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THE INTERACTION BETWEEN THE SWINE INFECTIOUS DISEASES AGENTS AND LOW LEVELS OF MYCOTOXINS IN SWINE FEED

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Abstract: The aim of the paper was to evaluate the possible interaction between the presence of swine infectious diseases and low levels of mycotoxins in swine feed. The material for this research included the samples from three swine farms, where health disorders in different swine categories were detected. The applied research methods included: epidemiological and clinical evaluation, pathological examination, bacteriological and virological laboratory testing and microbiological feed testing, in order to examine the presence of fungi and mycotoxins by the method of thin layer chromatography. Beside this, the molecular diagnostic method, reverse transcripton-polymerase chain reaction (RT-PCR) and viral isolation was included. The obtained results support the existance of positive interaction between the mycotoxins and causative agents of bacterial and viral swine infective diseases.

Key words: infective diseases, swine mycotoxicoses

Introduction

Mycotoxins are secondary metabolites of fungi that can cause serious health problems in animals, and may result in severe economic losses (*Greinier et al., 2013*). At the global level, it is considered that 25% of the world crop production is contaminated by mycotoxins, which may be a risk factor affecting human and animal health (*Bouhet and Oswald, 2005; Weaver et al., 2013*). A recent study investigated the occurrence of mycotoxins in European feed samples and concluded that 82% of the samples were contaminated with mycotoxins, indicating that mycotoxins are omnipresent (*Goossens et al., 2012*). Climatic conditions and growing of cereals on large areas in Republic of Serbia are conducive to the growth of toxigenic species, such as *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp., resulting in frequent contamination of animal food by their secondary metabolites. In Republic of Serbia, the most often isolated species

in animal food are fungi of *Fusarium* species, as well as their mycotoxins *(Krnjaja et al., 2011).*

Consumption of feed contaminated with mycotoxins may result in immunosuppresion, which represent a predisposing factor livestock to infectious diseases (*Oswald et al., 2005; Prodanov-Radulović et al., 2011*). All farm animals can experience a negative impact form a dietary intake of mycotoxins but pigs are one of the species which are highly sensitive (*Goossens et al., 2012*). The influence of mycotoxin on immune system is of special interest in swine industry. The technology on swine farms demands frequent vaccinations, which may be a problem in the case of immunocompromised animal (*Prodanov et al., 2009*). The economic impact of mycotoxins includes increased mortality, increased veterinary care costs, reduced livestock production, disposal of contaminated foods and feeds (*Goossens et al., 2012*). However, the major problem associated with animal feed contaminated with mycotoxins is not acute disease episodes, but rather the ingestion of low level of toxins which may cause an array of metabolic, physiologic, and immunologic disturbances (*Greinier et al., 2013*). The aim of the paper was to evaluate the possible interaction between the presence of swine infectious diseases and low levels of mycotoxins in swine feed.

Material and Methods

The material for this research included the samples from three swine farms, where health disorders i.e. clinical and patomorphological signs resembling to the problem with bacterial and viral infectious diseases in different swine categories were detected. Depending on the specificity of each evaluated case and available material, the applied research methods included: epidemiological and clinical evaluation, gross pathological examination, standard laboratory testing for detection the precence of aerobic and anaerobic bacteria, virological testing and microbiological feed testing.

History of the pig units

The following details were ascertained by the interview and from farm records: number and category of pigs on the unit, production details (breeding, finishing unit; nucleus or commercial), disease status, current veterinary health plan, (vaccination programmes, routine medication), biosecurity protocols and feeding system used. The control of indoor pig environment was inspected with regards to basic zootechnical conditions for swine: ambient temperature, lighting, ventilation, stocking density, bedding and hygiene (*Kyriazakis and Whittemore, 2006*). The animals were observed and inspected for clinical signs of disease and abnormal behaviour. The clinical inspection was followed by the necropsy of dead pigs for pathomorphological and laboratory diagnosis.

