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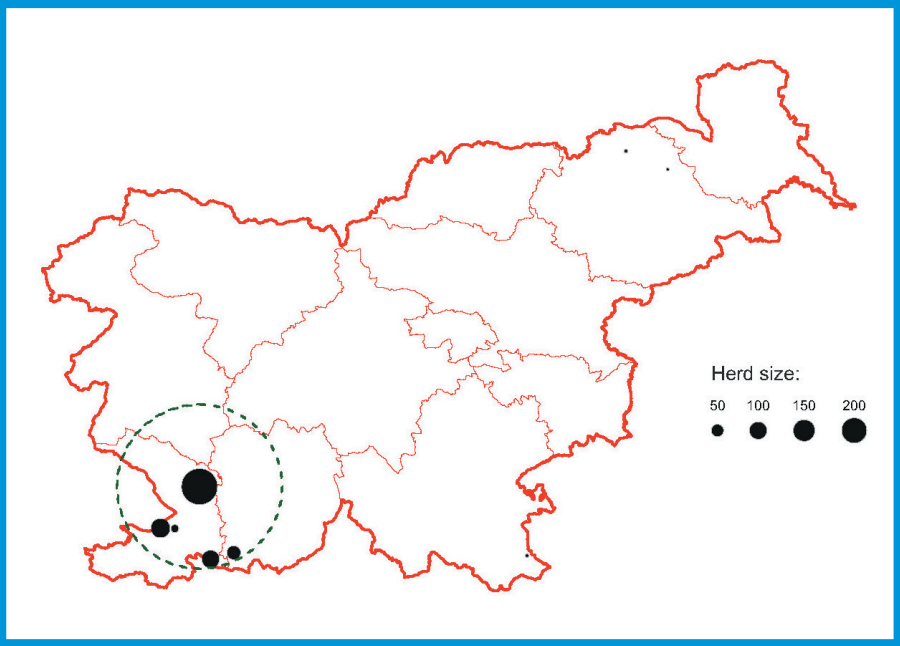

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Original Scientific Article

PRODUCTIVITY OF MILK AND MILK COMPOSITION OF ISTRIAN SHEEP IN CROATIA AND SLOVENIA

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Summary: The effects of country, flock nested within country, litter size, parity, lambing season, interval between lambing and the first milk recording, and year of lambing on production of Istrian sheep in Slovenia and Croatia was studied in period between 2005 and 2009. Data consisted of 3,347 lactations in Croatia and 1,788 in Slovenia that were collected as a part of separate breeding programs. The Croatian animals produced 124.87 kg total milked milk (TMM) with 7.31 % of fat (FC) and 6.05 % of protein content (PC). Slovenian ewes produced only 79.33 kg TMM with 7.25 % FC and 5.87 % PC. The increase of TMM was 5.40 kg per year ($P<0.05$). Ewes that lambed between October and the end of January had the highest TMM, fat yield (FY), and protein yield (PY). The productivity did not differ between countries. Flock effect nested within country caused differences ($P<0.05$) in TMM, FC, and PC. The effect considered genetic, management and climatic conditions (average temperature, above mean sea level (AMSL), and precipitation). Productivity was not affected by the litter size. Higher productivity was observed in ewes reared in the Mediterranean area. The exception was one flock coming from harsher continental conditions. The productivity in such conditions can be improved with earlier lambing, high quality forage before the grazing season and with an extra feeding on pasture after the dry weather begins.

Key words: Istrian sheep; dairy production; Croatia; Slovenia; environmental effects

Introduction

Istrian sheep (Istarska ovca, Istrska pramenka or Istriana) is an autochthonous dairy sheep breed originating from Istrian Peninsula and its hinterland. In the year 2009, estimated population size was between 2,600 and 3,100 animals in Croatia, 1,150 animals in Slovenia, and 920 animals in Italy (1). The population of Istrian sheep was divided after the Second World War to Yugoslav and Italian subpopulations. At the beginning of 1990s, the Yugoslav subpopulation was further split between Croatia and Slovenia. Migration of animals between the two countries has nearly stopped.

Geographic and climatic characteristics are changing very rapidly considering a rather small area. The altitude of breeding ranges from the sea to 1,000 meters above mean sea level (AMSL). The border between Slovenia and Croatia is also geographical. The Croatian part of breeding area consists mostly of the Istrian peninsula, therefore under influence of the sea. The temperature and

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the yearly rainfall levels are changing with the AMSL increase and with the distance from the sea. Pula (AMSL 30 m) on the southern coast of Istria has an average temperature of 14.7°C[1] [v "nogo" na ustreuno stran : The climatic data for period 1990 – 2010 were provided by Croatian Meteorological and Hydrological Service and Slovenian Environment Agency]. The coldest months are January and February with an average temperature of 6.0°C and 6.3°C respectively. Pazin (AMSL 242 m) is located in the middle of Istrian Peninsula with the average temperature of 11.5°C. Slovenian breeding area is placed around Ilirska Bistrica (AMSL 421 m), which is situated outside the Istrian peninsula in the typical Karstic valley. It is not influenced by Mediterranean climate. The average temperature is 9.9° C. The annual sum of precipitation for all three areas reaches the peak in winter months: 828 mm in Pula, 1,065 mm in Pazin and 1,356 mm in Ilirska Bistrica. In Istria, the pasture is available during the winter and especially in early spring period. However, in Ilirska Bistrica and its surrounding mountains, snowing is common and pasture season starts in April. The dry period lasts in Istria (Pula and Pazin) from spring to middle autumn, and from the middle July to the end of August in Slovenia (Ilirska Bistrica). Poor summer vegetation is a consequence of the low summer precipitation and porous Karstic pastureland on limestone bedrock with a very thin layer of soil.

Observed differences in climatic conditions between Croatian and Slovenian breeding area were not very important in the past. The larger flocks from Slovenian hinterland moved to the winter transhumance pasture (2) to the seaside of Trieste and Istria. At the beginning of April, the flocks returned home and prolonged good pasture to the middle of July. The exploitation of Mediterranean and continental vegetation was a comparative advantage of flocks from Slovenian territory. This practice was abandoned during the Second World War and since then animals are kept in the stables during the winter period. Under such conditions, early lambings are possible only with hay of high quality and a supplement of concentrates. Late lambings caused shorter lactation and consequently lower production in lactation. Most of Slovenian Istrian sheep flocks remained on less productive agricultural land, opposite to what was observed in Italy, Spain and France.

The aim of this study was to evaluate the effects of country, flock, lambing season, parity, litter size, interval between lambing and first milk recording, and year of lambing on the productivity of Istrian sheep.

Material and methods

Data were collected as part of separate breeding programs in Croatia and Slovenia for the period from 2005 to 2009. Breeding programs were established in the 1990-ties, 20 to 30 years later as in France, Italy or Spain (3). Daily milk yield and milk components were recorded according to the ICAR guidelines (4) using the AT4 method. The total number of recorded lactations was 3,347 in Croatia and 1,788 in Slovenia. The number of lactations per year did not change in the studied period in Slovenia. However, the number of recorded lactations per year increased more than six times (from 195 in 2005 to 1,266 in 2009) in Croatia.

The total milked milk (TMM) was calculated from recorded daily milk yield using test interval method (4). Fat (FY) and protein yields (PY) in the milking period were calculated with remodelled formula for TMM where daily yields of fat and protein were used instead of daily milk yield. Fat (FC) and protein (PC) contents in milking period were calculated from TMM, FY, and PY. The exact weaning date was not recorded. Therefore, the start of the milking period was set 15 days before the first milk record.

Lambing season (S) starts on the 1st October. The season was expressed as continuous variable and calculated as the number of days after October 1st. Data were analysed using the following statistical model:

$$y_{ijk} = \mu + C_i + H_{ij} + b_{11}\left(L_{ijk} - \overline{L}\right) + b_{21}\left(P_{ijk} - \overline{P}\right) + b_{22}\left(P_{ijk} - \overline{P}\right)^2 + b_{31}\left(S_{ijk} - \overline{S}\right) + b_{32}\left(S_{ijk} - \overline{S}\right)^2 + b_{33}\left(S_{ijk} - \overline{S}\right)^3 + b_{41}\left(I_{ijk} - \overline{I}\right) + b_{51}\left(J_{ijk} - \overline{J}\right) + e_{ijk}$$

where y_{ijk} is an observed trait i.e. TMM, FY, PY, FC, PC, and lactose (LC), μ is overall mean, C_i is the effect of the country (Slovenia, Croatia), H_{ij} is the flock effect nested within country, b_{11} is a linear regression coefficient for litter size (L_{ijk}), b_{21} and b_{22}

are linear and quadratic regression coefficients for parity (P_{ijk}), b_{31}, b_{32} and b_{33} are linear, quadratic and cubic regression coefficients for lambing season (S_{ijk}), b_{41} is linear regression coefficient for the effect of interval between lambing and the first recording (I_{ijk}), b_{51} is a linear regression coefficient for year of lambing (J_{ijk}), and e_{ijk} is the residual.

The statistical analysis was done by least square method using GLM (general linear models) procedure of statistical package SAS/STAT (5). The significance of parity effect was tested as composite quadratic, while the season of lambing effect was tested as composite cubic polynomial equation using the option CONTRAST in SAS/STAT (5).

Results

The average AMSL for six farms in Slovenia was 557.4 m and only 210.6 m for 32 farms in Croatia (Table 1). The average number of parities was 3.23 for Slovenian and 3.78 for Croatian ewes. The average litter size was 1.19 in Slovenia and 1.09 in Croatia. Ewes in Croatia lambed on average 104 days (middle of January) after the 1st October. Slovenian ewes lambed more than one month later. The lambing season is more variable in Croatia than in Slovenia (SD: 37.76 days vs. 19.14 days). The average interval between lambing and the first milk recording was 78 days in Croatia and 86 days in Slovenia.

Table 1: Mean and standard deviation (SD) for AMSL, parity (P), litter size (LS), lambing season (S), interval between lambing and first milk recording (I) and for TMM, FY, PY, FC, PC and lactose (LC) for Croatia and Slovenia

	AMSL	P	LS	S	I	TMM	FY	PY	FC	PC	LC
						Croatia					
Mean	210.6	3.78	1.09	103.91	77.79	124.87	8.91	7.46	7.31	6.05	4.30
SD	162.3	2.13	0.29	37.76	29.01	81.42	5.52	4.69	1.17	0.53	0.34
						Slovenia					
Mean	557.4	3.23	1.19	140.88	85.67	79.33	5.66	4.63	7.25	5.87	4.41
SD	132.5	1.94	0.43	19.14	34.55	48.61	3.28	2.81	0.94	0.52	0.29

Croatian ewes were more productive than the Slovenian ones. The later produced only 79.33 ± 48.61 kg milk compared to 124.87 ± 81.42 kg in Croatia. Large standard deviations were observed in both populations. FC and PC were slightly lower (7.25, 5.87) in Slovenia than in Croatia (7.31, 6.05). LC was higher (4.41 vs. 4.30) in Slovenia than in Croatia. FY and PY were higher in Croatian compared to Slovenian values due to larger milk yield and higher percentage of milk contents.

The productivity of flocks is presented on Map 1. Flocks are divided in four classes based on the average TMM per flock: up to 50 kg, 50.01 to 100 kg, 100.01 to 150 kg and more than 150 kg. Only two flocks (one in Slovenia and one in Croatia) milked less than 50 kg TMM, causing a large standard deviation in milk production. Four other Slovenian flocks produced between 50.01 and 100 kg TMM and one more than 150.01 kg. Nine Croatian flocks produced between 50.01 and 100 kg milk, ten between 100.01 and 150 kg and four flocks produced more than 150 kg TMM.

Map 1: Location of Istrian sheep flocks. Flocks are divided into four classes according to TMM: up to 50 kg (●), 50.01 to 100 kg (■), 100.01 to 150 kg (♦) and more than 150 kg (*)

Table 2: Coefficient of determination (R2) and p-values for model and effects of country (C), flock nested within country (H), litter size (L), parity (P), lambing season (S), interval between lambing and the first milk recording (I), and year of lambing (J)

	TMM	FY	PY	FC	PC	LC
Model	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
R^2	0.575	0.549	0.559	0.288	0.264	0.234
C	0.4348	0.7667	0.7967	0.5171	<0.0001	<0.0001
H	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
L	0.4792	0.8548	0.2703	0.1946	0.0350	0.0002
D	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
J	<0.0001	<0.0001	<0.0001	0.1458	0.0012	0.0373
P*	<0.0001	<0.0001	<0.0001	0.9865	0.4566	<0.0001
S**	<0.0001	<0.0001	<0.0001	<0.0001	0.0453	<0.0001

*composite linear and quadratic term; ** composite linear, quadratic and cubic term

Table 3: Linear regression coefficients and standard deviations for litter size (L), interval between lambing and the first milk recording (I), and year of lambing (J)

	L (b_{11})	I (b_{41})	J (b_{51})
TMM	1.45±2.05	-0.84±0.03	5.40±0.59
FY	0.03±0.15	-0.054±0.002	0.33±0.04
PY	0.14±0.12	-0.048±0.002	0.29±0.04
FC	-0.05±0.04	0.008±0.001	-0.016±0.011
PC	0.04±0.02	0.0046±0.0003	-0.018±0.006
LC	-0.05±0.01	-0.0025±0.0002	-0.0072±0.0035

As seen on Map 1, flocks with productivity between 50.01 and 100.00 kg TMM are spread over the whole territory. More productive flocks with TMM over 100 kg are mostly located on the southern part of the peninsula.

The coefficient of determination and p-values are shown in Table 2. The TMM, FY, PY, and FC were not affected by the country but by the effect of flock nested within the country (P<0.0001). For PC and LC, the effects of country and flock nested within the country were statistically significant (P<0.0001). The litter size effect influenced only PC and LC. The interval between lambing and the first milk recording had a statistically significant (P<0.0001) effect on all studied traits. The year of lambing significantly influenced (P<0.05) all traits with an exception of FC. The polynomial regression of the third degree, which describes the lambing season effect, was statistically significant for all traits (P<0.05), while the impact of parity affected TMM, FY, PY, and LC (P<0.0001).

The litter size caused 0.04 increase of PC and a 0.05 reduction of LC per liveborn lamb (Table 3). The prolonged interval from lambing to the first milk record contributed to 0.84 kg decrease of TMM, 0.054 kg of FY, and 0.048 kg of PY per day. The FC and PC increased 0.008% and 0.0045% respectively, if milk recording started one day later. The delay of one day decreased LC by 0.0025.

Total production of ewes increased during the studied period: TMM by 5.40, FY by 0.33, and PY by 0.29 kg per year. At the same time, FC, PC, LC decreased on average 0.018, 0.016, and 0.0072 % per year.

Estimated regression coefficients were used for the calculation of predicted yields and contents of milk (Figure 1). The largest TMM, FY, and PY were produced between the second and the forth lactation. The production of older ewes was rapidly reduced. The decrease of FY and PY during lactation was a consequence of reduced

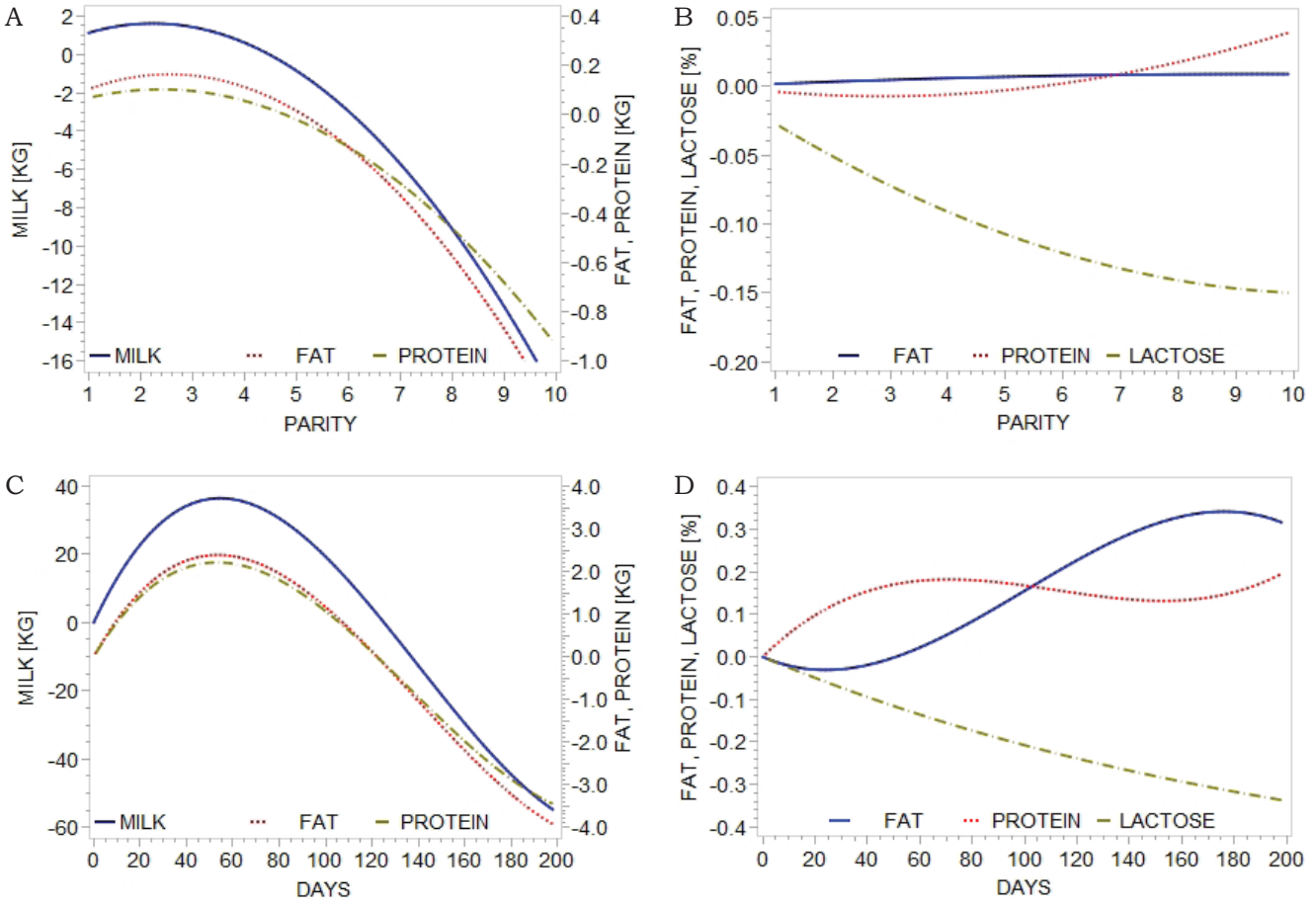

Figure 1: Predicted values of TMM, FY, and PY (A), FC, PC, and LC (B) between the first and tenth lactation, and predicted TMM, FY, and PY (C) and FC, PC, and LC (D) according to the season of lambing

TMM because there was a slight increase of FC and PC in older ewes. The LC decreased towards higher lactations.

The highest TMM, FY, and PY were observed in ewes that lambed 60 days after the beginning of lambing season, i.e. at the end of November and the beginning of December. Ewes that lambed until the end of January still retained satisfying TMM, FY, and PY. Lambings after that period were characterised with lower quantity of produced milk, with higher FC and PC, and lower LC.

