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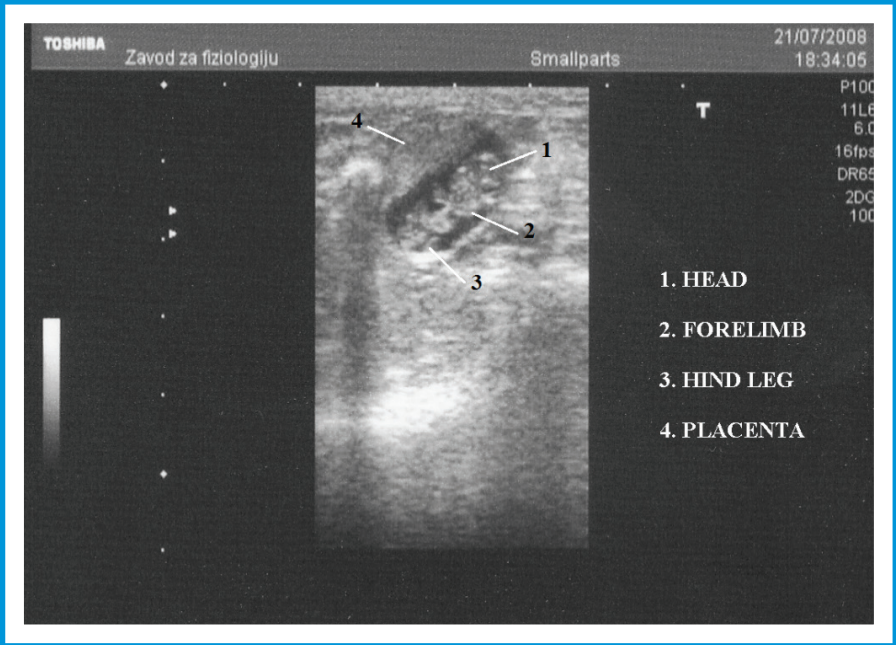

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Original Research Article

THE PREVALENCE OF TEN PATHOGENS DETECTED BY A REAL-TIME PCR METHOD IN NASAL SWAB SAMPLES COLLECTED FROM LIVE CATTLE WITH RESPIRATORY DISEASE

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Summary: Respiratory diseases often correspond to primary infections with different pathogens of cattle, causing heavy economic losses in young stock and breeding herds. Between 2012 and 2014, nasal swab samples were collected from twenty-eight herds from 133 affected live cattle that were clinically suffering from symptoms of respiratory disease, pyrexia, cough, serous nasal and lacrimal discharge, increased respiratory rate, and breath sounds. Individual swab samples were tested in the laboratory using three commercial and one in-house real-time PCR methods, to detect nucleic acids of a total of ten different respiratory pathogens. *Pasteurella multocida* (*P. multocida*) was detected in 58.65% of samples, *Mannheimia haemolytica* (*M. haemolytica*) in 15.04%, while *Mycoplasma bovis* (*M. bovis*) and *Histophilus somni* (*H. somni*) were positive in 9.77% of nasal swab samples. Among viral pathogens, the highest prevalence (40.60%) was observed for bovine respiratory syncytial virus (BRSV), followed by bovine coronavirus (BCV) 12.03%, bovine para-influenza 3 (PI-3) 3.01%, and bovine viral diarrhea virus (BVDV) with 1.50% of positive samples. The less frequently detected viral pathogens were bovine herpes virus type 1 (BHV-1) and bovine adenovirus (BAdV) with 0.75% positive samples each. The new implemented molecular methods can be an important diagnostic tool for laboratories and farmers to improve the therapy, control, and prevention of respiratory disease in cattle herds.

Key words: bovine respiratory disease; nasal swab samples; diagnostics; real-time PCR detection; cattle

Introduction

Bovine respiratory disease (BRD) is the major cause of serious respiratory tract infections worldwide, often leading to high morbidity and mortality rates in cattle. The disease is considered to be a multifactorial disorder, produced with either stress or reduced immunity, allowing several pathogens to emerge. It inflicts considerable mortality and financial losses mainly in calves in dairy and beef herds. Viral and bacterial pathogens together with mycoplasma and environmental risk factors are the most common cause of diseases, ranging from common colds to life-threatening pneumonia (1). A large number of both RNA and DNA viruses uses the respiratory tract to initiate host infection. Infection may be restricted to certain sections of the airway system such as the trachea, bronchi, or alveoli. For some viruses, the respiratory tract may merely serve as a primary entry site from where infection spreads to other organs or tissues. An important defense strategy is the mucociliary clearance system. While some epithelial cells are specialized to produce and release mucins, other cells are equipped with cilia

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that enable them to contribute to the transport of the mucus with pathogens out of the respiratory tract. The most important viral pathogens associated with BRD are bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (PI-3), bovine herpesvirus 1 (BHV-1), bovine adenovirus (BAdV), bovine coronavirus (BCV) and bovine viral diarrhea virus (BVDV) (2, 3). While BHV-1 and BVDV have already been eradicated in some European countries, infections with BRSV and BCV are endemic in the cattle population globally (4, 5). The infection of cattle with BAdV usually results in disease of the gastrointestinal or respiratory tract. Different serotypes of BAdV are divided into two subgroups. BAdV type 1, 2, 3, 9 and 10 comprise Group A, BAdV type 4, 5, 6, 7 and 8 comprise Group B (6). Only a few publications on the simultaneous detection of more than three different pathogens in case of respiratory or enteric diseases are available (7, 8, 9). Several studies present antibodies against etiologic agents of BRD, but these only indirectly confirm the previous infections with specific viral and bacterial agents in animals or herds (4-5, 10-12). Newly developed molecular methods significantly improved the diagnosis of respiratory tract infections, providing a fast and cost-effective tool for different pathogens, to determine the prevalence of respiratory viruses, bacteria, and mycoplasma in clinically affected cattle (13). Few reports about the prevalence on the respiratory disease of cattle in Slovenia exist, mainly obtained several years ago with conventional methods of bacteria or virus isolation (14–18). However, the traditional farming system in Slovenia with small isolated cattle herds is disappearing; herds are enlarging gradually and, in several cases, animals of different herds and ages are kept in a pen. In the new rearing system, young calves at the age of 1-3 weeks originating from several herds, are transported to beef units and grouping together, frequently with a combination of imported beef cattle. Sometimes, vaccines against respiratory disease are used, but almost no data about effectiveness is available. Antimicrobials are generally not used for disease prevention; sick animals are mostly treated individually with antibiotics.

The aim of this study was to determine the prevalence of ten respiratory pathogens detected by new implemented real-time PCR methods using nasal swab samples collected from affected cattle with respiratory disease.

Materials and methods

Sampling was conducted mostly in winter and spring periods from 2012 to 2014. All swab samples were collected into sterile swabs (Sigma Virocult®, MW 951S, UK) and were immediately sent to a laboratory. Nasal swab samples were collected from 133 live affected animals, originating from twenty-eight different Slovenian cattle herds identified with bovine respiratory disease. Five of them are feedlot cattle herds, four are dairy herds, and nineteen are traditional, combined herds, with milk and meat production. All of the sampled animals had abnormal sound on auscultation of the respiratory tract and most had either one or several of the following symptoms: fever >39 °C, elevated respiratory rate (> 40/min), cough or nasal and/or lacrimal discharge. In the case of an acute outbreak on a farm, 1 to 18 samples were collected from the same herd, only from clinically affected animals. As a control group of the study, ten animals from three farms without clinical signs of respiratory disease in the previous two months were selected, and nasal swab samples were collected from healthy animals and screened for ten pathogens.

After arrival at the laboratory, samples were homogenized and stored in a freezer at < -15 °C until testing. Total nucleic acids were extracted from 140 µl of homogenate using a commercial kit for RNA extraction (QIAamp® Viral RNA Mini Kit (Qiagen, Germany)) according to the manufacturer's instructions. Individual swab samples were tested by one in-house and three commercial real-time PCR methods, detecting specific nucleic acids of a total of ten different respiratory pathogens, including detection of endogenous internal positive control (IPC) for controlling the efficiency of extraction and the absence of inhibitors in individual samples. Samples were tested on a 96-tube microplate. On each microplate, the positive controls for all tested pathogens were included. A commercial TaqMan® real-time PCR kit for the detection of seven major ruminant pathogens (LSI VetMAX™ Screening Pack – Ruminants Respiratory Pathogens, LSI, France) allows the simultaneous detection of the *M. bovis*, *H. somni*, *P. multocida*, *M. haemolytica*, BCV, BRSV, and PI-3. For the detection of BVDV and BHV-1, another two commercial real-time kits (Kit TaqVet® BVDV "Screening" and LSI VetMAX™ IBR gB, both produced by LSI, France) were used

according to the producer's instructions. For the detection of BAdV, an in-house protocol was implemented with previously designed primers detecting BAdV, serotypes 4-8 (19). Real-time PCR was performed using the forward primer BAV4-8F 5'-CRA GGG AAT AYY TGT CTG AAA ATC-3', the reverse primer BAV4-8R 5'-AAG GAT CTC TAA ATT TYT CTC CAA GA-3' and the probe FAM-TTC ATC WCT GCC ACW CAA AGC TTT TTT-BHQ-1 targeting the hexon gene of BAdV (9). The reaction was performed in a total volume of 15 µl, using QuanTitec® Virus Kit (Qiagen, Germany) as follows: 8 µl of nuclease free water, 3 µl of 5x PCR Master Mix, 0,5 µl of the stock solution with 20 µM of BAV4-8F primer, 0,5 µl of the 20 µM of BAV4-8R primer, 0,5 µl of the stock solution with 10 µM of probe and 2,5 µl of the RNA/DNA template. The real-time PCR running program for BAdV was 95 °C for 15 min; followed by 45 cycles of 95 °C for 10 s, 54 °C for 30 s and 60 °C for 30 s. All real-time cyclings were performed on an Mx3005P thermocycler (Stratagene, USA) using protocol according to the manufacturer's instructions for commercial kits and the above-described protocol for BAdV detection. The fluorescent signal was detected after each annealing, and the results were presented as a cycle threshold value for individual samples. Analysis of real-time amplification curves was performed using commercial thermal cycler system software, and an "auto baseline" was used to determine fluorescence baselines.

