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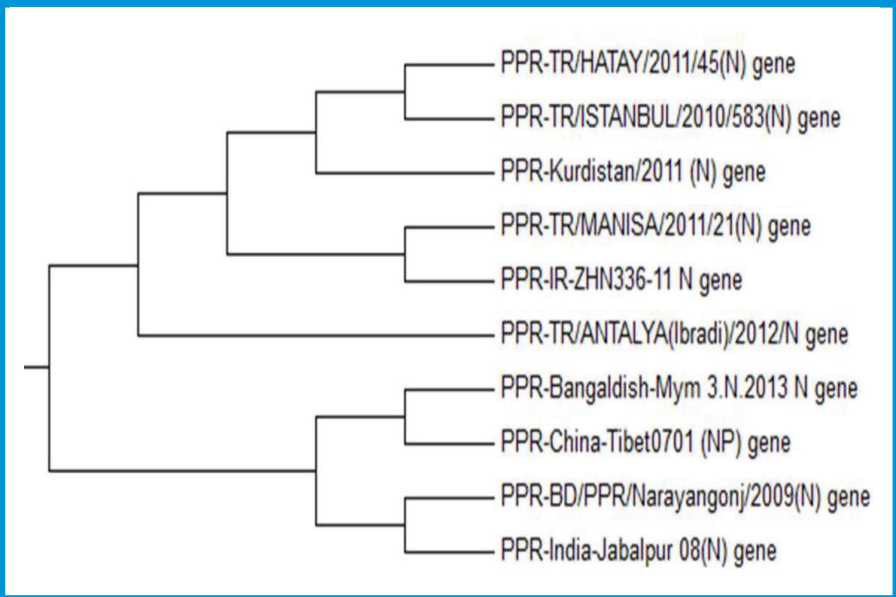

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Original Research Article

MOLECULAR EPIDEMIOLOGICAL STUDY ON PESTE DES PETITS RUMINANTS IN EGYPT 2015

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Abstract: Peste des petits ruminants (PPR) is one of highly contagious viral diseases of small ruminants with high economic losses due to the high morbidity and mortality. In Egypt, PPR in the last 10 years re-emerged again with high mortality in sheep flocks. There is no much data regarding the circulating Peste des petits ruminants' virus and the epidemiological distribution in small ruminants. The study was carried out on a sheep flock of 50 rams aged of 9-12 months with clinical signs suggestive to PPR infection (fever, erosions of the buccal mucosa, pneumonia, diarrhea high morbidity and mortality). Buffy coat and lymph nodes from diseased and dead animals were sent for diagnosis and molecular diagnosis was confirmed using RT-PCR with specific primers targeting three genes; nucleo-capsid (N), fusion (F), and hemagglutinin (H). Sequencing and phylogenetic analysis was carried out on the PCR products and revealed that, the circulating virus is belonged to lineage IV along with Ethiopian strain.

Key words: peste des petits ruminants; molecular; epidemiological; diagnosis; Egypt

Introduction

Peste des petits ruminants (PPR) is a highly contagious viral disease of domestic and wild small ruminants. It's caused by RNA virus belonged to genus Morbillivirus, family *Paramyxoviridae*. (1) and characterized by high morbidity and mortality in affected flocks. Clinically diseased animals suffered from fever (40-41 °C) for 5-8 days before returning to normal during recovery or to subnormal before death, ocular-nasal discharges which starts usually serous and might end with purulent discharges at late stages, erosions and ulceration of buccal mucosa, pneumonia, and ends with diarrhea which could be bloody in severe cases with high mortalities. The disease was first described in Cote d'Ivoire in 1947(2) and the infection is restricted to some African and Asian countries. Affected African countries extended from Egypt in the north to Kenya in southeast and Gabon in the west while, affected Asian countries include India, Pakistan, Bangladesh and Saudi Arabia, (3, 4). It has severe economic impact on animal wealth as it caused severe mortality with more than 50% of the affected animals died due to pneumonia, diarrhea and dehydration(5). PPR

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Virus is mainly transmitted by aerosol infection, and may also be spread through direct contact and/or consumption of contaminated water or feed(6).

There are four lineages of PPR. Three lineages I, II and III have been found in Africa [West Africa (I and II), East Africa (III)] while Lineage IV has been restricted to the Middle East and Asia. Lineage III is being reported in Yemen and Oman while mixed lineages of III and IV were reported in the United Arab Emirates and Qatar. Recently lineage IV is present across the PPR endemic areas which may replace other lineages(4, 7-9). The apparent expansion of Asian lineage IV across Africa is supported by a constant increase in the incidence of disease, suggesting an increase in its virulence (9).

The control strategy of PPR in Egypt depends mainly on control of animal movement combined with ring vaccination of 5 kilometers around the affected spot using live attenuated vaccines and symptomatic treatment of the affected animals (3).

The objectives of the study were to study different epidemiological parameters including the attack rate, mortality and case fatality in a clinically infected sheep flock and genetically characterize the circulating PPRV in Egyptian small ruminant population through the phylogenetic analysis of the infecting stain.

Materials and methods

Animals

The study was carried out between May 2015 and April 2016 on a fattening sheep flock consisted of 50 rams aged 9-12 months, diseased cases started 10 days after the last purchased animals. Diseased animals suffered from fever, salivation with severe erosions in the buccal mucosa swelling of lips, anorexia, nasal discharges, increased respiratory rate and signs of pneumonia in some cases and ended with severe diarrhea which was bloody in some cases, then dehydration and death. The signs were suggestive for PPR infection, blood samples were collected during viremia and tissue samples from dead animals. The samples were sent to the Virology Laboratory at Faculty of Veterinary Medicine, Sadat City University, Egypt for confirmation.

The affected flock was examined clinically according to Jackson and Cockcroft, 2002(10). The attack rate, mortality and case fatality rates were determined according to Stevenson, 2005 (11)

Treatment was done on symptomatic basis, fluid therapy (Ringer lactate dosed at 15 ml/kg), all the animals were given long acting oxytetracycline at a dose of 20 mg/kg (Oxitetraciclina 200LA®) , ketoprofen (Ainil®) at a dose of 3 mg/kg and mouth wash with betadine 1% till recovery or death according to Tariq et al. 2014 (12).

RNA extraction

RNA extraction was performed on the supernatant of pooled homogenate from tissues or buffy coat using viral gen-spin Viral DNA/RNA extraction kit (INTRON Biotechnology, Korea) according to the manufacturer's procedure. RNA concentration and integration were quantified by spectrophotometric method.

RT-PCR protocol

Stranded cDNA of the extracted RNA was firstly done using Hsien Script RH (-) cDNA synthesis kit (INTRON Biotechnology, Korea) following the manufacturer's instructions and reaction was as follow;10 µl 2x reaction solution, 1µl enzyme mix, 5 µl template RNA, 1µl specific reverse primer, DNase / RNase free water up to 20 µl and the cDNA was synthesis at 42 °C for 30min.

Conventional RT-PCR protocol was used with 3 µl of the resulted cDNA in 25 µl reaction mixtures using 2x PCR master mix solution (I-Taq) INTRON Biotechnology, Korea) with PPR-F, H, N genes specific primers as listed in Table 1. The thermal profile was as fellow; 94 C for 1 min, 50 C for 1 min, 72 C for 2 min extension for F gene, While with H and N genes, the conditions were as follow; 94 C for 30 seconds, 55 C for 30 seconds, 72 C for 30 seconds for total 35 cycles and final extension for 7 minutes.

PCR products were electrophoresed on a 1.2% agarose gel in 1x (TAE) buffer containing ethidium bromide, then visualized and photographed using gel documentation system.

Genetic analysis of F, H, and N protein gene

The purified RT-PCR products were sequenced directly using PCR primer specific for F, H and N genes. Cycle sequencing reaction was carried out

with Big Dye Terminator v3.1 Cycle Sequencing Kit on an Applied Biosystems 3100 automated DNA sequencer (Applied Biosystems, USA). The samples were sequenced in Animal Health Research Institute- Gene Analysis Unit. Egypt. The resulted nucleotide sequence and their transcribed amino acids were analyzed using Bio Edit (7.1.3) program Sequences, and phylogenetic trees were constructed by the neighbor joining method by bootstrap sampling of 1000 replicate using MEGA 7.

Results

Clinical Findings

The clinical examination of the diseased sheep flock revealed that, the affected flock was recently introduced in May 2015, all were rams and they were purchased from the animal market 10 days before the appearance of clinical signs. They were apparently healthy at the time of purchasing.

The attack rate was 80% (40 out of 50), mortality rate was 28% (14 out of 50), case fatality rate was 35% (14 out of 40) and the recovery rate was 65% (26 out of 40).

Affected animals suffered from fever, salivation with severe erosions in the buccal mucosa as in fig (1-a), ocular and nasal discharges. Some cases showed respiratory manifestations and the disease ended with diarrhea which was bloody in some cases as in fig. (1-b), then body temperature fall to subnormal and death occurred.

RT-PCR

All tissues and blood samples were RT-PCR positive with the three selected genes (N, F, H). The PCR products were at expected sizes 351 bp, 448 bp, 328 bp for N, F, and H genes respectively. The PCR bands of N gene are more clear and thick compared to PCR bands of F and H genes.

Table 1: Primers of peste des petite ruminants

Gene	Primer	Sequence	Position	Size	Reference
F-gene	PPRV F1b	5´-AGT ACA AAA GAT TGC TGA TCA CAG T-3´	760- 784	448 bp	(13)
	PPRV F2d	5´-GGG TCT CGA AGG CTA GGC CC GAA TA-3	1207-1183		
N-gene	NP3	5´-TCT CGG AAA TCG CCT CAC AGA CTG -3´	1232-1255	351 bp	(14)
	NP4	5´-CCT CCT CCT GGT CCT CCA GAA TCT -3´	1583-1560		
H-gene	Pprh_fr1	5´-TGT CAT GTT CTT ATA GAG TT-3´	1500-1519	328 bp	(15)
	Pprh_re2	5´-GAC TGG ATT ACA TGT TAC CT-3´	1847-1828		

Figure 1: a- Sheep has severe mouth lesions, nasal and ocular discharges; b- Sheep with severe diarrhea at the end of the disease

Table 2: Accession number for the PPR sequence from blood and tissue samples

	Strain	Accession number	Characters
1-	Egypt/Giza-1/2015	KX189061	PPR-Egypt-2016-blood(F-gene)
2-	Egypt/Giza-2/2015	KX189060	PPR-Egypt-2016-Tissue(F-gene)
3-	Egypt/Giza-3/2015	KX189062	>PPR-Egypt-2016-blood(H-gene)
4-	Egypt/Giza-4/2015	KX189063	>PPR-Egypt-2016-Tissue(H-gene)
5-	Egypt/Giza-5/2015	KX189064	PPR-Egypt-2016-blood(N-gene)
6-	Egypt/Giza-6/2015	KX189065	PPR-Egypt-2016-tissue(N-gene)

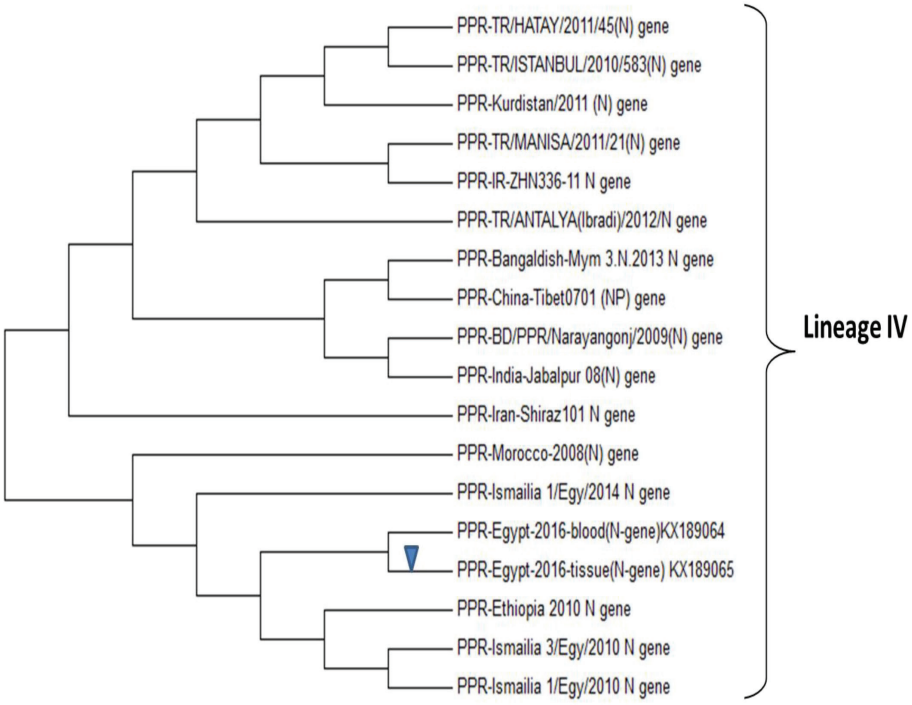

Figure 2: Phylogenetic tree of nucleotide sequences of partial nucleocapsid protein (N gene fragment of PPRV isolated from Egypt and reference strains from GenBank database. MEGA 7 program was used to generate the tree. Arrow head referee to isolates under study

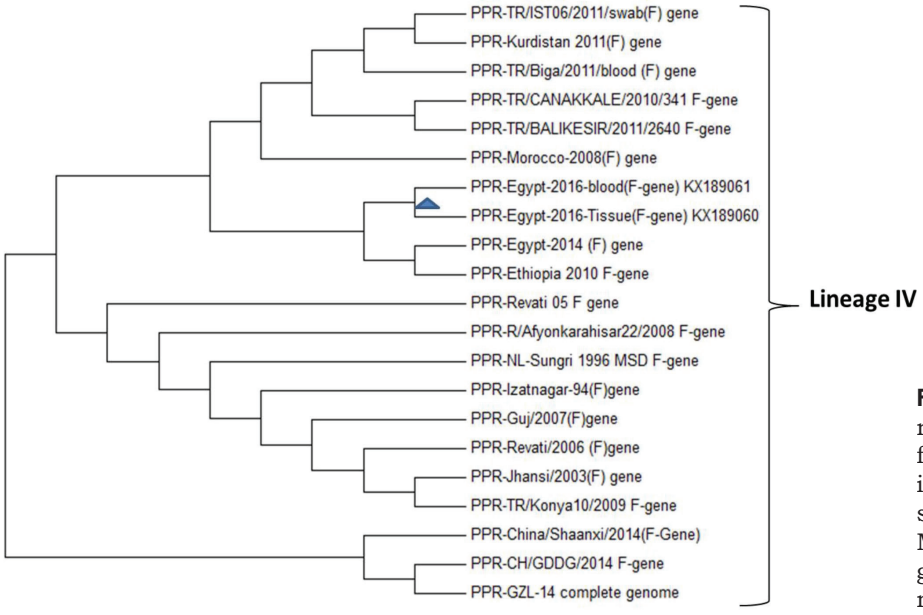

Figure 3: Phylogenetic tree of nucleotide sequences of partial fusion gene fragment of PPRV isolated from Egypt and reference strains from GenBank database. MEGA 7 program was used to generate the tree. Arrow head referees to isolates under study

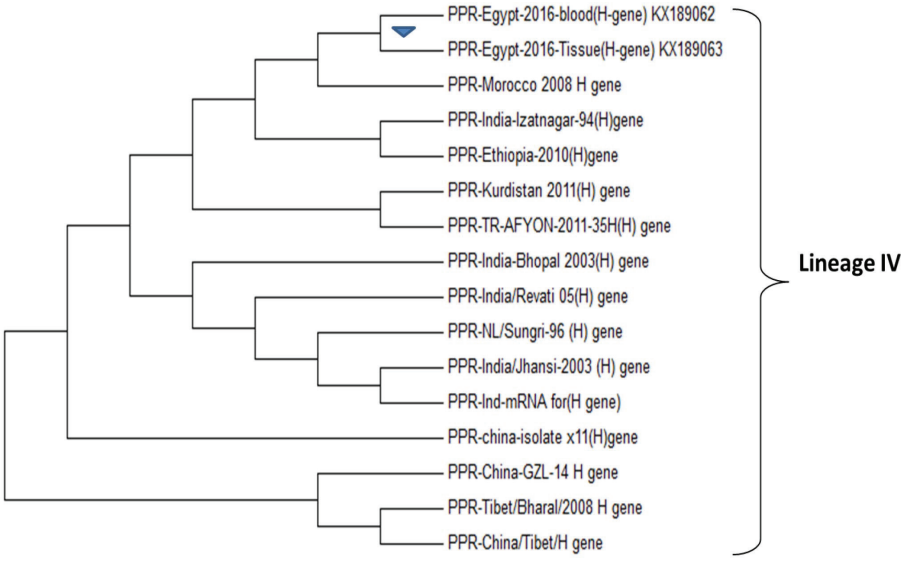

Figure 4: Phylogenetic tree of nucleotide sequences of partial hemagglutinin protein (H gene fragment of PPRV isolated from Egypt and reference strains from GenBank database. MEGA 7 program was used to generate the tree. Arrow head referes to isolates under study

The nucleotide and amino acid analysis of partial N, F, and H genes

The nucleotide sequence analysis of N gene showed no characteristic difference between the PPRV of the current study and other PPRV sequences except at position 1255 where PPRV of the recent study showed guanine at this position in contrast to adenine for other isolates. N gene sequences of PPRV strains detected in both tissues and blood of affected animals have shown no nucleotide difference. The amino acid analysis showed characteristic difference at position 452 between the detected strain of this study and the remaining isolates including previous Egyptian isolates.