Discussion

The production of Istrian sheep is comparable with the production of widespread Mediterranean breeds which have been selected for a longer period: Spanish Churra that produced between 89 (3) and 140 kg (6) or Italian Sarda with 168 (7), 186 (8) to 238 kg milk per lactation (3). However, Istrian sheep has lower TMM compared to the highly productive breeds like East Frisian sheep that produced 429 kg (9), Assaf, 431.6 kg (10) and 334 kg (11) or Awassi, 506 kg (11). The production conditions and average productivity per ewe differed between Slovenia and Croatia. However, the difference is caused mainly by flock within the country. Both effects, country and flock within the country, are related to the direct climate factors like heat stress (6) and indirect climate factors such as availability of feed on pasture. The flock management with high quality supplement feed will certainly improve productivity of housed animals before the beginning of grazing season or in dry period on pasture. Productivity of the best Slovenian flock on high AMSL was as good compared to the best flocks in Croatia and vice versa.

In spite of larger TMM of Croatian ewes, the FC and PC were surpassingly higher in Croatia (7.31, 6.05) than in Slovenia (7.25, 5.84). A higher FC and PC of less productive ewes is common within

the same breed (8), but also among breeds with different production levels (7, 9, 12). Higher PC and lower LC were observed in ewes with twins. Although, the ewes with twins produced more TMM, FY, and PY with higher PC, the differences were not statistically significant. The effect of litter size on milk production is not clear in literature. Some studies reported significant increase of milk production due to larger litter (13, 14). However, studies with non-significant effect of litter size were also found (15, 16). The PC and LC in our study were influenced by litter size. Our results are mostly different from the results of Peeters (17), who reports significantly higher FY and PY in milk of ewes with larger litter size. Longer period between lambing to first milk recording decreased the productivity due to shorter milking period, and increased FC and PC. During the studied period, TMM, FY, and PY were increased. The negative trend for PC and LC was observed in reported period. The increase of milk yield from the first to the third or fourth lactation was found in many researches (14, 18, 19). The increased FC and PC at higher parities was found in the literature (18). However, an increase of FC and PC was negligible and not significant in the present study. FY and PY were related only with TMM.

Late lambings after the end of January caused low TMM, increased FC and PC, and decreased LC. Although the increases of FC and PC in ewes that lambed late in season (after January) were statistically significant, lower TMM caused a decrease of FY and PY. Slovenian ewes were reared in the area with late vegetation. Therefore, late lambings with low productivity are common for Slovenian flocks. The productivity of Istrian sheep in Slovenia could be improved by earlier lambings and feeding with high quality forage before the grazing season and with an extra feeding on pasture after dry weather begins.

Conclusions

Productivity of Istrian sheep was studied for the period between 2005 and 2009 in Croatia and Slovenia. Milk production increased during the studied period. Litter size did not influence TMM, FY, and PY. The most productive ewes lambed between October and January. Ewes reared in Croatia were more productive compared to Slovenian ones. This is mostly the consequence of flock productivity and not the country effect. Most of the Slovenian flocks produced less than 100 kg TMM. However, the most productive flock in Slovenia had similar production to the best Croatian flocks with the productivity over 150 kg TMM. The Slovenian producers did not adapt their management to harsher climate in Slovenian hinterland, and pasture season is too short for high production. In the past, grazing season of Slovenian flocks was prolonged with transhumance pasture in Mediterranean conditions, which is nowadays not possible anymore. The productivity of Istrian sheep in Slovenia could be increased with earlier lambings and feeding with high quality forage before the grazing season starts and with an extra feeding on pasture after the dry weather begins.

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VPLIV GEOGRAFSKE LEGE NA PROIZVODNOST IN SESTAVO MLEKA ISTRSKE PRAMENKE

M. Kastelic, M. Špehar, Z. Barać

Povzetek: Proizvodnost istrske pramenke na Hrvaškem in v Sloveniji smo proučevali v obdobju med leti 2005 in 2009. Skupna količina namolzenega mleka na Hrvaškem s 7,31 % maščobe in 6,05 % beljakovin je znašala 124,87 kg. Slovenske ovce so dale 79,33 kg mleka s 7,25 % maščob in 5,87 % beljakovin. Količina namolzenega mleka je naraščala za 5,40 kg letno (P<0,05). Proizvodnost se med državama statistično značilno ni razlikovala. Učinek tropa, vgnezdenega znotraj države izvora živali, je povzročil razlike v količini in sestavi prirejenega mleka. Ta učinek vključuje tako genetske vplive kot vpliv menedžmenta črede in klimatske vplive. Ti so predvsem povprečna temperatura, nadmorska višina in letna količina padavin. Velikost gnezda ni vplivala na proizvodnost. Ovce, ki so jagnjile med oktobrom in koncem januarja, so dale večjo količino mleka z večjim odstotkom maščob in beljakovin. Menedžement črede in živali je bolje prilagojen klimatskim razmeram na Hrvaškem kot tršim pogojem reje v slovenskem zaledju.

Ključne besede: istrska pramenka; mlečna proizvodnja; Hrvaška; Slovenja; okoljski vplivi

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UDC 577.152.34:582.288:612.017

Original Scientific Article

THE EFFECT OF A PETIDE INHIBITOR OF CYSTEINE PEPTIDASES PRODUCED BY THE DERMATOPHYTE *Trichophyton mentagrophytes* ON A MOUSE IMMUNE SYSTEM

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Summary: A mouse model was used to determine a possible influence of the peptide cysteine peptidase inhibitor isolated from the dermatophyte fungus *Trichophyton mentagrophytes* (*T. mentagrophytes*) on a host immune system. The inhibitor was isolated from cytosol of mechanically and ultrasonically disintegrated fungal mycelia. BALB/c mice were divided into 4 groups. On days 0, 5 and 15, mice were treated with physiological fluid (Control group), isolated fungal inhibitor (I Group), fungal inhibitor and ovalbumin (I + OVA Group) and ovalbumin only (OVA Group). On day 29, blood samples were taken, animals were euthanized and their spleens were aseptically removed. Harvested spleen cells were cultured in microtiter plates and used for the measurements of cell proliferation and the level of apoptosis with commercially available kits. Mice sera were used in Western blot procedure to study the effect of the fungal inhibitor on the processing and presentation of ovalbumin as antigen. The method was used to determine whether antibodies from differently treated mice differ in their specificity. Experiments showed that the *T. mentagrophytes* inhibitor decreases apoptosis of mouse spleen cells, but has no effect on the proliferation of lymphocytes among them. It was concluded from the immunoblotting results that the fungal inhibitor probably affects the processing of ovalbumin.

Key words: cysteine peptidase; inhibitor; immune system; cell proliferation; apoptosis; antigen presentation

Introduction

Peptidases are enzymes that catalyse the cleavage of peptide bonds of proteins, digesting them into peptides or free amino acids and thus regulating their function and fate. On the basis of the mode of action and their active sites peptidases are classified into several groups, namely aspartic, cysteine, glutamine, metallo, asparagine, serine, threonine peptidases and those with an unknown catalytic mechanism (1, 2, 3, 4, 5). Despite their essential role, proteolytic enzymes can be potentially extremely damaging in living systems. Proteolysis is therefore stringently regulated by several distinct mechanisms. One of them is reversible or irreversible suppression of peptidase activity by an inhibitor which binds in the catalytic cleft of the protease and thereby prevents access to substrates (6, 7, 8, 9).

The endosome/lysosome compartments, together with the cytosolic proteasomes, are the two major

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protein degradation systems in cells. Endosome/lysosome–located proteases play key roles in antigen processing and presentation, cytokine regulation, natural killer T cell development, activation of serine protease zymogens in regulated secretory granules, integrin activation, induction of apoptosis and the Toll-like receptor signalling. Cysteine, partly serine as well as aspartic peptidases and their inhibitors play an important role in two processes in the immune system that require proteolysis, i.e. apoptosis and endocytotic pathway of antigen processing. They are involved in the regulation of apoptosis in neutrophils, monocytes and dendritic cells, in lymphocyte maturation in primary lymphatic organs and in killing of target cells by cytotoxic T cells and NK cells. In antigen processing, aspartic and cysteine proteases introduce cleavages in endocytosed antigens, which trigger unfolding of the polypeptide chains and the capture of processed antigens by newly synthesised MHC class II molecules. In addition, MHC class II molecules are processed by the same type of enzymes to degrade the invariant chain (Ii) that occupies the peptide-binding site (1, 10, 11, 12, 13, 14).

The described experiment assesses the effect of a peptide inhibitor isolated from the dermatophyte species *Trichophyton mentagrophytes (T. mentagrophytes)* on a mouse immune system. The research was focused on measuring its effect on immune cell proliferation and apoptosis as well as on antigen presentation and consequently on the specificity of antibody binding by the defined antigens.

Materials and methods

Peptide dermatophyte inhibitor

Fungal homogenate, prepared from mechanically and ultrasonically disintegrated dermatophyte mycelia, was centrifuged for 20 minutes at 10.000 × g and for 1 hour at 25.000 × g to remove cell debris (ultracentrifuge Beckman, Avanti J-301). The cytosol extract was ultrafiltered using Centriprep YM-3 concentrator (Amicon) with the nominal molecular weight limit of 3.000 Da. The obtained filtrate with inhibitory activity against cysteine peptidase papain was pooled and diluted with physiological solution.

Experimental animals, immunization and cell preparation

Adult female BALB/c mice were acquired at the Centre for animal genomics of the Veterinary Faculty, University of Ljubljana, Slovenia. They were kept in standard animals housing facilities and given free access to water and food. 12 BALB/c mice between 92 and 98 days old, weighing between 21.1 and 25.1 g were formed into 4 groups. On days 0, 5 and 15, mice in the Control group were i.p. treated with 300 µL of physiological fluid and mice in Group I with 300 µL of fungal inhibitor. At the same time, mice in the Group OVA were s.c. given 300 µL of ovalbumin (Chicken egg white, Grade V, Sigma; final concentration of 15 µg/L) and mice in the Group I + OVA were treated i.p. with 300 µL of fungal inhibitor and also s.c. with 300 µL of ovalbumin. On day 29, mice were anesthetized and their blood was removed from the retroorbital plexus using a glass Pasteur pipette (Brand, Wertheim). Blood was collected in Microtainer tubes with gel (Becton, Dickinson and Company). After centrifugation sera were frozen at –20 °C until analysis. Mice were then euthanized in accordance with the Slovene legislation on animal euthanasia and their spleens were aseptically removed. Animal experiment was approved by the Veterinary Administration of Republic of Slovenia (permit no. 34401-38/2007/3).

Aseptically removed spleens were teased apart between the frosted ends of two sterile microscope slides and resuspended in Lymphocyte Culture Medium (LCM). The cell suspension was centrifuged and the pellet resuspended in the ACK buffer for 5 minutes. Cells were washed twice with MEM (Gibco) and resuspended in LCM. Cells were quantified in Neubauer haemocytometer and appropriate cell dilutions were prepared.

Dermatophyte inhibitor, cell proliferation and apoptosis

Lymphocyte proliferation was measured using a commercially available kit (BrdU Cell Proliferation Assay, Calbiochem). It is an immunoassay for the quantification of bromodeoxyuridine (BrdU) incorporation into the newly synthesized DNA of actively proliferating cells. 50 µL of mononuclear cells in concentration of 2×10^6 cells/mL were cultured in triplicates in 96 well culture dish

(TPP). 100 µL of either medium (RPMI 1640 and MEM, 1:1 (v/v), Gibco), ovalbumin (Chicken egg white, Grade V, Sigma; final concentration of 5 µg/L), fungal inhibitor, the mix of the inhibitor and ovalbumin 1:1 (v/v) or phytochemagglutinin (PHA, Sigma) in the final concentration of 10 µg/mL was added to the cells. Cells stimulated with PHA were incubated for 5 days, whereas others were incubated for 7 days in the incubator at 37 °C, 100 % humidity and in 5 % CO_2. 24 hours before ending the incubation, 20 µL of BrdU (diluted 1:2000 (v/v)) was added to the wells. After the incubation period the assay was performed following manufacturer's instructions. Briefly, cells were fixed, denatured and incubated in the presence of anti-BrdU antibodies. After the incubation with peroxidase goat anti-mouse IgG/HRP conjugate, substrate solution was added to each well. In the end, the reaction was stopped and absorbance measured at dual wavelengths of 450 – 540 nm using a spectrophotometric plate reader (Tecan, Sunrise).

To measure the effect of the dermatophyte inhibitor on apoptosis in a culture of spleen cells the commercial kit (Cell Death Detection ELISA, Roche) was used again. It is based on a quantitative sandwich enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allows the specific detection and quantitation of mono- and oligonucleosomes that are released into the cytoplasm of cells that die from apoptosis. For the detection of cell death, mononuclear cells were prepared and treated as described previously. The assay was performed according to the manufacturer's instructions. Briefly, cells were centrifuged and lysed. Samples (in duplicates) were pipetted into wells of a coated microtiter plate. After adding the conjugate solution, the microtiter plate was washed and the substrate solution added. A photometric analysis was performed with a spectrophotometric plate reader at 405 nm (Tecan, Sunrise).

The results of measurements of cell proliferation and apoptosis were statistically analysed using the GrafPad Prism computer programme. The applied method was the one-way ANOVA followed by the Tukey's multiple comparison test. Data are presented as the mean ± SD. The P-value of ≤ 0.05 was considered statistically significant.

Dermatophyte inhibitor and MHC class II antigen presentation

To study the effect of the *T. mentagrophytes* cysteine peptidase inhibitor on antigen presentation, the method of Western blotting was applied. The antigen (ovalbumin, Chicken egg white, Sigma) was first enzymatically processed with papain and cathepsins B and L. The processed peptide fragments were transferred on the membrane, and mouse sera from the Control and experimental groups were used as primary antibodies. Secondary antibodies (anti-mouse IgG and IgM) were conjugated with horseradish peroxidase.

The SDS-PAGE was performed with the method of Laemmli using a 10 % resolving gel on a mini-Protean II Slab cell apparatus (Bio-Rad). Ovalbumin (Sigma) was digested *in vitro* with papain, cathepsin B or cathepsin L. 6 mg of ovalbumin was digested with papain (final concentration 0.2 mg/mL) in 0.1 M phosphate buffer and 1.5 mM EDTA, pH 6, for two hours at 37 °C. For papain activation, 1.4 mg of cysteine was added to the solution (all chemicals were from Sigma). 1.86 mg of ovalbumin was digested with cathepsin B (0.1 mg/250 µL) in 0.1 M of acetate buffer and 1.0 mM EDTA, pH 5.0 for 23 hours at 30 °C. 30 µL of cathepsin L (1.0 IU) was added to 0.65 mg of ovalbumin in 0.1 M acetate buffer with 2.0 mM EDTA and 20 mM DTT (dithiothreitol), pH 5.5 (all chemicals were purchased from Sigma). The digestion was performed for 20 hours at 30 °C. Prior to loading the samples onto the gel, fractions were mixed (1:1) with SDS Laemmli buffer (100 mM Tris/HCl buffer, pH 6.8; 4 % (w/v) SDS; 20 % (v/v) glycerol; 20 % (v/v) 2-mercaptoethanol; 0.025 % (w/v) bromophenol blue) and boiled for 5 minutes. The samples were visualized with Coomassie brilliant blue R-350. Molecular masses were determined using the LMW standards of 11 – 170 kDa (Fermentas).

The separated digested protein samples were transferred from the SDS-PAGE gels to polyvinylidene difluoride membranes (Immobilon-P, Millipore) using the same apparatus as mentioned above. Blots were incubated overnight at 4 °C in a blocking solution containing 2 % (w/v) Tween 20 in phosphate buffer (Sigma). After being washed three times with phosphate buffer, the membranes were incubated for 1.5 hours with primary antibodies

from the sera of mouse groups denoted Control, Group I, Group OVA and Group I + OVA (diluted 1:200). After the washing, blots were incubated with detection secondary goat HRP-conjugated anti-mouse IgG, diluted 1:1000 (Sigma) or goat HRP-conjugated anti-mouse IgM, diluted 1:1000 (Sigma). Antigen – antibody complexes were visualised by 3-amino-9-ethyl-carbazole (Sigma).

Results

The effect of the peptide T. mentagrophytes inhibitor on cell proliferation

Results depicted in Figure 1 show that the detected proliferation was the highest following the non-specific stimulation of cells with the plant lecithin PHA. The average O.D. values measured in cells of all groups of mice ranged from 2.596 (Group OVA) to 3.421 (Group I). As far as other treatments of cell cultures of all four groups of mice are concerned no statistically significant differences were observed. The measured O.D. values were very low ranging from 0.028 to 0.048.

The effect of the peptide T. mentagrophytes inhibitor on apoptosis

Results depicted in Figure 2 show that apoptosis was the strongest in all four groups of mice following the stimulation of cell cultures with ovalbumin. The average measured O.D. values in all groups of mice ranged from 0.622 (Group OVA) to 1.013 (Group I + OVA). Group I + OVA also had the highest standard deviation (0.588). Apoptosis was the lowest in all groups of mice after the cells were treated with the inhibitor or the mix of the inhibitor and ovalbumin. The average O.D. values were lower than 0.482 (Group OVA after the cells were treated with the inhibitor). The detected apoptosis was statistically significantly lower ($P \leq 0.05$) in Group I between the cells stimulated with ovalbumin and those treated with the inhibitor or the mix of ovalbumin and the inhibitor.

