herd no.3	Herd no.4	Herd no.5	Global
Minimum	3	4	4	3	4	3
Maximum	48	120	48	96	96	120
Mean	13	24	30	26	31	23
Std. Deviation	16	47	24	36	42	31
Std. Error of Mean	4,2	19	11	12	17	4,9
Lower 95% CI of mean	4,3	-25	0,48	-1,2	-13	13
Upper 95% CI of mean	22	73	60	54	75	33
Coefficient of variation	117%	196%	79%	136%	137%	138%

Table 3. Distribution of secondary infections amongst sex of the affected animal

Descriptive statistics	Herd no.1	Herd no.2	Herd no.3	Herd no.4	Herd no.5	Global
Mean	1,8	2	1,8	1,8	1,7	1,8
Std. Deviation	0,43	0	0,45	0,44	0,52	0,41
Std. Error of Mean	0,11	0	0,2	0,15	0,21	0,064
Lower 95% CI of mean	1,5	2	1,2	1,4	1,1	1,7
Upper 95% CI of mean	2	2	2,4	2,1	2,2	1,9
Coefficient of variation	24%	0,0%	25%	25%	31%	23%

Table 4. Percentage of isolated microflora in each herd

Bacteria involved	Herd no.1	Herd no.2	Herd no.3	Herd no.4	Herd no.5
<i>S.aureus</i>	52,17%	57,14%	57,14%	66,67%	66,67%
<i>C.pseudotuberculosis</i>	8,70%	14,29%	14,29%	11,11%	16,67%
<i>B.cereus</i>	21,73%	0%	28,57%	0%	0%
<i>Paeruginosa</i>	17,39%	28,57%	0%	22,22%	16,67%

Table 5. Lesion scores in each herd and at global level.

Minimum	1	3	1	1	2	1
Maximum	3	3	3	3	3	3
Mean	2,6	3	2,2	2,6	2,7	2,6
Std. Deviation	0,76	0	0,84	0,88	0,52	0,71
Std. Error of Mean	0,2	0	0,37	0,29	0,21	0,11
Lower 95% CI of mean	2,1	3	1,2	1,9	2,1	2,4
Upper 95% CI of mean	3	3	3,2	3,2	3,2	2,8
Coefficient of variation	29%	0,0%	38%	35%	19%	27%

Table 6. ONE Way ANOVA and Dunnett's multiple comparisons test between general lesion scores and scores obtained in every herd.

Model comparison	Probability it is correct			
Null H. All population means identical	98%			
Alternative H: Distinct population means	2,1%			
Dunnett's multiple comparisons test	Mean Diff	95,00% CI of diff	Significant?	Adjusted P Value
Global vs. Herd no.1	0,071	-0,53 to 0,67	No	0,9986
Global vs. Herd no.2	-0,36	-1,2 to 0,47	No	0,753
Global vs. Herd no.3	0,44	-0,45 to 1,3	No	0,6366
Global vs. Herd no.4	0,087	-0,62 to 0,79	No	0,9984
Global vs. Herd no.5	-0,024	-0,85 to 0,80	No	>0,9999
Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,
Global vs. Herd no.1	2,6	2,6	0,071	0,23
Global vs. Herd no.2	2,6	3	-0,36	0,31
Global vs. Herd no.3	2,6	2,2	0,44	0,34
Global vs. Herd no.4	2,6	2,6	0,087	0,27
Global vs. Herd no.5	2,6	2,7	-0,024	0,31

The average values of the lesion scores at the level of all samples were around 2.6, which means that in the whole population there is a tendency towards a severe evolution, the coefficient of variation (27%) obtained indicates that the average values of the population are around the average value of 2.6 (Table 5). The average values recorded separately for each herd were between 2.2 and 3. All values were above the value of 2.5 indicating averages that tend towards a severe evolution, except for herd no.3 where an average of 2.2 was calculated but at a significance level of 95% scores are variable between scores indicating moderate and severe lesions. In order to evaluate the evolution trends, the coefficients of variation were also calculated (see table no.3). In the case of herds no. 1, 3 and 4 the coefficients of variation, at a significance level of 95%, were higher than 25% meaning that the trend of the average population tends to vary.

The values for herds no.2 and no.5, however, tended to group around the averages indicating score 3.