For F gene, there were nucleotide differences between the PPRV of the current study and other PPRV sequences at positions 314, 552, 645. F gene sequences of PPRV strains detected in both tissues and blood of affected animals have shown high nucleotide homology.

The amino acid analysis showed characteristic difference at position 20 between the detected strain of this study (sequences detected from blood) and the remaining isolates including previous Egyptian isolates.

For H gene, there were nucleotide differences between the PPRV of the current study and other PPRV sequences at positions 1662, 1776, 1778. H gene sequences of PPRV strains detected in both tissues and blood of affected animals have shown no nucleotide difference. The amino acid analysis showed characteristic difference at positions 589, 596 between the detected strain of this study and the remaining isolates including previous Egyptian isolates.

The phylogenetic analysis of the detected PPRV based on partial sequences of N, F, and H genes

For N gene, the detected strain of the current study clustered with the previous Egyptian isolates in one cluster including PPRV isolated in Ethiopia 2010 (Fig. 2).

For F gene, the detected strain of the current study clustered with a previous Egyptian isolate (PPRV Egypt 2014) and PPRV Ethiopia 2010 in the same cluster (Fig.3).

For H gene, the detected strain of the current study clustered with a PPR Morocco 2008 in the same cluster which is very close to PPR Ethiopia 2010 and PPR India Izatnagar-94 (Fig.4).

Discussion

Peste des petite ruminants is one of the important economical viral diseases of small ruminants. In Egypt, PPRV was first detected in 1987 (13). Later, few reports described the disease in Egyptian small ruminants (1, 14, 15) and this might be due to absence of animal records of sheep and goats, PPR can be misdiagnosed with other diseases that cause respiratory problems and mortality of

small ruminants also animal health workers and livestock owners in the areas were not familiar with its clinical and pathological features (3, 16).

Clinical signs of PPR occurred 10 10 days after purchasing of the rams from the market which indicated that they were at the incubation period. The infection might occured at the market which could provide a potential source of transmission and spreading of different diseases. The attack rate of the disease was 80% which indicate the contagiousness of the disease, the mortality rate was 28% while the case fatality rate was 35% and these results are comparable with the results of the previous studies (17-20).

Overall, the data regarding the genetic characterizations and epidemiological distributions of PPR in Egypt are still relatively rare. The study aimed to genetically characterize the circulating PPRV in Egyptian small ruminant population.

Molecular diagnosis of PPRV was carried out through detection of fusion (F), nucleocapsid (N), and (H) hemagglutinin genes-based RT-PCR in tested clinical samples from the buffy coat and lymph nodes. All tested samples showed PCR bands at the expected sizes. The PCR bands of N gene were clearer than bands of other genes. It was suggested that N gene-based PCR is more sensitive. Because N messenger RNA is produced more during PPRV infection (2).

PPR viruses are classified into four lineages (I, II, III and IV) that vary genetically(21, 22). The partial regions of N, F or H genes are used for the phylogenetic tree analysis and it is clearly defined four different lineages of PPRV. In the current study, the genetic data generated, and RT-PCR results confirmed that the clinical disease observed in affected animals was caused by PPRV and the virus belonged to lineage IV(23).

The analysis of N gene sequence of PPRV is most suitable for phylogenetic difference of close viruses and provides an inclusive view of PPR molecular epidemiology (2, 21, 24). In this study, the N gene primers used encompass the region of nucleotides 1253 to 1470 of the N gene which is more variable. The amino acid analysis showed characteristic difference at position 452 between the detected strain of this study and the remaining isolates including previous Egyptian isolates. In the nucleotide sequence analysis, there was no characteristic difference between the PPRV of the current study and other PPRV sequences except at position 1255 where PPRV of the recent study showed guanine at this position in contrast to adenine for other isolates. In the phylogenetic analysis, the detected strain of the current study clustered with the previous Egyptian isolates in one cluster including PPRV isolated in Ethiopia 2010 (Fig. 2).

The analysis of F gene sequence of the PPRV of the study showed characteristic features like N gene analysis. In the nucleotide sequence analysis, there were differences between the PPRV of the current study and other PPRV sequences at positions 314, 552, 645. The amino acid analysis showed characteristic difference at position 20 between the detected strain of this study (sequences detected from blood) and the remaining isolates including previous Egyptian isolates. In the phylogenetic analysis, the detected strain of the current study clustered with a previous Egyptian isolate (PPR Egypt 2014) and PPR Ethiopia 2010 in the same cluster (Fig.3). It was reported the phylogenetic trees using the F gene data produced a limited number of clusters that although supported by bootstrap support, did not split isolates according to geographical isolation(2).

The analysis of H gene sequence of the PPRV of the study showed characteristic features as follow: In the nucleotide sequence analysis, there were differences between the PPRV of the current study and other PPRV sequences at positions 1662, 1776, 1778. The amino acid analysis showed characteristic difference at positions 589, 596 between the detected strain of this study and the remaining isolates including previous Egyptian isolates. In the phylogenetic analysis, the detected strain of the current study clustered with a PPR Morocco 2008 in the same cluster which is very close to PPR Ethiopia 2010 and PPR India Izatnagar-94 (Fig.4). H gene of PPRV is more variable than the F gene so the phylogenetic relatedness of isolates is more properly determined with respect to geographical isolation than when comparing the F gene data. It was also stated that although the H gene partial sequence data are rare compared to N and F gene partial sequence data, phylogenetic analysis using these data shows the formation of different clades that are divided into sub-clusters(2).

The PPR viruses detected in Egypt could not have been derived from the vaccine strain because the vaccine virus belongs to lineage II while the field isolates belongs to lineage IV.

Symptomatic treatment has a significant role in the recovery of the affected animals. Systemic antibiotics prevent the secondary bacterial infections and anti-inflammatory reduced the inflammation also fluid therapy restores the body fluids and nutritional supplement in absence of good appetite. The recovery rate in the study was 65% and these results agreed with Tariq et al. 2014 (12).

Conclusion

Peste des petite ruminants is detected in 2015 from an outbreak in sheep flock and the virus still circulating in Egypt and resulted in 28% mortality in the affected flock. Molecular data of the study provides an evidence for circulation of PPRV in Egyptian small ruminant flocks. The circulating PPRV is phylogenetically related to lineage IV which is in one cluster with the Ethiopian isolates in 2010. Further studies with large numbers of animals from different governorates are essentially required. Further epidemiological data regarding the prevalence of the disease, spatial and temporal distribution and evaluation of preventive measures are required. Symptomatic treatment plays a significant role in the recovery of diseased animals and need to evaluate the roles of different drugs and the effect on different hematological and biochemical parameters. All the Authors declare that there is no conflict of interest.

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MOLEKULARNA EPIDEMIOLOŠKA ŠTUDIJA KUGE DROBNICE V EGIPTU LETA 2015

W. R. ElAshmawy, A. I. Bazid, M. Aboelkhair, M. A. Sakr, A. M. Fayed, M. Fawzy

Povzetek: Kuga drobnice (PPR; iz angl. peste des petits) je ena izmed zelo nalezljivih virusnih bolezni malih prežvekovalcev, ki zaradi visoke obolevnosti in umrljivosti povzroča visoke gospodarske izgube. V Egiptu se je v zadnjih desetih letih PPR znova pojavila z visoko smrtnostjo pri ovcah.

O kugi drobnice in epidemiologiji te bolezni pri malih prežvekovalcih v literaturi ne najdemo veliko podatkov. Opisana raziskava je bila opravljena v čredi 50 ovnov, starih od 9 do 12 mesecev, s kliničnimi znaki, ki so kazali na prisotnost bolezni PPR (zvišana telesna temperatura, erozije ustne sluznice, pljučnica, driska, visoka obolevnost in smrtnost). Za natančno diagnosticiranje bolezni je bil uporabljen del krvi, pridobljen s centrifugiranjem, ki vsebuje veliko levkocitov in trombocitov (angl. Buffy coat) in bezgavke obolelih in mrtvih ovnov. Z uporabo specifičnih oligonukleotidov za določanje prisotnosti nukleotidne (N), fuzijske (F) in hemaglutininske (H). beljakovine v reakciji RT PCR smo na molekularnem nivoju potrdili prisotnost okužbe s povzročiteljem PPR. Analiza zaporedja genov in filogenetske analize so bile izvedene na produktih analize PCR, pri tem pa smo ugotovili, da virus pripada liniji IV skupaj z etiopijskim sevom.

Ključne besede: kuga drobnice; molekularna diagnostika; epidemiologija; Egipt

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Original Research Article

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF AVIAN POX VIRUS ISOLATED FROM PET BIRDS AND COMMERCIAL FLOCKS, IN IRAN

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Abstract: Avian pox (AP) is a viral disease with a wide host range. The aim of the study was molecular identification and characterization of field isolated pox virus from pet birds and commercial flocks in Iran, by polymerase chain reaction (PCR). Scab materials of skin and mucosal lesions were collected from five clinically affected cases. PCR was used to amplify a 578 bp fragment of the poxvirus 4b core protein. In order to determine the genetic relationships among the viruses, this conserved poxvirus genetic region was sequenced and analyzed. The Iranian Avipoxvirus isolates in this study grouped in clade A1 (commercial chicken and turkey flocks) and B1 (canary). Further studies on a larger scale need to be developed to have a better understanding of the molecular characterization of the Iranian APV strains.

Key words: avian pox; phylogenetic analysis; molecular characterization; Iran

Introduction

Avian Pox (AP) is a viral well-known disease in hens, turkeys and many other birds (278 species from 70 families and 23 orders), characterized by cutaneous lesions on the feather-less skin and/or diphtheritic lesions of mucous coats of the upper alimentary and respiratory tract. Moreover, concurrent systemic infection causing high mortality often occurs in canaries. AP lesions, however, may compromise vision, the ability to feed or lead to secondary bacterial or fungal infection leaving wild birds vulnerable to predation.The poxviruses which infect birds belong to the genus Avipoxvirus of the Poxviridae family. Avipoxviruses (APVs) within the family Poxviridae contain nearly 300-kilo base pair (kbp) of double-stranded DNA that replicate in the cytoplasm of infected cells and the members of the genus Avipoxvirus in the subfamily chordopoxvirinae (1). Pox infection usually occurs through the mechanical transmission of the virus to injured skin and also the bite of mosquitoes or mites. The incubation period and duration of APV infection is variable (from a few days to many months), but affected birds with mild lesions frequently recover and this is considered to be the most common

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situation in wild birds. Its incidence is variable in different areas because of differences in climate, management and hygiene or the practice of regular vaccination. It can cause drops in egg production, or retarded growth in younger birds (2).

The conventional laboratory diagnosis of APV is carried out by histopathological examination, electron microscopy, virus isolation on chorioallantoic membrane (CAM) of embryonated chicken eggs or cell cultures, and serologic methods (2). The 4b core protein gene (p4b) of APV that encodes the protein with molecular weights of 75.2 kDa is usually chosen for comparative genetic analysis (3,4,5). Also, amplification of the p4b of APV by PCR has often been used as a molecular tool for the detection of APVs (6). PCR in combination with restriction endonuclease enzyme analysis (REA) followed by sequence analysis of the amplified fragments is used for detection, differentiation and molecular characterization of fowl pox virus isolates (7). Even considering the decrease in problems caused by poultry production, avian pox is still a significant pathogen which can have serious effects on wild Galliformes. The incidence of AP in Iran is high in pet birds and also in fewer levels in commercial farms. In recent years, some outbreaks of skin lesions suspected to be avian pox were observed in the backyard poultry in different parts of western areas in Iran. Generally, the number of reports concerning incidence and characterization of avian pox viruses in Iran is very low. Gholami-Ahangaran et al. performed a survey on 328 backyard poultries with suspected signs of avian pox virus infection. Their results showed 217 and 265 out of 328 samples were positive for avian pox virus on histopathological and PCR examination, respectively (8). In the study of Fasaei et al. (2014), Avipoxvirus specific DNA was detected in all 10 different isolates from chicken, canary and mynah that were collected from Tehran province (3). The aim of this study was a characterization of AFPv isolates from canary, chicken and turkey flocks by PCR.

Materials and methods

Sampling

Samples (cutaneous scrub and caseous lesions) were collected from different species with characteristic clinical signs (5 samples from chicken, canary, turkey) had been submitted to PCR Veterinary Diagnostic Laboratory (Tehran, Iran), during 2012- 2014. The data of samples are available in table 1.

DNA extraction

DNA was extracted from the skin or pulmonary lesions of the clinical cases and lyophilized vaccines (as a positive control) by QIAamp DNAMini Kit (Qiagen) following the manufacturer's guidelines. DNA samples were stored at -20°C until analysis.

Amplification of 4b Gene

The AVP-specific PCR was performed using primer pairs described based on FPV 4b core protein (P4b) gene sequence of Fowl pox virus strain HP444 previously (9). The sequence of the primers was as follows: forward primer: 5′-CAGCAGGTGCTAAACAACAA and reverse primer: 5′- CGGTAGCTTAACGCCGAATA. PCR consisted of 25 µl reaction containing1.5 units of *Taq* DNA polymerase, 1.5 mM MgCl2, 200µM of each deoxynucleoside triphosphate, 6 pmol of each primer, DNA extracted from clinical samples and nuclease free water up to 25 µl. Amplification was performed after initial denaturation for 2 min at 94°C for 35 cycles and consisted of 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C. In this study, live fowl pox vaccine (Razi Vaccine and Serum Research Institute, Iran) was provided and used as positive control.Negative control includes all the reagents without a template.

Sequencing and Phylogenetic analysis

The positive PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized bye GelRed ™ (Biotium , USA) staining and ultraviolet transillumination. The PCR products were purified by the PCR AccuPrep® PCR Purification Kit (Bioneer Co., Korea) and purified PCR products were sent for sequencing (Source Bioscience, UK) with PCR primers for in a forward and in a reverse direction. Sequencing reactions were run on an ABI Prism 310 Genetic Analyzer. The sequence results were downloaded and analyzed using Chromas (Technelysium Pry Ltd., Australia). Phylogenetic analysis was carried out by analyzing the data obtained here with those of other sequences of FPVs

from the GenBank database. The phylogenetic analysis was performed with the MEGA5 (Phylogeny Inference Package) software, version5. Distance-based neighbor-joining trees were constructed using the Tamura–Nei model (10). The robustness of the phylogenetic trees was assessed by 1,000 bootstrap replicates. Bootstrap values lower than 50 were omitted. The FPV sequences tested in this study were deposited in GenBank under accession numbers KT003286 –KT003290.

Results

Because of the highly conserved nature of the analyzed genes, nucleotide sequences rather than amino acid sequences were used to determine divergence. Clades and sub clades have been named according to previous APV phylogenetic studies based on the P4b (11). The strains were placed in A1 and B1 sub clades. The homology between isolates was 67.3%-100% (Table 3).