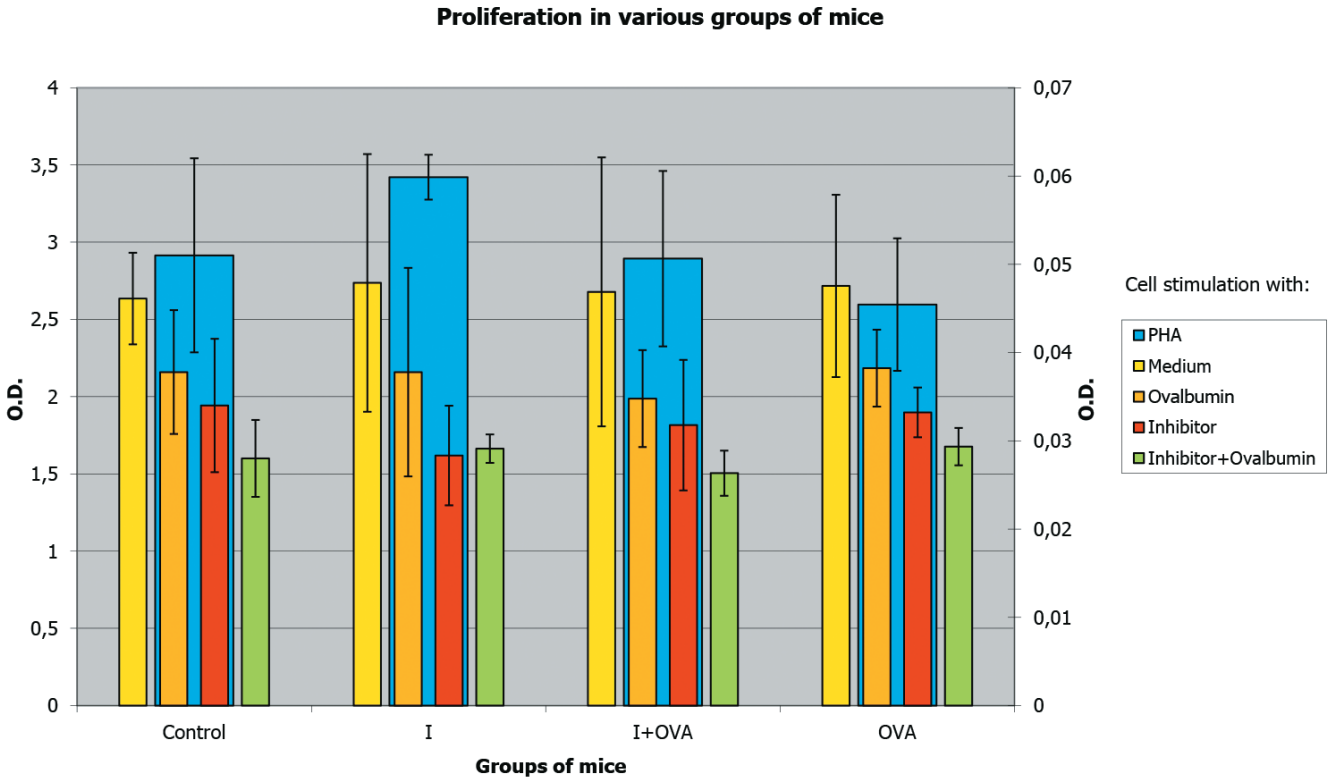

Figure 1: Comparison of results of proliferation of lymphocytes in the Control and experimental groups of mice. The results are represented as the mean OD value ± SD. Different columns represent treatments of cells in cultures with a medium (yellow), ovalbumin (orange), the inhibitor (red) or the mix of ovalbumin and the inhibitor (green) (OD scale on the right side). The wider blue-colored columns at the back (the OD scale on the left side) show unspecific proliferation of cells after on-plate stimulation with PHA

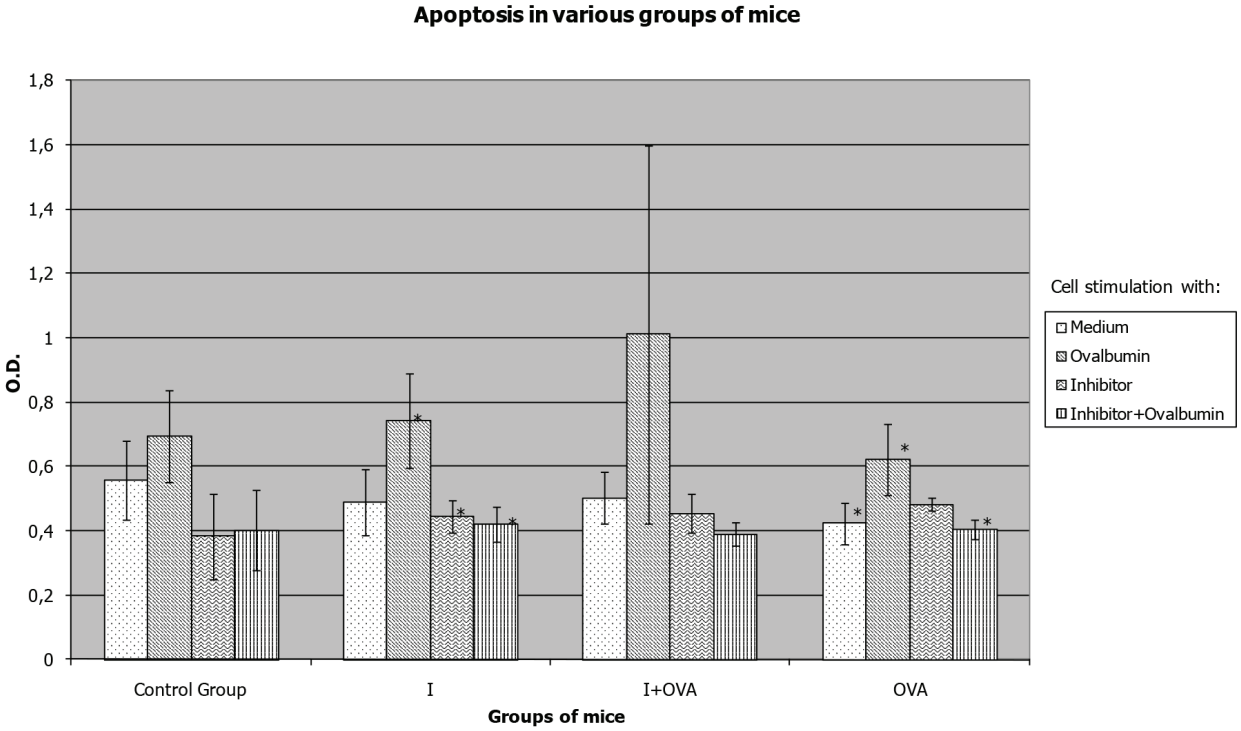

Figure 2: Comparison of results of apoptosis of spleen cells in the Control and experimental groups of mice. The results are represented as the mean OD value ± SD. Different columns represent treatments of cell cultures with a medium, ovalbumin, the inhibitor or the mix of ovalbumin and the inhibitor. * denotes statistically significant difference ($P \leq 0.05$)

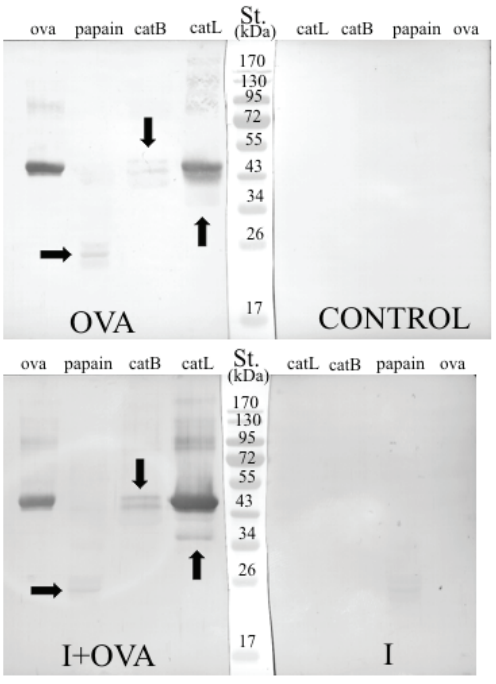

Figure 3: Immunoblot of unprocessed and processed ovalbumin visualised with goat HRP-conjugated anti-mouse IgG. Arrows represent a different pattern of bands in Groups OVA and I + OVA. Representative results are shown.

The detected apoptosis was also statistically significantly lower in the Group OVA between the cells stimulated with ovalbumin and those treated with a medium or the mix of the inhibitor and ovalbumin. The lowest detected average O.D. value was 0.384 in the cells of the Control group after the addition of the inhibitor.

Western blot analysis

The effect of the peptide inhibitor of *T. mentagrophytes* on antigen presentation was indirectly analysed by immunoblotting. Ovalbumin was processed *in vitro* with papain and cathepsins B and L. The processed peptides were loaded on the SDS-PAGE gel (the data not shown). Unprocessed ovalbumin with the molecular mass of 45 kDa was used as control. Digestion of ovalbumin was the greatest with papain since the highest number of fragments was observed there. The fragments had molecular masses from around 11 to 43 kDa. Digestion with cathepsin L gave fewer fragments with molecular masses of around 30 to 43 kDa and it appeared that some of the ovalbumin was not processed at all. Digestion

with cathepsin B (catB) resulted in only 3 bands with molecular masses of around 30 to 40 kDa.

Figure 3 shows the results of Western blotting. Fragments which were obtained after ovalbumin digestions *in vitro* were transferred from the SDS-PAGE gel to the PVDF membrane and detected with antibodies from control and experimental animals. As it can be observed, the number and intensity of the protein bands is different on the membrane where we used sera of Groups OVA (mice that were immunised with ovalbumin) and I + OVA (mice that were immunised with ovalbumin and treated with the fungal inhibitor).

Discussion

Dermatophyte colonization is characteristically limited to the dead keratinized tissue of the *stratum corneum.* Several steps are required for infection to take place, namely contact, adherence and invasion of keratin layers. The severity of the infection depends on the type of the agent, environmental factors and the host immunologic status and results in either a mild or intense inflammatory reaction. Although cornified layers of skin lack specific immune defense, both humoral and cell-mediated reactions as well as non-specific host defense mechanisms respond and eliminate the fungus (15, 16). There are reports on peptidases of various classes secreted mostly by parasitic organisms that functionally and structurally resemble the host endosomal proteases. In addition to their roles in the life cycle of pathogens, these peptidases act as virulence factors by proteolytically degrading components of the host immune system including immunoglobulins and components of the complement system. Helminth worms secrete proteases that can activate the Th2 type immune response, and, on the other hand, endogenous parasites and bacteria induce Th1 cell immune response (9, 17, 18). Among peptidase inhibitors expressed by pathogens, the best studied are those of filarial nematodes. Their immunoregulatory properties include inhibition of antigen presentation, induction of IL-10 expression and macrophage stimulation (9). For example, the intestinal nematode *Nippostrongylus brasiliensis* evades a host defense system by secreting the cysteine peptidase inhibitor nippocystatin, which modulates antigen processing in host antigen presenting cells (19). As for dermatophytes: certain dermatophyte fungi produce substances that supress the immune response. These mostly include mannans – glycoprotein constituents of the cell wall that bind to the cell surface of mononuclear phagocytes in suspension and can inhibit cell-mediated immunity and keratinocyte proliferation (20, 21).

Two important zoophilic dermatophyte fungi, namely *Microsporum canis* (*M. canis*) and *Trichophyton mentagrophytes* (*T. mentagrophytes*) were shown to produce *in vitro* peptide inhibitors that inhibit papain, cathepsins B and L, but not cathepsin H (22, 23). Different cysteine, serine and aspartic proteases and their inhibitors are involved in the immune response so we designed an experiment based on an animal model to study the effect of the fungal inhibitor on host immune response. Since the isolated peptide *T. mentagrophytes* inhibitor had no cytotoxic effect on baby hamster liver cells (BHL cells; the data not shown), its potential effect on proliferation of lymphocytes and apoptosis of immune cells in ovalbumin immunised mice was studied as well, as was its effect on the processing and presentation of ovalbumin as an antigen. To follow the 3 R rule in animal experiment, we used a minimal yet accepted number of mice which corresponds to small-sample statistics. We also tried to combine the *in vivo* and *in vitro* studies to test as much variables as possible.

Cell proliferation was studied using the commercial enzyme immune test BrdU Cell Proliferation Assay. The results (Figure 1) show that the measured proliferation was as expected the highest in cells that were stimulated with PHA. The PHA lecithin is a known mitogen that non-specifically stimulates the proliferation of lymphocytes T. Proliferation was not detected after all other specific stimulations. The results indicate that the treatment of mice with specific antigens did not activate the specific cellular immunity and that the peptide inhibitor had no effect on mice lymphocyte proliferation. The dermatophyte inhibitor was studied also regarding antibody immunity, where specificity of antibodies derived from mice of the Control and experimental groups was tested against the defined antigens applying the Western blot method.

Apoptosis was measured using a commercial photometric enzyme immunoassay for *in vitro* determination of cytoplasmic histone-associated DNA fragments. Figure 2 shows that the

measured apoptosis was the highest in all groups of mice (Control, Inhibitor, Ovalbumin, Inhibitor + Ovalbumin) after stimulation of cell cultures with ovalbumin. Cell cultures treated with the *T. mentagrophytes* inhibitor or the mix of ovalbumin and the fungal inhibitor exhibited lower detected apoptosis. The apoptosis between differently treated cell cultures was statistically significant. It can be concluded from the results of our experiment that the *T. mentagrophytes* peptide inhibitor supressed cell apoptosis. One could speculate that the role of the fungal inhibitor is to diminish cell apoptosis and thus influence the outcome of an infection and/or to modulate host inflammatory response during the infection. To elucidate the role of the *T. mentagrophytes* inhibitor further, the inhibition of caspases and not only cathepsins should be tested.

Helper T cells recognize antigens in the form of peptides bound to the MHC class II molecules on the surface of antigen presenting cells. Those peptide determinants are generated in acidic compartments of APC by a series of events including denaturation, reduction and proteolysis which are collectively referred to as endocytic antigen processing (24). To establish if the peptide *T. mentagrophytes* inhibitor affects processes in antigen presentation of ovalbumin, the method of Western blotting was applied. The method was used to determine whether antibodies from differently treated mice differ in their specificity as a result of modified antigen processing. Ovalbumin processed by different proteolytic enzymes (papain, cathepsins B and L) was transferred onto a membrane using electroelution. Membranes with ovalbumin digestions were incubated with sera belonging to all four groups of mice (the Control Group, the Group Ovalbumin, the Group Inhibitor and the Group Inhibitor + Ovalbumin). When anti-mouse IgG were used as detector antibodies, no bands were visible on the membrane incubated with serums of the Control Group of mice (Figure 3). This was expected since mice in the Control Group were not in contact with the antigen and did not develop antibodies against ovalbumin or its fragments. Similarly, we observed only a few non-specific bands on the membranes incubated with sera belonging to mice that were treated with the fungal inhibitor. This can be explained in the manner already mentioned above – mice did not develop the antibody immune response. The bands appear on the spot where ovalbumin digested with papain was applied. The cause of such staining could be cross-reactivity against antigens that are similar to ovalbumin. In this case, the exact staining pattern should have appeared also in the presence of antibodies from the Control Group of mice. A more probable cause for the presence of bands on the membrane incubated with the sera of the group that was treated with the inhibitor is the effect of a dermatophyte peptidase inhibitor on "daily" antigen presentation. This effect could have changed the specificity of antibodies against antigens that mice were exposed to and the above mentioned cross-reactivity could have appeared. More obvious differences were observed in the staining pattern of protein fragments between the group of mice that was immunised with ovalbumin (the Group OVA) and the group of mice that was treated with the fungal inhibitor and immunised with ovalbumin (the Group I + OVA). Mice in both groups were immunised with the ovalbumin so antibodies against it could have developed. The only difference between the two groups was that the mice in the Group I + OVA were also treated with the fungal inhibitor. The inhibitor could have inhibited or altered the activity of peptidases which are involved in the antigen presentation of ovalbumin so the recognition of peptides and consequently specificity of antibodies can be different. The differences in the band pattern and intensity were observed in all samples digested with peptidases. In the case of papain digestions, we noticed 3 bands with molecular masses of around 20 kDa on the membrane incubated with the serum from mice in the Group OVA while only one band was visible on the membrane incubated with sera of the Group I + OVA. In cathepsin B digestions, the membrane incubated in the presence of sera from the Group OVA, 3 bands are visible at around 43 kDa. On the other hand only 2 bands are seen on the membrane incubated with the sera of mice from the Group I + OVA. Major differences were observed in cathepsin L digestions. Two bands are visible at around 43 kDa on the membrane incubated with sera of mice from the Group OVA, while only one band is seen on the membrane incubated in the presence of antibodies from the Group I + OVA. In addition, two protein fragments at around 34 and 95 kDa are visible on the same membrane. It appears as if ovalbumin was processed differently in the Groups OVA and I + OVA due to the selective inhibition of proteolytic enzymes by the fungal

inhibitor. The same effect was not observed when anti-mouse IgM antibodies were used (the data not shown). There are two reasons for such a result, namely IgM antibodies develop early in the primary immune response and are not so specific and, on the other hand, specific IgG antibodies result from the secondary, more specific and more abundant immune response.

The results of our research can not fully answer which stages of antigen presentation the dermatophyte cysteine peptidase inhibitor affects. It is possible that due to the selective peptidase inhibition the fungal inhibitor influences the degradation of ovalbumin so different antigen peptides appear in the MHC II molecules. On the other hand, the fungal inhibitor might affect the processes in maturation of the MHC II molecules – the removal of Ii chain or loading of antigen peptides into the binding groove.

The biological function of a peptide cysteine peptidase inhibitor from the dermatophyte fungus *T. mentagrophytes* is still unknown. Although our studies demonstrated the possible effect of the fungal inhibitor on antigen processing and apoptosis, further experiments are needed to elucidate the physiological mechanisms involved in the complex host – the dermatophyte interaction during the infection. Also, selective inhibition of cysteine peptidases with the purified *T. mentagrophytes* inhibitor may have important therapeutic potential in modulating immune responses in the future.

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THE EFFECT OF A PETIDE INHIBITOR OF CYSTEINE PEPTIDASES PRODUCED BY THE DERMATOPHYTE *Trichophyton mentagrophytes* ON A MOUSE IMMUNE SYSTEM

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Povzetek: Za proučevanje vpliva zaviralca cisteinskih peptidaz iz dermatofita *Trichophyton mentagrophytes* (*T. mentagrophytes*) na gostitelja smo uporabili mišji model. Zaviralec smo izolirali iz citosola glive, ki smo ga pridobili z mehanskim in ultrazvočnim razbitjem micelija. Miši seva BALB/c smo razdellili v štiri skupine. Miši so bile 0., 5. in 15. dan tretirane s fiziološko raztopino (kontrolna skupina), izoliranim glivnim zaviralcem (skupina I), glivnim zaviralcem in ovalbuminom (skupina I + OVA), ali samo z ovalbuminom (skupina OVA). 29. dan smo mišim odvzeli vzorce krvi, jih nato evtanazirali in jim v aseptičnih pogojih odstranili vranico. Celice iz vranice smo gojili v mikrotitrskih ploščah in jih uporabili za meritve celične proliferacije in apoptoze. Meritve smo izvedli s komercialno dostopnimi testnimi kompleti. Serume mišk smo uporabili za imunski odtis, s katerim smo proučevali učinek glivnega zaviralca na procesiranje in predstavljanje ovalbumina kot antigena. Z omenjeno metodo smo poskušali preveriti, ali se protitelesa iz serumov različno tretiranih miši razlikujejo po svoji specifičnosti. S poskusi smo dokazali, da peptidni glivni zaviralec zavira apoptozo celic iz vranic miši, nima pa vpliva na proliferacijo limfocitov. Na podlagi rezultatov prenosa proteinov na membrano z imunodetekcijo smo ugotovili, da glivni zaviralec najverjetneje vpliva na procesiranje ovalbumina.

Ključne besede: cisteinska peptidaza; zaviralec; imunski sistem; celična proliferacija; predstavljanje antigenov; apoptoza

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Original Scientific Article

ESTIMATION OF *Yersinia enterocolitica* PREVALENCE IN SLAUGHTERED PIG TONSILS IN SLOVENIA BY USING THREE CULTURAL ISOLATION PROCEDURES

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Summary: A survey was conducted to collect preliminary data concerning the prevalence of *Yersinia enterocolitica* in slaughtered pigs in Slovenia. At the same time, culture procedures for its isolation were compared. During the period between January and May 2008, 1490 tonsil swabs from 149 slaughtered herds were taken from slaughterhouses in Slovenia. One swab was used to sample the tonsils of one slaughtered pig and ten swabs were taken in each sampled herd. For each swab, three protocols were used to isolate *Y. enterocolitica*. Firstly, swabs were tested by direct cultivation on Cefsulodin-Irgasan-Novobiocin (CIN) agar. Secondly, the swabs were incubated in enriching Yersinia PSB broth (PSB) and then inoculated on CIN agar. In the third case of isolation, the PSB enrichment after incubation was treated with the potassium hydroxide solution (KOH) and then streaked on CIN agar. Out of 43 (29.3%) herds, *Y. enterocolitica* was isolated from at least one of the 10 tested swabs by any of the procedures used. In the case of isolation, the enrichment with KOH treatment, was the most efficient (74.4%). Isolation using the combination of direct plating and the procedure using enrichment with the KOH treatment was successful in all positive herds. *Y. enterocolitica* were biotyped and serotyped. Most isolated strains (80.4%) belonged to the pathogenic biotype 4 serotype O:3.