Results

A total of 133 swab samples from live cattle with symptoms of respiratory disease and 10 swab samples from healthy cattle (control group) were successfully screened for 10 pathogens with the real-time PCRs method. In cattle with respiratory disease, *P. multocida* was detected in 78/133 (58.65%) of samples, *M. haemolytica* in 20/133 (15.04%), while *M. bovis* and *H. somni* were positive in 13/133 (9.77%) of nasal swab samples. The highest prevalence of viral pathogens was observed for BRSV 54/133 (40.60%), following BCV 16/133 (12.03%), PI-3 with 4/133 (3.01%) and BVDV with 2/133 (1.50%) of positive samples. The less frequently detected viral pathogens were BHV-1 and BAdV with 1/133 (0.75%) positive samples (Table 1).

At least one pathogen was detected in 110/133 (82.70%) of clinically affected cattle (Figure 1). In 43 samples (32.33%), only one pathogen was detected; *P. multocida* in 17 samples and BRSV in 16 samples, and *M. haemolytica* in five samples. The simultaneous detection of two different pathogens was observed in 46 samples (34.59%);

Table 1: The results of the detection of ten different pathogens in 133 nasal swabs samples, collected from live cattle suffering from respiratory disease together with a control group are presented. The ranges of cycle threshold values obtained by specific real-time PCR methods are presented for individual pathogens

Name of pathogen	Cattle with respiratory disease				Control group (healthy animals)		
	Number of tested samples	Number of positive samples	% of positive samples	Cycle threshold (Ct) range (mean)	Number of tested samples	Number of positive samples	Cycle threshold (Ct)
M. bovis	133	13	9.77%	20.88–37.81 (29.60)	10	0	-
H. somni	133	13	9.77%	26.63–41.97 (35.35)	10	1	35.35
P. multocida	133	78	58.64%	19.76–43.04 (30.41)	10	1	32.99
M. haemolytica	133	20	15.04%	27.12–43.54 (34.05)	10	0	-
BCV	133	16	12.03%	25.16–38.82 (31.61)	10	0	-
BRSV	133	54	40.60%	20.48–39.89 (29.53)	10	0	-
BPI-3	133	4	3.01%	20.81–42.05 (34.05)	10	0	-
BVDV	133	2	1.50%	28.73–35.34 (32,03)	10	0	-
BHV-1	133	1	0.75%	29.91 (29.91)	10	0	-
BAdV	133	1	0.75%	29.43 (29.43)	10	0	-

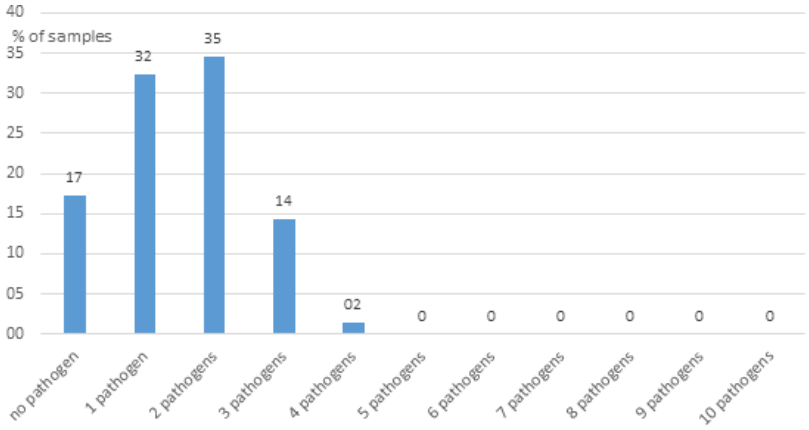

Figure 1: The percentage of negative and positive samples detected by real-time PCR method, with the presentation of the simultaneous detection of two to four different pathogens in nasal swab samples collected from live cattle suffering from respiratory disease

Table 2: The results of the detection of 10 different respiratory pathogens by real-time PCR methods in three different types of herds in which respiratory disease was observed

Type of herd	Number of nasal swab samples	Number of possible results	Number of positive samples	% of positive samples	Number of positive animals	% of positive animals
feedlot	29	290	67	23.1%	29	100.0%
combined	70	700	105	15.0%	61	87.1%
dairy	34	340	30	8.8%	20	58.8%

in 26 samples *P. multocida* and BRSV, in five samples *P. multocida* and *M. bovis*, while in four samples *P. multocida* with BCV or *P. multocida* with *H. somni* or *M. haemolytica* together with BCV. In 19 (14.29%) positive samples, the simultaneous detection of three pathogens was identified; in 13 samples, two bacteria and one virus were confirmed (Fig. 1). In two samples, four pathogens were detected, in one sample BHV-1, *M. bovis*, *H. somni* and *P. multocida*, while in one sample *M. haemolytica* with three different viruses (BRSV, BCV and PI-3) was detected. The high variability of cycle threshold (Ct) for different pathogens was observed from low to high viral loads in a sample; the summary results of these data are presented in Table 1 and for the individual samples in the supplementary file (Supplementary data). All 29 tested nasal swabs collected from feedlot herds were positive for at least one respiratory pathogen (100%), while samples from combined herds were positive for at least one respiratory pathogen in 87.1% and from dairy herds in 58.8% of the collected samples (Table 2). Up to five individually tested samples, which were collected from the same herd, showed a similar pattern of pathogens (data presented in Supplementary data). In the control group, out of ten healthy animals, only in one animal were *H. somni* and *P. multocida* detected with Ct values of 35.35 and 32.99, respectively.

Discussion

To our knowledge, this study represents the first report of the simultaneous detection of ten respiratory pathogens by using real-time PCR in cattle. According to the results of this study, *P. multocida,* BRSV, *M. haemolytica*, BCV, *H. somni* and *M. bovis* are common pathogens in Slovenian cattle herds with respiratory problems. BVDV is endemically present in about 30% of herds (20), while the occurrence of BHV-1 and PI-3 in Slovenia is low (16) and the previous observation was confirmed also in this study. A significant finding of our study was that the real-time PCRs approach used could detect the concurrent infection of five different viruses, four bacteria, and mycoplasma with positive results of at least one pathogen in 82.70% of nasal swabs samples. A high percentage (40.60%) of identified positive samples with BRSV confirmed that this virus is a significant problem of herds with respiratory infections,

frequently complicated with *P. multocida* and one or more other pathogens. In 67 samples, at least two respiratory pathogens were detected; in 39 (58.21%), *P. multocida* together with BRSV. Where BRSV resides in a population of cattle for the virus to survive is not well understood, but low biosecurity measures on farms may be the main reason for the high prevalence. Persistently infected calves may exist, and possible triggering mechanisms such as a change in temperature may induce shedding (21). The second most prevalent virus was BCV with 12.03%, confirming the regular circulation of this virus in the herds. In 2011 and 2013, the antibody status in 70 conventional dairy herds in Sweden ranged from 73.4 to 82.3% for BRSV and from 76.8% to 85.3% for BCV (10). According to the observation in our study, similar antibody prevalence could also probably be detected in Slovenia. Apparently, there are good chances for a herd to be free of these infections if good biosecurity is practiced, and the virus is not reintroduced, as was shown in a Norwegian survey (11). However, a significant proportion of Slovenian herds have low biosecurity standards with the introduction of animals from herds of unknown status. Imported calves can be the reason for the introduction of new respiratory pathogens and other infections. The circulation of very similar strains of BCV in Slovenia was confirmed with the sequencing of 21 positive samples collected between 2012 and 2013 with 99-100% nucleotide identity in RNA polymerase gene (17). Under the experimental condition, BRSV was detected in nasal secretions between Day 2 and Day 14, but the PCR methods may detect virus shedding for a longer period after infection than virus isolation, possibly due to neutralization by mucosal antibodies (22, 23). Although BVDV can be detected in persistently infected cattle or during acute infection of seronegative immunocompetent cattle, the results of our study with the detection of 1.5% of positive samples confirmed that this virus is only occasionally detected in cattle with respiratory disease and is not closely linked to occurrence of BRD, which is consistent with previous observation in Finland (12). The low percent of positive samples of PI-3 was also previously observed (12), confirming that PI-3 is not a significant causal factor in BRD. To our knowledge, the occurrence of BAdV 4-8 has not been studied earlier in Slovenia. The low detected prevalence of BAdV 4-8 is surprising in comparison to some previous observations and its global distribution (12), but also may be the result of the type of samples in this study and the limitation of used real-time PCR method, thus further research in Slovenia is needed, targeting all known BAdV (24). Previous observations that bacterial pathogens such as *M. haemolytica, P. multocida* or *M. bovis* may induce a response reaction in the epithelial cells that make them susceptible to virus infection was also confirmed in our study (25). Mycoplasmas are considered to be one of the causal factors of BRD, but often together with other pathogens. The detection of *M. bovis* in 9.77% of samples suggest that this pathogen might be significant in pathogenesis in feedlot cattle herds, especially in multiple pathogen infections. This observation is also supported with high pathogen loads in positive samples with Ct values between 20.88 and 32.32 for *M. bovis* in 11 out of 13 positive samples. Because no data regarding the prevalence of *M. bovis* is available for Slovenia, more research needs to be done in the future. In our study, at least one bacterial infection was detected in 92/133 (68.42%), frequently (64/133, 51%) in combination with one or more bacteria or viruses, which is consistent with previous observation (26). *P. multocida* is another common pathogen observed in 58.65% of affected animals in our study and, as was previously suggested, the antimicrobial treatments are necessary in all clinical cases of BRD, because all *Pasteurella* spp. isolates were susceptible to antibiotics (26). From our study, it was clearly shown that *H. somni* or *M. haemolytica* are also frequently present in affected animals, suggesting their importance for diagnosis of respiratory problems. In general, real-time PCR methods are more suitable for diagnostic applications than conventional methods of isolation. These techniques offer high sensitivity and provide specific results within a shorter period and for a larger number of pathogens in comparison to conventional methods, such as virus isolation or direct fluorescence antibody tests (8). Highly sensitive real-time PCR assays used in this study confirmed previous reports of naturally occurring viral and bacterial infection. Real-time RT-PCRs has also been used in disease pathogenesis studies to determine viral load and viral gene expression (23). From the obtained Ct values for individual pathogens and the comparison of Ct values of different pathogens, the laboratory interpretation of the results can provide valuable additional data to the on-field veterinarian regarding therapy. Na-