Table 1: Description of fowl pox virus strains investigated in this study

Strain name	Nam in Tree	Species	Type of Lesion	Year	Province	Accession No.
IR/Canary poxvirus/ H364/12	CP/H364/12	Canary	Cutaneous	2012	Alborz	KT003286
IR/Canary poxvirus/ H913/14	CP/H913/14	Canary	Cutaneous	2014	Kurdistan	KT003287
IR/Fowl pox virus/H252/12	FP/H252/12	Chicken	Caseous	2012	Isfahan	KT003288
IR/Fowl pox virus/H756/13	FP/H756/13	Chicken	Caseous	2013	Tehran	KT003289
IR/Turkey pox virus/ H335/12	TP/H335/12	Turkey	Cutaneous	2012	Alborz	KT003290

Table 2: Details of poxvirus sequences obtained from GenBank

Isolate name	Abbreviation (Tree)	Host	Country	Accession Number	Clades
Fowlpox virus isolate FPV-VR250	FP/FPV-VR250	Chicken	Norway	AY453172	A1
FowlpoxvirusNobilis Variole W (Intervet)	FP/ Nobilis Variole W	Chicken	United Kingdom	AM050379	A1
Fowlpox Mild (Websters; Fort Dodge)	FP/Websters Mild	Chicken	United Kingdom	AM050378	A1
Avipoxvirus isolate GB 134/01	TP/ GB 134	Turkey	Germany	AY530304	A1
FWPVD Diftosec CT (Merial)	FP/ Diftosec CT	Chicken	United Kingdom	AM050380	A1
pigeonpox PGPV TP-2	PP/ TP-2	Pigeon	Germany	AY530303	A2
Avipoxvirus isolate CVL 2/11/66	TP/ CVL 66	Turkey	United Kingdom	AM050387	A2
Avipoxvirus isolate CVL 10/12/98	TP/ CVL 98	Turkey	United Kingdom	AM050388	A2
Avipoxvirus HNPV/NZL/2002	PP/ HNPV	Pigeon	New Zealand	HQ701713	A3
AvipoxvirusCVL 353/87	AP/CVL 87	Albatross	United Kingdom	AM050392	A3
Falconpox FLPV GB362-02	FLP/ GB362-02	Falcon	Germany	AY530306	A4
Canarypox virus isolate CP10IR	CP/CP10IR	Canary	Iran	KC193679	B1
Canarypox virus isolate Yazd1	CP/Yazd1	Canary	Iran	KF673397	B1
Canarypox virus strain AT_Canarypox/1839/2009	CP/1839	Canary	Austria	GU108510	B1
anarypox virus isolate D98-11133	CP/ D98-11133	Canary	Canada	GQ487567	B1
Avipoxvirus CVL	SP/ CVL	Sparrow	United Kingdom	AM05038	B1
Pigeonpox PPV-B7	PP/PPV-B7	Pigeon	Norway	AY453177	B2
Parrot pox 364/89	PP/364/89	Parrot	United Kingdom	AM050383	C
Avipoxvirus isolate APIII	AP/ APIII	Agapornis	Germany	AY530311	C

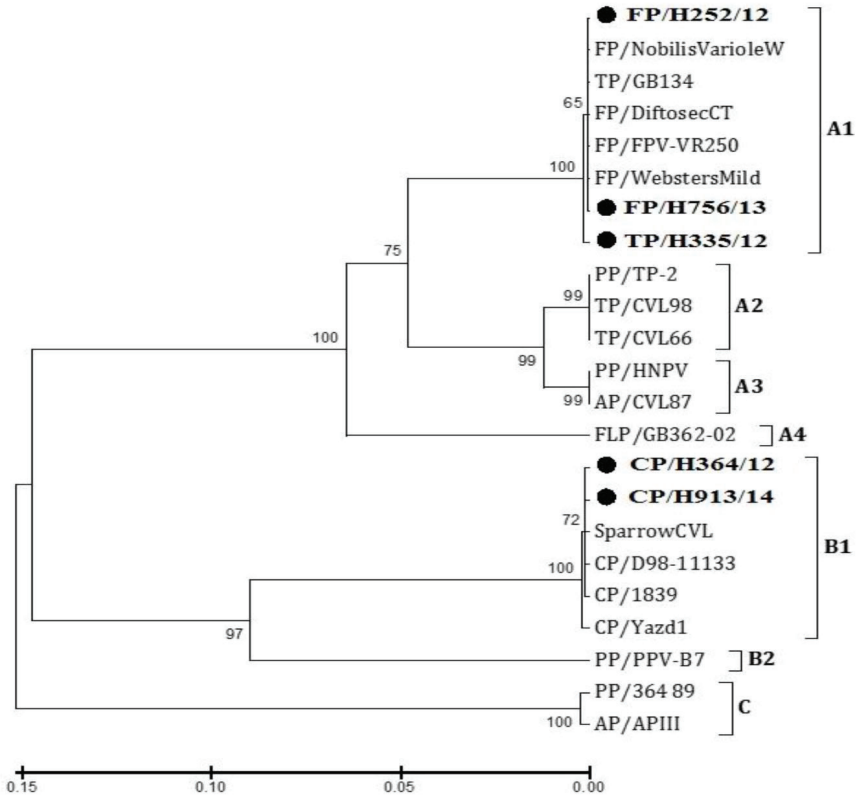

Figure 1: Phylogenetic tree of 578 bp nucleotide sequences of the 4b core protein gene of APV isolated in this study (marked with a black circle), reference APV sequences. The tree was obtained by the neighbour-joining method. Bootstrap testing of phylogeny was performed with 1000 replications and values equal to or greater than 70 are indicated on the branches (as a percentage).The length of each bar indicates the amount of evolution along the horizontal branches as measured by substitution per site. APV clades A–C and sub clades are labelled. You could find the details of viruses in tables 1 &2

Table 3: Percentage of 4b core protein sequence identity of APV isolated in this study and some selected APV isolates from GenBank

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	CP/GHPCRLAB.1																								
2	CP/GHPCRLAB.2	99.04																							
3	FP/GHPCRLAB.3	70.26	70.91																						
4	FP/GHPCRLAB.4	70.67	71.32	99.52																					
5	TP/GHPCRLAB.5	71.02	71.66	99.28	99.76																				
6	TP/GB134	70.67	71.32	99.52	100.00	99.76																			
7	FP/WebstersMild	70.67	71.32	99.52	100.00	99.76	100.00																		
8	FP/NobilisVarioleW	70.67	71.32	99.52	100.00	99.76	100.00	100.00																	
9	TP/CVL98	70.18	70.53	89.44	90.00	89.73	90.00	90.00	90.00																
10	FP/DiftosecCT	70.67	71.32	99.52	100.00	99.76	100.00	100.00	100.00	90.00															
11	FP/FPV-VR250	70.67	71.32	99.52	100.00	99.76	100.00	100.00	100.00	90.00	100.00														
12	PP/TP-2	70.18	70.53	89.44	90.00	89.73	90.00	90.00	90.00	100.00	90.00	90.00													
13	TP/CVL66	70.18	70.53	89.44	90.00	89.73	90.00	90.00	90.00	100.00	90.00	90.00	100.00												
14	AP/CVL87	69.11	69.37	90.54	91.09	90.82	91.09	91.09	91.09	97.58	91.09	91.09	97.58	97.58											
15	PP/HNPV	69.11	69.37	90.54	91.09	90.82	91.09	91.09	91.09	97.58	91.09	91.09	97.58	97.58	100.00										
16	FLP/GB362-02	70.91	71.99	86.28	86.86	86.58	86.86	86.86	86.86	87.65	86.86	86.86	87.65	87.65	87.10	87.10									
17	SparrowCVL	99.28	99.76	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62								
18	CP/D98-11133	99.28	99.76	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62	100.00							
19	CP/1839	99.28	99.76	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62	100.00	100.00						
20	CP/Yazd1	99.04	99.52	71.28	71.69	72.03	71.69	71.69	71.69	70.91	71.69	71.69	70.91	70.91	69.76	69.76	71.62	99.76	99.76	99.76					
21	CP/CP10IR	93.28	93.54	64.31	64.77	65.13	64.77	64.77	64.77	63.98	64.77	64.77	63.98	63.98	62.72	62.72	64.27	93.80	93.80	93.80	93.54				
22	PP/PPV-B7	81.26	82.15	73.29	73.69	73.36	73.69	73.69	73.69	75.15	73.69	73.69	75.15	75.15	75.15	75.15	75.88	82.15	82.15	82.15	81.83	75.12			
23	PP/364_89	71.66	71.62	70.77	71.16	71.14	71.16	71.16	71.16	69.95	71.16	71.16	69.95	69.95	69.26	69.26	67.17	71.99	71.99	71.99	71.62	65.17	72.37		
24	AP/APIII	70.91	70.87	70.39	70.80	70.77	70.80	70.80	70.80	69.57	70.80	70.80	69.57	69.57	68.87	68.87	66.36	71.25	71.25	71.25	70.87	65.17	72.37	99.52	

Discussion

Avian pox viruses have been isolated from a wide range of avian species including commercial poultry, wild and pet birds. Poxvirus infection is suspected when proliferative skin and/or oral and tracheal lesions are observed. In such cases, a diagnosis is made by histopathology examination of the lesions. Fowl pox vaccine is used in Iranian poultry industry in layer and breeder farms. We don't have any specific vaccine for pet birds. Canary pox has been known as the disease that can result in high losses in a short time, as a re-emerging disease that has not been present during recent years in canary flocks in Iran (12).

Most of our knowledge about the situation of APVs in Iran was obtained from very few reports concerning the epidemiology and the infection biology of the virus. In this study, APVs in clinical cases of affected commercial chickens, turkeys, and canary were identified and characterized by molecular methods to determine the etiology of APV in Iran, 2012. As for the molecular biological analysis, gene P4b amplification products of the expected size were obtained for all the strains of this study, thus confirming that PCR is an extremely valuable diagnostic method for APV infections. Phylogenetic relationships of Avipoxviruses have been analyzed based on the gene corresponding to *vaccinia* virus (VACV) P4b (fpv167, VACV A3L), indicating that all Avipoxvirus strains cluster into 3 major clades, namely, A (Fowl pox (FWPV)-like), B (Canary pox (CNPV)-like) and C (Psittacine). Clade A can be further divided into seven sub clades (A1-A7) and Clade B is comprised of three sub clades (B1-B3) (14). Based on the phylogenetic analysis of four conserved regions, the viruses characterized from Iranian columbiformes cluster into two groups. The viruses from turkey and commercial chickens grouped in sub clade A1 and the viruses from canary grouped in sub clade B1. Conversely, it has also been shown that the same viruses can infect different birds. Therefore, in this study as well as in others, APVs from the same species of bird are classified in different sub clades (4,13,14). Fasaei *et al* (2014) in phylogenetic analysis of Avipoxvirus strains isolated from different bird species in Iran showed that a similarity of 71-100% with the other sequences in the GenBank but they didn't submit their sequences in GenBank and didn't determine the clade of isolates (3). The research which did by Gholami-Ahangaran (2014) on avian pox of backyard poultry in Iran indicated that 66.1% and 80.7% of samples were positive for avian pox virus on histopathological and PCR examination, respectively (8).

The data presented in this research provide novel insights into the molecular characterization of avian pox viruses collected from the broad host range outbreaks in different geographical parts of Iran on particular period time.

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MOLEKULARNA KARAKTERIZACIJA IN FILOGENETSKA ANALIZA AVIARNEGA VIRUSA POX, IZOLIRANEGA PRI PETIH VRSTAH PTIC V KOMERICIALNIH JATAH V IRANU

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Povzetek: Osepnice ptic (AP, iz angl. avian pox) so virusna bolezen, ki lahko okužijo veliko različnih vrst ptic. Cilj predstavljene raziskave je bila molekularna identifikacija in karakterizacija izoliranih virusov pox, pridobljenih iz krast ljubiteljskih ptic in farmskih ptic v Iranu, s pomočjo metode PCR. Vzorci krast s področja sprememb na koži in sluznicah je bil zbran pri petih pacientih s kliničnimi znaki bolezni. S pomočjo metode PCR je bil pomnožen 578 baznih parov dolg odsek gena poxvirusa 4b. To ohranjeno območje poxvirusa je pomembno za določitev genskih razmerij med virusi, zato je bilo določeno zaporedje DNK za nadaljne analize. Iranska izolata Avipoxvirusa objavljena v tej študiji sta bila razvrščena v razred A1 (komercialne piščančje in puranje jate) in B1 (kanarčki). Za boljše razumevanje molekularne karakterizacije iranskih sevov virusa AP bo potrebno opraviti nadaljnje študije.

Ključne besede: osepnice ptic; filogenetska analiza; molekularna karakterizacija; Iran

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Original Research Article

THE EFFECT OF SUPRAPHYSIOLOGICAL DOSES OF VITAMIN E ON PERFORMANCE OF BROILER BREEDERS FLOCK

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Abstract: The effect of supplementary vitamin E levels on the performance of broiler breeder flocks was studied. A completely randomized design with four treatments (100, 500, 1000, or 1500 IU vitamin E/kg) and three replicates per treatment was used. Each replicate included seven females and one male broiler breeder. Rearing conditions, lighting, temperature, humidity, amount of feed, and the amount of other nutrients in the diet were equal for all treatment groups and according to specifications of the management guide for broiler production. Eggs from each replicate were collected up to six times daily for nine weeks. Every three days, eggs were transferred to a hatchery and their characteristics were determined. The results showed 5- to 15-fold Vitamin E levels over the recommended daily dose had significant negative effects on hatchability and related parameters as well as on herd economic index ($P<0.05$).

Key words: broiler breeder; hen; tocopherol; reproductive; performance

Introduction

Vitamin E has been shown to be an essential vitamin in the diet of poultry (1). Vitamin E, if given in sufficient amount in the poultry diet, can be stored in the body and distributed throughout different tissues and organs (2). In the past, there have been some reports on the vitamin requirements of broiler breeders (3, 4). However, genetic, management practice and environmental improvements with broiler breeder flocks has resulted in alteration of their vitamin requirements (5). Reports about the amount of vitamin requirements for broiler breeder are available (3, 4), but the vitamin formulation is no longer adequate for the actual broiler breeder flocks. (1). There are reports on role of vitamins on reproductive performance of broiler breeder hen and roosters (6, 7, 8, 9, 10). Based on most important findings it seems that absorption of vitamin E is facilitated by dietary fat, intestinal bile and pancreatic lipase, and enters the lacteals for transport. The main pathway of its excretion is through defecation. The main role of this vitamin is as an antioxidant, preventing the formation of peroxides. Vitamin E is protective for vitamin A and carotenoids in some tissues, e.g. gastrointestinal

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tract. The requirement of vitamin E increases whenever unsaturated fats are supplemented in poultry diet. Since there are different amounts of unsaturated fats added to commercial breeder broiler diets, the requirement of vitamin E can change based on fat contents (11). More research is needed in order to determine the requirement of vitamin E for the broiler breeder hen and rooster under different commercial conditions.

Vitamin E was recently shown to have antioxidant properties, protecting of cell membranes in both intra- and extracellular spaces (12). In the absence of sufficient vitamin E, breakdown of cell structure occurs, due to the formation of hydroperoxides from unsaturated fatty acids. There is a recent report on the positive effects of extra doses of this vitamin on performance, hatching process, and chick quality in broiler breeder flocks (5). The objective of this experiment was to evaluate the effect of supraphysiological concentration levels of vitamin E on the reproductive performance of broiler breeder hens.

Materials and methods

A total of 96 Ross-308 broiler breeders (84 hens and 12 roosters), aged between 61 and 69 weeks, were randomly assigned to four treatment groups. The birds were selected at the end of their production cycle, because it was expected that doses of vitamin E above standard recommendations would improve their performance and inhibit a decline in egg production. The observation period was nine weeks. Hens and roosters weighed, on average, 4.24 ± 0.1 and 4.96 ± 0.1 kg, respectively. Mean body weight for all replicates was the same at the start of the trial. There were three replicates per treatment with seven hens and one rooster.

The experimental facility was surrounded with fences and nets, and the cages (2.0 × 1.5 × 1.0 m) were placed on the ground, in a row, and each cage had one door to the outside. In each cage, there were nests for hens, one for the rooster and a two-floor egg-laying trap. A nipple system, in which water was supplied from a central repository, was created around the cages. In preparation for the trial, the poultry facility was cleaned, rinsed and disinfected. Poultry facilities had thermostatically controlled curtains and cross-ventilation. The lighting schedule consisted 16:8 h light:dark during the period of 61 to 69 weeks of the tests.

The four levels of vitamin E were 100 (Control), 500 (Treatment 2), 1000 (Treatment 3) and 1500 (Treatment 4) IU vitamin E/kg diet. 100 IU vitamin E/kg in the diet represents the recommended concentration of vitamin E. The effect of the doses was evaluated for 63 days. A standard broiler breeder's diet (2800 kcal of ME/kg and 14.5% CP for female and 2700 kcal of ME/kg and 13.5% CP for male) was fed to meet or exceed broiler breeder nutrient requirements (13). Feed was provided ad libitum and feed intake calculated weekly by difference between the quantities fed and left over. The ingredient composition and the energy content of the diet were kept similar for all the groups (Table 1).

Weekly egg production, egg counts, and egg weights per pen were recorded. Eggs were manually collected, weighted, and incubated at a commercial hatchery. At the hatchery, they were candled by a trained operator for eggshell defects and screened at day 12 for unhatched eggs. Unhatched and hatched eggs were sorted out, counted and weighted (hatchable produced egg number, hatchable total egg weight, hatchable egg weight average, dirty egg number, hatchable total egg weight at transfer incubator to hatcher, hatchable egg weight average at transfer incubator to hatcher, egg loss weight).

At the end of incubation, the number of live healthy chicks was counted ("chicks produced") and eggs weighed (total chick weight and mean chick weight). "Chick yield" was the percentage of initial eggs from which live chicks hatched (number of eggs hatched/number of initial eggs). "Percent hatchability: was calculated from eggs yielding live chicks (number of eggs hatched/ number of fertile eggs). "Economic index" was calculated as sum of the weekly chick number and half of the dirty eggs.

Statistical analysis was performed with SPSS (14). For statistical analysis, the replicate chicken pen was considered as the experimental unit. Data were subjected to a mixed linear model, with replicates as random factors, treatment as fixed factors and performance parameters as dependent variables. A completely randomized design, with four treatments and three replicates per treatment, was used. Alternatively, Kruskall-Wallis test was used. If both tests were significant, subsequent Pairwise testing was conducted using Bonferroni's method. Data between 0-0.5, or percentages between 0-30 were transformed into $x^{0.5}+0.5$.