Key words: *Yersinia enterocolitica*; slaughter pigs; tonsils; isolation

Introduction

Yersinia enterocolitica is well established as a foodborne pathogen of human concern (10). It is primarily a gastrointestinal tract pathogen acquired through the oral route and epidemiologically linked to porcine source (18). Healthy pigs are often carriers of *Y. enterocolitica* strains pathogenic for humans, in particular strains of biotype 4 (serotype O:3) and biotype 2 (serotype O:9) (6, 16, 18, 22). The most common biotype associated with human disease in Europe is biotype 4 (1, 12, 19). *Y. enterocolitica* is commonly found in pig tonsils, intestine and faeces (4, 14). Although pork and pork products are considered major transmission sources of pathogenic *Y. enterocolitica* (13), it has only rarely been isolated from pork products with the exception of fresh tongues (2). Strains of the nonpathogenic biotype 1A are widely spread in the environment and often isolated from food. When *Y. enterocolitica* is present in meat or meat products, it has, due to its psychrophilic character, a potential to multiply during storage. Since the

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population of *Y. enterocolitica* in food samples is usually low, isolation procedures usually involve enrichment in liquid media followed by plating on selective media (3). When pigs are slaughtered at the age of 135 days or more, the estimated prevalence of pathogenic *Y. enterocolitica* on the tonsils is greater than the prevalence in faeces or on the carcasses (5, 15, 23, 24, 25). Due to a higher prevalence and a higher amount of *Y. enterocolitica* in tonsils than in faeces, the tonsils were chosen as the sampling site for this study. The aim was to find the most appropriate protocol for isolation of *Y. enterocolitica* on pig tonsils and to collect preliminary data of its prevalence in slaughtered pigs in Slovenia.

Material and methods

The sampling was carried out by inspectors of the Veterinary Administration of the Republic of Slovenia in all slaughterhouses in Slovenia that slaughter more than 1000 pigs per year. From 1st January to 14th May 2008, 149 herd samples were taken and 10 animals were randomly sampled per herd. Eleven slaughterhouses were involved, covering 97% of pig slaughtering in Slovenia. Only the animals raised in Slovenia were sampled. For each slaughterhouse, the number of sampled herds was calculated based on the number of slaughtered animals per year. According to the slaughtering capacity, 1 to 50 herds were sampled and ten pigs were sampled per herd randomly. For each pig carcass, one individual cotton swab was used. This resulted in a final sample size of 1490 tonsil swabs.

The surface of the tonsils was wiped with dry cotton on a stick (Dolgi bris, Plastika Kavčič, Ljubljana, Slovenia) and enclosed in a sterile tube. Samples were transported to the laboratory in portable coolers and analysed within 24 hours after collection. In the laboratory, each swab was tested following three isolation protocols, namely:

Procedure 1: Swab was streaked onto the surface of Cefsulodin-Irgasan-Novobiocin (CIN) agar (Biolife, Milano, Italy) and incubated at 30±1°C for 24 hours.

Procedure 2: Five ml of the Yersinia PSB broth (Biolife, Milano, Italy) enrichment media was added to each swab and incubated at 23±1 °C for 5 days. After incubation, 10µl of PSB was streaked onto CIN agar.

Procedure 3: A half of a millilitre of PSB after incubation, which resulted from the second protocol, was transferred to 4.5ml of the potassium hydroxide solution (0.5% KOH prepared with 0.5% saline solution) and mixed for approximately 20 seconds. Using a loop, 10µl of the treated PSB was streaked on CIN agar plate.

All of the inoculated agar plates were incubated at 30±1 °C for 24 hours. When growth was weak or there were no characteristic colonies, incubation was prolonged for another 24 hours. *Y. enterocolitica* formed small colonies with a deep red centre and sharp border surrounded by a clear colourless zone. Characteristic colonies from CIN plates were streaked to nutrient agar (Biolife, Milano, Italy) and incubated for 24h at 30± 1°C. After incubation, detection of oxidase (BD, Sparks, USA), urease (Merck, Darmstadt, Germany) and fermentation of sugars by TSI agar (Biokar Diagnostic, Beauvais Cedex, France) was performed. Oxidase negative, urease positive, glucose positive, lactose negative and H_2S negative cultures were further tested. Additional biochemical confirmation tests were performed: indol (Biolife, Milano, Italy), lysine decarboxylase and ornithin decarboxylase (Merck, Darmstadt, Germany), fermentation of sugars (sucrose (Kemika, Zagreb, Croatia), trehalose, rhamnose, xylose (Merck, Darmstadt, Germany)), and aesculin hydrolysis (Kemika, Zagreb, Croatia). Antiserums for O:3 and O:9 (Statens Serum Institute, *Copenhagen,* Denmark) were used for serotyping.

Agreements between the procedures used were tested by Kappa statistic test. Kappa values below 0 indicate no agreement, 0–0.20 slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–1 almost perfect agreement (27).

Results

A herd was considered positive if at least one tonsil swab was positive for *Y. enterocolitica* by any of the procedures used. Forty-three (28.9%) out of 149 herds were positive. No one isolation procedure detected all of the positive herds. *Y. enterocolitica* was isolated from 16 herds (37.2%) using direct plating (procedure 1), from 16 using enrichment without treatment (procedure 2) and from 32 (74.4%) after enrichment using the treatment with KOH (procedure 3). Just one herd was found positive by all the procedures used. Nine

Table 1: Number of *Y. enterocolitica* positive herds and the rate (%) of successful isolations at positive herds by using each procedure or their combinations

Detection method	Number of positive results	% of positive results
Only procedure 1	9	20.9
Only procedure 2	0	0
Only procedure 3	14	32.6
Procedure 1 and procedure 2	2	4.7
Procedure 1 and procedure 3	4	9.3
Procedure 2 and procedure 3	13	30.2
All three procedures	1	2.3
All procedures and their combinations	43	100.0

Table 2: Coefficient of determination (R2) and p-values for model and effects of country (C), flock nested within country (H), litter size (L), parity (P), lambing season (S), interval between lambing and the first milk recording (I), and year of lambing (J)

Detection method	Number of positive results	% of positive results
Only procedure 1	22	25.6
Only procedure 2	4	4.7
Only procedure 3	36	41.9
Procedure 1 and procedure 2	1	1,1
Procedure 1 and procedure 3	3	3.5
Procedure 2 and procedure 3	20	23.2
All three procedures	0	0
All procedures and their combinations	86	100.0

herds were positive only by applying procedure 1 and 14 only by applying procedure 3. None of the herds was positive by using only procedure 2. In the case of 19 herds, isolation was successful using two of the procedures (Table 1).

Comparing procedures by Kappa test indicates statistical difference between procedures 1 and 2 ($x < 0$) and procedures 1 and 3 ($x < 0$), but just slight agreement between procedures 2 and 3 ($x = 0.16$). Between 32 herds detected positive by using procedure 3 were also 14 of 16 (87.5%) tested positive by procedure 2. This indicates better sensitivity of procedure 3, regarding procedure 2.

Within the positive herds, the number of positive swabs taken from individual animals varied from one to seven, with an average of 2 positives per herd. Out of 1490 individual swabs, 86 (6.0%) were positive by at least one of the methods used. Twenty-six swabs were positive after direct inoculation (procedure 1) and 64 using enrichment (procedures 2 and 3). By applying procedure 2, twenty-five swabs were positive and by applying procedure 3 fifty-nine. In the case of 20 swabs, *Y. enterocolitica* was isolated only by both enrichment procedures. In only four swabs *Y. enterocolitica* was isolated after direct plating (procedure 1) and after enrichment (procedure 2 or procedure 3). Numbers of positive isolations by one or more procedures used are presented in Table 2. *Y. enterocolitica* isolated from 37 herds belonged to the pathogenic biotype 4 serotype O:3 and from 9 herds to the non-pathogenic biotype 1A. In the case of 3 herds both biotypes were isolated.

Discussion

The results indicate that pigs are carriers of *Y. enterocolitica* in a significant proportion (28.9%) of herds in Slovenia. The prevalence reported in this study is comparable to the prevalence referred

by some other studies in which between 5% and 35% of positive animals, and from 20 to 80% of positive herds, have been reported (4, 5, 23, 24). Results of the studies, based on the prevalence of *Y. enterocolitica* in pig herds or slaughter batches from European countries reported by the European Food Safety Authority (EFSA) (9) varies greatly, from 1% reported in Germany to 48.4% in Spain. The highest prevalence of 52.0% indicated in pig tonsils was reported in Finland in 2007 (8). The reasons for such diverse data are probably due to thedifferent matrices and methods used. The results of this study are comparable to the results of Nesbakken et al. (23) from Norway, who found 5.2% positive animals and 25.0% positive herds. In many studies, the rate of contaminated animals and herds is higher (11, 14, 15). In the case of the French study (11) performed in one slaughterhouse by taking 20 swabs per batch, about 19.8% of positive tonsil swabs and 80% of positive pig herds, were reported. A higher number of tested swabs per herd could result in a higher number of positive herds due to the usually low number of infected animals per herd. They found 1 to 10 positive swabs out of 20 which agrees with this study's finding of 1 to 7 positives out of 10. Funk et al. (14) from the USA report that 92.2% lots and 23.9% of pigs, sampled by the swabbing of oral-pharyngeal surface, were infected with *Y. enterocolitica* and, 28.2% of lts and 13.2% of pigs with the pathogenic ones. In this study, high numbers of animals per lot were sampled (9 to 97 swabs at lots sized from 9 to 200 animals). They also found a low number (1 to 11) of animal carriers of pathogenic *Y. enterocolitica* per lot. Authors from Germany report that *Y. enterocolitica* persisted in tonsils in 38.4% (15). They also concluded, similarly to many of the previous studies, that tonsils are the most reliable tissue for *Y. enterocolitica* detection in slaughtered pigs (15, 20, 24) and that the relative difference of finding them in the tonsils and faeces is 6 to 1 (23). Due to this finding, sampling of tonsils by swabbing was chosen for this study. Protocols for enrichment procedures that were performed (procedures 2 and 3) are part of the International Standard Organization method (ISO 10273: 2003). In addition, direct plating on CIN (method 1) was implemented, due to the reports of good recovery rate (26), the ease of performing and a relatively fast result.

None of the herds were positive by only using procedure 2. With respect to the results of this study, and in agreement with Belgian findings (26), it can be concluded that the alkaline (KOH) treatment of enriched PSB cultures had a positive impact on *Y. enterocolitica* isolation. In this study, the procedure using the alkaline (KOH) treatment of enriched PSB was the most efficient procedure, with successful isolation at 74.4% of positive herds. *Y. enterocolitica* is able to resist weak alkaline treatment (3), and this property is used to select the organism while suppressing background flora. Successful isolation out of the treated enrichment broth (protocol 3) confirms the presence of *Y. enterocolitica* viable cells. Massive additional microflora which covered the target cells could be the reason for inability for isolation out of the enrichment broth without treatment (protocol 2).

In 9 out of 43 of the confirmed positive herds *Y. enterocolitica* were isolated only by applying direct plating (protocol 1). There is a theoretical possibility that all *Y. enterocolitica* cells were removed to the agar plates when direct plating or that the background microflora overgrew them during the following enrichment. By using both procedures in parallel both possibilities are overcome.

Other authors also indicate that the recovery rate at incubation in PSB for 5 days (procedures 2 and 3 of this study), is lower compared to the 2-day enrichment procedure, in particular as far as the method without KOH treatment (26) is concerned. Due to this finding, the 5-day incubation could be a minor reason for the relatively low isolation rate in this study.

Many surveys were performed using the PCR method and they reported a higher sensitivity of PCR compared with culture methods and in general they report a higher estimated prevalence (4, 12, 17, 21, 25).

The most frequently isolated serotype in the study was serotype 4 (O:3) which is the serotype most often recovered from pigs in Europe, Japan and Canada (2, 15, 18, 20, 23, 28) and the predominant serotype implicated in human illness (7, 18).

However, the study highlights the need for further development and improvement of the methods used for detection of pathogenic *Y. enterocolitica.* Among the procedures employed, a combination of the direct plating method (method 1) and enrichment using KOH treatment covered all positive isolations.

The number of animals sampled per herd could impact the estimation of herd contamination. It was confirmed that *Y. enterocolitica* is present in Slovenian pig herds, but an additional survey for its final estimation employing improved culture methods or PCR should be carried out.

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UGOTAVLJANJE PRISOTNOSTI BAKTERIJE *Yersinia enterocolitica* V TONZILAH KLAVNIH PRAŠIČEV V SLOVENIJI, Z UPORABO TREH POSTOPKOV IZOLACIJE

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Povzetek: Z raziskavo smo želeli pridobiti prve podatke o kontaminiranosti klavnih prašičev v Sloveniji z bakterijo *Yersinia enterocolitica*. Uporabili smo tri različne postopke izolacije ter njihovo uspešnost med seboj primerjali. V obdobju od januarja do maja 2008 je bilo v slovenskih klavnicah odvzetih 1490 brisov tonzil prašičev, ki so pripadali 149 klavnim serijam. Iz posamezne klavne serije je bilo vzorčenih po 10 živali. Postopki, ki smo jih uporabili za izolacijo *Yersinia enterocolitica*, so naslednji: 1. Vsebino brisa smo nanesli neposredno na gojišče agar Cefsulodin-Irgasan-Novobiocin (CIN); 2. Vsebino brisa smo inkubirali v PSB broth (PSB) Yersinia in jo nato po obogatitvi precepili na agar CIN; 3. Vsebini smo po obogatitvi v PSB dodali kalijev hidroksid (KOH) in jo nato precepili na agar CIN. V 43 (29,3 %) klavnih serijah smo bakterijo *Y. enterocolitica* izolirali iz brisa tonzil vsaj ene od 10 testiranih živali z vsaj enim od uporabljenih postopkov. Izolacija je bila največkrat uspešna če smo uporabili tretiranje s KOH (74,4 %). S kombinacijo neposrednega nasajanja na gojišče CIN in z uporabo obogatitve s tretiranjem s KOH, je bila izolacija uspešna pri vseh ugotovljeno pozitivnih serijah. Izolirani sevi bakterije *Y. enterocolitica* so bili biotipizirani in serotipizirani. Večina izoliranih sevov (80,4 %) je pripadala biotipu 4, serotipu O:3.

Ključne besede: *Yersinia enterocolitica*; klavni prašiči; tonzile; izolacija

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Original Scientific Article

DORAMECTIN DEGRADATION AND BACTERIAL COMMUNITY DURING SHEEP MANURE COMPOSTING

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Summary: In the present study doramectin degradation and bacterial community was investigated in sheep manure in experimental bioreactors during the most bioactive-thermophile phase of composting. Samples were artificially fortified with doramectin and composted for 21 days. On average, doramectin analysed by chromatographic determination (HPLC) was significantly reduced in all samples (K1-K3) by 31.8-41.8% during the 21 days of composting. The correlation between declination of doramectin and reduction of moisture in compost mixture samples (K1–K3) was also observed. It was assumed that the mean doramectin concentrations in samples were reduced in accordance with the decrease in water content. For this reason a parallel laboratory test was performed which resulted in lower mean values of doramectin in samples with lower contents of water. Doramectin indicated the influence on viable bacteria numerosity which was highest in the samples without doramectin compared to the samples fortified with doramectin. PCR amplification and phylogenetic analysis (T-RFLP) revealed the presence of the phyla *Fibrobacteres* and *Bacteroidetes*, while *MspI* restriction analysis indicated the potential presence of the genus *Pseudomonas* and *Rhodococcus* as potential decomposers of doramectin. This study indicated the effectiveness of biophysical factors on degradation of doramectin and its influence on the abundance of viable bacteria, but not on thediversity of the bacterial community.

Key words: composting; doramectin degradation; bacteria

Introduction

In the environment, biological degradation is the key process in the assimilation and reduction of waste, or hazardous substances (1, 2). Aerobic degradation, e.g. composting, is the fastest and most complete biotechnological process of actively controlled decomposition, often used as an important hygienic measure of organic waste and contaminated soil (3).

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Doramectin is an exceptionally potent endectocide drug with a broad spectrum of activity against nematodes and arthropod parasites in livestock. It belongs to a group of avermectins, a subfamily of macrocyclic lactones. Up to 98% of macrocyclic lactones are excreted through the faeces of medicated animals in a non-metabolised form as parent compounds or active metabolites (4, 5). Limited information is available about their fate and persistence in the environment (6). Their degradability and dissipation in the environment depends strongly on their physicochemical characteristics and environmental conditions (7).

Composting of livestock manures may lead to a reduction in veterinary drug residue levels before land application (8, 9, 10, 11). However, little is known about the reciprocal effect of pharmaceuticals on the composting process. As far as is known by the authors, the only such data available is for some veterinary antimicrobials, demonstrating their possible role in the inhibition of composting (12).

Due to the frequency of doramectin use, its high ecotoxicity (13) and its highly probable appearance as a parent compound in compost, this study was aimed at the examination of degradation of doramectin in relation to moisture levels, the impact of doramectin on the presence of different bacterial species, their number, viability, and the extracellular enzymatic activity in the process of composting sheep manure containing different concentrations of doramectin. Hence the test samples were fortified with doramectin before the onset of composting due to its known metabolism involving large excretion of a parent compound via faeces (14). The study was designed to maximally approximate the field situation during composting. However, tests of doramectin degradation were also carried out under laboratory conditions in sterilised compost mixtures to exclude the impact of biological processes and, in additionally moistened compost mixtures, to determine the influence of the compost moisture on doramectin degradation.

Methods

Doramectin inoculation in sheep manure compost mixtures in bioreactors

The experimental composting process in bioreactors was investigated during the most bioactive-thermophilic phase (21 days), using a mixture of sheep barn deep litter and pine bark chips (<30mm) with a moisture content of 60 to 65% (w/w) and a density of 570kg/m^3. To prepare the test samples, the compost mixture was fortified with doramectin, as the commercial drug Dectomax® (Pfizer, France), at a target test concentration of 2.0mg/kg corresponding to a maximum concentration of 2186µg/kg, which was determined in dry sheep faeces after a single subcutaneous administration of 0.2mg/kg b.w. of doramectin/kg b.w. (5). Two other investigated concentrations of doramectin in the tested compost mixtures were one half of, and double, the target test concentration. Therefore after homogenization, the final concentrations of doramectin in the compost mixtures were approximately 1.0mg/kg (sample K1), 2.0mg/kg (sample K2) and 4.0mg/kg (sample K3). Control compost mixture samples were not supplemented with doramectin (sample K0). The mixtures were homogenized, transferred to test boxes made of stainless steel mesh of dimensions 15 x 15 x 5cm and placed in the central layers of compost material in three 1m^3 polypropylene bioreactors. The bioreactors were insulated with a 6cm thick layer of expanded polystyrene and aerated using forced ventilation with radial fans enabling a positive pressure of 3kPa in compost to ensure effective aeration of the compost by ensuring at least 5% of oxygen in the material. Radial fans delivered air uniformly through the base of the entire compost, under control of oxygen concentration sensors Dräger Multivarn II (Dräger, Lübeck, Germany), and air speed sensors Testo 450 (Testo, Lanzkirch, Germany) certifying that each batch received the same volume of air. Collection of samples during the three composting batches (P1, P2, P3) as three separate experimental composting repetitions under the same conditions with a duration of 21 days within the thermophilic phase is presented in Table 1.

Table 1: Sampling of the compost mixtures (samples K0-K3) prepared with sheep manure and processed in composting reactors in three batches (P1, P2, P3) and of sterilised compost mixtures (samples K0-K3) processed in one experimental batch (P1)

	K0 (0.0 mg/kg*)	**K1** (1.0 mg/kg)	**K2** (2.0 mg/kg)	**K3** (4.0 mg/kg)	**Sterilised compost** (1.4 mg/kg)
0/7/14/21	**n=6/6/6/6	n=6/6/6/6	n=6/6/6/6	n=6/6/6/6	n=6/6/6/6

*doramectin addition in mg/kg of dry compost
**n=6 (number of samples of the sheep manure in composting reactors and in sterilised compost mixture samples in three experimental batches = P1(n=2)+P2(n=2)+P3(n=2)

Laboratory analysis of doramectin degradation in sterilised compost mixtures and in compost mixtures irrespective of additional amounts of water

A compost mixture was sterilised by autoclaving for 40 minutes at 200kPa and 121°C. After cooling, the suspension of doramectin was mixed into the sterilised compost mixture (mean value 1.4mg/kg of dry weight). Homogenized mixtures were placed in 250ml containers and incubated at 68°C. The samples were collected within 21 days according to Table 1.