sal swab samples are suitable for the examination of single or several affected animals in a herd and can also be performed by the farmer. This has an advantage in comparison to a sampling of lung lavage or blood collection, which can be done only by a veterinarian and because these samples frequently have lower detection rates in comparison to swabs (12). As was previously observed, the integrity of samples can be affected, if swab samples are not stored at low temperature and immediately send to the laboratory (27); the cold chain strategy also has to be followed for respiratory disease samples. If three to five animals are tested from the same herd, the probability of obtaining at least one pathogen is increased, as can be concluded from this study. Molecular methods also provide the pooling of up to five samples from the same herd to reduce the costs of testing. The observation of this study confirmed that BRD is frequently the result of infection with different pathogens, and their fast laboratory identification could help veterinarians towards selecting the correct therapy for animals with BRD. Our results indicate that the nasal swabs sampling in combination with real-time PCR methods can be a useful tool for the rapid and cost-effective diagnosis and surveillance of viral and bacterial respiratory infection in cattle.

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UGOTAVLJANJE PRISOTNOSTI DESETIH PATOGENOV Z METODO PCR V REALNEM ČASU V ODVZETIH VZORCIH NOSNIH BRISOV PRI ŽIVEM GOVEDU Z ZNAKI RESPIRATORNEGA OBOLENJA

T. Paller, P. Hostnik, M. Pogačnik, I. Toplak

Povzetek: Bolezni dihal so pri govedu pogosto posledica primarne okužbe z različnimi patogeni, ki pri teletih in tudi v plemenski čredi povzročijo veliko gospodarsko škodo. V letih od 2012 do 2014 smo v 28 govejih čredah odvzeli vzorce nosnih brisov pri obolelih živih živalih. Vzorčenje smo izvedli pri 133 živalih, ki so klinično kazale enega ali več znakov obolenja dihal, povišano telesno temperaturo, kašelj, serozni nosni in očesni izcedek, pospešeno dihanje in povišan zvok ob pregledu pljuč. Vzorce nosnih brisov smo testirali s tremi komercialnimi in eno novo uvedeno laboratorijsko metodo PCR v realnem času na prisotnost nukleinskih kislin desetih različnih patogenov. Prisotnost bakterije *Pasteurella multocida* smo ugotovili v 58.65 % vseh vzorcev, bakterije *Mannheimia haemolytica* v 15.04 %, pristonost bakterij *Mycoplasma bovis* in *Histophilus somni* pa smo ugotovili v 9.77 % vzorcev. Med iskanimi virusi smo bovini respiratorni sincicialni virus ugotovili v 40.60 % vzorcev, bovini koronavirus v 12.03 % vzorcev, virus parainfluence 3 v 3 %, virus bovine virusne diareje v 1.5 % vzorcev; najmanj pogosto smo ugotovili prisotnost bovinega herpesvirusa 1 in bovinega adenovirusa (0.75 % vseh vzorcev). Novo uporabljene molekularne metode predstavljajo pomembno diagnostično orodje za laboratorije in rejce in v goveji čredi pomagajo pri izbiri ustreznejše terapije, nadzoru in preprečevanju bolezni dihal.

Ključne besede: respiratorno oboljenje; vzorci nosnih brisov; diagnostika; PCR v realnem času; govedo

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Original Research Article

DETECTION OF *Lawsonia intracellularis* DNA IN ILEAL TISSUES OF DEAD WILD BIRDS IN THE REPUBLIC OF KOREA

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Abstract: *Lawsonia intracellularis* is an etiological agent that causes proliferative enteropathy in various species. Little is known about the mechanisms of transmission of *L. intracellularis*, especially in wild bird species. The presence of *L. intracellularis* in dead wild birds in the Republic of Korea was investigated using the polymerase chain reaction method. *L. intracellularis* DNA was identified in the mucous membrane of the ileum in one Eurasian eagle-owl (*Bubo bubo*, Strigidae), two black-billed magpies (*Pica pica sericea*, Corvidae), and one jungle crow (*Corvus macrorhynchos*, Corvidae) among 745 dead wild birds examined. Although few wild birds in this study were exposed to *L. intracellularis*, the exposure was likely to be epidemiologically relevant. Regarding the ecological behavior of the bird species found to be exposed to *L. intracellularis* in the current study (Eurasian eagle-owl, black-billed magpie, and jungle crow), swine or horse farm facilities, which are reservoirs for *L. intracellularis*, might be easily accessed by such wild birds. Thus, these and similar species could have increased chances of exposure to *L. intracellularis* and could serve as biological vectors of proliferative enteropathy. Wild bird feeding patterns and previous reports of wild and feral animals exposed to *L. intracellularis* could be an alternative explanation for the association between *L. intracellularis* and wild birds.

Key words: *Lawsonia intracellularis*; gene; diagnosis; surveillance; infectious disease; PCR

Introduction

Proliferative enteropathy is an intestinal disease that is characterized by thickening of the distal small and proximal large intestinal mucosa due to enterocyte proliferation associated with the presence of an intracellular bacterium (1). One such bacterium is the highly fastidious, obligate intracellular Gram-negative bacterium *Lawsonia intracellularis* (2). A salient feature of the biology of *L. intracellularis* is its ability to produce a chronic infection that persists in the host, thus making control of proliferative enteropathy difficult in infected animals.

We previously reported an overall 4-year average true prevalence of *L. intracellularis* infection of 40.0% (CI: 39.4 - 40.6%) at the individual animal level and 71.9% (CI: 70.3-73.4%) at the herd level in 8,008 swine serum samples obtained from 1,001 herds (3). Although proliferative enteropathy is currently present in all swine-producing areas worldwide, including the Republic of Korea (ROK) (4), the epidemiology of proliferative enteropathy is still poorly understood. Although *L. intracellularis* has been most frequently recognized and studied by serology and molecular methods in swine and horses (5-8), diseases that closely resemble porcine

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proliferative enteropathy and are also caused by *L. intracellularis* have been described in a range of host species as single case reports, including rodents, deer, emus, wolves, foxes, non-human primates, and rhesus macaques (9-16). Much of the available information regarding *L. intracellularis* is still rudimentary, despite its worldwide spread, high prevalence, economic impacts on the swine industry, and newly identified susceptible animal hosts. The fastidious conditions required for the isolation and in vitro cultivation of this pathogen also impede the progress of discovery and make *L. intracellularis* difficult to study. Therefore, previous surveillance for proliferative enteropathy caused by *L. intracellularis* has focused on the swine and equine industries, while reports in other species are in the format of case studies (10, 15-17).

L. intracellularis has been reported in animals living in the wild, most frequently in wild pigs in the Czech Republic (15, 18) and in the feces of wolves (*Canis lupus*), red foxes (*Vulpes vulpes*), and red deer (*Cervus elaphus*) in the Slovak Republic (16). Recent studies of *L. intracellularis* in wild and feral animals caught on pig farms suggested a potential environmental spill-over from swine to wildlife (19, 20). However, little is known about the mechanisms of transmission of *L. intracellularis,* especially in wild bird species, which could be important vectors for this bacterium. Proliferative enteropathy has not been reported in chickens or other avian species, with the exception of ratite birds (11, 17). Although *L. intracellularis* DNA was recently detected in turkey flocks (21), the disease has been confirmed only in emus (11) and ostriches (17) and has been reported as being absent in chickens and wild birds (22-24). The aim of the present study was to screen for the presence of *L. intracellularis* in the ileal tissues of dead wild birds in the ROK.

Materials and methods

Samples

The study was carried out from 2010 to 2013. Carcasses of wild birds submitted to the Conservation Genome Resource Bank for Korean Wildlife (CGRB, Seoul National University, Seoul, ROK) and the Animal Disease Diagnostic Center of the Animal, Plant, and Fisheries Quarantine and Inspection Agency of the Ministry of Food, Agriculture, Forestry and Fisheries of the ROK, were used in the study. A total of 745 dead wild birds (belonging to 70 species) from all geographical regions of the ROK were submitted for examination, comprising 51 samples from 2010, 167 samples from 2011, 239 samples from 2012, and 288 samples from 2013. Table 1 shows the taxonomic families of the birds collected. All of the birds were received as carcasses, which were then stored at -20 ℃, until required. At necropsy, the gastrointestinal tracts were examined, with special attention paid to gross pathological findings, such as swelling, congestion, and hemorrhage of the ileum and cecum. The mucous membrane of the ileum was sampled by scraping the intestinal walls of each carcass; in addition, a 1-cm sample was taken from the middle of each ileum.