Results

Neither hen nor rooster weights differed significantly among the treatment groups at the beginning and at the end of the trial. Hens produced 1.82 dirty eggs/hen in the Control group, whereas in Treatment 4 only 0.81 dirty eggs/hen were produced (p=0.013) (Table 2). The percentage of egg loss weight differed significantly (p=0.052) between the Control group and Treatment 4. Hens produced 16.71 chicks/hen in the Control group, whereas in Treatment 4 only 7.05 chicks/hen were produced (p=0.023). Hens in the Control group produced 1103.81 g total chick weight, which was significantly superior (p=0.003) compared to 756.21 g, 783.76 g and 387.28 g in Treatments 2, 3, and 4 respectively. The hatchability percentage was significantly lower in Treatment 4 (35.58%) compared to the other groups. The economical index confirmed the significantly inferior results (p=0.008) reported for Treatment 4 (7.45).

Other traits were not significantly affected by the treatments. Briefly, hens in Treatment 3 had the greatest number hatchable eggs, hatchable total egg weight, hatchable total egg weight at transfer from incubator to hatchery,

Table 1: Experimental diets fed to broiler breeder hens and roosters

	Hens	Roosters
Ingredients, %		
Maize	69.64	66
Soybean meal (44% protein)	18	11
Wheat bran	2.47	18.37
Vitamin and Mineral premix	0.5	0.5
Calcium carbonate	6.5	1.5
Oysters shell	0.5	0.5
Dicalcium phosphate	1.4	1.4
DL-methionine	0.4	0.08
L-lysine HCL	0.01	0.07
Salt	0.2	0.2
Sodium bicarbonate	0.15	0.15
Natuzyme P	0.03	0.03
Toxin binder	0.1	0.1
Formycine Gold	0.1	0.1
Calculated analysis		
Metabolizable energy, kcal kg^{-1}	2800	2700
Crude protein, %	14.0	13.5
Calcium, %	3.0	1.1
Available Phosphorus, %	0.4	0.4
Sodium, %	0.16	0.16
Chloride, %	0.16	0.18
Lysine, %	0.6	0.6
Methionine, %	0.033	0.3
Ether extract, %	2.9	3.15
Fibre, %	2.9	4.2
Linoleic acid, %	1.65	1.8

[1] Supplied per kilogram of feed - Vitamin A: 12500 IU; vitamin D_3: 1250 IU; vitamin E: 18 IU; vitamin K_3: 3.7 mg; thiamine: 1.8 mg; riboflavin: 6.6 mg; calcium pantothenate: 10 mg; niacin: 37.5 mg; pyridoxine: 32.5 mg; vitamin B12: 2.5 mg; Mn: 50 mg; Zn: 37.5 mg; Fe: 25 mg; Cu: 7.5 mg.

Table 2: Effects of vitamin E on reproductive performance of Ross broiler breeder hens during the trial

		Hatchabale produced egg number (N/female broiler breeder)	Hatchable total egg weight (g/female broiler breeder)	Hatchable egg weight average (g/egg)	Dirty egg (n/female broiler breeder)	Hatchable total egg weight at transfer incubator to hatcher (g/female broiler breeder)	Hatchable egg weight average at transfer incubator to hatcher (g/egg)	Egg loss weight (%)	Produced chick (N/female broiler breeder)	Total chick weight (g/female broiler breeder)	Chick weight average (g/chick)	Chick yield (%)	Hatchability (%)	Economic index **
Vitamin E*	100	21.96	2230.68	71.81	1.82^{a}	2035.93	63.63	11.37^{a}	16.71^{a}	1103.81^{a}	48.72	67.82	75.67^{a}	17.63^{a}
	500	20.48	1928.57	72.27	1.19^{ab}	2042.86	64.98	10.09^{b}	13.38^{a}	756.21^{b}	48.65	67.36	67.36^{ab}	13.98^{a}
	1000	22.95	2314.67	70.04	1.19^{ab}	2369.05	62.73	10.43^{ab}	13.14^{a}	783.76^{b}	48.08	68.69	56.39^{b}	13.74^{a}
	1500	22.95	2220.76	70.94	0.81^{b}	2193.90	64.19	9.51^{b}	7.05^{b}	387.28^{c}	47.71	67.32	35.58^{c}	7.45^{b}
SEM		2.270	253.994	0.949	0.427	234.396	0.723	0.476	1.640	85.188	0.966	0.712	4.344	1.474
P-Value		0.839	0.743	0.434	0.013	0.672	0.199	0.052	0.023	0.003	0.895	0.528	0.001	0.008

Values within the same column followed by different superscript letters differ significantly (P<0.05).
SEM: Standard Error of Means. * = % relative to standard catalogue; ** = chick + 1/2 dirty egg

and chick yield compared to other treatments, but these were not significantly different from the other treatments. Hens in Treatment 2 had only a numerically greater mean hatchable egg weight and hatchable egg weight at transfer from incubator to egg hatcher, compared to other treatments. Finally, the Control group had the highest chick weight average compared to other treatments, but again the difference was not statistically significant (Table 2).

Discussion

Next to the cost of feeding, the major benchmark for a broiler breeding farm is the number of chicks produced per hen. Furthermore, these chicks must be of optimal weight for successful future meat production. Thus, presuming management conditions are optimal, the total number of chicks delivered per hen for rearing and the total weights of these of chicks per hen are two important parameters. Hens supplemented with a normal level of vitamin E in their ration (control) produced 16.71 chicks per hen, which was better than that of hens fed with overdoses. Furthermore, the total weight of all chicks produced per hen of the Control group was significantly more that of the three treatment groups and resulted in a better economic index. Since mean chick weight was around 48 grams, the high numbers of chicks produced in the control group was the major success factor.

The numbers of hatched chicks per hen depend on the numbers of eggs laid, as well as on the numbers of eggs that are fertilized by the roosters. Thus, health is important for both parents.

One explanation for the increased production of chicks per hen could be due to increased egg production per hen, but the number of hatchable eggs per treatment group was not significantly different. Thus, in our studies, egg production was not significantly affected by excessive vitamin E level in the feed.

More important for hatchability is the quality of the hatchable and fertilized eggs per hen. It appears that above normal doses of vitamin E did not affect egg production per se, but dramatically decreased hatchability.

Other research did not find positive effects of high levels of vitamin E supplementation on chick yield and weight in laying breeders (3). Yet another study showed that a deficiency of vitamin E lead to impairment of the reproductive organs, such as degeneration of the seminiferous tubules, degenerative spermatogonia and testicular damage (15). Vitamin E as a free radical scavenger can prevent the oxidation of readily oxidized substances. The potential of vitamin E to act as a lipid-based radical chain-breaking agent and protect against free-radical attack is considered fundamental to its effects in biological systems such as broiler breeders (16). Apparently, the expected beneficial antioxidant activity of larger than normal doses of vitamin E compromises the development of the chicken embryo, resulting in poor hatchability. Negative effects of "hyperdoses" of vitamin E have been linked to impairment of absorption of vitamins A and D3. Furthermore, weak bone calcification was observed when birds received "hyperdoses" of vitamin E, and body weight decreased after an increase of dietary vitamin E (17). At "hyperdoses" of vitamin E, there was decreased pigmentation of the shanks, feet and beak and waxy feathers. There are similar studies about positive effects of optimum doses and negative effects of "hyperdoses" of vitamin E (18, 19, 20, 21, 22).

Our results may be explained by findings of meta-analyses of clinical studies in man that showed that supplementation of vitamin E as antioxidants did not result in the presumed health benefit, but was rather associated with increased mortality (22). The conclusion of this trial is that the vitamin E levels of 100 IU/kg, as currently advised in broiler breeder production, should not be increased.

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UČINEK SUPRAFIZIOLOŠKIH ODMERKOV VITAMINA E NA PRIRAŠČANJE VZREJNIH BROJLERJSKIH JAT

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Povzetek: V študiji smo raziskovali učinek dodajanja vitamina E na hitrost prirasta v vzrejnih brojlerskih jatah. Študija je bila zasnovana naključno s štirimi koncentracijami dodatka (100, 500, 1000 ali 1500 i.e. vitamina E/kg telesne mase) in tremi ponovitvami tretiranja za vsako koncentracijo dodanega vitamina E. Vsaka ponovitev je vključevala sedem samic in enega brojlerskega samca. Pogoji reje, razsvetljava, temperatura, vlaga, količina krme in količina drugih hranilnih snovi v prehrani so bili enaki za vse poskusne skupine in v skladu s specifikacijami reje pitovnih piščancev. Jajca iz vsake ponovitve tretiranj po skupinah so bila zbrana do šestkrat dnevno devet tednov. Vsake tri dni so bila jajca prenesena v valilnico, kjer smo določili njihove značilnosti. Rezultati so pokazali da so imele 5- do 15-kratne vrednosti vitamina E nad priporočenim dnevnim odmerkom značilno negativne učinke na sposobnost valjenja in sorodnih parametrov, kakor tudi na na čredni gospodarski indeks ($P<0,05$).

Ključne besede: brojlerji; kokoš; vitamin E; razmnoževanje

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Original Research Article

TRENDS IN VETERINARY EXPERT OPINIONS ON ANIMALS

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Abstract: This paper analyses veterinary expert opinions and determines the most common reasons for appointing veterinarians as expert witness in cases related to different animal species. The paper also summarises twenty one years of services provided by the Department of Forensic Veterinary Medicine and Administration, University of Warmia and Mazury in Olsztyn in 1995 - 2015. The analysis was based on 319 expert opinions, of which 172 concerned various animal species and is presented in this work. Criminal judicial bodies were the most common ordering party, followed by civil judicial bodies, natural persons, public administration authorities and insurance companies. To determine the trends in conflicting situations in the expert opinions issued over 21 years, two periods of opinions were distinguished: the first period covering the years 1995 – 2005 (73 expert opinions) and the second one covered 2006 – 2015 (99 expert opinions). The authors demonstrated that in the second period, companion animals (mainly dogs) were far more often the subject of expert opinions than in 1995 – 2005 and the cases predominantly referred to animal cruelty issues. The second most common group were production animals (cattle, horses and pigs) followed by wild living animals. The expert opinions concerned the animals themselves but also animal products, particularly the observance of sanitary and hygiene measures at different manufacturing stages. The diversity of cases and conflicts in which a veterinarian acts as the expert witness is also increasing.

Key words: veterinary forensic medicine; expert opinions; animals; conflicts; veterinary practice

Introduction

In its complexity and diversity, animal breeding and rearing gives rise to numerous conflicting situations which can generate economic losses. This, together with breeders' increasing awareness of legal issues, has led to increasing numbers of conflicts reaching the courts (1, 2). Such cases, as they are specific in nature and legal background and need a thorough evaluation of the facts, often require expert opinions based on specialized knowledge (2, 3, 4, 5). For this purpose, judicial bodies appoint veterinarians with theoretical and practical expertise of veterinary medicine as an expert witness (6, 7, 8, 9, 10).

An expert witness is an auxiliary entity in pre-trial proceedings (investigation or probe) and in the court (3, 9). The police, prosecutors and the court itself can question the expert witness, thereby defining the scope of her or his activities (11, 12, 13). The expert opinion issued by an expert witness equals other forensic evidence and is arbitrarily evaluated by the judicial bodies, whereas an opinion ordered by other entities (a so-called "private opinion") does not serve as evidence in a conflict situation but it is only considered by investigating bodies (5, 10, 13, 14). The role of a veterinarian as an expert witness is to assess the factual circumstances of a given event

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together with specifying its causes and identifying its consequences (11, 15, 16, 17).

The objective of the paper is to analyse the expert opinions issued over twenty years by the Department of Forensic Veterinary Medicine and Administration, University of Warmia and Mazury in Olsztyn and identify the main source of conflicts.

Materials and methods

The analysis was based on 172 opinions issued in 1995 - 2015 by the Department of Forensic Veterinary Medicine and Administration, University of Warmia and Mazury (UWM) (formerly of the Agricultural and Technical Academy in 1995 - 1999) in Olsztyn, for ordering parties from all over Poland. The analysis included the animal species (except for poultry, due to the specificity of husbandry), the ordering parties, the reasons for conflicts or production failures and decisions/propositions of expert witness. To determine the trends in conflicting situations in the expert opinions issued over 21 years, two periods of opinions were distinguished: the first period covering the years 1995 – 2005 (73 expert opinions) and the second one covered 2006 – 2015 (99 expert opinions).

Results and discussion

Animal species as the subject of conflicts, and entities initiating veterinary expert opinions

It was found that of 172 expert opinions issued in 1995 - 2015 by the Department of Forensic Veterinary Medicine and Administration, UWM, 70 documents referred to dogs, 25 to cattle, 18 to horses, 9 to cats and 10 to pigs. The remaining 40 opinions were related to different animal species, such as wild boars, eagles, rabbits, chinchillas, roe deer, cormorants, turtles and foxes. The animal species and number of expert opinions are presented in Fig. 1.

Judicial bodies involved in criminal investigations (i.e. the courts, police and prosecutors - 70 and civil courts - 57) were the most common ordering parties. A significantly smaller number of expert opinions were issued for private persons (13), different public administration authorities (10), insurance companies (10) and other entities, e.g. hunting clubs, veterinary tribunals and screeners for veterinary professional liabilities (12 expert opinions) (Fig. 2).

Expert opinions on companion animals

The expert opinions issued for cases in which companion animals were the subject of conflicts were the most numerous group as compared with the opinions on the other animal species. This was particularly true for the second group of expert opinions. Most companion animals were the subject of a conflicting situation in the criminal proceedings. Some of the opinions were associated with necropsies; these included 64 opinions, of which in 58 documents dogs were necropsies and six opinions presented necropsy findings on cats, which constitutes approximately 37% of all issued expert opinions. During the first analysed period, 21 expert opinions referred to dogs and four to cats while the number of opinions on dogs significantly increased (about 133%) in the second period.

Almost half of the expert opinions on companion animals focused on animal cruelty issues (37 opinions) and single cases were related to negligence of animal welfare (lack of provision of veterinary care, food deprivation, keeping in poor conditions). In one opinion on poor transport conditions, the expert witness, further to necropsy, discovered that the death of a puppy resulted from PDA (patent ductus arteriosus; a congenital defect) (18). The most common causes of deaths were gunshot wounds (approximately 18%), poisonings (about 12%) and drowning/suffocation (approximately 7%). Most of the cases described were associated with animal abuse. There included mechanical injuries, including hard objects and stab wounds. There was also a case of a dog buried alive and a death due to pylorus obstruction with pieces of blood-moistened material (such an approach is used to "feed" dogs trained for dog-fighting). In two cases of dogs, necropsy was performed following cadaver exhumation; the causes of death were skull trauma in one case and suffocation in the other case. However, regarding poisonings, in two dogs and three cats the characteristic changes were caused by anticoagulant rodenticide intoxication (17). The origin of those substances was not proven.

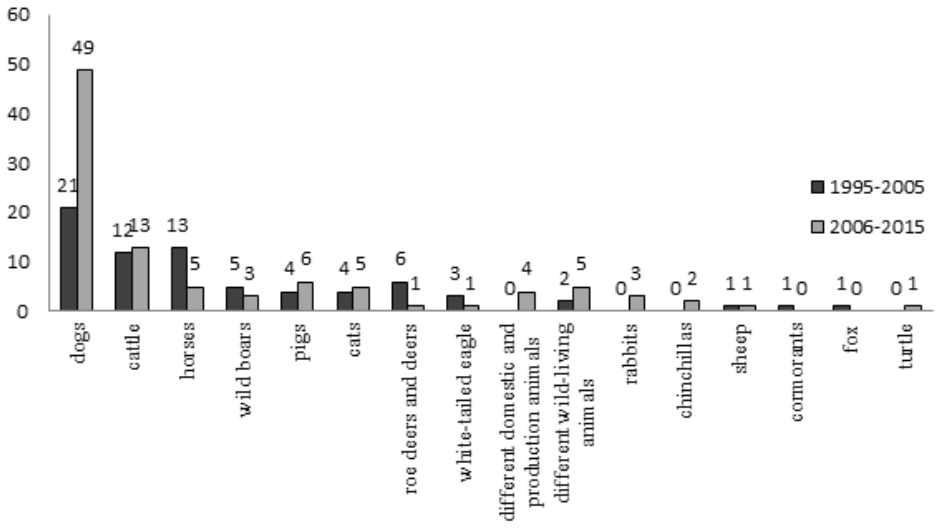

Figure 1: Animal species as the subject of expert opinions and the number of opinions issued by the Department of Forensics Veterinary Medicine and Administration, University of Warmia and Mazury in Olsztyn in 1995 - 2015

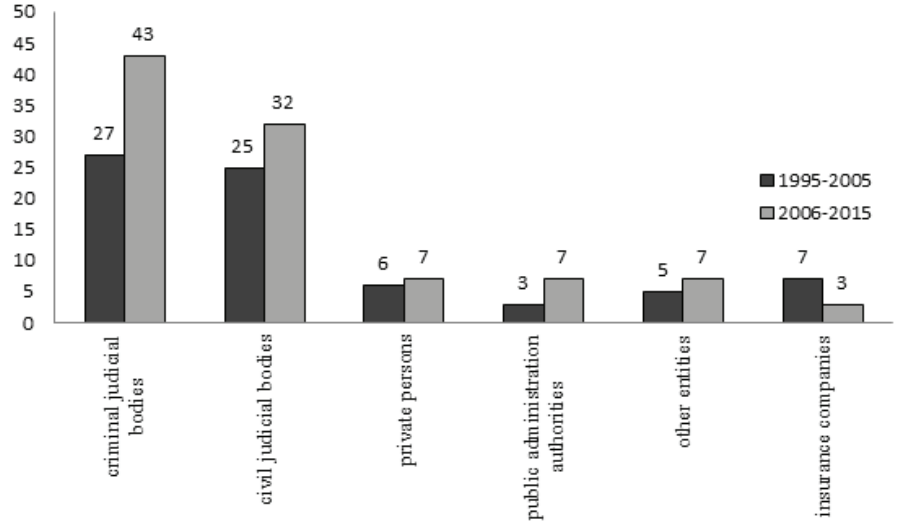

Figure 2: List of the ordering parties and number of expert opinions issued by the Department of Forensics and Veterinary Administration, University of Warmia and Mazury in Olsztyn in 1995 – 2015

In cases with veterinarians involving being sued, nine expert opinions were issued, with only one included in the first period, concerning a Ragdoll cat suffering from FIP (5, 18). In the second decade, eight cases referred to a medical error and the majority of them were linked to negligence during surgical procedures. It was found in two cases that the veterinarian was guilty of post-surgical complications (hernia removal and ovariohysterectomy). One case of internal haemorrhage after orchiectomy in cat was revealed, due to improper diagnosis and treatment. For one Bernese Mountain Dog, elbow dysplasia was not diagnosed and therefore no appropriate treatment was applied. In another case, the veterinary surgeon's error involved excessively tight bandaging on the dog's pelvic limb, after the removal of the skin lesion. As a result, a soft tissue necrosis occurred, complicated by an anaerobic bacterial infection. The consequence was the amputation of the limb. The patient was a valuable stud dog, which, as a result of the veterinarian's malpractice, could not be used properly.