Bacteriology testing

Isolation of bacteria from tissue samples deriving from dead pigs was performed by standard aerobic and microaerophilic cultivation. Microscopic examination determined whether the isolated bacteria were Gram positive or not and whether it is a coccoid or rod-like organisms. The determination was carried out by determining the biochemical characteristics of the isolated bacteria (*Quinn et al., 2011*).

Microbiological and mycotoxicological feed testing

The feed sampling were done according to The Official Gazette of SFRY, No 15/87. Microbiological examination of food animals for the presence of total molds and yeasts were performed by standard methods Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and molds, Part 2: Colony count technique in products wit water activity less than or equal to 0.95. Determination of the isolated fungi was performed macroscopic examination of colonies in terms of their color and appearance and the microscopic examination of a certain shape of vesicles and conidia, or what the structure of conidia isolated molds (*Quinn et al., 2011*). The presence of mycotoxins in examined feed samples was determined by the method of thin layer chromatography (*Balcer et al., 1978*).

Virological testing

Isolation of Aujeszky's disease virus (ADV; pseudorabies virus PrV) was done by cultivation of tissue samples on cell culture line PK-15 (porcine kidney - ATCC CCL-33). Samples of brain, tonsil, and lung of died animals were homogenized by mortar and pestle and diluted in PBS 1:10 (1g of tissue and 9 mL of PBS) supplemented with antibiotics (200 IU/ml penicillin; 100 μg/ml gentamicin and 5 μg/ml amphotericin B) to prevent bacterial grow. The tissue homogenate is centrifuged on 2000 *g* for 10 min and 1 mL of supernatant was used for inoculation of 24 hours old PK-15 cell culture with 75% confluent cell layer in 25 cm^2 tissue culture flask. Before inoculation, the cell culture growing medium is decanted from the flask and 1 mL of tissue homogenate was added to the cell monolayer, gently shaking to distribute the inoculated material over the whole cell monolayer, and incubated for 1 hour on 37°C. After the incubation 10 mL cell growing medium (Eagle MEM, Sigma) with 10% fetal calf sera (EU grade, PAA, Austria) was added to the cells and cell monolayer was microscopically observed daily for the development of the characteristic herpes virus cytopathic effects (CPE - with rounded birefringent cells, followed by complete detachment of the cell sheet) in the next 7 days. In the absence of any obvious CPE, after the 7 days incubation period, one blind passage into the new 24 old cell monolayer was performed with 1 mL suspension of the first cell passage after 3 cycles of freezing-towing steps. If the visible CPE is observed, the virus presence was confirmed by neutralization with specific antiserum. Beside this, the molecular diagnostic

method, real time reverse transcriptase - polymerase chain reaction (RT-PCR) for for detection of *Mycoplasma hyopneumoniae* (*Strait et al., 2008*) and detection of ADV (*Ma et al., 2008*) were applied.

Serology testing

The serum neutralising test (SNT) was applied in order to estimate the specific antibody titer in farm pigs (sows), following standard procedure as described before (*O.I.E. Manual of Epizootic, 2004*). A total number of 15 blood samples were examined.