To evaluate the differences between scores in each flock and scores obtained at global level, we used the One Way ANOVA test. We compared the averages obtained in herds with the average of the entire batch of samples. The test confirmed the null hypothesis, namely that the herd averages tend to be equal to the averages of the whole batch of samples with a probability of 98% (Table 6). The smallest differences were found when comparing the values in the case of herds no.1, 4, 5. For multiple comparisons Dunnetts multiple comparisons test was used showing almost insignificant differences between global values and herds 1, 4, 5.

Table 7 shows the values of comparisons between the values of lesional scores, the difference between the averages being very close to the value 0 in the case of comparisons between herd no.1 and herds 4 and 5. And in this case the probability of population averages trend to be equal was 96 %. Comparisons between herd no.1 and herd no.4 and 5 showed a very small difference and higher difference between herd no.2 and 3. The p value higher than $\alpha=0,05$ showed that differences between herds were small having a trend to be equal.

Correlation between the individual characteristics of sheep such as sex, lesion score and age proved that at a 95% confidence level there is

no correlation between sex, lesion score and the number of bacteria isolated (see Table 8). A very weak correlation was observed between age and the number of isolated bacteria, in the sense that at small ages the trend to become infected with two bacteria increases. But the value of P that is not less than 0.05 indicates that we cannot generalize, and this slight negative correlation is because can be attributed to coincidence.

For the same reason, Pearsons correlation was performed in each herd. Thereby in herd no. 1 there was no correlation between lesion scores, sex, age and the number of species of bacteria that caused secondary infections in one sample. But P values higher than 0,05 suggest that at a 95% confidence level we cannot generalize and secondary infections with two bacteria at once can be influenced by other factors (see table 9).

In flocks 2, 4 and 5, the correlation coefficient did not return any results, indicating that there is no link between age, sex, lesion scores and the number of bacteria that populate the orf lesions..

In herd no. 3, however, there was a reasonable correlation between lesion scores and the number of isolated bacteria and a strong negative correlation between age and the number of isolated bacteria. Thus in herd no.3 it was observed the existence of a trend that number of bacterial species populating lesions to influence the evolution of lesion scores. At the same time, as age decreases, the tendency to contract two bacteria increases. In this case the values of P were higher than 0,05 indicating that these trends were due to chance and results are valid only for herd no.3 (Table 10). Correlations between age and number of species that cause secondary infections returned no results, the two parameters evolving totally independently one from the other.

Discussion and Conclusions

Following the determinations, we found that *S. aureus* and *P. aeruginosa* dominated in secondary infections, underlying their highly infectious character if they encounter a breach in the organism (Zecconi and Scali, 2013). Contagious ecthyma being a disease that affects the skin, it creates breaches for *S. aureus* and can complicate the evolution of this disease (Chi *et al.*, 2017). Being a bacteria that easily adapts to any biological environment can even cause treatment-resistant rebellious mastitis (Weese *et*

Table 7. Anova comparisons and Dunnet's multiple comparisons test results between herds

Model comparison	Probability it is correct			
Null H. All population means identical	96%			
Alternative H: Distinct population means	4%			
Tukey's multiple comparisons test	Mean Diff	95,00% CI of diff	Significant?	Adjusted P Value
Herd no.1 vs. Herd no.2	-0,43	-1,4 to 0,57	No	0,733
Herd no.1 vs. Herdno.3	0,37	-0,70 to 1,4	No	0,8535
Herd no.1 vs. Herd no.4	0,016	-0,86 to 0,89	No	>0,9999
Herd no.1 vs. Herd no.5	-0,095	-1,1 to 0,90	No	0,9987
Herd no.2 vs. Herd no.3	0,8	-0,44 to 2,0	No	0,3606
Herd no.2 vs. Herd no.4	0,44	-0,64 to 1,5	No	0,7609
Herd no.2 vs. Herd no.5	0,33	-0,85 to 1,5	No	0,9258
Herd no.3 vs. Herd no.4	-0,36	-1,5 to 0,79	No	0,8971
Herd no.3 vs. Herd no.5	-0,47	-1,7 to 0,77	No	0,8149
Herd no.4 vs. Herd no.5	-0,11	-1,2 to 0,97	No	0,9982
Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,
Herd no.1 vs. Herd no.2	2,6	3	-0,43	0,35
Herd no.1 vs. Herdno.3	2,6	2,2	0,37	0,37
Herd no.1 vs. Herd no.4	2,6	2,6	0,016	0,3
Herd no.1 vs. Herd no.5	2,6	2,7	-0,095	0,35
Herd no.2 vs. Herd no.3	3	2,2	0,8	0,43
Herd no.2 vs. Herd no.4	3	2,6	0,44	0,38
Herd no.2 vs. Herd no.5	3	2,7	0,33	0,41
Herd no.3 vs. Herd no.4	2,2	2,6	-0,36	0,4
Herd no.3 vs. Herd no.5	2,2	2,7	-0,47	0,43
Herd no.4 vs. Herd no.5	2,6	2,7	-0,11	0,38