In three analysed cases, no errors on the part of a veterinarian were discovered. In one of the analysed cases, the death of a bitch after a caesarean section occurred due to toxic shock. In another – a cat died during castration preparations, following anaphylaxis or shock after the administration of the proper aesthetic dose. Another case concerned a dog, in which the *causa mortis* was pancreatitis and intestinal perforation – but not due to a diagnostic laparotomy carried out by the veterinarian.

In four cases (5%), the expert opinions concerned conflicts associated with the sale transactions of animals (19). In two of them it was found that the purchased animals were not suitable for reproduction purposes due to a urogenital tract disease in a bitch and hip dysplasia in a male dog. Two further cases concerned puppies: in one case, the expert witness demonstrated in a necropsy that gastric torsion, and not a congenital defect, was the cause of death (18). In the second case, it was shown that incorrect prophylaxis resulted in an infectious disease (canine parvovirus infection).

One of the expert opinions was issued in relation to a veterinary technician who performed surgery on an animal in breach of his authorisation. His actions resulted in a prolonged healing time of the surgical site and, consequently, to potentiated suffering of the animal and higher treatment costs. In another case, the expert witness identified a missing dog based on the analysed materials. In one of the issued opinions, the case was initiated at the request of a screener for veterinary professional liability who claimed that a veterinarian had made medical errors during a caesarean section surgery resulting in the death of a bitch. Both the opinion and pre-trial proceedings proved that labour assistance was performed in the correct manner (19, 20).

Expert opinions on production animals

Of the analysed expert opinions, 25 documents concerned cattle (14.5%), mainly dairy cows (19), calves (3) and heifers (3). The rate of conflicts related to this species was similar over two periods.

It was found that 11 expert opinions were requested by criminal judicial bodies. Four of these opinions concerned animal cruelty (lack of veterinary care and of proper nursing, starvation, excessive animal compaction), two were associated with identifying the cause of death and one expert opinion referred to an epidemiological risk created by bovine enzootic pneumonia. Another opinion was aimed at determining the losses caused by a driver involved in a car accident involving a cow. In one of the opinions, the veterinarian was found to have acted correctly in a case involving the diagnostic slaughtering of five cows. Another case involving criminal proceedings concerned the use of a falsified health certificate that was issued in an erroneous manner by a veterinarian. As a result, a claimant purchased cattle infected with rhinotracheitis infections. The last expert opinion in criminal proceedings referred to a cow which was in labour when transported to a slaughterhouse together with a calf with a broken limb; the animals were exposed to stress and suffering (21).

Fourteen expert opinions were requested by civil judicial bodies. In seven cases, the ordering parties were insurance companies and in four of such situations, a medical error by a veterinarian was proven and, in one case, reduced utility value of dairy cows was determined. Two expert opinions concerned the suitability of dead cow meat for consumption (the cows suffered from fatal electric shock). One case was aimed at determining the cause of a high somatic cell count in milk and another case involved determining whether it had been possible to prevent the death of a cow during labour. Issues related to veterinary malpractice concerned both complications due to surgical procedures (causing heavy haemorrhage after the uterine artery cut, during caesarean surgery; incorrect birth assistance) and non-compliance with the asepsis rules of vaccination.

An analysis of one case concerned damages to an owner who did not meet his contractual duties, resulting in the euthanasia of an animal. In one of the expert opinions, the expert witnesses were requested to determine whether incorrect installation of silos resulted in diseases and losses of animals. An analysis of factual circumstances demonstrated that the losses were the result of intoxication caused by improperly stored feed. One opinion was requested by a pharmaceutical company. The case concerned determining the occurrence of post-vaccine adverse effects such as local reactions and general malaise of animals. The expert witness found that the poor health condition of cattle was due to *Trueperella pyogenes* bacterium. The infection probably resulted from bacteria entering the body at the vaccine injection site (20, 22). In one of the cases it was shown that an incorrectly formulated feed ration which triggered ketosis was complicated by a BVD/MD virus infection and *Clostridium perfringens* enteric toxin (22). In other cases, analyses were conducted to determine the cause of reduced milk production and fertility as well as deaths in cows due to incorrectly balanced feed rations.

Ten expert opinions concerned pigs (5.8% of the analysed cases), of which six cases were requested

by civil judicial bodies, two by criminal judicial bodies and the other two by private persons. Seven of sale transactions and one referred to settling the health status of weaners brought onto a farm by a petitioner. In one of the expert opinions, the cause of death and reduced growth rates in pigs was determined (salmonellosis) (22). Another case concerned determining the losses incurred by a producer who caused a fire in a swine facility.

Two expert opinions concerned sheep (1.2%). In one of them, the issue was to determine the cause of losses in sheep production (pulmonary adenomatosis was found to be the cause). The other case referred to the death of sheep resulting from spinal cord insult and multiple bite wounds.

A comparison of two periods (1995 - 2005 and 2006 - 2015) demonstrates that the proportion of expert opinions on production animals was at a comparable level.

Expert opinions on horses

The analysis showed that 18 (10.5%) expert opinions concerned horses. Of them, eight opinions referred to poor husbandry and management with negligence, beating and work overload. In two criminal proceedings, it was proven that horses (including Konik horses) were kept under inadequate husbandry conditions in autumn and winter seasons. They were incorrectly fed and deprived of proper veterinary care (20). Seven expert opinions questioned the actions of a veterinarian. In one case, a case of death was analysed (a person kicked by a Hucul horse). In three opinions, fraud was revealed in sale and purchase transactions, i.e. selling horses with physical defects preventing the stated purpose of the animals (4, 19). Over 21 years, the proportion of cases involving horses has changed significantly: in the second time period (2006 - 2015), the number of such expert opinions decreased by over 60%.

Other expert opinions

Other expert opinions constitute approximately 20% of cases (34 opinions). Among them, over half concern wild-living animals such as roe deer, deer, wild boars, eagles or foxes. Most of them were related to determining the cause of death, of which shotgun wounds were the most common (23). In one expert opinion on a white-tailed eagle, the necropsy revealed cardiorespiratory failure due to mechanical trauma as the cause of death. The expert witnesses were equally often requested to identify an animal species from which meat and illegally owned hunting trophies (such as skin or antlers) were obtained. One opinion was requested by a natural person and concerned a comparison of submitted canine hair with a hair coat found on the fence of premises. It was identified as fox hair. In another case, the role of expert witness, as requested by an insurance company, was to determine whether damage to a car was caused by a traffic accident involving a wild boar (9).

Veterinarians also act as expert witness in cases involving people or their property. Three such opinions concerned damage for traffic accidents involving a wild boar, a cow and a dog. In one case of an expert opinion requested by a poultry slaughter house, the role of the expert witness was to determine whether the retina of an employee could have been damaged by turkey bile. Within the investigated time period, two cases concerning the deaths of humans were analysed. In the first case, the expert witness was requested to determine whether the injuries of a victim were inflicted by a horse kick (Hucul horse). In the second case, the court questioned the possibility of isolating human DNA from the faeces of dogs suspected of the fatal biting of a person.

Among cases involving rabbits, one expert opinion was requested by a pharmaceutical company on the adverse effects of a vaccine. In the second case, the expert witness assessed the correctness of a necropsy procedure performed by a veterinarian. In another case, the expert witness ruled out any impact of poor quality feed on animal deaths (in fact, infectious rhinitis was the aetiology).

In ten cases, products of animal origin were questioned. One expert opinion requested by an investigation body concerned the species origin and quality of a meat bath. The expert witness found significant discrepancies in storage conditions (hygiene aspects) and the quality of the tested material. In three expert opinions (including two complementary opinions), the expert witness was asked to estimate the value of meat after a road traffic accident. Due to the disruption of the cold chain, prolonged transport time and significant contamination, the meat was labelled as unsuitable for consumption or processing. Two expert opinions (major and supplementary)

concerned the infection of 225 persons with trichinosis. In that particular case, the expert witness did not find any negligence on the part of the employees of a slaughterhouse from where the meat had originated. Another two opinions were linked to the evaluation of an animal-derived product: pork fat contaminated with bristles and skin fragments. In two other cases brought against a district veterinary office, the expert witness found that the inspection frequencies in the abattoirs were insufficient and the records were erroneous.

Exotic animals were a rare subject of veterinary expert opinions. Over 21 years, only one opinion was issued and it regarded the species identification of a turtle. The expert witness stated that submitted photos represented a red-eared slider, a species that presents a risk to the native European pond turtle if it adapts to local climatic conditions.

The expert opinions issued on different animal species most often concerned animal cruelty, welfare negligence and poor husbandry conditions for production and companion animals. Within the selected time period, four such opinions were issued, including one brought against an animal shelter (2, 9, 16, 18).

Discussion

It was shown that the expert opinions were most often issued on companion animals (45.9%), mainly on dogs (40.7%), although in 1995 – 2005 34.2% of the expert opinions concerned companion animals and this number increased to 54.5% in the years 2006 - 2015. This is partly due to the increase in the number of companion animals in Poland. During this period, the population of these animals increased by approximately 2% per year (24). In addition the data indicate that conflicts based on animal treatment are increasing in Poland. An increasing tendency, as a reflexion of the presented facts, can be observed in issuing expert opinions as well. An analysis of the literature also indicates that this is a fairly common trend all around the world (9, 12, 16). The literature also indicates that awareness in providing animals with adequate welfare and, consequently, the need to respond to their harm is increasing (1, 16, 25, 26). It can be concluded that the increasing amount of expert veterinary opinions being issued are due to these, and not from a rise in human cruelty towards accompanying animals (25). This thesis is also supported by the increase in the number of shelters and accompanying animals staying in them (24, 27).

Production animals (such as cattle, horses and pigs) were the second-most common group of animals involved in these reports (32%). The cases of wild-living animals constituted 16.3% and mainly referred to poaching, illegal ownership of hunting trophies or identification of carcasses or meat from game animals. The other 5.8% of all expert opinions issued by the Department of Forensic Veterinary Medicine and Administration, University of Warmia and Mazury in Olsztyn encompassed varied aspects, such as animal origin products and compensation for traffic accidents involving animals.

An analysis of conflict etiology regarding companion animals shows that almost half of the opinions concerned animal cruelty (31 opinions). This fact indicates a relatively steady elimination of this phenomenon. The authors quoted emphasize the growth of common empathy for the suffering animals (1, 25). On the other hand, the recorded rise in the number of opinions related to the gunshot injuries to animals can be explained by changes in legal regulations facilitating weapon possession (28). The widespread availability of firearms results in both intentional and accidental injuries to animals (29, 30, 31).

A growing trend in issuing opinions concerning veterinarian malpractice can also be observed (9, 32, 33). In this paper, 2/3 of such cases revealed the veterinary surgeon's culpability. Relatively often, these cases involved insurance companies taking legal action.

The data indicates that the knowledge, expertise and experience of veterinarians serve both judicial bodies and different administration authorities, institutions and private persons and the diversity of cases and conflicts in which a veterinarian acts as an expert witness is also increasing (1, 2, 5, 7, 12, 15, 16, 25, 26).

Recapitulation

The analysis of the expert opinions issued by the Department of Forensic Veterinary Medicine and Administration, University of Warmia and Mazury in Olsztyn in 1995 - 2015 demonstrated

that most of the opinions were requested by criminal judicial bodies (40.7%) followed by civil judicial bodies (33.1%), natural persons (7.6%) and public administration authorities and insurance companies (5.8% each).

A comparison of the expert witness opinions issued in two investigated decades 1995 - 2015 demonstrated that the total number of opinions on different animal species was higher (by approximately 26%) in the second decade than in the first one.

The expert opinions that were issued concerned, apart from an assessment of prophylaxis and treatment choices, evaluation of husbandry conditions, animal welfare, feeding and animal identification as well as the biological materials of animal origin, entering into sale transactions and observing legal veterinary regulations.

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TRENDI STROKOVNIH VETERINARSKIH MNENJ O ŽIVALIH

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Povzetek: Prispevek presoja veterinarska strokovna mnenja in določa najpogostejše razloge za imenovanje veterinarjev kot strokovnjakov v primerih, povezanih z različnimi živalskimi vrstami. V prispevku je povzetih 21 let službe Oddelka za forenzično veterinarsko medicino in administracijo na Univerzi Warmia in Mazury v Olsztynu, in sicer od 1995 do 2015. Analiza je temeljila na 319 strokovnih mnenjih, od katerih jih je 172 obravnavalo različne živalske vrste, ki so predstavljene v tej študiji. Najpogostejši naročnik so bili kriminalni pravosodni organi, nato pa so sledila civilna pravosodna telesa, fizične osebe, organi javne uprave in zavarovalnice. Za določitev trendov v konfliktnih situacijah v strokovnih mnenjih, izdanih v zadnjih 21 letih, je bila raziskava razdeljena na dve ločeni obdobji: prvo obdobje od leta 1995 do leta 2005 (73 strokovnih mnenj) in drugo obdobje, ki zajema čas od leta 2006 do leta 2015 (99 mnenj strokovnjakov). Avtorji so pokazali, da so v drugem obdobju ljubiteljske vrste živali (predvsem psi) veliko bolj pogosto predmet strokovnih mnenj kot v zgodnejšem obdobju (1995 – 2005), pri čemer so se mnenja v največ primerih nanašale na vprašanja krutega ravnanja z živalmi. Druga najpogostejša skupina so bile proizvodne živali (govedo, konji in prašiči), tem pa so sledile divje živali. Strokovna mnenja so se nanašala na same živali, pa tudi na živalske proizvode, zlasti na upoštevanje sanitarnih in higienskih ukrepov na različnih stopnjah proizvodnje. Raznolikost primerov in konfliktov, v katerih veterinar deluje kot izvedenec se je prav tako povečalo.

Ključne besede: veterinarska forenzična medicina; strokovna mnenja; živali; konflikti; veterinarska praksa

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Original Research Article

ANTIMICROBIAL ACTIVITY AND ENZYMES ON SKIN MUCUS FROM MALE AND FEMALE CASPIAN KUTUM (*Rutilus frisii kutum* Kamensky, 1901) SPECIMENS

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Abstract: The mucus layer covering the surface of fish contain a high number of antimicrobial compounds that provide a first line of defense against aquatic pathogens. In the present study, bactericidal activity present on skin mucus of Caspian kutum *(Rutilus frisii kutum)* broodstock was tested against six pathogenic bacterial strains *(Streptococcus iniae*, *Yersinia ruckeri*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Escherichia coli)*. Furthermore, fungicidal activity was assessed against four pathogenic fungi *(Saprolegnia* sp., *Fusarium solani*, *Candida albicans* and Aspergillus fla*vus)*. Maximum and minimum antibacterial activity was observed against Y. ruckeri and S. iniae, respectively, while maximum and minimun fungicidal activty was detected against *F. solani* and *C. albicans*, respectively. Curiously, antimicrobial activity was higher in the fish mucus of female than male against most tested strains. In addition, minimum inhibitory concentration test showed that minimum concentrations of mucus ranged between 125 to 500 µg/L were able to inhibit the growth of the selected bacterial and fungal pathogens. Alkaline phosphatase, lysozyme, protease and esterase activities were also studied on mucus samples being the observed activities very similar between both sexes, although higher lysozyme activity was detected in the mucus of female fish in comparison to the values recorded on male samples. Skin mucus of this fish species (especially females) could be a potential source of newer and more effective antibacterial components.