Results and Discussion

The first examined farm represent the modern commercial swine farm, located in Južnobački district in Vojvodina. In the time of examination, on the farm the following swine categories were included: 1400 sows, 7 boars, 120 growing gilts, 290 breeding gilts, 2060 suckling piglets, 5051 weaned piglets and 6050 fatteners. The farm represent the one-site production system (farrow-to finish) i. e. all production stages occuring at one site. Applying control of all production stages (farrowing, weaning, finishing), the correct stocking densities and housing requirements (ventilation, temperature, bedding and hygiene) (*Kyriazakis and Whittemore, 2006*) were detected. The farm have organised own veterinary services and swine health control programm include vaccination against *Classical Swine Fever (CSF), Porcine Parvovirus (PPV), Mycoplasma hyopneumoniae, Circovirus type 2 (PCV-2), Erysipelas* and sows vaccination against enteric bacterial infections (*Clostridium perfringes* and *Escherichia coli).* The last mentioned vaccination of dams is alpplied during gestation with the aim to prevent disease in piglets in the first days of life. In the case of disease outbreak (pneumonia, digestive problems), the sucklings, growers and fatteners are therapeutically treated (parenteral injection for clinically diseased animals and water/feed medication is given for in-contacts). Recently, the health disturbances in the youngest swine categories on the farm were registrated. Clinically, the diarrhoea in suckling piglets already in the first 3 days of life after farrowing were detected. After supervision of the farm records several facts were discovered: diarrhoea occurs in the piglets of normal birth body weight, the percent of mortality is higher in animals in good body condition and on the weaning there is 30% of small piglets. Therapeutic treatment of piglets by oral and parenteral antibiotics aplication did not improve health problems. By clinical examination the certain number of suckling piglets the clinical sign of vulvovaginitis (swelling and reddening of the vulva) were discovered. Carriyng health control in the weaned piglets the diarrhoea and signs of pneumonia (dyspnea, cough, purulent nasal discharge) were detected. The gross pathological examination of the dead suckling piglets revealed lesions dominantly

on the mucosal surface of the digestive tract *(Haemorrhagiae mucosae ventriculi, Enteritis catharralis acuta et haemorrhagica*). In dead weaners the prominent pathological changes in lungs were discovered (*Pleuropneumonia, Pneumonia fibrinosa in statu hepatisationis rubrae et griseae*). By bacteriological testing on tissue samples deriving from dead animals the following bacteria was detected: *Escherichia coli haemolytica, Arcanobacterium pyogenes, Pasteurella multocida, Haemophilus parasuis.*

Table 1. The clinical, gross pathology and bacteriology results overview for swine farm No. 1

Swine category	Clinical signs	Gross pathology	Bacteriology testing
Suckling piglets	Diarrhoea, vulvovaginitis	*Haemorrhagiae mucosae ventriculi, Enteritis catharralis, Enteritis haemorrhagica*	*E. coli haemolytica*
Weaned piglets	Diarrhoea, dyspnea, cough, purulent nasal discharge	*Pleuropneumonia, Pneumonia fibrinosa in statu hepatisationis rubrae et griseae*	*A. pyogenes, Pasteurella multocida, Haemophilus parasuis*
Pregnant sows	Not detected	Not observed	Not isolated

Table 2. The results of testing swine feed samples from farm No.1

Complete feed mixture for:	Results of swine feed testing			
	Microbiological testing	Levels of mycotoxines		
	Total fungi number *Fungi Species*	Investigated Mycotoxins	Level (mg/kg)	mpl (mg/kg)
Suckling piglets	**$163x10^3$/1g** *Fusarium sp, Penicillium sp., Aspergillus sp., Rhisopus sp.* mpn: $50x10^3$/1g	AF	**0.018**	0.01
		OCT-A	< 0.02#	0.1
		ZEA	< 0.05#	0.5
Weaned piglets	**88 $x10^3$/1 g** *Fusarium sp, Penicillium sp., Aspergillus sp., Rhisopus sp.* mpn: $50x10^3$/1g	AF	<0.005#	0.01
		OCT-A	**0.12**	0.1
		ZEA	< 0.05#	0.5
Pregnant sows	Not detected	AF	<0.01#	0.02
		OCT-A	< 0.02#	0.2
		ZEA	**0.75**	0.50

Legend: **mpl**- maximum permissible level and **mpn** - maximum permissible number according to Serbian national regulations (The Official Gazette of RS, No. 4/2010); # - limit of detection

Microbiological testing of complete feed mixture for piglets (starter) detected 3-fold increase in the number of fungi genera *Fusarium sp, Penicillium, Aspergillus, Rhisopus sp.* as compared to the level set by the regulation. Applying further laboratory testing, the presence of mycotoxins was detected: zearalenon (ZEA) in the feed for pregnant sows (0.75 mg/kg), total aflatoxin (AF) in the complete feed mixture for piglets (0,018 mg/kg) and ochratoxin (OCT) A in the grover (0.12 mg/kg).