Polymerase Chain Reaction (PCR)

Ileal tissues were processed for nucleic acid purification within 48 hours of the carcasses having thawed. Phosphate-buffered saline (2 mL) was added to 1 g of ileal tissue in a conical tube. Each sample was vortexed for 10 sec. Nucleic acid purification from 180 µL of the supernatant was performed using an automated nucleic acid extraction system (BioRobot M48 Workstation, Qiagen, GmBH, Hilden, Germany) according to the manufacturer's recommendations. The purified DNA was then amplified by PCR using a previously described assay targeting an *L. intracellularis* gene, GenBank ID L08049 (25), and using a Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). The following primer sequences were used: LIA (5′-TATGGCTGTCAAACACTCCG-3) and LIB (5′-TGAAGGTATTGGTATTCTCC-3′). Positive (DNA from a pure culture of *L. intracellularis*) and negative (*L. intracellularis*-free DNA from ileal tissue samples) DNA controls were used in each run. After the PCR reaction, amplification products (5 µL) were analyzed by electrophoresis on a 3% agarose gel containing 0.5 µg/mL ethidium bromide. A 319-bp product indicated that *L. intracellularis* DNA was in the original sample. To prevent cross-contamination, the lab areas used for sample preparation, DNA extraction using automated nucleic isolation/processing, and amplification/post-PCR analysis

were physically separated. To prevent false positives, individual reagents and PCR-related consumables were screened before use to test for unknowns, especially oligonucleotides. Moreover, negative controls were run for every step using fresh reagents and disposables.

Cloning, Sequencing, and Analysis of Nucleotide Sequences

PCR and sequencing analysis of the 16S rRNA gene were performed. The amplified PCR products of DNA extracted from the ileal tissues were used for sequence analysis. Briefly, the PCR products were purified using a PCRquick- spin™ PCR Product Purification Kit (Intron Biotechnology, Seongnam-si, ROK) and cloned into the pGEM-T cloning system (Promega Corp., Madison, WI, USA). The plasmid clones were purified with a DNA- spin™ Plasmid DNA Extraction Kit (Intron Biotechnology), and the sequence analysis was conducted by Macrogen (Seoul, ROK). Nucleotide sequence homology searches of the cloned products of *L. intracellularis* in ileal tissues from the dead birds were analyzed by the National Center for Biotechnology Information (NCBI) BLAST network service.

Results and discussion

Of the 745 birds examined, *L. intracellularis* DNA was present in the mucous membrane of the ileum from one Eurasian eagle-owl (*Bubo bubo*; 5.0% among 20 samples), two black-billed magpies (*Pica pica sericea*; 2.0% among 96 samples), and one jungle crow (*Corvus macrorhynchos*; 3.0% among 33 samples) (Table 2). The amplified 16S rRNA gene sequences from all four infected wild birds were found to be 100% identical to a strain designated *L. intracellularis* PHE/MN1-00 (GenBank accession no. AM180252.1) by sequence analysis. In some wild birds, gross pathology, such as swelling, congestion, or intestinal hemorrhage, was found, but was not correlated with the molecular detection results. No pathological findings were observed in the intestines of wild birds that tested positive for *L. intracellularis*. The lack of molecular pathogen detection in those wild birds with pathological findings may be related to a potential intermittent mode of pathogen shedding or recovery from *L. intracellularis* infection.

All of the wild birds that were found to be positive for *L. intracellularis* in this study were collected in provinces where the prevalence of farm animals was relatively high. For example, the *L. intracellularis* DNA-positive Eurasian eagle-owl was collected in Gyeonggi province, where a 34.8% pig prevalence and 77.3% herd prevalence of *L. intracellularis* were previously reported (3). A black-billed magpie was found in Gangwon province (46.5% pig and 63.6% herd prevalence) and in Chungnam province (44.4% pig and 89.1% herd prevalence). In addition, the jungle crow was found in Jeju province (40.6% pig and 64.7% herd prevalence). All of the wild birds collected in areas with a lower prevalence of pigs and herds were negative in this study, e.g., Chungbuk (26.4% pig and 52.9% herd prevalence), Jeonbuk (30.8% pig and 38.2% herd prevalence), and Gyeongnam (20.3% pig and 47.9% herd prevalence).

The demonstration of *L. intracellularis* as a causative agent is difficult (4, 26), because its in vitro cultivation is complicated and not widely available. For these reasons, methods of molecular biology are widely used to detect this pathogen (25, 27, 38). Our surveillance method for the molecular detection of DNA demonstrated evidence of *L. intracellularis* in tissue samples of the small intestine in dead wild birds (Eurasian eagle-owl, black-billed magpie, and jungle crow) during the surveillance period. However, there are a few reports that detail a lack of evidence for the presence of *L. intracellularis* in other avian species, such as sparrows (*Passer domesticus*) and domestic poultry (*Gallus gallus*) (29, 24). McOrist *et al.* could not find evidence of *L. intracellularis* DNA in chickens with enteric disease and considered that the bacterium appears to be associated with malabsorption syndromes in these birds (23).

We previously reported that 40.0% (CI: 39.4-40.6%) of pigs and 71.9% (CI: 70.3-73.4%) of swine herds (3) were serologically positive for *L. intracellularis*. Lim et al. published that a total of 13/137 healthy rabbit feces were positive for *L. intracellularis* in the ROK (30). In addition, Hossain et al. reported that a total of 35 (25.74%) out of 136 sera and 36 (33.03%) out of 109 feces were positive for *L. intracellularis* in wild animals, such as the Korean water deer (*Hydropotes inermis*), Siberian roe deer (*Capreolus pygargus*), and raccoon dogs (*Nyctereutes procyonoides*), in

Table 1: Seven hundred forty-five dead wild birds from 70 species were tested for the presence of *Lawsonia intracellularis* infection

Family	Species	Common name	Samples
Accipitridae	*Aegypius monachus*	Cinereous vulture	1
	Buteo buteo	Common buzzard	8
	Accipiter nisus	Eurasian sparrowhawk	1
Alcedinidae	*Alcedo atthis*	Common kingfisher	3
Anatidae	*Anas formosa*	Baikal teal	6
	Anser fabalis	Bean goose	2
	Anas platyrhynchos	Mallard	60
	Aix galericulata	Mandarin duck	2
	Psittacidae	Parrot	1
	Anas acuta	Pintail	2
	Anas poecilorhyncha	Spot-billed duck	16
	Anser albifrons	White-fronted goose	6
Anatinae	*Anas crecca*	Common teal	7
Ardeidae	*Nycticorax nycticorax*	Black-crowned night heron	5
	Bubulcus ibis	Cattle egret	10
	Ardea alba	Great egret	6
	Ardea cinerea	Gray heron	9
	Mesophoyx intermedia	Intermediate egret	1
	Egretta garzetta	Little egret	8
	Butorides striatus	Striated heron	3
Caprimulgidae	*Caprimulgus jotaka*	Gray nightjar	5
Ciconiidae	*Ciconia boyciana*	Oriental white stork	1
Columbidae	*Columba livia*	Feral pigeon	3
	Columba rupestris	Hill pigeon	39
	Streptopelia orientalis	Rufous turtle dove	19
Coraciidae	*Eurystomus glaucurus*	Broad-billed roller	3
Corvidae	*Cyanopica cyanus*	Azure-winged magpie	1
	Pica pica sericea	Black-billed magpie	96
	Garrulus glandarius	Jay	6
	Corvus macrorhynchos	Jungle crow	33
Cuculidae	*Cuculus canorus*	Common cuckoo	1
	Cuculus optatus	Oriental cuckoo	1
Emberizidae	*Emberiza rustica*	Rustic bunting	2
Falconidae	*Falco tinnunculus*	Common kestrel	11
	Falco subbuteo	Eurasian hobby	10
Fringillidae	*Carduelis spinus*	Eurasian siskin	1
	Carduelis sinica	Gray-capped greenfinch	1

Family	Species	Common name	Samples
Gaviidae	*Gavia stellata*	Red-throated diver	1
Halcyonidae	*Halcyon pileata*	Black-capped kingfisher	2
Hirundinidae	*Hirundo rustica*	Barn swallow	1
Laridae	*Larus crassirostris*	Black-tailed gull	2
	Larus argentatus	Herring gull	1
Muscicapidae	*Cyanoptila cyanomelana*	Blue-and-white flycatcher	1
Oriolidae	*Oriolus chinensis*	Black-naped oriole	2
Paridae	*Parus major*	Great tit	2
Passeridae	*Passer montanus*	Tree sparrow	18
Phasianidae	*Gallus gallus domesticus*	Chick	1
	Chrysolophus pictus	Golden pheasant	1
	Gallus gallus var. domesticus	Korean black chicken	1
	Phasianus colchicus	Ring-necked pheasant	39
Picidae	*Dendrocopos major*	Great spotted woodpecker	1
	Picus viridus	Green woodpecker	1
	Dendrocopos kizuki	Japanese pygmy woodpecker	1
Procellariidae	*Calonectris leucomelas*	Streaked shearwater	1
Pycnonotidae	*Microscelis amaurotis*	Brown-eared bulbul	11
Rallidae	*Fulica atra*	Coot	1
	Gallinula chloropus	Moorhen	1
Scolopacidae	*Numenius phaeopus*	Whimbrel	1
	Scolopax rusticola	Woodcock	6
Strigidae	*Ninox scutulata*	Brown hawk owl	37
	Otus lettia	Collared scops owl	7
	Bubo bubo	Eurasian eagle-owl	20
	Otus scops	Eurasian scops owl	33
	Asio otus	Long-eared owl	2
	Strix aluco	Tawny owl	1
Sturnidae	*Sturnus cineraceus*	Gray starling	1
Sylviidae	*Paradoxornis webbiana*	Vinous-throated parrotbill	1
Turdidae	*Turdus hortulorum*	Gray-backed thrush	1
	Zoothera dauma	White`s thrush	13
Zosteropidae	*Zosterops japonicus*	Japanese white-eye	1
Unidentified			142
Total			745

the ROK (31). However, a molecular survey of *L. intracellularis* in wild birds was lacking.