Key words: *Rutilus frisii kutum*; skin mucus; bactericidal activity; antifungal activity; fish.

Introduction

The mucus layer is suggested to be multifunctional by displaying traits and actions important in osmoregulation, reduction of friction between fish and the aquatic environment and disease resistance (1). The major components of the mucus layer are produced by goblet cells. Goblet cells start to differentiate in the basal part of the epidermis, and then grow in size and move towards the surface where they release their content (1). In this way, the mucus is a dynamic coat, which passively flows over and covers the fish (2).

In fish mucus, the predominant gel-forming macromolecules are glycoproteins called mucins (2). Other known components are involved in fish immunity such as lysozyme, immunoglobulins, complement, carbonic anhydrase, lectins, crinotoxins, calmodulin, flavoenzymes, acute-phase proteins such as C-reactive protein, antimicrobial peptides, Apolipoprotein A-1, peroxidases, trypsin like proteases and proteolytic enzymes like alkaline phosphatase and esterases, among others (3). In previous studies, it has been shown that skin mucus plays an important role in the prevention of colonization

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by parasites, bacteria and fungi (3, 4). The antibacterial properties of mucus has been studied in several fish species such as *Oncorhynchus mykiss*, *Plecoglossu altivelis*, *Scopthalamus maximus*, *Cyprinus caprio, Catla catla*, *Labeo rohita*, *Hypophthalmichthys molitrix* and *Ctenopharyngodon idella* (1, 3, 5, 6) among others.

Caspian kutum or Caspian white fish is an economically and historically important species in the Caspian sea as well as in Iranian fisheries. It is allocated about 12,500 tons of catching bony fish in 2015 (7). To the best of our knowledge, there is very few available results focus on the immune parameters of this fish species and, curiously, all of them focus on the effects of different dietary components on fish growth, chemical body composition, haematological parameters and humoral immune activities. Among the tested substances are found probiotics such as *Bacillus licheniformis* and *B. subtilis* (8), prebiotics (9, 10), some food additives (*e.g.* sodium propionate) (11) and even extracts of medicinal plants, such as peppermint (12).

Recently, there is a great interest in applied non-invasive techniques to measure the fish immune status and numerous studies have been carried out to explore the immune activities present in fish mucus (1, 3, 5, 6, 13, 14). There is a work focused on the comparison of the total protein and lysozyme levels in serum and mucus, being the data obtained for males and females Caspian kutum specimens, in relation with three parameters related to the reproductive period (seasonal temperature, gonadal growth and reproductive migration). Results demonstrated that significant differences were observed in mucus total proteins for male and female specimens (4). Taken into account all these considerations, the main objective of the current study was to know the antimicrobial activity (both bactericidal and fungicidal) and the levels of important enzymes involved in mucosal immunity of skin mucus of Caspian kutum specimens. The results recorded from male and female specimens for the studied activities are discussed.

Materials and methods

Fish

A total of 100 Caspian kutum (with the average length of 40 ± 5 cm and average weight of 4 ± 1 kg) specimens were captured from Shirood river (west of Mazandaran province, North of Iran) by a local fisherman during reproductive period (in April 2014). Fish were transferred to the laboratory of Fish Diseases at the Caspian Sea Ecology Research Center (Sari, Iran) providing a constant aeration supplied by a portable air pump. The sex and stage of maturity of fish was recognized by the macroscopic examination of the gonads. Female brooders were recognized as stage VI (spawning) maturity and male brooders recognized as in the spermiation stage (15). A quarantine period before the start of the study ensured the absence of disease in the fish. Furthermore, fish were allowed to acclimatise to the laboratory conditions for 7 days prior to mucus collection in aerated fiber glass tanks (with 2000 L capacity). Fish were fed with commercial diet (Mazan, Iran) at a rate of 2% body weight day^{-1} three times a day.

Mucus collection

Mucus samples were collected from individuals from each sex following previously described protocol (3). Briefly, after being kept 24 h without feeding, 20 males and 20 females were randomly netted, individually placed in a bathtub tank and anesthetized with clove powder (150 mg/L). Mucus was scraped from the anterior to posterior direction on dorsal body surface using a sterile spatula. Mucus was not collected in the ventral side to avoid anal and sperm contamination. The collected mucus were thoroughly mixed with equal quantity of sterilized physiological saline (0.85% NaCl) and centrifuged (30,000 × *g*, 4 °C, 30 min) (Beckman coulter, Avanti J-26 XPI, Brea, CA, USA). Supernatant was then collected, filtered with Whatman No.1 filter paper and kept frozen at -70 °C to avoid bacterial growth and degradation until used. After mucus collection, the fish were released in Shirood river.

Microbial strains

In vitro bactericidal activity of Caspian kutum skin mucus was examined against five bacterial strains including: *Streptococcus. iniae* (ATCC29178), *Yersinia ruckeri* (KC291153), *Staphylococcus aureus* (ATCC25923), *Listeria. monocytogenes* (ATCC1143), *Pseudomonas aeruginosa* (ATCC27583) and *Escherichia coli*

(PTCC 1037). These bacterial strains were obtained from the Persian Type Culture Collection, which were prepared as lyophilized stocks. Furthermore, antifungal activity of skin mucus was determined against four fungi: *Saprolegnia* sp., *Fusarium solani*, *Candida albicans* (PTCC1023) and *Aspergillus flavus*. Fungal strains were obtained from Department of Aquatic Animal Health and Diseases, Research Organization of Caspian Sea.

Selective bacterial and fungal cultures were grown in Tryptic soy Agar (TSA) and PDA (Potato Dextrose Agar) medium, respectively for 24 h at 37 ºC, then, pure colonies with 2-3 mm diameter were diluted in 2.5 ml of appropriate liquid culture medium and cultured for 18 h at 25 ºC.

Antimicrobial assay

The disc diffusion method as described by Subramanian et al. (16) was used to determine the growth inhibition effect of skin mucus extract of Caspian kutum on selective microbial collection. Bacterial suspensions (1.5×10^8 CFU mL^{-1}) with McFarland Standard 0.5% were inoculated in Mueller-Hinton agar medium with the help of sterile cotton swabs. For fungal studies, PDA medium was dispensed in Petri plates for different strains of fungi. Whatman No.1 filter paper discs with 4 mm diameter were impregnated with different known amount of test skin mucus while sterile paper disc and standard antibiotic discs were applied as negative and positive controls, respectively. The impregnated discs along with the controls were kept on agar plates, previously seeded with test bacterial and fungal cultures, separately. The bacterial plates were incubated for 24 h at 37 ºC while the fungal plates were incubated for 72-96 h at 30 ºC to reveal any antimicrobial activity. The antimicrobial activities were determined by measuring the diameter of zone of inhibition in mm. All tests were performed in triplicate in order to confirm the reproducible results.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Mucus extracts that showed antimicrobial activity was further subjected to the test of minimum inhibitory concentration (MIC). The MIC is defined as the lowest concentration of the mucus extract of Caspian kutum at which the microorganism does not demonstrate visible growth. MIC test for bacteria was carried out by using broth microdilution method as described by Wei et al. (17). Briefly, mucus samples were serially two-fold diluted with 100 µl of Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) in order to determine the MIC. Fifty µl of this inoculum was then added into each tube containing different concentrations of mucus samples and/or Enrofloxacin (positive control) and then the samples were incubated for 16 to 24 h at 37 ˚C. Growth inhibition was monitored by visual inspection of the turbidity of the mixture. After incubation the MIC was determined by the lowest concentration of the mucus extract of Caspian kutum at which the microorganism did not demonstrate visible growth. Ten µL of MIC and higher concentrations were reinoculated to each blood agar plates and incubated for 24 h at 25 °C. The MBC was defined as the lowest concentration of the mucus extract at which incubated microorganisms are completely killed (17).

Minimum fungicidal concentration (MFC)

Determination of MFC for fungal collection was carried out following the method described by Hellio et al. (18). Briefly, fungal suspension (2×10^8 CFU m/L) were placed in a liquid medium consisting of RPMI 1640 (with L-glutamine buffered to pH 7.0 with 0.165 MOPS buffer) and various known concentrations of skin mucus or ketoconazole (positive control). Fungal cultures were then incubated at 30 ºC for 24h and MFC values were recorded.

Evaluation of enzyme activities

Alkaline phosphatase, lysozyme, protease and esterase activities were determined in skin mucus samples. Alkaline phosphatase activity was estimated using Pars Azmoon kit (Tehran Company, Iran) and absorbance was read at 405 nm with an spectrophotometer (19).

Lysozyme activity was determined based on the method described by Ellis (20) with slight modifications. Briefly, aliquots of 50 µL of mucus samples were added to 2 ml of a suspension of *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA) (0.2 mg m/L in a 0.05 M sodium phosphate buffer (pH 6.2) and absorbance was measured at 450 nm after 0.5 min and 3 min in

a spectrophotometer (Biophotometer, Eppendorf). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min. The units of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the results were expressed as U mg^{-1} mucus or serum proteins.

Finally, protease and esterase activities were determined using the methods described by Sheikhzadeh et al. (21). The absorbance was measured continuously for 2 h at 405 nm by ELISA reader. The activity was defined as the amount of enzyme required to release 1 µmol of para-nitrophenyl product in 60 s.

Statistical Analysis

The data were subjected to statistical analysis using the SPSS software version no. 20 (SPSS Inc., Chicago, IL, USA). Differences between both sexes were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P* value of < 0.05 was considered statistically significant.

Results

Bactericidal activity

The growth of all the tested bacteria was affected by the incubation with skin mucus of Caspian kutum. The *in vitro* bactericidal activity of skin mucus of Caspian kutum against selective microbial collection is shown in Table 1. Zones of inhibition for female fish mucus were a little higher (17.4 mm, 25.4 mm, 25.1 mm, 24.0 mm, 18.4 mm and 22.6 mm) than those observed for mucus from male specimens (16.2 mm, 23.2 mm, 24.7 mm, 24.3 mm, 16.7 mm and 20.2 mm) for the same bacterial species ($p < 0.05$).

Maximum antibacterial activity was observed against *Y. ruckeri* (24.7 mm) and *P. aeruginosa* (25.4 mm), respectively in male and female fish. This was followed by *E. coli* (24 mm), *P. aeruginosa* (23 mm), *L. monocytogenes* (22 mm) and *S. aureus* (18 mm). While the minimum bactericidal activity was observed against *S. iniae* incubated with mucus from either male or female specimens (16.2 mm and 17.4 mm, respectively) (Table 1). In addition, minimum inhibitory concentration test showed that minimum concentrations of mucus ranged between 125 to 500 µg l^{-1} were able to inhibit the growth of the selected bacterial and fungal pathogens.

Fungicidal activity

Similarly to the previous results described for the bacteria, the growth of all the tested fungi was also affected by the incubation with skin mucus of Caspian kutum (Table 2). The highest antifungal activity of skin mucus of Caspian kutum was observed against *F. solani* with 19 mm diameter of inhibition zone, followed by Saprolegnia sp. (17 mm) and *A. flavus* (16 mm). On the contrary, the minimum antifungal activity was observed against *C. albicans* (14 mm) (Table 2).

Enzymes activities

No significant differences were observed in the mucus alkaline phosphatase, protease and esterase activities between male and female fish specimens ($p>0.05$) (Table 3). Curiously, only higher lysozyme activity was detected in the mucus of female fish in comparison to the values recorded on mucus from male specimens ($p <0.05$).

Discussion

In the present work, the antimicrobial activity of skin mucus of Caspian kutum was studied against common fish pathogens. The studied microorganisms were selected because they cause infections and mortalities on eggs, larvae and juvenile specimens of Caspian kutum (12). Our results suggest that antimicrobial components are present in the skin mucus. All bacteria and fungi tested in the current study presented more sensitivity to the skin mucus samples than that of the control antibiotics. The resulting zones of inhibition for control (Enrofloxacin) against S*treptococcus, Pseudomonas, Yersinia, Escherichia, Staphylococcus* and *Listeria* species, were 13 mm, 16 mm, 17 mm, 15 mm, 13 mm and 17 mm, respectively. For the same bacterial species, zones of inhibition for female fish mucus were always a little higher than those observed for mucus from male specimens. The obtained

Table 1: Bactericidal activities of skin mucus of Caspian kutum. MIC, minimum inhibitory concentration; MBC, minimum bacteriocidal concentration

Bacterial strains	Zone of inhibition (mm)				MIC (µg/mL)	MBC (µg/mL)
	Sex Male	Female	Positive control (Enrofloxacin)	Negative control		
Streptococcus iniae	16.2± 0.9^{b}	17.4± 1.1^{b}	13± 0.9^{a}	0	>500	1000
Pseudomonas aeruginosa	23.2± 1.4^{b}	25.4± 1.5^{c}	16± 0.7^{a}	0	250	500
Yersinia ruckeri	24.7± 2.1^{b}	25.1± 2.5^{b}	17± 0.8^{a}	0	125	250
Escherichia coli	24.3± 1.9^{b}	24.0± 1.8^{b}	15± 0.7^{a}	0	125	250
Staphylococcus aureus	16.7± 1.2^{b}	18.4± 1.5^{c}	13± 0.6^{a}	0	500	750
Listeria monocytogenes	20.2± 1.3^{b}	22.6± 1.7^{c}	17± 1.1^{a}	0	250	500

*Data are mean ± SD (n = 20). Those within a row superscripted by different letters are significantly different ($p < 0.05$).

Table 2: Antifungal activities of skin mucus of Caspian kutum. MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration.

Fungal pathogens	Zone of inhibition (mm)				MIC (µg/mL)	MFC (µg/mL)
	Sex Female	Male	Positive control (Ketoconazole)	Negativ e control		
Saprolegnia sp.	17.0± 0.8^{b}	18.9± 1.1^{c}	13± 0.5 ^{a}	0	>125	250
Fusarium solani	19.2± 1.2^{b}	18.6± 0.9^{b}	14± 0.8^{a}	0	125	250
Aspergillus flavus	16.1± 0.8^{b}	16.5± 0.9^{b}	11± 0.6^{a}	0	>250	500
Candida albicans	14.2± 0.6^{b}	14.5± 0.7^{b}	10± 0.4^{a}	0	500	1000

*Data are mean±SD. Those within a row superscripted by different letters are significantly different ($p < 0.05$).

Table 3: Enzyme activities in skin mucus samples of Caspian kutum

Enzyme	Male	Female
Alkaline phosphatase (IU/L)	73.58±4.2 ^{a}	73.86±4.8 ^{a}
Lysozyme (IU/ mg)	22.72±0.34 ^{a}	25.28±0.58 ^{b}
Protease (IU/ mg)	30.94±2.3 ^{a}	31.12±0.16 ^{a}
Esterase (IU/ mg)	2.94±0.08 ^{a}	3.20±0.12 ^{a}

*Data are mean±SD (n = 20). Those within a row superscripted by different letters are significantly different ($p < 0.05$). IU: International unit.

results agree with the findings of Ghafoori et al. (4) denoting significant higher total protein in the skin mucus of female fish than male fish. However, future studies should demonstrate what specific proteins or molecules are responsible of the differences of antibacterial activity detected in the mucus of both sexes.

Compared to the results obtained from previous studies, the current work underline that the microbicidal activity present in mucus vary among the fish species, even for the same bacteria. In this sense, Kuppulakshmi et al. (24) reported high antibacterial activity against ten pathogenic bacteria in skin mucus of *Channa punctatus*. They reported an inhibition zone diameter 25 mm for *S. aureus* which is higher compared to our finding (18 mm). However, in agreement to our results, similar antibacterial activity against *Pseudomonas aeruginosa* (24 mm) was reported (24). Conversly, lower antibacterial activity was demonstrated in the mucus of *C. punctatus* against *S. aureus* (inhibition zone diameter 8.75 mm compared with inhibition zone diameter 24 mm in our study). Interestingly, no bactericidal activity was observed against the *E. coli* and *P. aeruginosa* bacterial strains in the skin mucus of the freshwater fishes, *Rita rita* and *Channa punctatus* (27).

Previous findings confirm that the antibacterial activity in fish skin mucus differ from fish species to species and even that can be specific toward certain bacteria (22). However, one reason for different results obtained in different studies may be due, at least in part to the type and the concentration of solvents used in mucus extraction.

In fact, some studies revealed that the antimicrobial activity of fish mucus extract is higher in acidic solvents (*e.g.* in 0.1% trifluoroacetic acid or 3% acetic acid) than that crude extracts or in aqueous medium (16, 17, 28).

Skin mucus of Caspian kutum specimens tested in the present work also presented highest antifungal activity against *F. solani* (19.2 mm) and *Saprolegnia* sp. (18.9 mm) in mucus from male and female fish specimens, respectively. On the contrary, this fungicidal activity was lowest against the *A. flavus* (14.2 mm) and *C. albicans* (14.5 mm) in skin mucus of male and female fish specimens, respectively. Balasubramanian et al. (3) demonstrated that epidermal mucus of Indian carps (*Catla catla, Labeo rohita,* and *Mugil cephalus*) showed the higher antifungal activity than that of exotic Chinese carps such as *Ctenopharyngodon idella* and *Hyphophthalmichthys molitrix* while mucus of *M. cephalus* presented moderate antifungal activity in all the tested fungi. Very similar to our findings were observed against *A. flavus* and *C. albicans* in epidermal mucus from *C. catla* and *L. rohita* (3).