Research investigating the influence of mycotoxins on the animal susceptibility to infectious diseases focuses mainly on exposure to single major mycotoxins. However, limited information is available on the interaction between low levels of mycotoxins and causative agents of swine infectious diseases (*Antonissen et al., 2014*). In our research we noticed the presence of various infections, which react poorly or do not react on the applied antimicrobial therapy (gastroenteritis, pneumonia). Also, the chronic disturbances and presence of infections of low intensity suggest on the potential presence of mycotoxins. As a consequence of immunosuppresive action of mycotoxins (*Kabak et al., 2006*), clinical and pathological lesions that correspond to the infective diseases of different ethiology occured on the examined farm. From the obtained results an example of potential immunosuppresive effect can be presented i.e. the occurence of enteroxemia in piglets, despite the fact that dams were vaccinated twice during gestation. The enterotoxemia is caused by pathogenic bacterial strains and occurs frequently as a cause of mortality in the examined production phase (*Prodanov et al., 2009*). It can be provoked with the feed quality i. e. the presence of mycotoxins. The gastrointestinal tract represents the first barrier against ingested food contaminants and natural toxins (*Bouhet and Oswald, 2007*). Stability of the intestinal flora appeared to be an important factor for animal health (*Oswald et al., 2005*). Thus an impaired balance of the intestinal microbiome, such as dysbiosis condition, could have many adverse effects on the health of the host. However, data on the influence of toxins on the intestinal microflora are still limited (*Greinier et al., 2013*). The biggest challenge with mycotoxicoses is the non-specific nature of symptoms in the affected animals (*Kabak et al., 2006*). Consequently, the health disorders due to mycotoxins in the feed are difficult to diagnose (*Prodanov et al, 2009*). Great potential in prevention of the diarrhoea syndrome of piglets and subsequent improvement in animal growth and feed conversion has been attributed to organic acids, probiotics or/and prebiotics. Although some studies do show little response, a number of studies have shown at least trends for improvements in growth performance, decrease in variation, mortality and morbidity, or decreased medicine costs when prebiotics are fed (*Živković et al., 2011*)

The second evaluated swine farm represent one-site production system i. e. on the farm there is only fatteners production, capacity 2000 fatteners. The pigs are delivered from one large farrow-to-finish, commercial swine farm at the body

weight 20-25 kg. The farm is located in Sremski district in Vojvodina. Applying control of fatteners production, the correct stocking densities and housing requirements (ventilation, temperature and hygiene) (*Kyriazakis and Whittemore, 2006*) were detected. The local veterinary service provide all necessary medication and vaccination against CSF. Anamnestically and clinically, the health problems included increased incidence of respiratory diseases. Analysing the existing data on the farm, the high incidence of morbidity in grovers and fatteners was noticed, which did not decreased after medical treatment. Therapeutic treatment of the diseased animals was intensive and multiple: the antibiotics were given through feed, water and parenterally. In the grover pigs the disease was clinically characterised with the signs of severe coughing, dyspnoea with open-mouth breathing, pyrexia. Prolonged non-productive coughing, worsened by exercise was the main clinical sign of the disease in affected fatteners. Appying gross pathological examination on the dead pigs, the prominant changes on the respiratory tract were detected: acute necrotizing and fibrinous pneumonia, areas of grey-pink consolidation in apical, cardiac and diaphragmatic lung lobes. By bacteriological testing on tissue samples from dead pigs the following bacteria were isolated: *Pasteurella multocida*, *Haemophilus parasuis*, *Streptococcus uberis*. Applying RT-PCR method on the lung tissue derived from dead fatteners, *Mycoplasma hyopneumoniae* was detected. By laboratory feed testing for grovers and fatteners the increase presence of total AF (0.04 mg/kg and 0.038 mg/kg) was discovered.