In order to determine the influence of the compost moisture on doramectin degradation, four (4) amounts of water (0.0, 0.375, 0.75 and 1.25ml) were added into four (4×2g) samples of homogenised compost mixture from experimental bioreactors which already contained the moisture of 342g/kg. Thus samples contained 0.34ml/g, 0.53ml/g, 0.71ml/g and 0.96ml/g of water. Samples were incubated at room temperature for 42 hours. The moisture level remained almost constant throughout the investigation of the sterilised compost since the air in the room, where composting was performed, had at least 80% of relative air moisture, likewise, containers were covered with semipermeable coverings during incubation.

Doramectin analysis - extraction, clean-up and derivatization

Homogenized, moist compost samples (2g) were extracted with 25ml of acetonitrile (Merck, Darmstadt, Germany, p.a.) by shaking for 1 min using a vibromix and for 5 min using a horizontal shaker at 400rpm (Vibromix 313 EVT, Tehtnica, Železniki, Slovenia). After centrifugation at 2100×g for 10min (centrifuge Rotixa/RP, Hettich, Germany) the supernatant was filtered through paper with pores < 2µm. 50µl of triethylamine was then added to 15ml of supernatant. The mixture was diluted with deionized water to 50ml volume and cleaned-up using a solid phase extraction (SPE), Bakerbond columns with C_8 sorbent (500mg, 6ml, J.T. Baker, Phillipsburg, NJ, USA), previously conditioned by 10-15ml of acetonitrile and 10ml of a mixture of acetonitrile, water and triethylamine (30:70:0.1, v/v/v). After applying the extract, the columns were washed with 15ml of a mixture of acetonitrile, water and triethylamine (50:50:0.1, v/v/v). Doramectin was eluted with 5ml of acetonitrile and concentrated at 50°C under a stream of nitrogen (evaporator Organomation: N-evap No111, Berlin, MA, USA). Dry extracts were derivatized at room temperature with 100µl N-methylimidazole solution in acetonitrile (1:1, v/v) and 150µl trifluoroacetic anhydride solution in acetonitrile (1:2, v/v). After 30s 750µl of acetonitrile was added to the formed conjugated fluorescent derivative, of which 50µl was injected into the HPLC system.

Chromatographic determination

The HPLC system was a Thermo Separation Product (Thermo Scientific, Waltham, MA, USA) and consisted of the Spectra Systems P2000 pump and an AS300 auto injector. The fluorescence detector was RF-535 from Shimadzu (Nakagyo-ku, Kyoto, Japan). The chromatographic separation was performed at 28°C on the Phenomenex Luna C18 (2) 150 ×4.6 mm (3µm) reversed analytical column with a 4cm pre-column filled with C18 stationary phase (5µm) (Phenomenex, Torrance, CA, USA). The mobile phase was a mixture of ultra HPLC grade acetonitrile, methanol and water (475:475:60, v/v/v), pumped at a flow rate of 1.1 ml/min. Excitation and emission wavelengths were 365 and 470 nm, respectively. The results were evaluated according to the external standard method.

The analytical method was validated. A detection limit of 1.0µg/kg of dry sample, reproducibility of the analytical method (RSD < 15%), linearity in the range between 20 and 500 µg/kg of the moist sample ($r > 0.98$) and the mean recovery of the analytical method > 80% were assured for the analysis. To approach the real values, the measured concentrations were corrected for mean recoveries of the respective series and used as final results.

Bacterial count, PCR amplification and T-RFLP analysis for identification of microbial communitie.

Bacterial cell counts were determined by quantitative estimations of direct counting using fluorescence microscopy. For this purpose LIVE/DEAD® BacLight™ (Invitrogen, USA) dye was used, which selectively stained live and dead bacteria.

Microbial communities were identified by

means of a polymerase chain reaction (PCR) with subsequent restriction analysis (15). Prior to PCR, microbial DNA was extracted using the PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, USA). Fluorescence-labelled eubacterial primers 27F (16) and 1392R (17, 18) were used in PCR, targeting the 16S rRNA gene. PCR amplification was performed using the GeneAmp PCR System 2700 (Applied Biosystems, USA). The obtained PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions and were subjected to *HhaI*, *MspI* and *RsaI* (Promega, USA) restriction. Restriction fragments were cleaned using the QIAquick Nucleotide Removal Kit (Qiagen, Germany). The length of fluorescence-labelled terminal restriction fragments was determined using the ABI PRISM 310 genetic analyser (Applied Biosystems, USA), employing standard size marker 2500 ROX, and the GENEMAPER 2.0 analysis software programme (Applied Biosystems, USA). Results were compared with restriction patterns from the public database using the web based tool MiCA (Microbial Community Analysis, http://mica.ibest.uidaho.edu/trflp.php) (MiCA) (19).

Temperature, moisture, dry matter, pH value, nitrogen and carbon determination

Temperature and air flow in the composted substrate were monitored using PT 100 and hot wire probe (Testo 450, Germany) directly in the compost and analysed using the computer software VisiDAQ™ runtime (Advantech®, Sunnyvale, USA). The moisture content and dry matter of the compost were analysed by drying (for 24 hours at 105°C) and weighing. The content of nitrogen and carbon were determined by a Vario MAX CNS analyzer (Elementar, Hanau, Germany) after incineration of the sample at 900°C. The pH values of a liquid phase of the material were measured by a pH meter (Hanna HI 221, Germany).

Data evaluation

Statistical evaluation of results was carried out by ANOVA, t-test and correlation analysis using the SPSS (Statistical Package for the Social Sciences) 17.0 statistics software (Rainbow Technologies, USA). The Pearson product-moment correlation and linear regressions ABS versus time were accepted for r >0.95, and values of P <0.05 of the slopes were considered statistically significant.

Results

Temperature profile, moisture content, carbon, nitrogen, and pH value characteristics

Temperatures of the compost in bioreactors varied from 24 to 69.3°C. (Table 2). The mean moisture content of the compost was 612g/kg before composting (day 0) and decreased to 461g/kg at the end of composting (day 21) (Table 2).

The ratio of carbon (C) : nitrogen (N) in the compost mixtures decreased significantly (P<0.05) from the start to the end of composting: day 0 = 27:1, day 21 = 21:1. The pH value in the compost mixtures within 21 days of composting ranged from 7.4 to 6.4.

Temperature / moisture levels in the compost samples with additional water were constant for 21 days (21 ± 0.5°C/ 0,34, 0,53, 0,71 and 0,96 ± 0,05 ml/g). The C : N ratio and the pH value were the same as in the compost in bioreactors.

Temperature in the sterilised compost samples was constant for 21 days (68 ± 0.2°C), The mean moisture level in the sterilised compost was 670.9g/kg before composting (day 0) and decreased to 653.0g/kg by the end of composting (day 21). The C : N ratio in the sterilised compost mixtures was the same as in the compost in bioreactors. The pH in the sterilised compost mixtures during 21 days of composting ranged from 6.8 to 7.7.

Degradation of doramectin in compost mixtures

The results of tests showed that the mean concentration of doramectin in all samples (K1-K3) declined significantly (P<0.05) by 31.8-41.8% during 21 days of composting. The mean concentrations of doramectin in samples with initial concentration K1 and K2 declined significantly (P<0.001) with the time of composting, thus from day 0 to day 21 the mean concentration of doramectin declined by 37.0-37.8%. The most pronounced decrease in doramectin (P<0.001) in the samples K1 and K2 was observed between day 0 and day 14 of composting (41.9-42.6%).

Table 2: Temperature profile and moisture content of the compost mixtures (K0-K3) in three experimental batches (P1-P3).

		Temperature (°C)				**Moisture content** (g/kg)			
Batch	n	Lowest	Highest	Average	Standard deviation	day 0	7	14	21
P1	21	24.0	69.3	55.0	12.9	634.9	511.6	545.6	486.8
P2	21	25.7	68.3	56.3	11.5	632.8	466.7	459.1	555.4
P3	21	29.2	65.1	48.9	12.8	572.0	517.6	517.3	342.1

Table 3: Doramectin concentrations in the compost and sterilised compost mixture samples (n) with initial concentrations K1-K3, according to the sampling time (day 0 – day 21)

Doramectin concentration day	n	Lowest	Highest	Average	Standard deviation	Average doramectin reduction from day 0 (%)
		(µg/kg dry matter)				
K1						
0	6	962	1232	1108.3	100.5	/
7	6	777	1202	1022.3	161.1	7.7
14	6	606	714	643.2	41.2	41.9
21	6	595	845	698.7	90.4	30.8
K2						
0	6	1709	2134	1878	152.3	/
7	6	1147	1969	1470	272.6	21.8
14	6	936	1352	1078	171.6	42.6
21	6	1014	1330	1167	127.1	37.8
K3						
0	6	3896	6056	4625	998	/
7	6	2772	3146	2980	154	35.5
14	6	2245	3444	2976	486	35.6
21	6	1112	3584	2688	1102	41.8
Sterilised compost mixture						
0	6	906	1986	1428	/	/
7	6	748	1932	1316	/	7.8
14	6	660	1667	1312	/	8.2
21	6	697	1863	1398	/	2.1

The highest decrease of doramectin (41.8%) was observed in the compost samples with initial concentration K3 but this reduction was insignificant. Nevertheless, doramectin decrease in samples K3 was significant (P<0.05) (35.6%) from day 0 to day 14 of composting (Table 3).

Degradation of doramectin in sterilised compost mixtures

The degradation of doramectin in sterilised compost mixtures was 2.1% and within the accuracy of the respective analytical method.

Degradation of doramectin in relation to compost moisture

Declination of doramectin and reduction of moisture in compost mixture samples (K1 – K3) during the composting (0 – 21 day) were in significant correlation (P<0.001; r=0.512), thus higher concentrations of doramectin were found in compost with higher concentrations of water. The same was asceratained in the laboratory test since mean values of doramectin were significantly (P<0.05) lower in samples with the addition of water.

Number of viable bacteria in compost mixture samples

Irrespective of doramectin concentration (K1-K3) in all groups of samples, the mean count of viable bacteria was lowest prior to composting (day 0) and highest on day 7 of composting. The difference was significant (P<0.05). The number of viable bacteria gradually decreased in the period between days 7 and 21. The mean total count (n = 7.65 x 10^8) of viable bacteria in the samples without doramectin (K0) was higher in comparison to the samples with doramectin (K1-K3) and steadily grew from the day 0 to day 21 (Fig. 1).

Phylogenetic analysis of bacterial community in compost mixture samples

Most taxonomic groups of bacteria in the samples (K0-K3) belonged to the phyla *Fibrobacteres.* Other bacteria belonged mostly to the rumen bacteria from the phylum *Bacteriodete*s and its genera *Porphyromonas, Tannerella, Prevotella, Cytophaga, Bacteroides, Alistipes, Microscilla* and *Rikenella* and to the unidentified bacteria as presented in Figure. 2.

Regarding the T-RFLP analysis among all thedoramectin supplements (K0-K3), 2.7 to 11.6 times higher number of molecular fragments were found using the *MspI* enzyme compared to the number of fragments acquired by the enzyme *HhaI*, and 53.6 to 128.8 times higher than that acquired by the enzyme *RsaI*. Identification of fragments using the enzyme *MspI* indicates the presence of numerous potential bacterial taxa belonging to the phylum *Proteobacteria,* genus *Pseudomonas*, phylum *Actinobacteria,* genus *Rhodococcus* and to the phylum *Firmicuites,* genus *Streptococcus, Enterococcus* and *Lactobacillus.* The potential presence of bacteria belonging to the phylum *Spirochaees* was also determined.

Discussion

The results of the experiment indicate a gradual reduction of doramectin in thermophilic phase of composting and suggest an impact on the number of live bacteria, but not on their taxonomic diversity. Namely the average concentration of doramectin in all the samples (K1-K3) significantly (P < 0.05) decreased during 21 days of composting (mainly between days 7 and 14) meanwhile doramectin reduction did not differ significantly among samples K1–K3. Doramectin degradation was obviously associated with the biological processes of composting, since the test results of doramectin degradation in sterilised compost mixtures demonstrated that in the absence of viable bacteria the degradation of doramectin was

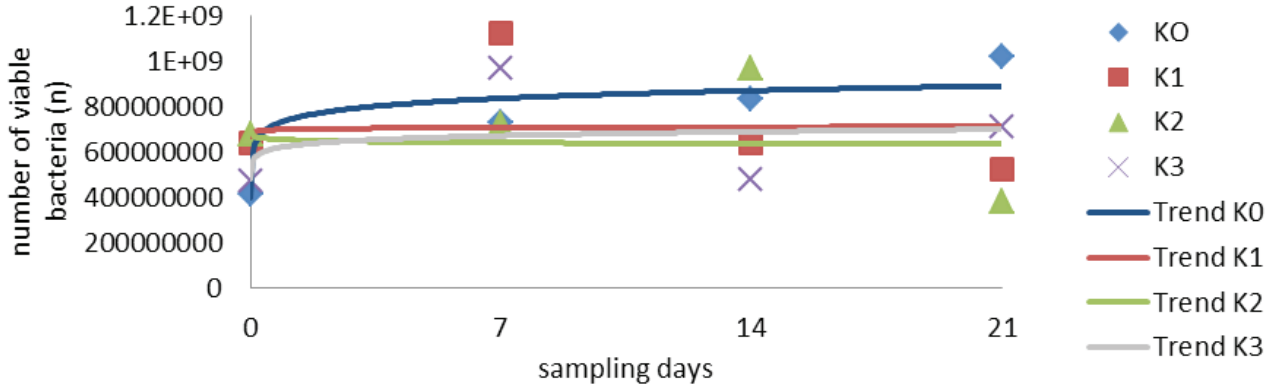

Figure 1: Count of viable bacteria (n) in the compost mixture samples depending on doramectin concentration (K0-K3)

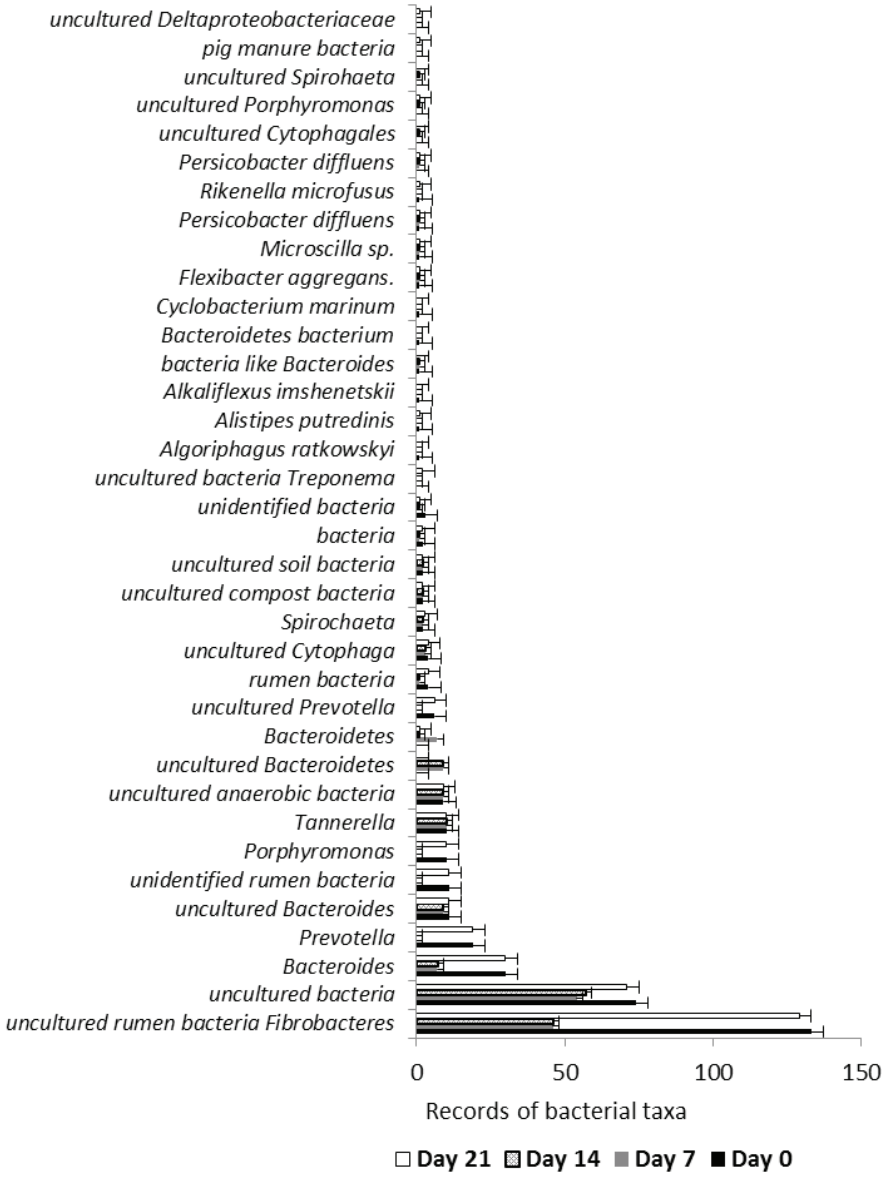

Figure 2: Records of bacterial taxa (taxonomic units) in the compost mixture samples according to the time of composting

very low. The highest counts of viable bacteria in bioreactors were observed in the samples without doramectin (K0) compared to the samples with doramectin (K1-K3). Namely the viable bacterial population did not alter significantly in the samples with doramectin addition (K1-K3) while the number of viable bacteria in the samples without addition of doramectin (K0) slightly increased with the time of the composting process (day 0 to the 21st day).

Beside biological degradation of doramectin it can be hypothetically concluded that declination of doramectin was related to the moisture content of the compost. It is assumed that the reason for this relationship is based on the physicochemical properties of the compost, even though doramectin has low water solubility and low vapour pressure (20). Therefore it was assumed that the mean doramectin concentrations were reduced in accordance with decreasing water content in the compost. This hypothesis was confirmed by significant (P<0.05) correlation between doramectin content and moisture content in the samples K3 (r=0.969) K1 and K2, although in insignificant positive correlation (r>0.80). This was also proved in the laboratory

test in which the mean values of doramectin correlated significantly (P<0.05) with the amount of added water, since doramectin concentrations in the samples with no addition of water were lower by 63-118% than those in the samples to which some water was added. On the other hand, concentrations of doramectin in sterilised compost mixtures remained almost the same where the mean moisture level remained almost constant throughout the investigation. These results are comparable to the previous studies (21, 7) which demonstrated a parallel increase in the concentrations of avermectins and the moisture content in sheep faeces. However, those samples were not treated as in the composting process.