Table 8. Correlation between individual characteristics and the number of bacteria isolated at global level

Pearsons correlation	Lesion scores vs. Number of bacterial species isolated in a sample	Sex vs. Number of bacterial species isolated	Age vs. Number of bacterial species isolated
r	-0,048	-0,11	-0,24
95% confidence interval	-0,35 to 0,27	-0,41 to 0,21	-0,51 to 0,080
R squared	0,0023	0,013	0,057
P (two-tailed)	0,7687	0,4915	0,1395

Note: Correlations are being calculated at a 95% level of significance, P values higher then 0,05 indicating that correlations between the two sets of values cannot be generalized and every correlation between two datasets is the result of hazard
 r- correlation coefficient

Table 9. Correlations of individual factors with the number of isolated bacteria in herd no.1

Pearsons correlation	Lesion scores vs. Number of bacterial species isolated in a sample	Sex vs. Number of bacterial species isolated	Age vs. Number of bacterial species isolated
r	-0,11	-0,1	-0,012
95% CI	-0,61 to 0,44	-0,60 to 0,45	-0,54 to 0,52
R squared	0,013	0,01	0,00015
P	0,6999	0,7325	0,9665

Note: Correlations are being calculated at a 95% level of significance, P values higher then 0,05 indicating that correlations between the two sets of values cannot be generalized and every correlation between two datasets is the result of hazard
r- correlation coefficient

Table 10. Correlations of individual factors with the number of isolated bacteria in herd no.3

Pearsons correlation	Lesion scores vs. Number of bacterial species isolated in a sample	Sex vs. Number of bacterial species isolated
r	0,33	-0,61
95% confidence interval	-0,78 to 0,94	-0,97 to 0,59
R squared	0,11	0,38
P (two-tailed)	0,5908	0,2722

Note: Correlations are being calculated at a 95% level of significance, P values higher then 0,05 indicating that correlations between the two sets of values cannot be generalized and every correlation between two datasets is the result of hazard
r- correlation coefficient.

al., 2010). As mentioned by Mombeni *et al.*, 2013 and Venkatesan *et al.*, 2014, secondary infections with *S. aureus*, are among the most common forms of suprainfection, in orf episodes as confirmed in this study. This is also underlined by the fact that secondary infections with *S. aureus* also predominated in every single flock, not only at global level. *Paeruginosa* that is a very versatile and resistant bacterium, due to its capsule, was as well very present in secondary infections, but was not detected in all herds. Since they cause abscesses, abortions and other lesions that are very difficult to heal (Quinn *et al.*, 2011) increases the concern, especially since these bacteria are rebellious to antibiotic therapy (Kerr *et al.*, 2009). The different distribution of bacteria highlighted in the herds was probably due to the microbism present in each flock. Even if in two herds it was observed the existence of a link between sex and the severity of clinical signs and the number of bacteria detected in lesions (herd no.3) and

between age and the number of species of bacteria found (herd no.1), at global level, there were no correlations between these parameters. We can thus say that there is no connection between the sex, age or severity of the lesions and the number of species of bacteria that infect lesions caused by orf virus. Lesional scores of sheep with suprainfected orf lesions tended to cluster around 2 and 3 confirming that the bacteria that populate such lesions also complicate the course of the disease as noted by Fleming *et al.*, 2015 and Chi *et al.*, 2017. However, as regards sex of the affected animals, females seemed to be more susceptible to a secondary infection, probably due to the fact that the disease developed in late spring, during the shearing when the stress caused by lamb suckling and shearing can decrease the immunity of mothers. There was no connection between sex and number of bacteria causing secondary infections.

As shown in literature (Chi *et al.*, 2017, Quinn *et al.*, 2011) orf virus can create breaches for opportunistic bacteria to develop secondary bacterial infections. In our study it is stated that such bacterial infections can often occur, regarding that all 4 bacterial species isolated can develop infections if they encounter discontinuities in natural barriers of the hosts. To avoid such infections prevention measures should be used such as isolating affected sheep, disinfection of the lesions and antibacterial prevention if it is necessary.

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