In the second evaluted case, the presence of AF in the feed for grovers and fatteners was detected. Consequently, on the farm an evident decrease in the swine immunity against infective diseases of the respiratory tract was noticed and no positive respond on the applied antibiotic therapy. Respiratory disease in pigs are often caused by the combined effects of multiple pathogens and predisposing factors (*Antonissen et al., 2014*). For AF, swine are one of the most sensitive species. When consumed, this mycotoxin can cause immune dysfunction or damage organs, even when consuming moderate concentrations of contaminating grains (*Weaver et al., 2013*).

The last examined farm represent the modern commercial swine farm, located in Južnobački district in Vojvodina. In the time of examination, on the farm the following swine categories were included: 650 sows, 10 boars, 155 pregnant gilts, 400 breeding gilts, 1400 suckling piglets, 2600 weaned piglets and 4000 fatteners. The farm represent the one-site production system (farrow-to-finish) i. e. all production stages occuring at one site. Applying control of all production stages (farrowing, weaning, finishing), the correct stocking densities and housing requirements (ventilation, temperature, bedding and hygiene) (*Kyriazakis and Whittemore, 2006*) were detected. The farm have organised own veterinary services and swine health control programm include: imunoprophylaxis i.e. the vaccination against *Classical Swine Fever (CSF), Porcine Parvovirus (PPV), Mycoplasma*

hyopneumoniae, Circovirus type 2 (PCV-2), Erysipelas and sows vaccination against *Clostridium perfringes* and *Escherichia coli.* In the case of health distrurbance, the animals are therapeutically treated (parenteral injections and in-water medication). However, recently the connection between the presence of mycotoxins in swine feed and an outbreak of viral infection of swine, *Morbus Aujeszky* (MA) was established. By microbiological testing in feed for lactating sows the precence of fungi (in 1g x 10^3 85,5 *Fusarium sp., Mucor*) and AF (0.09 mg/kg) were detected. Anamnestically, the health disorders in sows and in their litters were observed. By epidemiological investigation it was discovered that on the swine farm 4 months before in total 15 new sows had been introduced. Serologically, in most of the sows the presence of specific antibodies against MA by SNT was detected. However, despite the fact that these animals were serologically positive, the origin of that immunological status from the aspect of MA remained unknown: vaccination or infection. By clinical examination in sows the signs of inapetence, mild apathy, constipation and agalactiae were observed. In suckling piglets the sings of severe disturbance of the central nervous system (paddling, trembling, ataxia, paresis and paralysis) were clinically detected. In some cases the whole litter of piglets died within 48 hours. Clinically the fatteners also become anorectic, listless and apathic. The gross pathological changes that were detected in dead sucklings indicated the lesions characteristic for MA infection (*Necroses miliares hepatis et lienis, Tonsillitis diphtheroides necroticans).* Applying virological testing (VI on the susceptible cell culture PK-15) and RT-PCR from the tissues deriving from dead piglets the *Morbus Aujeszky* virus (ADV) was isolated.

Table 3. The clinical, gross pathology and bacteriology results for swine farm No. 3

Swine category	Clinical signs	Gross pathology	Virology testing
Suckling piglets	paddling, trembling, ataxia, paralysis	*Necroses miliares hepatis et lienis, Tonsillitis diphtheroides necroticans*	isolated ADV RT-PCR positive result
Lactating sows	inapetence, mild apathy, agalactiae	No dead sows	SNT positive antibody titer 1:16- 1:128

Suppressed immune functions by mycotoxins may decrease resistance to infectious diseases, reactivate chronic infections and reduce therapeutic efficacy (*Oswald et al., 2005*). In the last examined case, where the outbreak of MA on the farm were examined, AF in the feed can be connected with the possible reactivation of chronic (latent) infection in sows. Even when is present in low

doses, AF alters the immune response and this may predispose pigs to infectious diseases (*Prodanov-Radulović et al., 2011*).