Results of this study showed that among all the taxonomic groups of bacteria determined in test samples of compost mixtures (K0-K3), most of them belonged to the phyla *Fibrobacteres, Bacteroidetes* and to the unidentified bacteria. Very similar results were reported by Green et al. in the research on cattle manure composting, which found that as many as 19 (out of 31) groups belonged to the phylum *Bacteroidetes*. However, regarding the potential degradation of the environmental pollutants, bacteria from genera *Pseudomonas* and *Rhodococcus* were described as extremely important decomposers (3). The experiment showed that the highest number of molecular fragments were found using the enzyme *MspI* indicating the potential presence of bacterial taxa belonging to the phylum *Actinobacteria,* genus *Rhodococcus*. This is an important indicator to the assumption of biological degradation of doramectin in the experiment. For this reason, reliable future research is essential to confirm the presence of bacteria from the genus *Rhodococcus* in sheep manure composts.

To conclude in this study the degradation of doramectin in relation to biophysical composting factors was demonstrated, and reciprocally the impact of doramectin on the count of viable bacteria was proved. In addition, the correlation between doramectin degradation and compost moisture was proved. Due to the complexity of the composting process, a multi-variable analysis should be performed in the future to elucidate the mutual impacts of avermectin drugs and composting processes. Moreover, the development of specific micro-organisms for avermectin decomposition in the composting process should be examined in the future.

Acknowledgements

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DEGRADACIJA DORAMEKTINA IN BAKTERIJSKE ZDRUŽBE MED KOMPOSTIRANJEM OVČJEGA GNOJA

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Povzetek: Raziskana je bila razgradnja doramektina in bakterijskih združb v ovčjem gnoju med najbolj bioaktivno-termofilno fazo kompostiranja v poskusnih bioreaktorjih. V vzorce komposta, ki smo ga kompostirali 21 dni,so bile dodane različne koncentracije doramektina. V povprečju so se koncentracije doramektina v vseh vzorcih (K1-K3), analiziranega po postopkih kromatografske analize (HPLC), s časom kompostiranja (21 dni) znižale za 31,8 - 41,8 %. Ugotovljena je bila značilna korelacija (P<0,001; r=0,512) med koncentracijo doramektina in znižanjem vlage v vzorcih (K1-K3). Domnevamo, da se povprečne koncentracije doramektina v vzorcih znižujejo skladno z zniževanjem vsebnosti vode. Iz tega razloga je bil vzporedno izveden laboratorijski preskus, v katerem so bile ugotovljene značilno (P<0,05) nižje povprečne vrednosti za doramektin v vzorcih z nižjo vsebnostjo vode. Doramektin je vplival na številčnost bakterij, ki je bila najvišja v vzorcih brez doramektina v primerjavi z vzorci, obogatenimi z doramektinom. PCR in filogenetska analiza (T-RFLP) sta pokazali prisotnost bakterij iz rodov *Fibrobacteres* in *Bacteroidetes*, medtem ko je analiza z restrikcijskim encimom *MspI* pokazala morebitno prisotnost bakterij iz rodu *Rhodococcus*, ki so odgovorne za potencialno razgradnjo doramektina. Študija je pokazala učinkovanje biofizikalnih dejavnikov na degradacijo doramektina in njegov vpliv na številčnost živih bakterij, ne pa tudi na raznolikost bakterijske združbe.

Ključne besede: kompostiranje; degradacija doramektina; bakterije

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Original Scientific Article

GEOGRAPHICAL DISTRIBUTION OF SHEEP AND GOAT BREEDS IN SLOVENIA

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Summary: Geographical distribution of small ruminant breeds kept in Slovenia and included into the National selection program was studied. Analyses of the population size and its structure were made together with the geographical distribution (geographical coordinates of flocks locations upon Gauss-Krueger coordinate system; the values of x and y). For individual flocks the radial distance from the geographic centre of gravity was calculated, and a distribution graph was made where cumulative distribution of animals depending on their distance from the geographic centre of gravity is presented. The calculated geographical centre for the autochthonous breeds is in the area of their origin, while traditional and foreign breeds gravitate towards the central part of Slovenia. It is characteristic of autochthonous breeds that the majority of their population is located within a small radius, compared to foreign and traditional breeds. So, the autochthonous breeds are mostly concentrated just to a smaller geographical area. Three Slovenian autochthonous breeds of sheep (Bela Krajina Pramenka, Istrian Pramenka and Bovec sheep) have 90 % of their population within a distance of less than 25 km, while the Slovenian autochthonous breed of Drežnica goat has 90 % of the total population within a radius of less than 30 km. Traditional and foreign breeds are not confined to only one region or area. Due to the occurrence of natural disasters or sudden outbreak of diseases the scarce Slovenian autochthonous sheep and goat breeds are considered as most endangered population.

Key words: sheep; goats; breeds; geographical distribution; endangered breeds; endemic area

Introduction

In the report entitled "The State of the World´s Animal Genetic Resources for Food and Agriculture" prepared by the International Organisation of the United Nations for Food and Agriculture [1] an urgent need is stressed to improve the overview of each animal breed population (size, endangerment, etc.). However, other important factors such as geographical concentration should also be taken into account [2]. This involves the study of the distribution of individual species, breeds and flocks of farm animals which is particularly important for the "*in situ*" conservation in an environment where the farm animal genetic resources originate from and where they developed their distinctive traits [3].

An assessment of the risk status of livestock breeds or populations is an important element in the planning of animal genetic resources management. The risk status of each breed informs stakeholders whether, and how urgently, actions need to be taken [1]. Gandini et al. [4] define "degree of endangerment" as a measure of the likelihood that, under current circumstances and expectations, the breed will

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become extinct. Accurately estimating degrees of risk is a difficult undertaking and incorporates both demographic and genetic factors. Clearly, current population size is important factor in determining risk status. A small population concentrated in one area is at greater risk of being wiped out by natural disasters, disease, or inappropriate management [1]. An essential aspect of risk is the geographical distribution of flocks because potential threat for farm animal genetic resources is positively associated with high geographical concentration. Carson et al. [5] stresses that in determining the level of risk status of certain breed besides the number of population, the number of flocks within particular breed etc., special attention should be given also to other factors such as geographical concentration of an individual breed. The example of a large impact of the geographical concentration of animals on one area was clearly visible in the UK during the outbreak of foot-and-mouth disease in 2001 [5].

Small numerous autochthonous breeds are often kept in a well-defined (original) geographical area where they were selected to be maximally adapted to the local conditions. However, some breeds can be present in very small areas, while other breeds are more dispersed. The species, which is tied to a specific geographical area, can be considered as endemic [6]. Breed diversity in small ruminants in Slovenia is relatively large. Slovenian autochthonous (Jezersko-Solčava sheep, Bovec sheep, Istrian Pramenka, Bela Krajina Pramenka, Drežnica goat) and traditional (Improved Jezersko-Solčava sheep, Slovenian Saanen goat and Slovenian Alpine goat) breeds of sheep and goats and the foreign Boer goat are included into the National selection program according to the breeding program of each individual breed. The goal of the breeding program are selected purebred females and males that are suited to meet the individual needs and requirements of the sheep and goat breeders. This goal also helps in the recovery of endangered species by preserving the existing gene pool and prevents inbreeding especially in endangered breeds.

The aim of this work was to evaluate the potential threat for the spread of infection in purebred flocks due to geographical distribution in case the infection would arise in the vicinity. Based on these results the conservation policy could be modified or improved by considering the geographical component. The risk of losing an endangered breed due to infectious diseases could therefore be reduced.

Material and methods

Population data of sheep and goat breeds in Slovenia for 2011 were obtained from the database of National selection program for small ruminants. The database includes selection aspects for each individual animal (e.g. identification number, birth date, pedigree information and production recording) and is being updated daily. The proportion of sheep and goat breeds covered by the database of National selection program differs among breeds. For traditional and foreign breeds the number of animals included in the database is less than half of the whole population kept in Slovenia, while the percentage of animals by autochthonous breeds included in the database is as follows: Bovec sheep = 76.4 %; Istrian Pramenka = 83.74 %; Bela Krajina Pramenka = 88.86 %; Drežnica Goat = 83.12 % and Jezersko-Solčava sheep – the only non-endangered autochthonous breed = 29.22 %. All purebred males of individual breeds are used as breeding males. We analysed the population size and structure together with the geographical distribution (geographical coordinates of flocks according to the Gauss-Krueger coordinate system, values x and y). Gauss-Krueger coordinates system is commonly used in the Republic of Slovenia (http://www.spatialreference.org/ref/sr-org/7011/), where meter is used as a unit for x and y. A potential danger of endemic diseases for a particular breed is best show on the geographical map, where it is clearly seen how individual breed flocks are dispersed or concentrated in a particular geographic area. Data processing and presentation of the results was performed with the software package R [7]. By using coordinates (pairs of values x and y) we indicated the locations as well as flock size on the map of the Republic of Slovenia. Flock size is represented by a point size [8]. Due to the proximity of certain flocks we sketched a partially transparent point. In the case of multiple overlapping of nearby points it is plotted as a darker colour. Like Carson *et al.* [5] we calculated the geographical centre of gravity for flock locations and marked it on the map. The centre of gravity was calculated as the weighted median of coordinate values: X_T = median (x, w) and Y_T = median (y, w), where x and y are vectors of coordinates of individual flocks, while w is the vector of relative weights calculated in relation to the size of an individual flock (population number of a particular breed per farm). Compared to Carson *et*

al. [5] we used the median instead of the mean. It is clearly seen that a single strong isolated point can greatly affect the location of the centre of gravity, which is determined by the arithmetic mean. The median is insensitive to an individual remote flock and therefore more suitable for the evaluation of the centre of gravity, mostly for concentrated flocks. For individual flocks the distance from the centre of gravity was calculated, and a distribution graph was made where cumulative proportion (distribution) of animals depending on their distance from the centre is shown. Apart from the points on the map which represent location and flock size, there are also circles drown to show the distance from the calculated geographic centre of gravity, where 50, 90 and 95 % of all animals included into the National selection program for the individual breed are located.

Results

Geographical distribution of flocks and flock size for individual breeds of sheep and goats kept in Slovenia is presented in Figures 1-4. Individual flocks are presented by circle, while the calculated geographic centre of gravity is marked by an x. From the images it is clear that the geographical distribution differs among breeds.

Bela Krajina Pramenka

In 2011, there were 41 purebred males, 652 purebred females and 115 purebred young females included in the National selection program. Bela Krajina Pramenka is characterized by the concentration of a large part (95 %) of the population at a distance calculated from the geographic centre of gravity of less than 30 km while 90 % of the population is located within the radius of ~ 18 km (Figures 1a and 2a).

Jezersko-Solčava sheep

Jezersko-Solčava sheep is the only non-endangered Slovenian autochthonous small ruminant breed. In 2011, there were 126 flocks of 5202 purebred animals included in the National selection program which means 203 purebred males, 4247 purebred females and 752 purebred young females. The average flock size was relatively small, amounting only to 36.6 animals. Jezersko-Solčava sheep is otherwise scattered throughout the entire territory of Slovenia. However, most of the flocks are in the area of two Slovenian statistical regions (Gorenjska and Savinjska). The breed is therefore in its native region, as well as outside and it is not geographically confined to only one statistical region or area (Figure 1b). A relatively uniform distribution of flocks depending on the distance from the calculated geographic centre of gravity is shown in Figure 2b. It is necessary to take into account the fact that the percentage of animals included into the National selection program according to the number of animals in the entire Jezersko-Solčava population is different in comparison to other Slovenian autochthonous breeds of sheep and goats. In less numerous breeds there are more than 80 % of the population included into the National selection program, while less than 30 % belong to Jezersko-Solčava sheep.

Bovec sheep

In 2011, there were 148 purebred males, 1886 purebred females and 640 purebred young females included in the National selection program. The average flock size was 64.6 animals. There are only a few flocks of bigger size. Most farmers keep a small number of animals, therefore the median for the flock size is 36.0 animals.

Istrian Pramenka

In 2011 there were 43 purebred males, 650 purebred females and 270 purebred young females included in the National selection program. The average flock size was 151 animals. Figure 1d shows the small size of Istrian Pramenka population and a local distribution in the Karst and Istria region. A little less than a half of the total population of Istrian Pramenka were bred in a single flock. For this reason a median for flock size is only 41.5 animals.

Improved Jezersko-Solčava sheep

Improved Jezersko-Solčava sheep is the most numerous sheep breed in Slovenia. In 2011, there were 151 purebred males, 4052 purebred females and 670 purebred young females included into the National selection program. Improved Jezersko-Solčava sheep is the most geographically dispersed breed in Slovenia (Figure 1e).

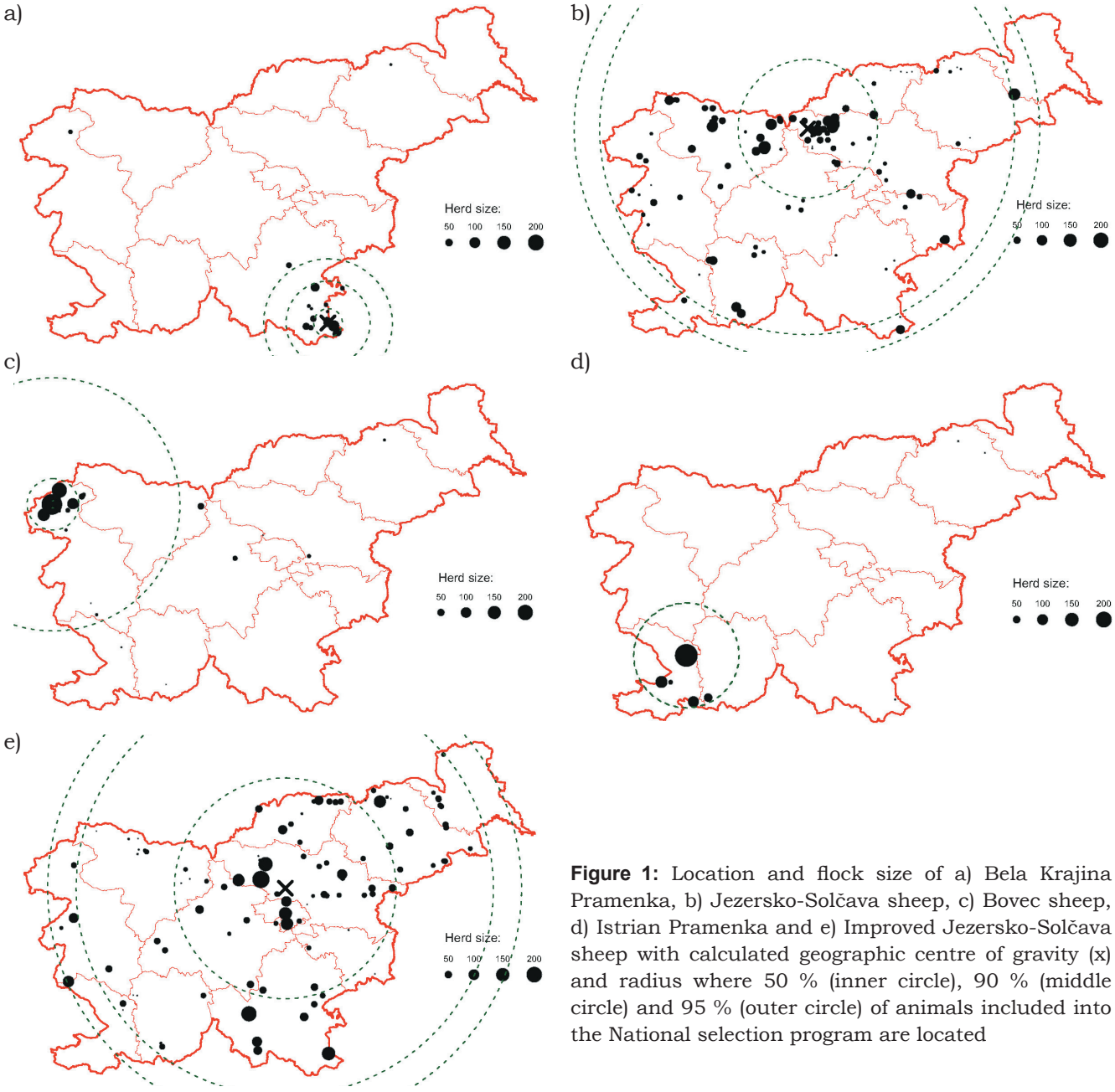

Figure 1: Location and flock size of a) Bela Krajina Pramenka, b) Jezersko-Solčava sheep, c) Bovec sheep, d) Istrian Pramenka and e) Improved Jezersko-Solčava sheep with calculated geographic centre of gravity (x) and radius where 50 % (inner circle), 90 % (middle circle) and 95 % (outer circle) of animals included into the National selection program are located

Table 1: Flock dispersion of sheep breeds from the calculated geographic centre of gravity

Breed	Purebred females (n) in NSP* in 2011	Distance (km) from the calculated geographic centre of gravity			Calculated geographic centre of gravity by Gauss-Krueger coordinate system	
		Percentage of animals (%)				
		50	90	95		
Bela Krajina Pramenka	767	~ 6	~18	~ 28	x=523488, y=41375	Mala sela close to Adlešiči
Jezersko-Solčava sheep	4999	~ 35	~ 95	~ 103	x=135715, y=477431	Zg. savinjska dolina and Luče
Bovec sheep	2526	~ 1	~ 12	~ 55	x=133610, y=389289	Bovec
Istrian Pramenka	920	0	~ 25	~ 100	x=62814, y=423325	Gabrče close to Divača
Improved Jezersko-Solčava sheep	4722	~ 49	~90	~105	x=121970, y=502424	Gomilsko

*National selection program

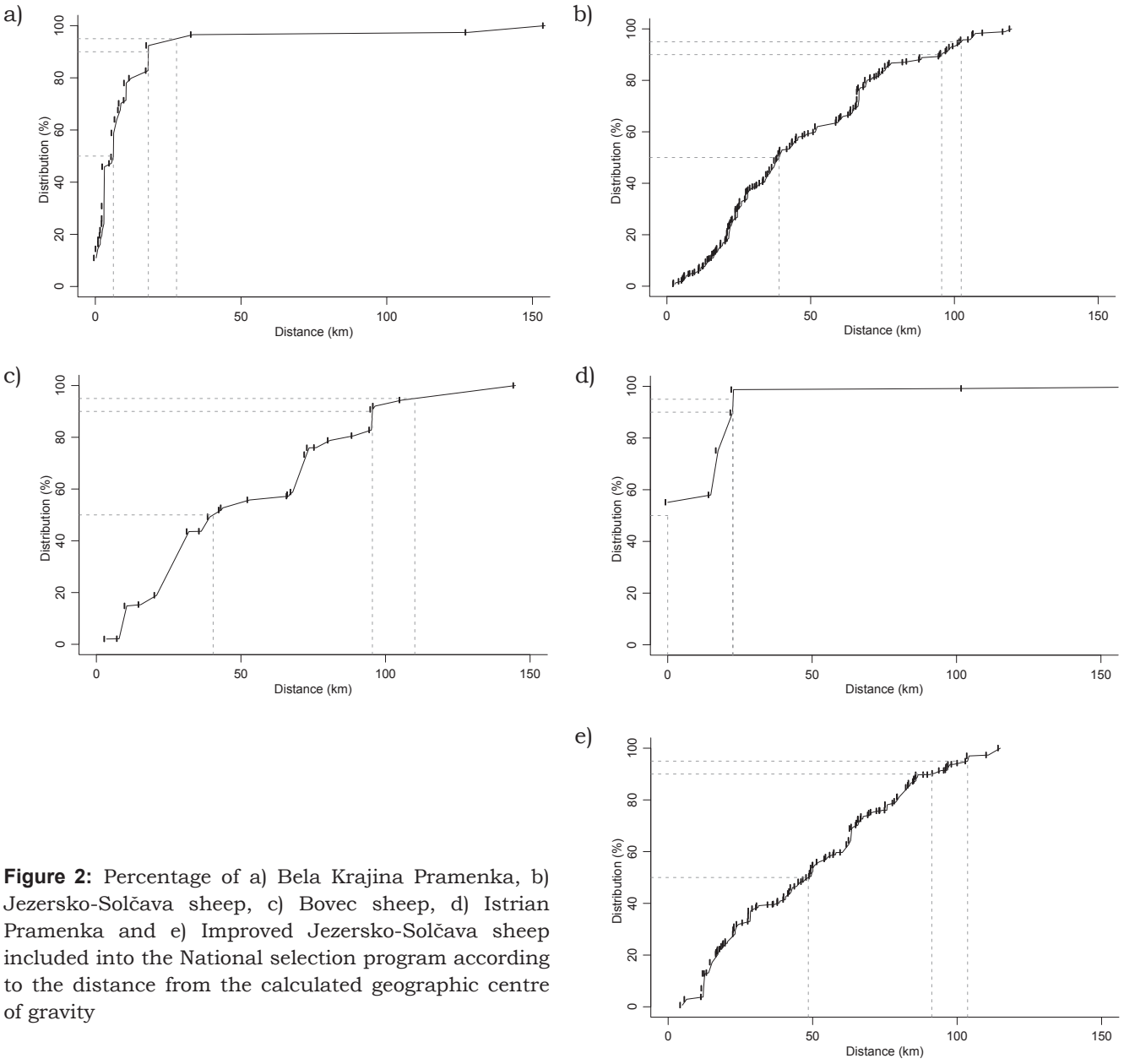

Figure 2: Percentage of a) Bela Krajina Pramenka, b) Jezersko-Solčava sheep, c) Bovec sheep, d) Istrian Pramenka and e) Improved Jezersko-Solčava sheep included into the National selection program according to the distance from the calculated geographic centre of gravity

Based on the results for individual sheep breeds the distance (km) from the calculated geographic centre of gravity and the calculated geographic centre following Gauss-Krueger coordinate system is presented in Table 1.