This is the first report of the detection of *L. intracellularis* in wild birds in the ROK. In this study, very few wild birds were infected with *L. intracellularis*, but the infections are likely to have epidemiological relevance. One Strigidae (Eurasian eagle-owl) and two Corvidae (black-billed magpie and jungle crow) were exposed to *L. intracellularis*. The Eurasian eagle-owl is known to feed mainly on small mammals, such as voles, rats, mice, and hares. However, prey the size of foxes, marmots, and young deer (up to 17 kg) can also be killed, if taken by surprise (32). Another significant group of prey is other birds, and almost any type of bird is potential prey. Common avian prey includes corvids, grouse, woodpeckers, and other raptors. These feeding behaviors could be a reason why the Eurasian Eagle-owl had been exposed to *L. intracellularis*, given that recent studies have shown exposure to *L. intracellularis* among wild and feral animals, e.g., cats, rabbits, foxes, and wild rodents, that were caught on pig farms (16, 19, 20). The black-billed magpie is an opportunistic omnivore, known for eating many types of insects, carrion, seeds, rodents, berries, nuts, eggs, and garbage and food from pets that are fed outside (33). Its chicks are fed animal matter almost exclusively. Crows are also omnivorous (34), and will eat a variety of both plant and animal foods, whether alive or dead, including fruits, nuts, mollusks, earthworms, seeds, frogs, eggs, nestlings, mice and carrion (35, 36). In rural areas of the ROK, these two Corvidae species, the black-billed magpie and jungle crow, scavenge livestock feeding areas in large numbers, and obtain much of their food from grains spilled or wasted by livestock feeders or from undigested grain in horse manure (37). These foraging habits may be responsible for the positive PCR results for *L. intracellularis*, because horses are one of the most important susceptible animal species in the epidemiology of proliferative enteropathy. The feeding patterns of the Eurasian eagle-owl, black-billed magpie, and jungle crow and previous reports of wild and feral animals exposed to *L. intracellularis* could be possible alternative explanations for the association between *L. intracellularis* and wild birds. Further study will be necessary to determine the relationship between susceptible animal species and avian species, given that increasing numbers of new susceptible animal hosts being identified.

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DOLOČANJE DNK BAKTERIJE *Lawsonie intracellularis* V TKIVU VITEGA ČREVESA MRTVIH PTIC V REPUBLIKI KOREJI

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Povzetek: Vrsta bakterije *Lawsonia intracellularis* je vzrok proliferativne enteropatije pri različnih vrstah živali. O mehanizmih prenosa *L. intracellularis*, še zlasti pri divjih ptičjih vrstah, je na voljo malo podatkov. Prisotnost *L. intracellularis* pri mrtvih prosto živečih pticah v Republiki Koreji je bila raziskana z metodo verižne reakcije s polimerazo (PCR). DNK *L. intracellularis* smo dokazali v sluznici vitega črevesja pri veliki uharici (*Bubo bubo*, Strigidae), dveh korejskih srakah (*Pica pica sericea,* Corvidae) in eni velekljuni vrani (*Corvus macrorhynchos*, Corvidae) izmed 745 preiskanih mrtvih divjih ptic. Čeprav je bilo v tej študiji le nekaj prosto živečih ptic izpostavljenih *L. Intracellularis,* kaže, da je izpostavljenost epidemiološko pomembna. V povezavi z običajnim obnašanjem različnih vrst ptic je verjetno možno domnevati, da so vrste izpostavljene *L. intracellularis* (velika uharica, korejska sraka in velekljuna vrana) na različnih farmah zlahka dostopale do prašičev ali konj, ki pa so znani rezervoarji za *L. Intracellularis*. Tako je verjetno, da imajo te in podobne vrste visoko možnost izpostavitvi *L. intracellularis* in bi lahko služile kot biološki vektorji za proliferativno enteropatijo. Drugo možnost izpostavitve teh ptic *L. intracellularis* pa so lahko načini prehranjevanja divjih ptic in njihovi stiki z divjimi živalmi, okuženimi z *L. Intracellularis.*

Ključne besede: *Lawsonia intracellularis*; geni; diagnoza; nadzor; nalezljiva bolezen; PCR

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Original Research Article

THE INFLUENCE OF ISOFLURANE ANAESTHESIA ON INTESTINAL PERMEABILITY IN HEALTHY DOGS

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Abstract: This study investigated the most appropriate blood sampling time for determining the lactulose/mannitol (L/M) index, and whether isoflurane anaesthesia increases intestinal permeability in dogs, in terms of changes in the L/M index. Six dogs were given 100 ml of sugar solution (3.6 g lactulose and 3.4 g mannitol) with orogastric tube. Blood samples for determination of basal plasma L/M index were taken 90, 120, and 180 minutes later. The next day, the dogs were administered methadone, induced with midazolam mixed with ketamine, followed immediately by propofol, and anaesthesia maintained with isoflurane in oxygen for 200 minutes. The same sugar solution as the day before was administered at the end of anaesthesia and 12 and 24 hours post-anaesthesia. Blood samples were taken 90, 120 and 180 minutes after administration of sugar solution. HPLC-MS was used for plasma determination of lactulose and mannitol and the results expressed as L/M index. The highest concentrations of lactulose and mannitol were detected 120 minutes after administration of sugar solution. Lactulose/mannitol index significantly increased at the end of anaesthesia, regardless the sampling time, when compared to basal values from non-anaesthetized dogs. The increase of L/M index due to anaesthesia with isoflurane was short-lived as there was no significant difference between L/M index at 12 hours after the anaesthesia and basal values. The most appropriate blood sampling time for determining the L/M index is 120 minutes after oral administration of dual sugar solution.

Key words: dog; isoflurane; intestinal permeability; L/M index

Introduction

The gastrointestinal tract forms a barrier between the contents of its lumen and the systemic circulation. Epithelial cells lining the digestive tube, linked together by tight junctions, form the basis of the gastrointestinal barrier. The tight junctions are the main determinant of gastrointestinal permeability. The intestinal barrier allows absorption of nutrients while preventing passage of many potentially harmful particles such as bacteria, bacterial and food antigens, and compounds that could be toxic, antigenic or carcinogenic (1).

Intestinal damage may be assessed by non-specific and non-invasive intestinal permeability tests, where one or more probes are given orally and then measured in urine or blood (2, 3, 4). Collection of urine sample is impractical when compared to blood sampling because it takes five to six hours (5) and the results of test may be false due to incomplete urine collection (6). One of the tests used for determination of intestinal permeability is the dual sugar test. The advantage of this test is that all variables that alter intestinal permeability or absorption will equally affect both

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markers and are cancelled out when results are expressed as index (2, 4, 7, 8).

Conventionally, a disaccharide and monosaccharide are used together. Lactulose is the most widely used disaccharide probe, and is completely metabolised in the colon. Its absorption reflects small intestinal permeability (7). Lactulose is absorbed paracellularly, between the enterocytes through pores in the area of the tight junctions (10) or via damaged epithelium (11). A maximum of 0.4 – 2% lactulose permeates through pores (10). This amount can be increased when lactulose is given in hyperosmolar solution (12). The monosaccharide mannitol is a low molecular weight sugar alcohol, which is thought to diffuse through water-filled pores in the enterocyte membrane (13). The ratio between plasma lactulose and mannitol concentrations is expressed as lactulose/mannitol (L/M) index.

Changes in intestinal permeability may occur due to major surgery (14) or trauma (15), ingestion of non-steroidal anti-inflammatory drugs (16), and intestinal ischemia. Partial intestinal ischemia, where blood flow is reduced to one third of the resting control level, induces increased mucosal permeability to macromolecules within one hour and obvious morphological injury to the small intestinal villi. In disease states that compromise the mucosal barrier, microorganisms and their toxins may escape from the intestinal lumen to the lymph, the portal vein and systemic circulation or to the peritoneal cavity, producing deleterious effects (8, 17).

General anaesthesia with isoflurane decreases intestinal tissue perfusion to varying degrees during systemic hypotension in the dog (18), which in turn might increase intestinal permeability. The aim of this study was to investigate whether general anaesthesia with isoflurane increases intestinal permeability in dogs, in terms of changes in the L/M index, and to establish the most appropriate blood sampling time for determining the L/M index.

Materials and methods

Animals

Six healthy, intact adult male beagle dogs weighing 15.3 to 21.7 kg were included in the study. The dogs were judged to be healthy on the basis of clinical examination and normal blood work, i.e., complete blood count, white cell differential count and serum biochemistry profile including blood urea nitrogen, creatinine, inorganic phosphate, total protein, albumin and electrolytes, i.e., potassium, sodium, and chloride (data not shown). The dogs were housed in couples, fed a commercial dry and canned diet twice a day with unlimited access to water and walked in pairs at least 20 minutes three times per day. Social contacts between the caretakers and dogs were carried out during the day.

The study complied with applicable Slovenian governmental regulations (Animal Protection Act UL RS, 43/2007) and obtained ethical approval by the Ministry of Agriculture, Forestry and Food, Veterinary Administration of the Republic of Slovenia; license No 323-02-80/01.