Table 4. The results of testing swine samples farm No. 3

Complete feed mixture for:	Results of swine feed testing			
	Microbiological testing	Levels of mycotoxines		
	Total fungi number *Fungi Species*	Investigated Mycotoxins	Level (mg/kg)	mpl (mg/kg)
Suckling piglets	< $50x10^3$/1g *Fusarium sp, Penicillium sp., Aspergillus sp., Rhisopus sp.* mpn: $50x10^3$/1g	AF	< 0.005#	0.01
		OCT-A	< 0.02#	0.1
		ZEA	< 0.05#	0.5
Lactating sows	85.5 $x10^3$/1g *Fusarium sp, Mucor sp.* mpn: $200x10^3$/1g	AF	**0.09**	0.02
		OCT-A	< 0.02#	0.2
		ZEA	< 0.05#	0.50

Legend: **mpl**- maximum permissible level and **mpn** - maximum permissible number according to Serbian national regulations (The Official Gazette of RS, No.4/2010); # - limit of detection

One should remember that detected concentrations of mycotoxins in the feed are approximations, because sampling is never completely representative. Applying chemical analyses we can identify mycotoxin, but sometimes causative cereal that iniciated the problem is no longer available or representative sample (*Kabak, 2006*). From the other side, when we are discussing the mycotoxin problem, feed dilution may reduce exposure initially, but care must be taken that wet or contaminated grain can introduce new fungi, and conditions that eventually lead to the entire mixture being contaminated (*Oswald et al., 2005*).

Because of detrimental effects of mycotoxins, a number of strategies have been developed to decontaminate and detoxify mycotoxin-contaminated feed (*Krnjaja et al., 2011*). They may include inhibition of mycotoxin adsorption in the gastrointestinal tract. One of the most recent approaches to the prevention of mycotoxicoses is the addition of non-nutritionally adsorbents in the feed that bind mycotoxins in the gastrointestinal tract and reduce their bioavailability. The activated carbons, aluminosilicate, zeolites, bentonites and certain clays are well known (*Kabak et al., 2006*). Strategy which includes application of good agricultural practice and good storage practice with reduced effect of mycotoxins, with implementation of all regulations and measures, can provide production of safe food for humans and animals *(Krnjaja et al., 2011)*.

Conclusion

The achieved results support the existance of possible positive interaction between the mycotoxins and causative agents of bacterial and viral swine infective diseases. The continuos intake of small amounts of mycotoxins may leads to chronic intoxication which is clinically characterized by the loss of weight, insufficient weight gain and increased susceptibility for infectious diseases. The basic preventive measures in order to protect animals are usage of healthy feed and proper storage and managemant conditions for animals feed. Certainly, when mycotoxicosis occurs or is suspected, the first action should be to change the source of feed. Mycotoxicoses is generally a herd problem and not amenable to individual treatment. Practical preventive program should be part of every swine management program.

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Interakcija između uzročnika infektivnih bolesti svinja i niskih vrednosti mikotoksina u hrani za svinja

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Rezime

Cilj rada je bio ispitivanje mogućnosti interakcije između uzročnika infektivnih oboljenja svinja, bakterijske i virusne etiologije i niskih vrednosti mikotoksina u hrani za svinje. Materijal za ispitivanje je obuhvatao uzorke poreklom sa tri farme svinja, na kojima su registrovani zdravstveni problemi kod različitih kategorija svinja. Primenjene metode ispitivanja su obuhvatale epizootiološka i klinička ispitivanja, patomorfološki pregled, standardne laboratorijske bakteriološke i virusološke metode i mikrobiološko ispitivanje uzoraka hrane u cilju ustanovljavanja prisustva plesni i mikotoksina, metodom tankoslojne hromatografije. Pored toga, primenjena je i molekulatna metoda dijagnostike, reverzna transkripcija-lančana reakcija polimeraze (RT-PCR). Postignuti rezultati ispitivanja ukazuju na postojanje pozitivne interakcije između mikotoksina i uzročnika bakterijskih i virusnih infektivnih bolesti svinja.

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