Differences in flock dispersion among various sheep breeds occur mainly due to the group of breeds (autochthonous, traditional) (Table 1). Thus, the Slovenian autochthonous sheep breeds are mostly concentrated in their own calculated geographic centre of gravity. At the same time these centres are also their area of origin according to the historical sources on development of the individual breed. For example, in Istrian Pramenka a half of the population is located in its own geographic centre of gravity, while a larger share of animals is located within a distance of about 25 km. The most numerous Slovenian autochthonous sheep breed (Jezersko-Solčava sheep) has a half of its population in a distance of about 35 km from the calculated geographic centre of gravity, whereas practically all animals are located within a distance of about 103 km from the calculated geographic centre of gravity. Traditional sheep breed (Improved Jezersko-Solčava sheep) is fairly evenly dispersed throughout the territory of Slovenia.

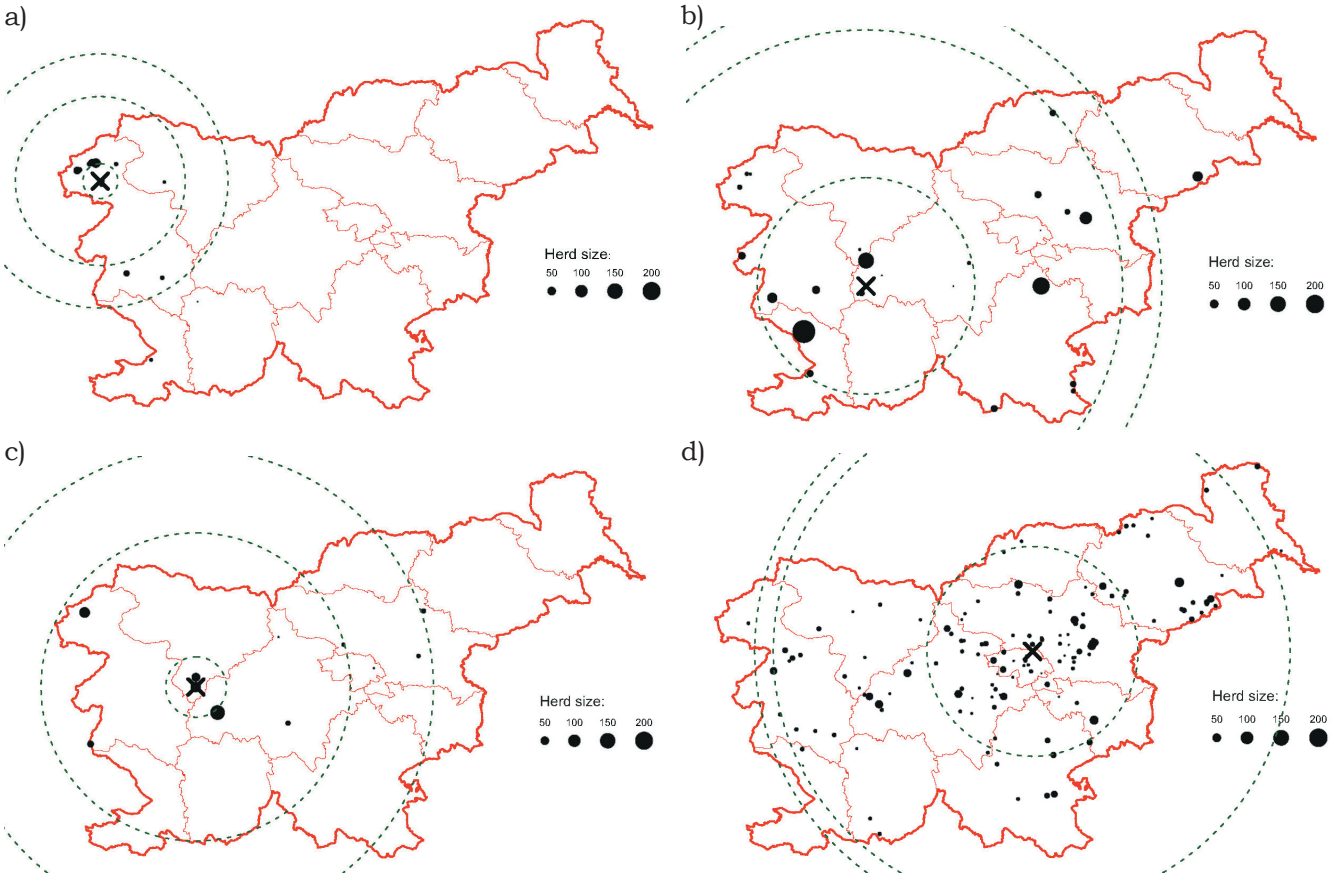

Figure 3: Location and flock size of a) Drežnica goat, b) Slovenian Alpine goat, c) Slovenian Saanen goat and d) Boer goat with calculated geographic centre of gravity (x) and radius where 50 % (inner circle), 90 % (middle circle) and 95 % (outer circle) of animals included in the National selection program are located

Drežnica goat

In 2011, there were 39 purebred males, 355 purebred females and 105 purebred young females included in the National selection program. Almost all flocks of Drežnica goat breed are located in the area of breed origin (Figure 3a). Within a distance less than 30 km there are 90 % of all population of Drežnica breed included in the National selection program (Figure 4a).

Slovenian Alpine goat

In 2011, there were 1241 animals; 37 purebred males, 976 purebred females and 228 purebred young females included in the National selection program. Based on the distance from the calculated geographic centre of gravity, flocks of Slovenian Alpine goat breed have a relatively even distribution throughout the country (Figures 3b and 4b).

Slovenian Saanen goat

In 2011, there were 526 animals; 26 purebred males, 324 purebred females and 176 young purebred females included in the National selection program. Slovenian Saanen goat breed is small in number, therefore it is difficult to speak of greater or lesser dispersion. Depending on the distance from the focus the flocks of Slovenian Saanen goat breed are relatively evenly distributed (Figures 3c and 4c).

Boer goat

Boer goat is the most numerous goat breed in Slovenia and it is spread throughout the country (Figures 3d and 4d). In 2011, there were 297 purebred males, 1465 purebred females and 640 young purebred females included in the National selection program.

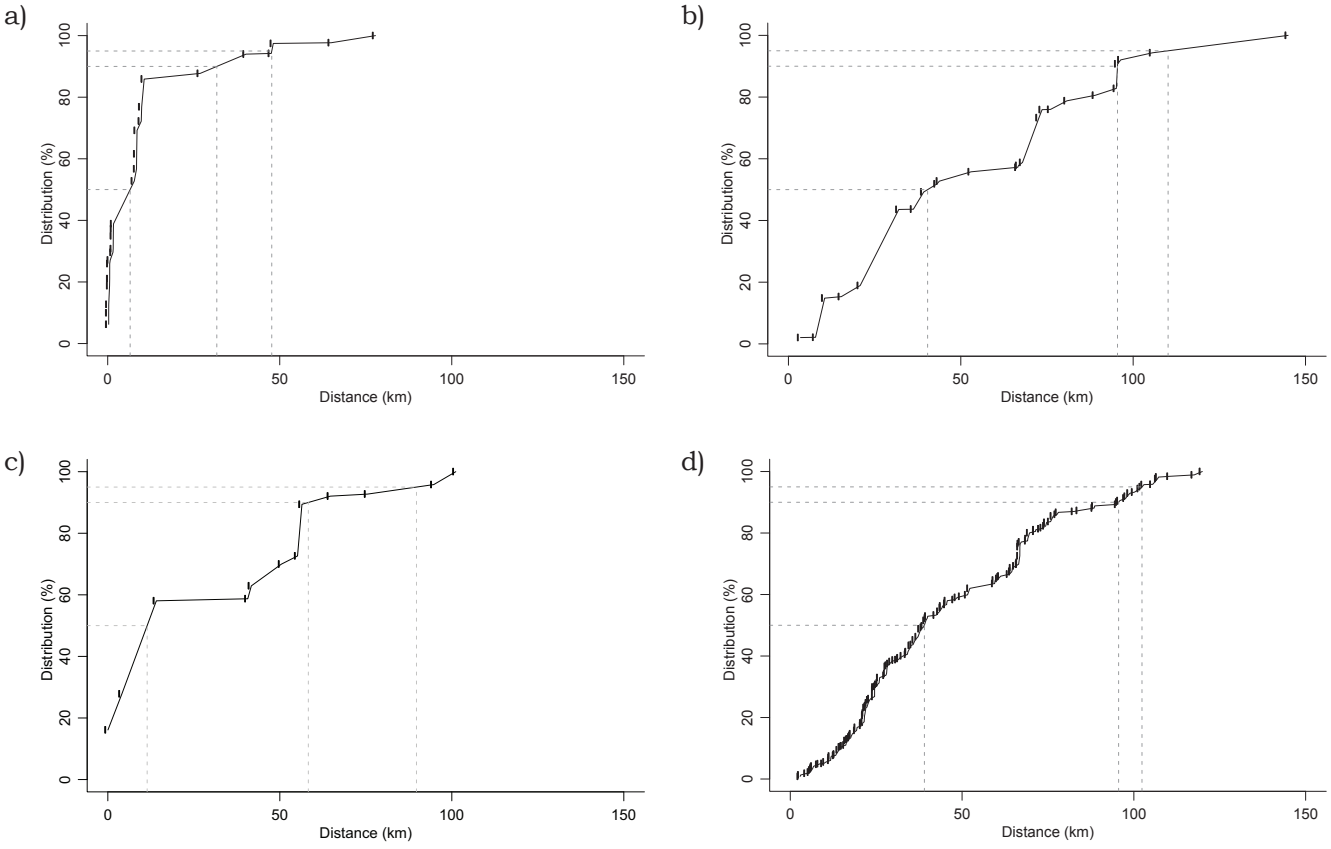

Figure 4: Percentage of a) Drežnica goat, b) Slovenian Alpine goat, c) Slovenian Saanen goat and d) Boer goat according to the distance from the calculated geographic centre of gravity

Table 2: Flock dispersion of goat breeds from the calculated geographic centre of gravity

Breed	Purebred females (n) in NSP* in 2011	Distance (km) from the calculated geographic centre of gravity			Calculated geographic centre of gravity by Gauss-Krueger coordinate system	
		Percentage of animals (%)				
		50	90	95		
Drežnica goat	460	~ 5	~ 29	~ 48	x = 126070, y = 392751	Drežniške Ravne
Slovenian Alpine goat	1204	~ 38	~ 96	~ 110	x = 87495, y = 437278	Žibrše close to Logatec
Slovenian Saanen goat	500	~ 10	~ 60	~ 90	x = 102600, y = 434683	Žirovski vrh
Boer goat	2105	~38	~ 95	~ 102	x = 115563, y = 505911	Marija Reka close to Trbovlje

*National selection program

The smallest distance from the calculated geographic centre of gravity has Slovenian autochthonous Drežnica goat breed (Table 2). A half of the entire population of this breed included in the National selection program is located within a distance of about 5 km. The calculated geographic centre of gravity of Drežnica goat is the area of its origin. Other goat breeds included in the analysis have a similar distance from the geographic centre of gravity calculated for 50, 90 and 95 % of the animals. Their calculated geographical focus gravitates away from the central part of Slovenia.

Discussion

Slovenian autochthonous breeds of sheep and goats are mostly concentrated in the area of their origin. The small numerous breeds of sheep such as Istrian Pramenka, Bela Krajina Pramenka and also Bovec sheep have 90 % of their population within a distance less than 25 km. Drežnica goat, the only Slovenian autochthonous goat breed has 90 % of their population within a distance less than 30 km. The calculated geographic centre reflects a single rearing centre of all Slovenian autochthonous sheep and goat breeds. In more dispersed traditional breeds like Improved Jezersko – Solčava sheep, Slovenian Saanen goat, Slovenian Alpine goat and the foreign Boer goat the calculated geographic centre is not a single rearing centre. Istrian Pramenka sheep is characterized by a small geographical dispersion (Figure 1d) and it is small in flock number (Figure 2d). In the event of any natural disaster or outbreak of infectious and communicable diseases this breed could suffer serious consequences, so we must emphasise the risk in this respect. It may be noted that due to the high concentration of flocks in smaller geographical areas in Slovenia a small population number is particularly vulnerable and dangerous for autochthonous breeds with the exception of Jezersko-Solčava sheep. It has its calculated geographic centre range in the area of its origin, but as a relatively productive meat type it is extended to other parts of Slovenia. In case of transfer of infectious diseases within a narrow range, the concentration of animals of certain species within the area is also important because in high concentrations animal diseases spread quickly. Geographical concentration of breeds indicates that the animals are very well adapted to their specific environment and to the traditional uses and farming management, as well as the attachment of breeders to them. Increased geographical dispersion of breeds to larger area of Slovenia means lesser possibility for spread of infection, while increased geographical concentration of breeds means higher possibility for spread of infectious diseases. The traditional Improved Jezersko – Solčava sheep breed dispersed in a large area of Slovenia and not confined to only one region or area, is therefore not highly at risk. Density of the same and different species located in the specific area is a risk factor for disease transmission, therefore further investigations on density would be necessary.

Acknowledgment

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GEOGRAFIJA RAZŠIRJENOSTI PASEM OVC IN KOZ V SLOVENIJI

M. Žan Lotrič, G. Gorjanc, D. Kompan

Povzetek: Proučevali smo geografijo razširjenosti pasem drobnice v Sloveniji, ki so vključene v Rejski program. Analizirali smo velikost populacije, njeno strukturo in geografijo razširjenosti (geografske koordinate rej po Gauss-Kruegerjevem sistemu; vrednosti x in y). Za vsako rejo smo izračunali zračno razdaljo oddaljenosti od geografskega težišča ter prikazali porazdelitveno sliko, ki prikazuje delež živali glede na oddaljenost od geografskega težišča pasme. Izračunano geografsko težišče za avtohtone pasme je njihovo izvorno območje, za tradicionalne in tujerodne pasme pa je usmerjeno proti osrednjemu delu Slovenije. Za avtohtone pasme je značilno, da večji del populacije obstaja znotraj manjšega radija, kot je le-ta v primerjavi s tujerodnimi in tradicionalnimi pasmami in da so reje večinoma skoncentrirane v manjšem geografskem območju. Tri slovenske avtohtone pasme ovc (belokranjska pramenka, istrska pramenka in bovška ovca) imajo 90 % populacije znotraj razdalje, ki je manjša od 25 km, slovenska avtohtona pasma koz drežniška koza pa znotraj radija, ki je okoli 30 km. Tradicionalne in tujerodne pasme niso omejene le na eno regijo ali območje. Zaradi ogroženosti in tveganj ob naravnih nesrečah ali nenadnih izbruhih bolezni so najbolj izpostavljene maloštevilne slovenske avtohtone pasme ovc in koz.

Ključne besede: ovce; koze; pasme; geografija razširjenosti; ogroženost; endemično področje

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Original Scientific Article

AN ATTEMPT TO ELIMINATE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS) BY SERUM INOCULATION ON SMALL PIG FARM

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Summary: The great heterogeneity among porcine reproductive and respiratory syndrome virus (PRRSV) isolates is probably the main obstacle to its effective control using current commercial vaccines, since the induced immunity by one strain is specific only to this strain. Exposure of all breeding pigs to the PRRSV circulating on the farm is an option for elimination of PRRS in breeding herd. Adoption of strict biosecurity measures is essential. The objective of this study was to eliminate the PRRS from a farrow-to-finish small pig farm (130 breeding pigs) by serum inoculation. The owner was acquainted with the biosecurity measures (strict biosecurity protocols and herd closure for at least 200 days). Breeding pigs were immunized with serum obtained from weaners. The number of high positive breeding pigs decreased from six months after the II. serum inoculation till the end of the study, but the prevalence of antibody were almost the same comparing the sampling before serum inoculation to last sampling 13 months after the II. serum inoculation. The breeding herd were free of virus during all testing, but PRRSV circulated in the two-month old weaners. The owner did not implement herd closure and other required biosecurity measures and a new strain of PRRSV was introduced. Hence, serum inoculation proved to be unsuccessful for the elimination of PRSS from the farrow-to-finish farm. Implementation of biosecurity measures in field conditions is a much more difficult challenge than what was expected at the beginning.

Key words: control; immunization; pig; PRRS; serum inoculation

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a viral infection endemic in most swine-producing countries and leads to major economic losses (1). The disease is characterized by reproductive failure, including late-term abortions, early farrowing, stillbirths, weak born piglets and increased mortality in neonates, nursery and growing pigs, and respiratory tract illness that can be especially severe in neonatal and nursery-age pigs (2). PRRSV infection is difficult to control due to large heterogeneity among the isolates. A variety of strategies have been described for PRRS elimination, including total depopulation/repopulation, partial depopulation (3), isowean (4) or segregated early weaning (5), test and removal (6), mass vaccination with unidirectional pig flow and herd closure (7). Elimination of a disease is disappearance of all clinical cases of a specific disease (8) which is the consequence of desistance of virus replication and circulation in the population of pigs. No single strategy for elimination will work on infected farms; therefore, the program must be individually designed based

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on the unit's pig flow and facility design as well as serological results (9).

PRRS elimination is a long term goal, and the first step is stabilization of the breeding herd. Herd stability is defined as a herd which lacks clinical signs and in which a virus is not actively circulating and transmitting between pigs (9). Stabilization can be achieved with simultaneous immunization of the breeding herd (10). Immunization can be achieved with commercial vaccines, serum inoculations and natural exposure. It appears that currently available vaccines may not be effective in protecting against infections with genetically different strains of PRRSV (11). Numerous studies have shown some cross-protection against different strains which are reflected only in the reduction of clinical signs and lesions but not in elimination of the virus (12). Moreover, inoculation with a homologous strain provides a high level of protection against the same or nearly the same virus strain (13). It is, however, readily accepted that homologous immunity is more protective than heterologous immunity. In fact serum inoculation is the intentional immunization of pigs with the strain of PRRS virus originating from the same, infected farm (homologous herd strain). This method consists of intramuscular injection of complete breeding herds with serum derived from acutely infected pigs that contain the particular farm-specific PRRSV (14). Shibata et al. (15) showed that, after exposure to a homologous PRRSV strain, pigs subsequently challenged with that strain did not develop clinical signs, and virus replication was reduced in both the titer and the length of infection.