Experimental design

The dogs were given 100 ml iso-osmolar sugar solution containing 3.6 g lactulose (Portalak, Belupo, Koprivnica, Croatia) and 3.4 g mannitol (Manit 20 %, Pliva, Zagreb, Croatia) via the orogastric tube. Blood samples for determination of basal plasma lactulose and mannitol concentrations were collected in heparinized tubes (Vacutainer Systems, Becton Dickinson, Franklin Lakes, New Jersey, USA) at 90, 120 and 180 minutes after administration of dual sugar solution.

The influence of general anaesthesia on intestinal permeability was investigated the next day. The same sugar solution as the day before was administered at the end of anaesthesia that lasted 200 minutes and 12 and 24 hours post-anaesthesia and blood samples were taken at 90, 120 and 180 minutes after each administration of dual sugar solution.

Access to water was unlimited until the dogs were given dual sugar solution, while the food was withheld for 12 hours before administration of dual sugar solution on both days. After the last blood sampling at 180 minutes, the dogs were offered food and water.

Determination of L/M index

Blood samples were centrifuged at 3000 *g* for 15 minutes at 4 °C immediately after collection and stored at –70 °C until analysis.

Plasma lactulose and mannitol concentrations were determined by high performance liquid chromatography-mass spectrometry (HPLC-MS) and L/M index calculated as the ratio between plasma lactulose and mannitol concentrations. Plasma samples (150 µL) prepared for quantitative determinations of sugars were diluted with 750 µL distilled water and freeze-dried. Dehydrated samples were redissolved in 150 µL of methanol (Merck, Darmstadt, Germany), centrifuged and supernatant injected into HPLC–MS system. Separation and quantitative determinations of lactulose and mannitol were performed with a Surveyor LC system (Thermo Finnigan, Riviera Beach, CA, USA) equipped with LCQ mass detector (Finnigan MAT, San Jose, CA, USA). Quantitative determinations of lactulose and mannitol were done in one run on Thermo Hypersil APS-2, 150 x 4 mm, 3 µm column (Thermo Electron Corporation, CA, USA) at room temperature. The isocratic mobile phase consisted of methanol/ethanol/water (MeOH/EtOH/H_2O), 52:35:13 (v/v/v), the run time was 9 minutes, and the flow rate was 1.0 mL/minute. The retention time of mannitol was 4.33 ± 0.2 minutes, and for lactulose 6.63 ± 0.2 minutes. MS identification and quantification was done in negative APCI ionization mode. Ionization discharge current was 6.0 µA, and source temperature 500 °C. Capillary voltage was 23.0 V, tube lens offset was 35.0 V, capillary temperature was 200 °C, sheath gas pressure was 2.7 bar, and auxiliary gas flow was 1.6 L/minute.

Anaesthesia

The dogs were premedicated with methadone (Heptanon, Pliva, Zagreb, Croatia) 0.2 mg/kg subcutaneously and 15 minutes later induced to anaesthesia with midazolam (Dormicum, F. Hoffmann-La Roche, Basel, Switzerland) 0.2 mg/kg mixed with ketamine (Ketanest 10%, Parke-Davis, Freiburg, Germany) 1 mg/kg, followed immediately by propofol (Diprivan, Zeneca Pharmaceuticals, Wilmington, Delaware, USA) 3 mg/kg, all of them given intravenously. The dogs were endotracheally intubated and anaesthesia maintained with isoflurane (Forane, Abbott Laboratories, Baar, Switzerland) at end-tidal isoflurane concentrations (EtIso) of 1.1 - 1.4% in oxygen for 200 minutes. The dogs were mechanically ventilated (Ventilog, Dräger, Lübeck, Germany) with tidal volume of 15 mL/kg. The respiratory rate was adjusted to maintain end-tidal carbon dioxide ($EtCO_2$) in the normal range (35 - 45 mmHg). Lactated Ringer's solution (Sestavljen natrijev laktat, B Braun Melsungen AG, Melsungen, Germany) was infused at a rate of 10 mL/kg/h during anaesthesia. The dogs were positioned in dorsal recumbency on a heated surgical table (33 °C) during the anaesthesia. The temperature of the air in the operating theatre was maintained at 20 to 24 °C.

EtIso, $EtCO_2$, arterial oxygen saturation measured with pulse oximetry (SpO_2), respiratory rate and airway pressure within the breathing circuit were continuously monitored during anaesthesia (RGM 5250, Ohmeda, Louisville CO, USA). Tidal volume and minute volume of respiration were measured by means of mechanical volumetry (Ventilog, Dräger Tiberius 800, Lübeck, Germany). Direct arterial blood pressure (catheter placed in the femoral artery), heart rate (Table 3) and body core temperature were measured (HP Model 78354A, Hewlett Packard GmbH, Hamburg, Germany). At the end of anaesthesia, the dogs were allowed to recover from anaesthesia and returned to their cages.

Statistical analysis

Data were analysed with commercial software (SPSS 15.0, Chicago, Illinois, USA). Results are expressed as means ± SD. To test whether the data were normally distributed, histograms were generated and inspected visually and Shapiro-Wilk tests were performed. Repeated measures ANOVA (RMANOVA) with Bonferroni correction was used to compare basal values of lactulose, mannitol and L/M index to later measurements (end of anaesthesia and 12 and 24 hours later). The same method (RMANOVA with Bonferroni correction) was used to test for statistically significant differences of lactulose and mannitol between different blood sampling times (90, 120 and 180 minutes after administration of sugar solution) at each measurement (end of anaesthesia and 12 and 24 hours later). The value of $p < 0.05$ was considered significant.

Table 1: Plasma mannitol concentration (mol/L; mean ± SD) 90, 120 and 180 minutes after administration of sugar solution at basal values, end of anaesthesia, 12 and 24 hours after the end of anaesthesia

time	basal values	end of anaesthesia	12 h	24 h
90 min	3.985E-05 ± 1.64E-05	2.278E-05 ± 1.08E-05[a]	3.643E-05 ±1.09E-05	3.292E-05 ± 9.78E-06
120 min	5.910E-05 ± 2.47E-05[b]	3.348E-05 ± 1.62E-05[a,b]	5.398E-05 ± 1.63E05[b]	4.860E-05 ± 1.47E-05[b]
180 min	5.475E-05 ± 2.16E-05[b]	2.995E-05 ± 1.44E-05[a,b]	4.817E-05 ±1.45E-05[b]	4.350E-05 ± 1.31E-05[b]

[a] significantly lower plasma mannitol concentration compared to basal value ($p < 0.05$)
[b] significantly higher plasma mannitol concentration compared to blood sampling at 90 minutes ($p < 0.05$)

Table 2: Plasma lactulose concentration (mol/L; mean ± SD) 90, 120 and 180 minutes after administration of sugar solution at basal values, end of anaesthesia, 12 and 24 hours after the end of anaesthesia

time	basal values	end of anaesthesia	12 h	24 h
90 min	2.29E-07 ± 3.31E-08	2.24E-07 ± 2.43E-08	1.83E-07 ± 7.41E-09[a]	1.80E-07 ± 6.75E-09[a]
120 min	2.92E-07 ± 5.97E-08[b]	2.83E-07 ± 4.4E-08[b]	2.09E-07 ± 1.33E-08[a,b]	2.02E-07 ± 1.26E-08[a,b]
180 min	2.85E-07 ± 5.7E-08[b]	2.64E-07 ± 4.16E-08[b]	2.6E-07 ± 1.27E-08[a,b]	2.01E-07 ± 1.12E-08[a,b]

[a] significantly lower plasma lactulose concentration compared to basal value ($p < 0.05$)
[b] significantly higher plasma lactulose concentration compared to blood sampling at 90 minutes ($p < 0.05$)

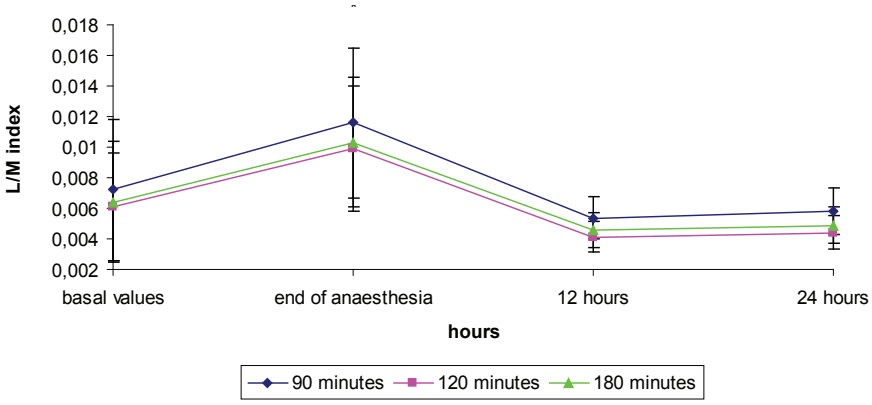

Figure 1: L/M index (mean ± SD) 90, 120 and 180 minutes after administration of sugar solution. *Significant difference compared to basal values ($P < 0.05$)

Table 3: Systolic (SAP), diastolic (DAP) and mean (MAP) blood pressure (mean ± SD) and heart rate (HR; mean ± SD) during anaesthesia

Time (min)	20	40	60	80	100	120	140	160	180	200
SAP (mm Hg)	103 ± 9	107 ± 12	127 ± 13	128 ± 11	124 ± 13	132 ± 22	128 ± 17	138 ± 15	132 ± 11	133 ± 16
DAP (mm Hg)	57 ± 7	61 ± 8	65 ± 8	64 ± 5	64 ± 7	70 ± 11	64 ± 6	71 ± 7	67 ± 7	67 ± 12
MAP (mm Hg)	71 ± 7	73 ± 5	80 ± 7	82 ± 5	79 ± 7	86 ± 13	81 ± 8	88 ± 7	84 ± 6	80 ± 9
HR (beats/minute)	115 ± 26	107 ± 19	106 ± 22	107 ± 20	106 ± 18	109 ± 18	105 ± 20	110 ± 17	104 ± 23	113 ± 24

Results

The highest concentrations of lactulose and mannitol were detected 120 minutes after administration of sugar solution. Plasma mannitol and lactulose concentrations, determined at both 120 and 180 minutes after administration of sugar solution, were significantly higher than at 90 minutes (Table 1 and 2). Comparing to basal values, plasma mannitol concentration

was significantly lower at the end of anaesthesia at all three sampling times (Table 1), while the concentration of lactulose was significantly lower 12 and 24 hours after anaesthesia at all three blood sampling times (Table 2).