Curiously, Ikram and Ridzwan (29) only find antifungal activity in water extract of fish skin mucus whereas the PBS extracts and even the pure mucus failed to produce any positive result. A possible explanation for these results was based on the fact that the extracts obtained using higher polarity solvents were more effective radical-scavengers and microbial inhibitors than were those obtained using less polar solvents (29).

The antifungal activity of mucus from fish might be due to different mechanisms such as pore formation or disruption of fungal cell membrane in salt dependent and energy independent situations, or to the formation of reactive oxygen species depletion or binding to a receptor on the fungal cell membrane (3). On the other hand, the antifungal activity of anti-microbial peptides (very abundant on fish mucus) are due to the inhibition of germination of conidia or inhibition of chitin synthesis (28).

In the present study, the results of MIC determination showed that minimum concentrations of aqueous extract of mucus ranged between 125 to 500 µg L^{-1} was able to inhibit the growth of the bacterial and fungal pathogens tested. Our results about MIC values are very similar to others obtained when using mucus extracts to inhibit the growth of *E. coli, P. aeruginosa* and *S. aureus* (17, 18).

Different enzymes with a putative antimicrobial effect (such as lysozyme, protease, esterase and alkaline phosphatase) have been identified in several fish species including Caspian kutum (4).

In the present study, the presence of alkaline phosphatase, lysozyme, protease and esterase activities as antibacterial agents were studied on mucus from males and females specimens and some variations were observed among them and perhaps, these differences also contribute to the differences observed between the bactericidal activities recorded (30). However, among the studied enzymes, only lysozyme presented significantly higher activity in the mucus of females, when compared with the activity found in mucus of males. Lysozyme is believed to be the most powerful bacteriolytic protein since it has

the ability to cleave the bacterial peptidoglycan (31, 32). Due to the multiple effects known of such important enzyme in fish, we postulate the implication of this enzyme to the overall higher antimicrobial effect observed in the skin mucus of Caspian kutum females. In accordance with present data, higher lysozyme activity was also recorded in skin mucus of female Caspian kutum (respect to the values found on males) during reproductive period. Authors related the observed differences to environmental (seasonal temperature) and physiological (reproductive activity and migration) conditions (4).

In overall, the observed dissimilarities in the antimicrobial activity of fish skin mucus seen in the different studied species may be due, at least in part, to the great variation in the quality and quantity of mucus composition among fish species that cause different anti-bacterial and anti-fungal effects. These differences, in addition to the impact of genetic factors related to fish species, can also be caused by age, sex, nutritional impact and environmental factors or even differences in laboratory protocols or conditions of sample storages (4, 22, 31, 33-35). Furthermore, the denoted variation in the antimicrobial activity of skin mucus in the same fish species, against different pathogens, inhabiting different geographical regions could be due to diverse ecological and physiological conditions (27).

Conclusion

Diverse studies on innate immunity in fish have demonstrated that fish epidermal mucus can inhibit the growth of some bacteria and fungi, therefore may have a potential source of novel antimicrobial components (3, 17, 18, 23). The mechanism by which antimicrobial substances in fish mucus kill microbes are still unclear, but it is currently thought that different peptides involved in such activities could employ different strategies. These include the fatal depolarization of the cell membrane (25), cytoplasmic membrane disruption, pore or channel formation (26, 27) or inhibition of cell wall and nucleic acid synthesis (26). Independently of the isolated effector molecules and the mechanisms involved in the microbial killing, the measurement of the microbicidal activity is a very realistic approximation. Therefore, the current research demonstrated the antimicrobial activities of epidermal mucus from Caspian kutum. Higher lysozyme activity and antimicrobial activities was recorded in the mucus of female fish in comparison to the values recorded on male fish specimens. As a result, skin mucus of this fish species (especially from female specimens) could be considered as a potential source of newer and more effective antibacterial components. Further studies are needed to purify, fractionate and characterize those antimicrobial compounds from the mucus of Caspian kutum.

Acknowledgements

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PROTIBAKTERIJSKA AKTIVNOST IN ENCIMI V VZORCIH SLUZI KOŽE SAMCEV IN SAMIC KASPIJSKEGA KUTUMA (*Rutilus frisii kutum* Kamensky, 1901)

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Povzetek: Sloj sluzi, ki pokriva površino rib, vsebuje veliko količino protimikrobnih spojin, ki zagotavljajo prvo obrambo pred škodljivimi mikroorganizmi v vodi. V študiji je bila testirana baktericidna aktivnost sluzi s površine kože kaspijskega kutuma (*Rutilus frisii kutum*) na šestih patogenih bakterijskih sevih (*Streptococcus iniae*, *Yersinia ruckeri*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* in *Escherichia coli*). Ocenjena je bila tudi fungicidna aktivnost proti štirim patogenem vrstam gliv (*Saprolegnia* sp., *Fusarium solani*, *Candida albicans* in *Aspergillus flavus*). Največja in najmanjša protibakterijska aktivnost je bila opažena proti *Y. ruckeri* in *S. iniae*, medtem ko je bila pri *F. solani* in *C. albicans* odkrita največja in najmanjša fungicidna aktivnost. Zanimivo je, da je bila protimikrobna aktivnost višja pri sluzi samic kot pri sluzi samcev. Minimalni zaviralni preizkus koncentracije je pokazal, da so najmanjše koncentracije sluzi, ki so zavirale rast izbranih bakterijskih in glivičnih patogenov med 125 do 500 µg/L. V vzorcih sluzi so bili proučevani tudi aktivnosti alkalne fosfataze, lizocima, proteaz in esteraz. Pri večini meritev ni bilo razlik med spoloma, le pri aktivnosti lizocima je bila ta aktivnost višja v sluzi samic v primerjavi z vrednostmi, izmerjenimi v vzorcih samcev. Kožna sluznica te vrste rib (zlasti samic) bi lahko bil potencialni vir novih in učinkovitejših antibakterijskih sredstev.

Ključne besede: *Rutilus frisii kutum*; kožna sluznica; baktericidna aktivnost; proti glivično delovanje; ribe

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Original Research Article

EFFECTS OF HUMIC ACIDS ON POULTRY UNDER STRESS CONDITIONS

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Abstract: The transportation of chickens from the poultry farm to the slaughterhouse causes stress conditions that influence the oxidative status of the whole organism and subsequently change the organoleptic properties of the meat delivered to the consumer. The aim of this work was to investigate how administering 0.6% humic acids to broiler chickens for a period of 42 days affects the level of selected enzymes directly involved in oxidative stress elimination. For the most objective estimation of the oxidative state, parameters were determined in liver and kidney mitochondria, and in the blood plasma. With regards to the chelating properties of humic acids, our interest was in monitoring the effects on the distribution of the transition metals Fe, Zn, Cu, Mn, which serve as cofactors of antioxidant enzymes. We have found that, under normal conditions, 42 days of humic acid administration do not cause significant metal redistribution. It has a significant effect on Se excretion, according to the pronounced deposition of Se in kidney tissue, without significantly increased activity of the corresponding enzyme. This led to compensation by changes in other antioxidant enzyme activities. This is a noteworthy finding, especially after administration of longer than 42 days. In conditions caused by sudden stress, according to the detected element levels, it is possible to expect a better response in the case of humic acid administration. The effect of humic acid supplementation appeared to be organ-specific and may ultimately be beneficial for the chickens' health, stress elimination and, finally, the quality of the meat.

Key words: antioxidant enzymes; humic acids; chicken; metal cofactors; oxidative stress

Introduction

Meat from poultry is deemed a suitable commodity for the production of functional foods for human consumption. This is currently of interest for human, agricultural and scientific research. From the perspective of the consumer, poultry is a very attractive and important element in the human diet due to its nutritional, dietetic, and sensory properties, and its rapid culinary preparation. Poultry consumption has risen dramatically across the world, including Slovakia. Thanks to modern factory farming, poultry production does not require a long period of fattening and can be purchased at any time. Achieving the most appropriate final quality of the meat is actually the purpose of all studies dealing with the impact of conditions to which it is exposed before reaching the final consumer.

Chickens slaughtered and processed in the meat processing industry are often transported a few hundred kilometres from a farm just before slaughter. However, even before the animals are slaughtered, they are subjected to a sequence of different events, such as cessation of feeding, capture, and placement in boxes or containers. Subsequently, broilers are transported to a slaughterhouse where they usually have to wait some time for slaughter (1). Transportation

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includes another set of stressors such as loading, social deprivation, restricted movement, vibration, noise, temperature, humidity, poor ventilation and often lack of food and water during transport, unloading, and subsequent handling in a new and unfamiliar environment. All of these factors compromise meat quality through formation of pale, soft and exudative meat or even death on arrival (2-5). The reaction of animals during transport depends on the length and intensity of the stressors and their physiological state (6). The animals respond to stress with changes in behaviour and in haematological, physiological, and neurohormonal parameters (7). The first reactions to stress are associated with enhanced secretion of a number of hormones including glucocorticoids, catecholamines, growth hormone and prolactin. The effect of this is to increase mobilisation of energy sources and help the individual adapt to its new circumstances (8). In particular, the enzymatic-mediated degradation of catecholamines by monoaminooxidases in mitochondria, as well as catecholamine auto-oxidation, contributes significantly to the increased levels of reactive oxygen species (ROS) under stress conditions. The mitochondria have their own elimination mechanisms against ROS, consisting of the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and tripeptide glutamine (reduced form, GSH). The actions of these enzymes are particularly relevant to the function of mitochondria as an energy formation centre and also as a non-controllable source of ROS under stress conditions.

Humic acids (HAs) are structurally very complex high-molecular-weight natural compounds arising through the process of humification, which involves a number of biochemical reactions (9). Owing to their structure, HAs have the ability to interact with many compounds and structures in their natural environment (organic and inorganic molecules, ions, minerals and microbial surfaces) which are suitable for sorption, ion exchange, and biodegradation processes. They have a potential use in the detoxification of contaminants present in the environment (10). Humic substances have been shown to be an excellent means of reducing the bioavailability of hazardous substances. They may avert the formation of mutagenic and carcinogenic substances, protect DNA in cells from damage, and decrease the rate of gene mutation (11). The anti-viral, anti-inflammatory, anti-oxidant properties and binding properties of toxic substances have been well described in *in vitro* and *in vivo* systems (12,13).

Since HAs have the ability to significantly influence the redox state of the body, there is also need to consider their chelating properties. The connection between these two properties in *in vivo* systems is yet to be described in the literature available. The aim of our study was therefore to determine the effects of the activities of antioxidant enzymes and levels of trace element co-factors after a 42-day supplementation of humic acids in normal breeding conditions and under stress conditions caused by transportation to the slaughterhouse.

Materials and methods

To verify the potential properties of HAs, the Vinica poultry farm in Veľký Krtíš (Slovakia) was selected. This poultry farm is located 220 km from a poultry slaughterhouse in Košice. Chickens were fed conventional feed mixtures (FM) by corresponding growth phase (LUX DKAS, DKAG-LUX 1, DKAG-LUX 2, DKAF LUX) and water *ad libitum* over 42 days. The entire brood was divided into two groups. The group without prophylactic doses of humic acids, designated as a control – C, contained a total of 15,700 birds. The second group, identified as HA, consisted of a total of 20000 birds which had been fed conventional feed mixtures enriched with 6 g/kg humic acid FM (Humac® Natur, Humac Ltd., Kosice, Slovakia) from the first day of fattening. All chicks were subjected to standard management and health. The chicks were randomly selected by three people for 10 birds from each group before and after transport to a slaughterhouse. Liver, kidney and plasma were collected. The mitochondria were isolated from parenchymatous organs according to Fernández-Vizzara et al. (14). In isolates and plasma, the activity of glutathione reductase (GR; E.C.1.6.4.2) was measured according to a method previously described by Calberg and Mannervik (15), while that of glutathione peroxidase (GPx; E.C. 1.11.1.9) was measured as described by Flohe and Gunzler (16). The measurements of SOD activities were provided by an SOD-Assay Kit-WST (Sigma-Aldrich, Switzerland) set. The levels of reduced glutathione were measured

by the method previously described by Floreani et al. (17). All the measured parameters were calculated per mg or g of mitochondrial protein (mg_{prot}, g_{prot}) determined using the bicinchoninic acid assay. The total content of zinc and iron was then determined by flame atomic absorption spectroscopy and that of copper, manganese and selenium by graphite furnace atomic absorption spectrometry (Shimadzu AA7000). The measured levels of elements were calculated to the value of µg (ng) per mg of proteins in mitochondrial homogenate or plasma. All measurements were performed in triplicate and the measured parameters are expressed as the mean ± SD. Statistical significance between the two groups (C vs. HA) was determined using an unpaired Student's t-Test. Differences between the groups and bodies in the measured parameters were compared by one-way ANOVA followed by Tukey HSD test.

Results

The assessment of the activities of SOD revealed that a significant increase in activity was only detected in liver mitochondria when comparing control and HA groups after transportation (Figure 1).

A significant difference was also found between SOD activity in the liver of the control group prior to transportation and of the HA group after transport (p=0.0074). In the mitochondria of the liver, kidney, and plasma of chickens prior to transport, significantly lower GPx activities were observed when HA was administered when compared to controls (Figure 2).

Multiple comparisons of groups revealed significant differences in GPx activity in the plasma of the group with HAs administered prior to transport and of the control group after transportation (p = 0.0264) as well as between the two control groups (p = 0.0034). GR activity was analogous to GPx, significantly increased in the control group in liver mitochondria, before and after transport, and in the kidney mitochondria before transport. Also, multiple comparisons of groups showed significant differences in GR activity in plasma (Figure 3). These were observed between the control group and the HA group prior to transport (p = 0.0023), between the two control groups (p = 0.0366), between both groups with administered HA (p = 0.0002), and between the control group and HA group after transportation (p = 0.0035).

Comparison of GSH concentrations revealed similarity between the significant changes in the activities of GPx and GR and the significant changes in plasma (Figure 4). Significant differences were found between the HA group prior to transport vs. the control group after transportation and between HA vs. controls after transport (p = 0.0001). Differences were also revealed between the control group and the HA group prior to transport and control group and HA group after transport at p = 0.0057 and p = 0.0068, respectively.

The average concentrations of Zn, Cu, Mn, Fe, and Se were normalised to the protein content in the mitochondria of the liver, kidney and plasma. Differences between the control group and HA-administered chickens were found in the mitochondria and plasma for Cu, Mn, and Fe before and after transport (Table 1). Levels of metals showed specific changes. In the HA groups, concentrations of Zn were lower than in controls, whereas the copper concentration was increased after transport in the HA group. The concentrations of Mn, Fe and Se generally declined after transport. Multiple comparisons of groups confirmed significant differences between the concentrations of elements in the HA group prior to transport, the control group after transportation, and the HA group after transportation (Table 1). The analyses of correlations between enzyme activities and concentrations of metals only showed strong positive correlation (r=0.8223) between the increasing concentrations of Cu and SOD activity.

The average carcass weight was lower in both groups prior to transport than after transport, but the % yield was higher (Table 2). After transport, the HA group gave higher % yield in comparison to control and the frequency of carcasses rejected from human consumption was significantly lower when compared to the control group.