In addition, herd closure is also required to achieve herd stability. In the period of herd closure new pigs cannot be introduced to the farm. This applies also to internal replacements of gilts to the breeding herd (7). The success of PRRS elimination depends on biosecurity practices and cooperative work (9). Consequently, one very important measure is to follow strictly biosecurity protocol, which includes preventing direct routes of spread as well as indirect and miscellaneous routes, as authored by Pitkin et al. in the American Association of Swine Veterinarians Foundation (AASV) website (www.aasv.org/aasv/PRRSV_BiosecurityManual.pdf).

The objective of this study was to eliminate PRRS from a small farrow-to-finish pig farm with herd closure, improved biosecurity and serum inoculation.

Materials and methods

Farm

The study was carried out from June 2010 until March 2012 on one farrow-to-finish farm consisting of four boars and 130 breeding sows. Six months after the second round of serum inoculations, the owner reduced the number of breeding sows to 88 due to the lower price of pigs on the Slovenian market and not due to our request as a measure for the elimination of PRRSV. Semen originated from their four boars. Serum inoculation was performed twice on the farm: the first being after the conformation of PRRS and the second three months after I. serum inoculation.

Herd closure

The introduction of new pigs to the farm was prohibited for 200 days. Also in this period gilts from the farm could not enter the breeding herd.

Biosecurity measures

The owner was acquainted with obligatory measures: strict biosecurity protocols (entering the farm after changing clothes; having personnel aid in the changing of coveralls and boots; the washing of hands; using footbaths; maintaining individual responsibility for each pig category; use of the all in/all out system; one age category of pigs in one room; one way pig flow; the cleaning and disinfection of pens, pig equipment kept on the farm; deratization and disinsection).

Preparation of inoculum for serum inoculation of breeding pigs

The weaners at age 8 to 14 weeks of age were bleeding and tested by RT-PCR. Inoculum was prepared from positive serum samples. The PRRSV positive serum samples were pooled. To one part of each pool four parts of RPMI-1640 medium (Gibco, Germany) were added and mixed with 1% of Antibiotic-Antimycotic (100x), (Invitrogen, Germany). The inoculums contained 10^2 to 10^4 $TCID_{50}$ PRRSV/ml. All breeding pigs were inoculated intramuscularly with 2 ml/pig on the same day.

Samplings procedure

All together 704 blood samples were collected for serology and 456 for molecular testing. The sequencing of PRRSV positive samples were performed 6 times.

Enzyme-linked immunosorbent assay (ELISA)

The HerdChek, IDEXX Laboratories, PRRS X3 ELISA test was used for detecting antibodies in serum samples. The ELISA was performed according to the manufacturer's instructions. Sample results were divided in four groups: samples with S/P less than 0.4 (negative), samples with S/P between 0.4-1 (low positive), samples with S/P between 1 and 2 (positive) and samples with S/P more than 2 (high positive).

Detection of PRRSV with gel-based RT-PCR and direct sequencing of PRRSV positive samples

Total RNA was extracted from 140 µl of serum samples using the QIAamp® viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. 456 samples were tested individually or as pools (maximum 5 samples in pool) by one-step RT-PCR (One-Step RT-PCR Kit, Qiagen, Germany) using sequences based on the open reading frame 7 (ORF7), which detect Type 1 and Type 2 PRRSV strains respectively (16, 7). The PRRS strain VR-2332 (Type 2) and the Lelystad viruses (Type 1) were used as positive controls. Reaction mixtures without RNA served as negative controls. Fifteen PRRSV positive samples were directly sequenced in both directions using the Macrogen sequencing service (Macrogen, South Korea) and the RT-PCR amplification primers. For each sample, 258 nucleotide long sequences were aligned with the published data using BLAST (available at http://www.ncbi.nlm.nih.gov/) at the National Centre for Biotechnology Information (NCBI), and PRRSV sequences obtained were compared using the sequence analysis software Lasergene® (DNASTAR Inc., Madison, WI, USA).

Results

PRRS was confirmed on the farm via testing of 10 animals which showed positive or high positive results in ELISA. Before I. serum inoculation 13 (9.7%) samples were negative, 24 (17.9%) were low positive, 43 (32.1%) were positive and 54 (40.3%)

Table 1: Number of tested sera for serology (ELISA for detection of PRRS antibodies), PRRSV detection (RT-PCR method for PRRSV genome detection) and times of sequencing

Sampling	No. of tested sera by ELISA		No. of tested sera with RT-PCR		Sequencing of PRRSV positive samples	
	Breeding pigs	Pigs	Breeding pigs	Pigs	Breeding pigs	Pigs
Confirmation of PRRS	10	-	-	-	-	-
Before I. serum inoculation	134	-	-	15	-	yes
3 months after I. serum inoculation	134	-	-	15	-	yes
3 months after II. serum inoculation	133	-	133	-	-	-
6 months after II. serum inoculation	88	20	88	20	-	yes
10 months after II. serum inoculation	20	30	20	30	-	yes
13 months after II. serum inoculation	97	13	97	13	-	yes
17 months after II. serum inoculation	-	25	-	25	-	yes

Footnote: In the "Pigs" column, all categories from weaning pigs to fatteners are included

Table 2: Results of PRRSV Detection By RT-PCR and Sequencing of PRRSV

Sampling	Results of RT-PCR		Age of positive pigs (weeks)	Identification number of sequence	Nucleotide identity to 08066t/2010
	Breeding pigs	Pigs			
Before I. serum inoculation	-	positive	10	**08066t/2010** 06088t/2010	100% 100%
3 months after I. serum inoculation	-	positive	8-12	**Meol/2010**	98.4%
3 months after II: serum inoculation	negative	-	-	-	-
6 months after II: serum inoculation	negative	positive	10	2768-81/2011 2768-83/2011 2768-84/2011 2768-86/2011 2768-87/2011 2768-89/2011	99.6% 97.7% 99.6% 98.1% 97.7% 98.1%
10 months after II. serum inoculation	negative	positive	10	Meol15/2011 Meol19/2011	97.3% 96.9%
13 months after II: serum inoculation	negative	positive	10	Meol20/2011 Meol21/2011	99.2% 99.2%
17 months after II. serum inoculation	-	positive	10	0803-1/2012 0803-2/2012	99.2% 99.2%

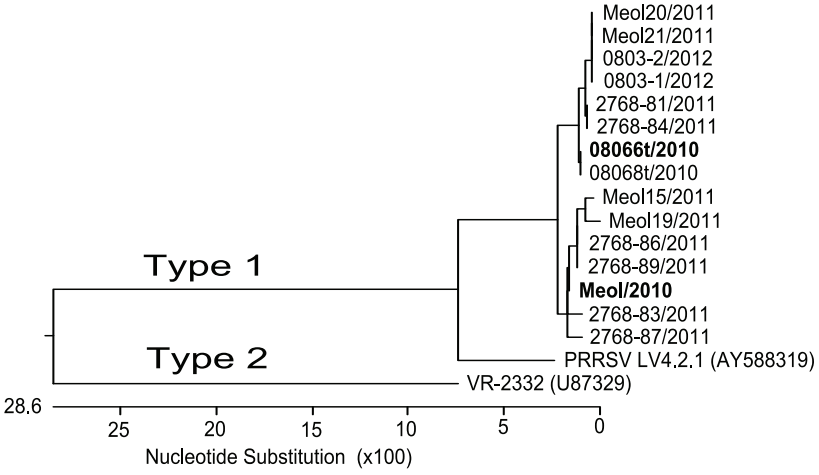

Figure 1: The Phylogenetic Tree of 15 Sequenced PRRSV Samples. The strains 08066t/2012 and Meol/2010 which were used for serum inoculation are presented in bold

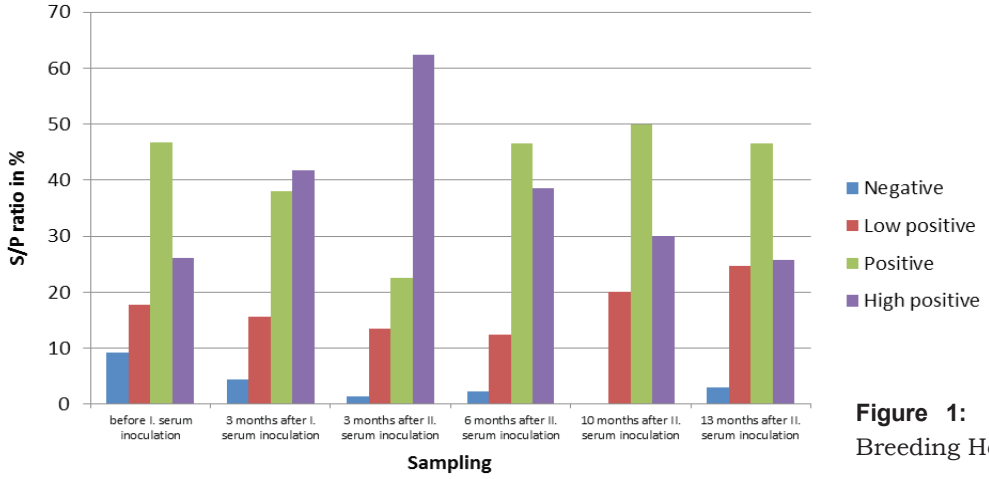

Figure 1: Serological Results of the Breeding Herd

Table 3: Results of PRRSV Detection By RT-PCR and Sequencing of PRRSV

Biosecurity measures	Required	Implemented
Herd closure (introducing replacement gilts into the breeding herd)	yes	no
Herd closure (introducing new pigs to the farm)	yes	yes
Entering the farm after changing clothes	yes	no
One age category of pigs in one room	yes	yes
One way pig flow	yes	yes
Changing of coveralls between pig category	yes	no
Changing of boots between pig category	yes	no
Washing hands between pig category	yes	no
All in/ all out in farrowing and fattening units	yes	no
Individual responsibility for each pig category	yes	no
Cleaning and disinfection of pens	yes	no
Footbath	yes	yes
Pig equipment kept on the farm	yes	no
Deratization and disinsection	yes	yes

were high positive (figure 1). Three months after the I. serum inoculation 6 (4.5%) breeding pigs were still negative and 21 (15.7%) breeding pigs were low positive therefore the second serum inoculation was performed. Three months after II. serum inoculation 2 (1,5%) samples of breeding pigs were negative; 18 (13.5%) were low positive; 30 (22.5%) positive and 83 (62%) high positive, which points to improved immune response of breeding herd. Six months after II. serum inoculation the number of high positive pigs decreased to 35 breeding pigs (39.8%); the number of positive breeding pigs increased to 39 (44.3%); and the percentage of low positive breeding pigs remain at the same level (13.6%) compared to prior sampling. Six months after II. serum inoculation we also checked the status of weaners. Two were negative; three, low positive; seven, positive; and six were high positive which indicate a persistent circulation of the wild type of PRRSV. Samplings at 10 and 13 months after II. serum inoculation still present a trend of decreasing high positive and positive pigs in the breeding herd while the low positive increased. One possible reason is that the breeding herd developed protective immunity; and, in spite of the new introduction of the homologous PRRSV, the titers of antibodies (high positive and positive) decreased. The last sampling—17 months after II. serum inoculations— was performed only in weaners and fatteners to check if PRRS was eliminated from the herd. Results showed the following: 2 negative, 4 low positive, 7 positive and 12 high positive which indicated persistent circulation of the PRRSV.

During the whole period of the study, the breeding herd was negative for PRRSV by the RT-PCR method in all samplings. Sample 08066t/2010 (Fig 1) was used for the I. serum inoculation of the entire breeding herd (134 breeding pigs). A second serum inoculation of the entire breeding herd (134 breeding pigs) was carried out using the detected PRRSV strain Meol/2010. Observed sequence homology between detected strains in herd and topology from phylogenetic tree obtained from fifteen sequenced positive samples in the period of study suggested that the second strain was introduced into the farm between I. serum inoculation and II. serum inoculation.

The owner implemented only 5 of the required biosecurity measures, and the rest of the measures were neglected.

Discussion

To be successful, any PRRS herd elimination strategy must stop replication of the virus within a population of breeding pigs and this will prevent infection of neonates (18). PRRSV circulates in

endemically infected herds because, at any given time, animals are in various stages of infection and immunity (19). Considering the heterogeneity of PRRS serotypes and the importance immunity of homologues, serum inoculation can be a successful measure to eliminate PRRS from the farm (20). With simultaneous serum inoculation of homologues PRRSV strain (strain 08066t/2010) of the breeding herd, we tried to stop the circulation of PRRSV in the breeding herd which would lead to stabilization of breeding herd and which resulted in, as much as possible, uniform S/P ratios (between 1 to 2) and production of negative fatteners. Three months after I. serum inoculation, 4.5% breeding pigs were still negative and 15.7% positive, which was too high for the number of breeding pigs to ensure good protection. We decided to use the II. serum inoculation (strain Meol/2010) in order to ensure the stoppage of shedding the PRRSV. From the beginning of the study we implemented one additional method, herd closure. PRRSV elimination through herd closure is based on the fact that naturally developed immunity eliminates virus infection from the farm (7, 21). With serum inoculation we try to enhance the development of homologous immunity which did not prove as good a protection, according to our serological results. One good candidate for the elimination of the PRRSV is the three-site farm (7); moreover, the success rate is above 85% for farms with segregate production (22), keeping in mind that our study was performed on farrow-to-finish farm. According to the results of RT-PCR method and sequencing, we confirmed the introduction of a new closely-related strain PRRSV after I. serum inoculation. The observed sequence homology between 08066t/2010 strain (used for I. serum inoculation) and Meol/2010 strain which we used for II. serum inoculation was 98.4% (table 3, figure 1). Both strains were detected also 6, 10 and 13 months after II. serum inoculation in weaners age of 10 weeks, confirming long period of circulation of strains in farm, although the breeding herd was negative in all testing during the study. The results of serology 6 months after II. serum inoculation show a trend of decreasing high positive breeding pigs which continued until the end of the study. On the other hand, the prevalence of antibody is almost the same comparing the sampling before serum inoculation (90.3%) to sampling 13 months after II. serum inoculation (96.9%). Merely the percentage of high positive breeding pigs decreased from 40.3 to 25.8. We expected the number of high positive breeding pigs to be much lower or non-existent. The results 17 months after serum inoculation indicated by the periodical introduction of PRRSV into the breeding herd resulted in persistently high S/P ratios.

The results of RT-PCR of weaners aged 8 weeks from testing 6, 10, 13 and 17 months after II. serum inoculation were negative, but the virus constantly persisted in group of 10 week old weaners. Pigs born from PRRSV infected dams maintain maternal antibody until 4 to 8 weeks of age using the indirect ELISA (23). Thus it is obvious that after decreasing maternal immunity, the weaners got infected. Shortly, 25% of breeding pigs were high positive 13 months after II. serum inoculation and at the age of 10 weeks, the virus was persistently circulating among the weaners. In this category of weaners, they were in various stages of infection and immunity. While some developed antibodies, the virus replicated in others due to not following the all-in/ all-out protocol. Newly incoming weaners were infected from prior weaners that remained in the room. In every visit to the farm we checked if the biosecurity measures were implemented according to our written guidelines. On the basis of owner assurance, the owner followed all required measures. But the facts presented a completely different picture. He equivocated on lack of time and personnel. We established that only 5 of the 14 required biosecurity measures were followed. Probably, it is very difficult to change the daily routine, which takes one more time and energy. The owner introduced his own replacement gilts into the breeding herd. In the case of introducing the negative replacement gilt, that animal can be a source of virus replication and transmission. The herd closure was not implemented as proposed at the beginning of the study and consequently this can be one of the reasons that the breeding herd could not reach stabilization. So the first goal in the process for achieving the elimination of PRRSV was not accomplished. Although some breeding pigs were identified as negative during the study, when we analysed the individual data the same animals did not remain negative. Hence this suggested that we did not stop the circulation of the virus in breeding herd despite all of the negative results of RT-PCR. One very important measure was the all-in/all-out protocol which

was not followed and thus resulted in pore pen hygiene due to non-vacant pens being thoroughly cleaned and disinfected. It follows that both pigs and pens were the source of PRRSV. Moreover, additional staff were not appointed to a single pig category and did not change coveralls or boots between pig categories, nor wash hands between pig categories. Hence these factors were the reason as well as the route of transmission of PRRSV between categories and between facilities. Pig equipment was not kept on the farm but rather brought to the farm without prior sterilisation (tattooing pliers). From the results of serology, molecular testing and biosecurity measures, we can conclude that the owner did not follow the required biosecurity measures nor carry out strict herd closure which proved to be the reasons for the unsuccessful elimination of PRRSV from the farm. In order to eliminate PRRSV from the farm, the proposed measures should be strictly followed and additional measures, immunization of fatteners and partial depopulation should be implemented since we are dealing with a one-site farm. Dee et al. (3) reported that partial depopulation and strict biosecurity measures can stop the circulation of PRRSV in weaners.

Thus it can be concluded that the serum inoculation did not prove itself as a successful measure for elimination of PRRSV from the farrow-to-finish farm and implementation of herd closure and biosecurity measures in field conditions is a much more difficult challenge than expected. Nonetheless, further study focusing on the education of farmers must be undertaken.

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POSKUS ELIMINACIJE PRAŠIČJEGA REPRODUKCIJSKEGA IN RESPIRATORNEGA SINDROMA NA MANJŠI FARMI Z INOKULACIJO SERUMA

M. Štukelj, I. Toplak, Z. Valenčak

Povzetek: Poglavitni razlog za veliko genetsko raznolikost virusov PRRS je verjetno neučinkovita kontrola bolezni s komercialnimi cepivi, ki vsebujejo samo en sev virusa, saj je zaščita po preboleli okužbi homologna. Ena izmed možnosti za eliminacijo PRRS je prekužitev plemenske črede s farmskim sevom virusa. Za uspešno eliminacijo je nujno upoštevati biovarnostne zahteve. Namen študije je bil eliminirati virus PRRS iz manjše farme (130 plemenskih prašičev) z inokulacijo seruma. Rejec se je obvezal, da bo izvajal stroge biovarnostne ukrepe in zaporo reje vsaj za 200 dni. Plemensko čredo smo imunizirali s pozitivnim serumom tekačev. Šest mesecev po drugem vnosu seruma je število visoko pozitivnih prašičev padlo in trend padanja se je nadaljeval do konca študije, vendar pa je prevalenca protiteles pred serumizacijo v primerjavi s prevalenco na koncu študije (13 mesecev po vnosu seruma) ostala skoraj enaka. Plemenska čreda je bila v vseh testiranjih negativna na prisotnost virusa, virus pa smo stalno dokazovali pri kategoriji tekačev, starih 10 tednov. Rejec se ni držal zapore reje in ostalih predpisanih biovarnostnih zahtev, saj je med drugim vnesel na farmo nov sev virusa PRRS. Eliminacija PRRS z inokulacijo seruma zato ni bila učinkovita. Ugotovili smo, da je izvajanje biovarnostnih zahtev v praksi za rejca zelo velik izziv.

Ključne besede: kontrola; imunizacija; prašiči; PRRS; vnos seruma

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