Compared to basal values in non-anaesthetized dogs, the L/M index increased significantly due to a decrease of plasma concentration of mannitol at the end of anaesthesia at all blood sampling times (90, 120 and 180 minutes). Lactulose/mannitol index decreased as early as 12 hours after anaesthesia on account of significantly lower plasma lactulose concentration and there was no significant difference when compared to the basal values (Figure 1).

Discussion

In the present study, dual sugar test with iso-osmolar solution of lactulose and mannitol was used, as described by Papasouliotis at al. (19). The advantage of determining the ratio between disaccharide and monosaccharide is the enhanced sensitivity of the test, since the ratio evaluates not only the raised permeability to a disaccharide, due to opening of intercellular pathway, but also the effect of decreased absorption of a monosaccharide, due to reduced surface area or villous atrophy (2, 4).

Most investigators use physiological iso-osmolar tests rather than hyperosmolar test solutions (7, 20) because permeation of lactulose is markedly increased when the osmolarity of sugar solution increases beyond 1500 mOsm/L (12). Hyperosmolar sugar solution may also cause osmotic diarrhoea, distended abdomen and flatulence, while large volumes of iso-osmolar solutions may affect intestinal motility and alter the contact time between the sugar and the intestinal mucosa (21).

Lactulose and mannitol were detected in plasma with HPLC-MS at all three sampling times, the highest values being obtained 120 minutes after sugar administration. The concentrations of mannitol and lactulose were significantly higher at 120 and 180 minutes than at 90 minutes after sugar administration, which is in agreement with the results of Cox et al., (6) who used lactulose and mannitol to test intestinal permeability in humans. The levels of lactulose and mannitol in serum in their study were relatively stable from 60 to 120 minutes, and the lowest values were detected at 30 minutes after administration. An increased concentration of lactulose in dog serum from 30 to 180 minutes after administration of iso-osmolar sugar solution was also demonstrated by Rodriguez et al. (22).

Factors that can increase intestinal permeability to lactulose include increased perviousness of the intercellular tight junctions and increased accessibility of the luminal content to the intestinal crypts (21).

In the present study, L/M index increased at the end of the anaesthesia on account of decreased plasma mannitol concentration, despite a decrease of plasma lactulose concentration. Splanchnic ischemia is one of the factors that increase gut permeability by reduction of villous flow of up to 10% to 30%. Permeability abnormalities are mainly reflected by a decrease in the excretion of mannitol, while a decrease in the absorption of mannitol suggests a decrease of the functional absorptive area. The decrease in the absorption of mannitol with no increase in lactulose absorption, as observed in the present study, suggests a less severe injury of the mucosa than when both types of absorption are affected (23).

Anaesthesia induced hypotension, mainly due to decreased systemic vascular resistance, may be responsible for splanchnic hypoperfusion (18) and intestinal mucosal damage (24). Although in the present study the mean arterial pressure was maintained above 70 mmHg (Table 3), a value that enables adequate renal and splanchnic perfusion 25), the absorption of mannitol decreased.

Transitory increase in L/M index at the end of anaesthesia in this study may be a consequence of decreased gut motility and therefore reduced delivery of mannitol to the absorptive surfaces. Isoflurane results in a reduced frequency of occurrence of motility periods in rats (26) and gastro-caecal transit time in humans. The addition of ketamine to isoflurane anaesthesia delays gastric emptying and small-bowel transit time in humans (27). Moreover, a delay in gastric emptying and intestinal transit time can be found after intravenous, but not epidural morphine administration in dogs (28). A prolonged delay in gastro-caecal transit time has been also reported for other opioids, such as nalbuphine (27) and pethidine (29) in humans. Long-term methadone use prolongs oral-caecal transit time in humans (30), while no data on single-dose methadone

influence on gastro-caecal transit time is available. We may only assume that methadone which was used in our study, similarly as other opioids, contributed to the reduced delivery of mannitol to the absorptive surfaces.

Conclusions

The most appropriate time for determining plasma lactulose and mannitol concentration in dogs was determined to be 120 minutes after oral administration of dual sugar solution. The transitory increase of L/M index in the present study suggests that general anaesthesia with isoflurane after premedication with methadone and induction with midazolam, ketamine and propofol increases intestinal permeability in healthy normotensive dogs for less than 12 hours.

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VPLIV ANESTEZIJE Z IZOFLURANOM NA PREPUSTNOST ČREVESJA PRI ZDRAVIH PSIH

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Povzetek: Namen raziskave je bil ugotoviti najprimernejši čas odvzema krvi za določanje razmerja med laktulozo in manitolom (indeks L/M) in za ugotavljanje ali anestezija z izofluranom zveča indeks L/M in s tem prepustnost črevesne bariere pri psih. Šestim psom smo z orogastrično sondo dali 100 ml sladkorne raztopine, in sicer 3,6 g laktuloze in 3,4 g manitola. Za določitev bazalne vrednosti indeksa L/M smo psom odvzeli kri 90, 120 in 180 minut po dajanju sladkorne raztopine. Naslednji dan smo jih premedicirali z metadonom, v anestezijo pa uvedli z midazolamom s ketaminom in propofolom. Anestezijo smo vzdrževali z izofluranom v kisiku 200 minut. Enako sladkorno raztopino smo psom dali še trikrat, in sicer na koncu anestezije ter 12 in 24 ur po anesteziji. Po vsakem dajanju sladkorne raztopine smo po 90, 120 in 180 minutah odvzeli kri za določitev indeksa L/M. Laktulozo in manitol v plazmi smo določali z metodo HPLC-MS in rezultat izrazili kot indeks L/M. Najvišjo koncentracijo laktuloze in manitola smo zaznali 120 minut po dajanju sladkorne raztopine. Indeks L/M se je, v primerjavi z bazalnimi vrednostmi pri neanesteziranih psih, na koncu anestezije značilno zvišal ne glede na čas odvzema krvi. Anestezija z izofluranom kratkotrajno zveča indeks L/M, saj že 12 ur po anesteziji ni bilo značilne razlike v primerjavi z bazalno vrednostjo. Najprimernejši čas odvzema krvi za določitev indeksa L/M je 120 minut po dajanju sladkorne raztopine.

Ključne besede: pes; izofluran; prepustnost črevesja; indeks L/M

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Original Research Article

THE ORF5 VARIATION OF VIETNAMESE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS STRAINS

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Abstract: Porcine reproductive and respiratory syndrome is a devastating disease that causes heavy losses to the economy and the development of agriculture. In this study, we aimed to assess the genetic variation of the ORF5 gene from 12 Vietnamese porcine reproductive and respiratory syndrome virus (PRRSV) strains. The phylogenetic analysis of the ORF5 sequences of Vietnamese strains and other strains indicated that the Vietnamese strains belong to type II. The Vietnamese strains were also separated into two clusters. Five strains BG/12, TG1/12, TG2/12, TG3/12, and TG4/12 were grouped in cluster 1 with a 98% bootstrap value, while the other seven strains HCM/14, TG5/15, TG6/15, TG7/15, ST1/15, ST2/15, and ST3/15 belonged to cluster 2. The alignment of the deduced amino acid sequences demonstrated that the identity between Vietnamese strains with CH-1a, JXA1, and VR2332 strains were 87–93%, 91–98%, and 83–89%, respectively. The mutation of the N21 glycosylation site (N□S) of the GP5 sequence was observed in five Vietnamese strains from cluster 1. The core sequence of the neutralizing epitopes (including five positions at H25, Q27, I29, Y30, and N31) in GP5 was presented in all Vietnamese strains except strain TG1/12. The hydrophobicity plots of GP5 revealed two different positions of BG/12 strain from CH-1a strain and VR2332 strain. The first difference was the missing of a hydrophilic peak from position amino acid 85 to 95. In this region, the CH-1a and VR2332 strains have 3 hydrophilic peaks. The second difference was the loss of another hydrophilic peak at position amino acid 100.

Key words: genotype; ORF5; phylogenetics; PRRSV; Vietnam

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most dangerous pathogens for the swine industry (1). PRRSV is a member of the *Arteriviridae* family that comprises equine arteritis virus (EAV), simian haemorrhagic fever virus (SHFV), and lactate dehydrogenase-elevating virus (LDV) (2). Genomic sequence comparisons have revealed that PRRSV includes two genotypes: type I and type II (3). These two genotypes share only approximately 60% sequence identity (4). PRRSV is a spherical, enveloped virus containing a single, positive-sense RNA genome. The PRRSV genome is approximately 15 kb in length and contains nine open reading frames (ORFs), ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF6, and ORF7. ORF1a, and ORF1b (~12 kb) encode 12 non-structural proteins (nsp), nsp1–nsp12, which play major roles in viral replication (5). The remaining ORFs encode structural proteins (6).