Discussion

Previous studies experimenting with the addition of humic acids confirmed higher profitability and meat quality (18-20). With regards to the number of parameters that create

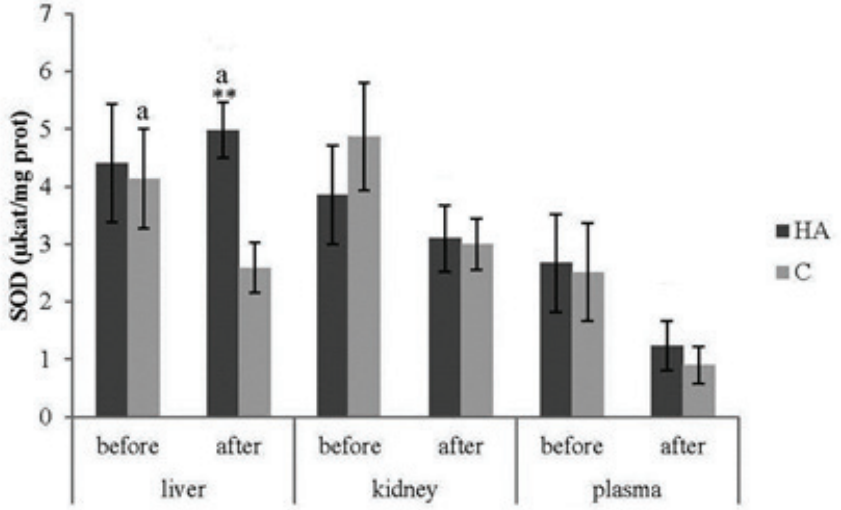

Figure 1: Superoxide dismutase activity measured in plasma, liver and kidney mitochondria before and after transport of chickens

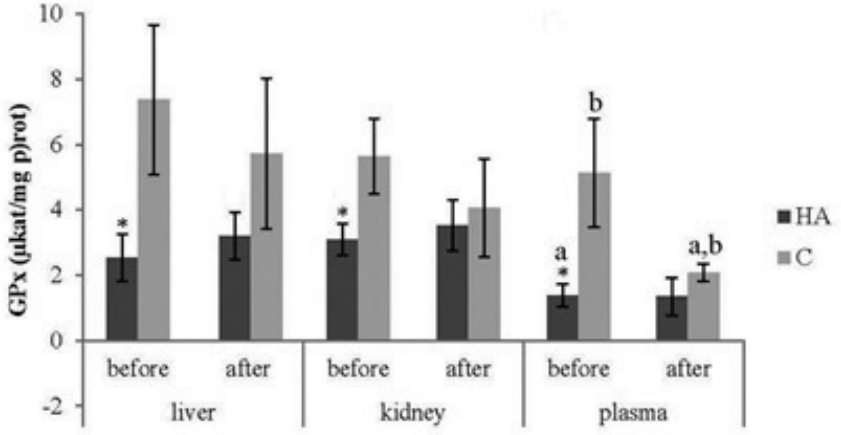

Figure 2: Changes in glutathione peroxidase activities measured in control group and group administered with humic acids for 42 day before and after transport

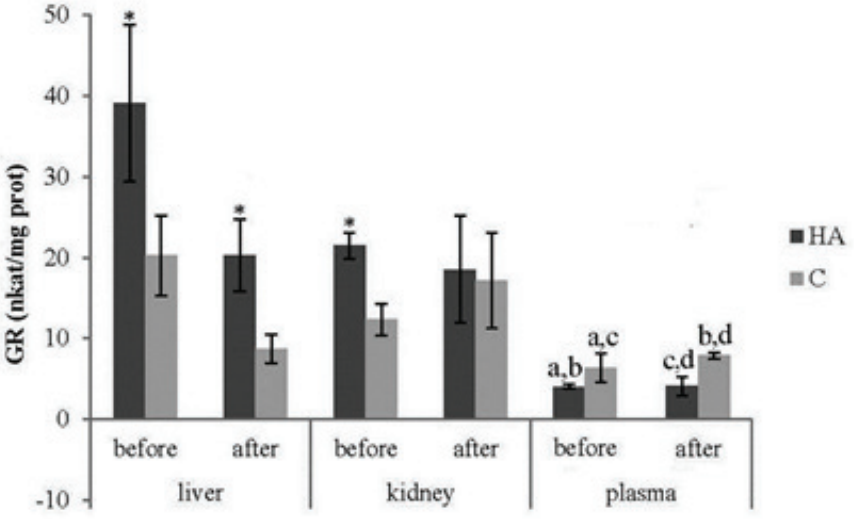

Figure 3: Changes in glutathione reductase activities measured in control group and group administered with humic acids for 42 days before and after transport

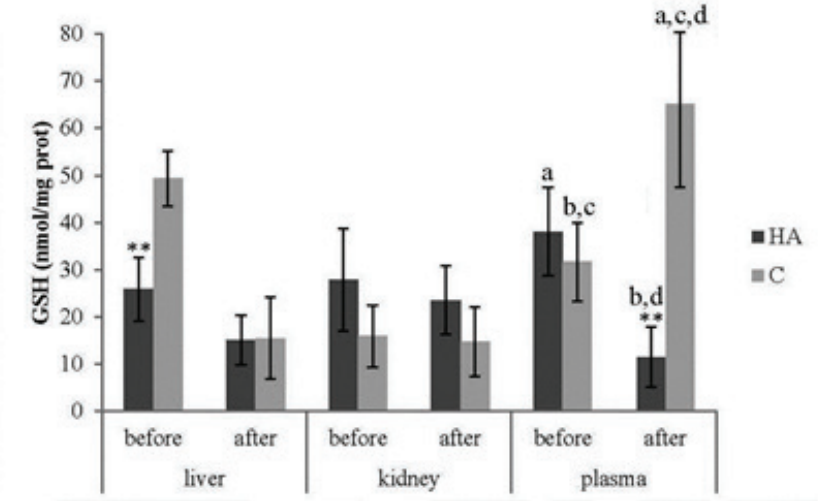

Figure 4: Reduced glutathione levels measured in plasma and liver and kidney mitochondria before and after stress from transport to the slaughterhouse

Table 1: Average values of trace elements measured in plasma, and liver and kidney mitochondria expressed per mg of protein

	Body	Before transport		After transport		Significance
		C	HA	C	HA	
Zn	liver	2.199 ± 0.155	1.239 ± 0.0819	0.362 ± 0.0098	0.291 ± 0.0517	
(μg/mg prot)	kidney	1.365 ± 0.0852	0.650 ± 0.0448	0.253 ± 0.0579	1.223 ± 0.0517*	
	plasma	1.326 ± 0.066	1.180 ± 0.0152[a]	1.509 ± 0.0349[a,b]	0.202 ± 0.0101[b]	[a]p=0.002, [b]p=0.0042
Cu	liver	1.148 ± 0.010	2.769 ± 0.0010**	12.478 ± 0.1965	16.812 ± 0.0412	
(ng/mg prot)	kidney	2.010 ± 0.0011	1.230 ± 0.0018***	9.806 ± 0.1368	10.455 ± 0.1024	
	plasma	1.134 ± 0.0002	1.103 ± 0.0006***	7.875 ± 1.6756	8.221 ± 0.0419	
Mn	liver	4.298 ± 0.0038[a]	1.231 ± 0.0076**[b,c]	0.004 ± 0.0001[b]	0.010 ± 0.0002***[a,c]	[a,b,c]p<0.001
(ng/mg prot)	kidney	3.497 ± 0.0179[a]	1.037 ± 0.0125**	0.003 ± 0.00001	0.007 ± 0.0001***[a]	[a]p<0.001
	plasma	0.981 ± 0.0060	1.393 ± 0.0065**	0.004 ± 0.00001	0.003 ± 0.0001***	
Fe	liver	0.073 ± 0.0028[a]	0.086 ± 0.0005**[b,c]	0.004 ± 0.0002[b]	0.005 ± 0.00011***[a,c]	[a,b,c]p<0.001
(μg/mg prot)	kidney	0.022 ± 0.0003[a]	0.066 ± 0.0005***	0.003 ± 0.00018	0.004 ± 0.00014***[a]	[a]p<0.001
	plasma	0.034 ± 0.0007[a]	0.075 ± 0.0009***[b,c]	0.004 ± 0.00007[b]	0.003 ± 0.00015***[a,c]	[a,b,c]p<0.001
Se	liver	under LOD	1.278 ± .00025	under LOD	0.642 ± 0.0004	
(ng/mg prot)	kidney	2.945 ± 0.0014	25.921 ± 0.0009***	under LOD	1.353 ± 0.00004	
	plasma	11.899 ± 0.0009	3.907 ± 0.0006***	under LOD	1.524 ± 0.0006	

* **s**tatistical significance of T-test comparison between control and HA group.
[a,b,c] Represent statistical significance between groups from multiple comparison by one-way ANOVA followed by Tukey post-hoc test.

Table 2: Comparison of some parameters before and after transport

Parameter	Before transport		After transport	
	C	HA	C	HA
Carcass yield (g)	1344.8 ± 233.28	1370 ± 97.24	1489 ± 109.61	1398 ± 106,77
% yield	83.46 ± 2.47	80.83 ± 4.16	71.36 ± 3.45	73.98 ± 3.21
Death on arrival	-	-	18	39
Carcass rejected from human consumption	20	51	125	89

stress conditions for birds before slaughter and consequently affect the quality of the meat, the transport of poultry to the place of slaughter is the one that is impossible to avoid. Cetin et al. (21) presented humic acids as a suitable supportive supplement in managing social stress. It is also worthwhile monitoring the parameters of antioxidant defence in central metabolically active organs as well as in circulation when assessing the effect of administered substances on the redox state of the organism. With regards to the site of distinctive ROS and energy generation, the mitochondria isolated from these organs have proved to be even more suitable for assessing redox status in poultry (22).

SOD activity was found to be significantly increased in liver mitochondria after transportation (Figure 1). The role of SODs against ROS is in the catalysis of the disproportionation of superoxide radicals ($O_2^{\cdot-}$) into O_2 and H_2O_2. As O_2 and $O_2^{\cdot-}$ differ in a single electron, the enzyme must have a high specificity and be finely tuned to perform its catalytic role (23). Increased SOD activity after transport clearly indicates increased formation of $O_2^{\cdot-}$ in liver mitochondria. As transition metals have the ability to readily pick up and transmit electrons, they are very effectively used in ROS elimination reactions in the body. SOD is a collective term for a total of 3 unrelated enzymes that combine only the same catalytic activity. A localised form containing Mn is found in the mitochondrial matrix, while Cu/Zn-SOD is found in the cytosol, between the outer and inner membranes of the mitochondria, and in the extracellular space (24). Concentrations of metals were also measured and then normalised to the protein content in tissue samples taken. Extrapolation of the detected values to the activities of the enzymes measured which elements are contained in the active site, and provided an explanation of the nature of the effect of the administered substances. Concentrations of Mn in HA groups were lower compared to control and even more pronounced after transport (Table 1). At the same time, Zn concentrations were found to be decreased in HA groups, while copper concentrations increased, markedly so after transport (Table 1). The properties of Has, described by Mezes et al. (25), have shown that organic metal complexes, like HAs, have higher bioavailability. This is largely due to the structure that protects and stabilises trace elements in the passage through the gastrointestinal tract, allowing their passage through the intestinal wall via amino acid transport systems, and providing for a higher rate of passive diffusion due to lowered interaction with other nutrients. Although the high bivalent cation binding capacity of HAs improves their absorption, similar to Mezes et al. (25), we cannot confirm these properties after 42 days of HA administration, nor those of Herzig et al. (26). In part, this unexpected effect in the HA group without stress of transport is eliminated by the findings of Islam et al. (27), where concentrations of Cu and Zn were also reduced with recovery after 60 days. Under stress conditions, however, concentrations of Mn and especially Cu (Zn in kidney mitochondria) are higher in the HA group when compared to stressed control and even the control group of unstressed birds (Table 1). In this situation, it is possible only to monitor the beneficial effect of HAs based on the nature of these enzymes. As Brown et al. (28) pointed out, Cu/Zn-containing SOD exists as an apoenzyme that is readily activated by copper without new protein synthesis. A copper-load form of copper chaperone has been shown as a higher order type of physiological regulation in response to oxidative stress (29), changing the enzyme disulphide status and forming an active dimer state. Binding of Zn ions is not essential for the dismutation reaction but confers higher stability (30). The same is not true for MnSOD, as Mn insertion only occurs with newly synthesized and imported molecules into mitochondria and it should stay sufficiently unfolded to allow Mn entry (31). After transport, there were generally lower concentrations of Mn. However, they were a little higher in the HA-administered stressed group than in stress control. The mechanism of maintaining this protein structure under stress conditions is not entirely clear. Despite the slight increase in Mn concentrations in mitochondria, there is a clear and apparent benefit in SOD activity support when administered with HAs, especially Cu/ZnSOD, under stress conditions.

The resulting product of dismutation $O_2^{\cdot-}$ is hydrogen peroxide. This is the substrate for GPx, the next enzyme monitored. Essentially, in the catalytic cycle of all peroxidases, H_2O_2 is used as a specific electron acceptor, which is reduced to water. In terms of defence against oxidative stress, however, non-haem GPx catalyses the reduction of H_2O_2 or organic peroxides to water or corresponding alcohols, with the simultaneous oxidation of GSH

playing an important role. The oxidised glutathione is subsequently reduced by the catalytic activity of GR. Taking these three parameters together allows a more objective assessment of antioxidant efficiency. Some GPx isoforms with specific distribution between tissues and subcellular compartments (cytosol, nucleus, mitochondria, plasma) contain selenium in their catalytic centre. The distribution of these selenoenzymes affects the level of ROS, the relative level of glycolysis in ratio to oxidative phosphorylation, the level of redox-sensitive transcription factors, and the resulting rate of important cellular processes (32). In humans, endemic diseases and increased virulence of coxsackie virus have been linked to selenium deficiency, and high concentrations of humic acids in drinking water among other possible etiological factors (33). Therefore, it was interesting to compare GPx activity and Se levels. According to these measurements, it is obvious that the activities of GPx were significantly lowered when administered with HA and stress-free (Figure 2). Concentrations of Se, however, were markedly increased in the kidney (Table 1, before transport). After the effect of transport stress, GPx activity was lower but without prominence in liver and kidney mitochondria, and the Se concentration remained measurable unlike in controls. Considering the changes in GR activity (Figure 3) and GSH levels (Figure 4), it cannot be assumed that oxidative stress conditions have been created. Through the action of a stressor, differences are no longer statistically significant, except for GR in liver. These *in vivo* results confirm the antioxidant activity of HA in mitochondria under *in vitro* conditions (13). Due to higher Se concentrations upon HA administration, it can be assumed that the effect of HA is through maintaining the activities of Se-GPx isoforms in mitochondria. Significant differences in all three parameters were only found comparing groups in plasma, which ultimately resulted in markedly reduced levels of reduced glutathione in the stressed group administered with HA (Figure 4).

Interestingly, lower Se concentrations were detected in the plasma following administration of HA versus control, still without changes in plasma GPx activity. After stress from transport, however, the highest Se concentration was found in plasma. Kidneys are the main location for the synthesis of selenium-containing GPx before its secretion into plasma and extracellular fluids (34). Therefore, as an explanation we can state that humic acids used in this study did not cause deficiency but increased the bioavailability of Se. Se accumulates in the kidney and is likely to allow for the synthesis of Se-GPx isoforms under stress conditions. Still, levels of reduced GSH were significantly lowered in plasma in the HA group after transport. The liver is the main source of GSH in circulation, followed by complicated inter-organ transfer. Circulating GSH and GSSH are particularly used by the kidneys (35). That could be one of the possible explanations for the renal load of Se after HA administration. According to results of copper concentrations in connection with SOD, it seems more appropriate that its role lies in the metabolism of copper ions for the biosynthesis of copper-containing proteins (36). GSH serves as a carrier for Cu+, and is involved in copper mobilisation with the complex Cu(I)-GSH being used for incorporation into Cu/Zn-SOD (37). This explanation is also supported by a strong positive correlation between the increased concentration of Cu and SOD activity.

Significantly elevated Fe concentrations were found after administration of HAs in both mitochondria and plasma, which is consistent with the findings of Ipek et al. (38) and the described properties of HA. After transport, Fe concentrations decreased in both monitored groups. Regarding the measured parameters, oxidative stress conditions are not created due to Fe concentrations, and its bioavailability is associated with better use.

Overall, the administration of humic acids at 0.6% in feed mixtures over 42 days is considered to be beneficial for stress from transport, since oxidative stress conditions have not been demonstrated in the main organs responsible for nutrient metabolism and energy production. Finally, in terms of losses and meat excluded from consumption, the group with HAs fared better. According to measurements of metals, we cannot fail to notice the rapid differences between mitochondrial concentrations caused by stress, which have yet to be mentioned at all. However, based on the results, HAs administered as feeding additives are shown to cause metal redistribution in terms of the unexpected requirements of the organism.

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UČINKI HUMINSKE KISLINE NA PERUTNINO V STRESNIH POGOJIH

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Povzetek: Prevoz piščancev s perutninske farme v klavnico povzroča stresne razmere, ki vplivajo na oksidativni status celotnega organizma in kasneje spremenijo organoleptične lastnosti mesa, dostavljenega potrošniku. Cilj raziskave je bil proučiti, ali dodajanje 0,6 % huminske kisline v hrano pitovnim piščancem za 42 dni vpliva na aktivnost nekaterih encimov, ki so neposredno vključeni v urejanje in zmanjševanje oksidativnega stresa. Za objektivno ocenjevanje oksidativnega stanja so se določevali parametri v jetrnih in ledvičnih mitohondrijih ter v krvni plazmi. Kelacijske lastnosti huminske kisline so bile proučevane s spremljanjem učinkov na porazdelitev prehodnih kovin Fe, Zn, Cu, Mn, ki služijo kot kofaktorji antioksidantnih encimov. Ugotovili so, da v normalnih pogojih 42-dnevno dodajanje huminske kisline ne povzroči bistvene prerazporeditve kovin. Dodajanje pa pomembno vpliva na izločanje Se glede na izrazito usedlino Se v tkivu ledvic, brez bistveno povečane aktivnosti ustreznega encima. To je privedlo do sprememb, ki so nadomestile aktivnosti drugih antioksidantnih encimov. To je pomembna ugotovitev, še posebej pri dodajanjih več kot 42 dni. V pogojih, ki jih povzroča nenadni stres, je glede na ugotovljene ravni elementov mogoče pričakovati boljši odziv pri uporabi huminske kisline. Učinek dopolnjevanja s huminsko kislino se je izkazal za organsko-specifičnega in je lahko koristen za zdravje piščancev, odpravo stresa in končno kakovost mesa.

Ključne besede: antioksidativni encimi; humične kisline; piščanec; kovinski kofaktorji; oksidativni stres

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