In Vietnam, the first cases of PRRS were recorded in 1997 (7). Since then, PRRS has quickly spread and seriously affected almost all

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provinces. The PRRS outbreaks have significant economic impacts on the swine industry of Vietnam in 2008, 2010, and 2012. Highly pathogenic PRRSV is the causative agent of porcine high fever syndrome and characterized by high fever and high death rates in pigs of all ages (8). Furthermore, the coding region for ORF5 of PRRSV displays substantial genetic variation (9). Thus, ORF5 has become the regions of choice for monitoring the evolution of PRRSV and for molecular epidemiology research on PRRSV (10). Glycoprotein 5 (GP5) is an envelope glycoprotein of porcine reproductive and respiratory syndrome virus (8). The N-linked glycosylation in GP5 may be associated with the antigenicity of the neutralization epitopes located in the ectodomain (15). GP5 acts as a major inducer of neutralizing antibodies in vivo, containing three putative N-linked glycosylation sites (N34, N44, and N51), where a major neutralization epitope is located (15). The major neutralization epitope of PRRSV is located in the middle of the GP5 ectodomain (aa 36-52) (16). This report describes the investigation of genetic variation of ORF5 gene of Vietnamese PRRSV strains. The phylogenetic analysis was assessed to determine the genotype of Vietnamese PRRSV strains.

Materials and methods

Sample collection

The blood samples (n=12) were collected from the PRRSV-infected pigs with the clinical displaying of PRRS from 2012 to 2015 (Figure 1). All samples were stored in ice boxes and transported to the laboratory. Subsequently, the samples were kept at -80 ºC.

Figure 1: Sample collecting locations: 1: Bac Giang Province (1 sample), 2: Tien Giang Province (7 samples), 3: Ho Chi Minh City (1 sample), 4: Soc Trang Province (3 samples)

RNA isolation and RT-PCR

Total RNA was extracted using Rneasy Mini Kit (74104, Qiagen) according to the manufacturer's instructions. The RT-PCR reaction was carried out with a 1-Step RT-PCR Kit (PB10.52-05, PCR Biosystems) in a total volume of 50 µl containing 25 µl 2x PCRBIO One-Step Mix, 2 µl primers (400 nM), 2.5 µl 20x RTase, 2.5 µl RNA template (1 ng), 18 µl RNase-free H_2O. The RT-PCR was performed in a thermal cycle under the following conditions: the reverse transcription was performed at 42 ºC for 45 min; an initial denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 90 s; and the final cycle at 72 °C for 10 min. The primers specific for amplification of the ORF5 gene are ORF5-F: 5'-CAT GAG GTG GGC AAC TGT TT-3' and ORF5-R: 5'-GTC ATG TAC CCG AAG GTG AA-3' (13). Amplified products of ORF5 genes were estimated as 800 bp.

Table 1: PRRS strains

No.	Strain	Location - year	Reference	Type	Subtype
1	01UD6	Thailand - 2003	AY297113	Type 2	
2	02PB1	Thailand - 2003	AY297116		
3	FJ-1	China - 2005	AY881994		
4	GDCZ2	China - 2004	AY857636		
5	Ingelvac	USA - 2004	AY656991		
6	Jis2	Japan - 2004	AB175695		
7	Gu922M	Japan - 2004	AB175721		
8	Jeh1	Japan - 2004	AB175691		
9	CH-1a	China - 2001	AY032626		
10	CH-1R	China - 2008	EU807840		
11	JXA-1	China - 2006	EF112445		
12	VR2332	USA - 2007	EF536003		
13	01CB1	Thailand - 2003	AY297119	Type 1	Subtype 1
14	03RB1	Thailand - 2003	AY297124		
15	361-4	Denmark - 2001	AY035915		
16	Upa-13	Poland - 2005	DQ324688		
17	Amervac	Spain - 2005	DQ324668		
18	Porcillis	Netherlands - 2005	DQ324678		
19	2567/96	Denmark - 2001	AY035932		
20	Bor-41	Belarus - 2005	DQ324671		Subtype 2
21	Bor-54	Belarus - 2005	DQ324672		
22	Sid	Lithuania - 2005	DQ324682		
23	Aus	Lithuania - 2005	DQ324667		
24	Sno-4	Belarus - 2005	DQ324683		
25	Zap-46-50	Belarus - 2005	DQ324697		Subtype 3
26	Soz-6	Belarus - 2007	DQ324686		
27	Soz-8	Belarus - 2005	DQ324687		
28	Zad-1	Belarus - 2007	DQ324694		
29	Zad-14	Belarus - 2005	DQ324695		
30	Yuz-34	Belarus - 2005	DQ324692		
31	Bel-42	Belarus - 2007	DQ324669		Subtype 3/2
32	Bel-43	Belarus - 2005	DQ324670		
33	Okt-35	Belarus - 2005	DQ324677		Subtype 4
34	BG/12	Vietnam - 2012	KY310596	This study (Type 2)	
35	TG1/12	Vietnam - 2012	KY310597		
36	TG2/12	Vietnam - 2012	KY310598		
37	TG3/12	Vietnam - 2012	KY310599		
38	TG4/12	Vietnam - 2012	KY310600		
39	HCM/14	Vietnam - 2014	KY310601		
40	TG5/15	Vietnam - 2015	KY310602		
41	TG6/15	Vietnam - 2015	KY310603		
42	TG7/15	Vietnam - 2015	KY310604		
43	ST1/15	Vietnam - 2015	KY310605		
44	ST2/15	Vietnam - 2015	KY310606		
45	ST3/15	Vietnam - 2015	KY310607		

Table 2: Matrix of Tamura & Nei genetic distance among PRRSV strains using deduced amino acid sequence of GP5 protein. Lower triangular matrix values were mean genetic distances; upper triangular matrix values were standard errors

		Type 1					Type 2	Vietnam
		Subtype_1	Subtype_2	Subtype_3	Subtype_3/2	Subtype_4		
Type 1	Subtype_1		0,020	0,016	0,016	0,019	0,035	0,036
	Subtype_2	0,256		0,020	0,021	0,022	0,034	0,035
	Subtype_3	0,198	0,252		0,011	0,019	0,036	0,038
	Subtype_3/2	0,167	0,239	0,107		0,022	0,037	0,039
	Subtype_4	0,212	0,254	0,199	0,199		0,040	0,044
Type 2		0,513	0,510	0,522	0,499	0,535		0,010
Vietnam		0,496	0,490	0,513	0,477	0,547	0,114	

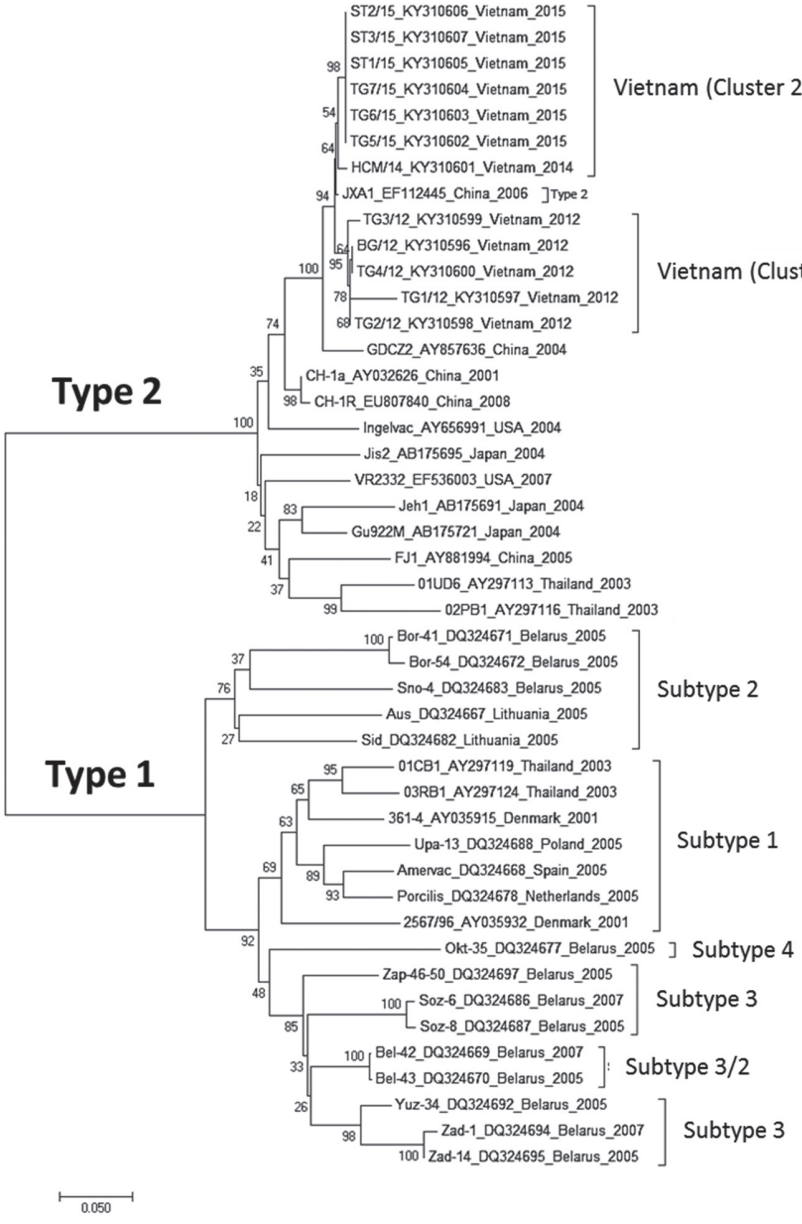

Figure 2: Phylogenetic tree constructed from ORF5 sequences of PRRSV strains using the neighbour-joining analysis method. Bootstrap resampling was done 1000 times, and the resulting bootstrap values are shown on the corresponding branches

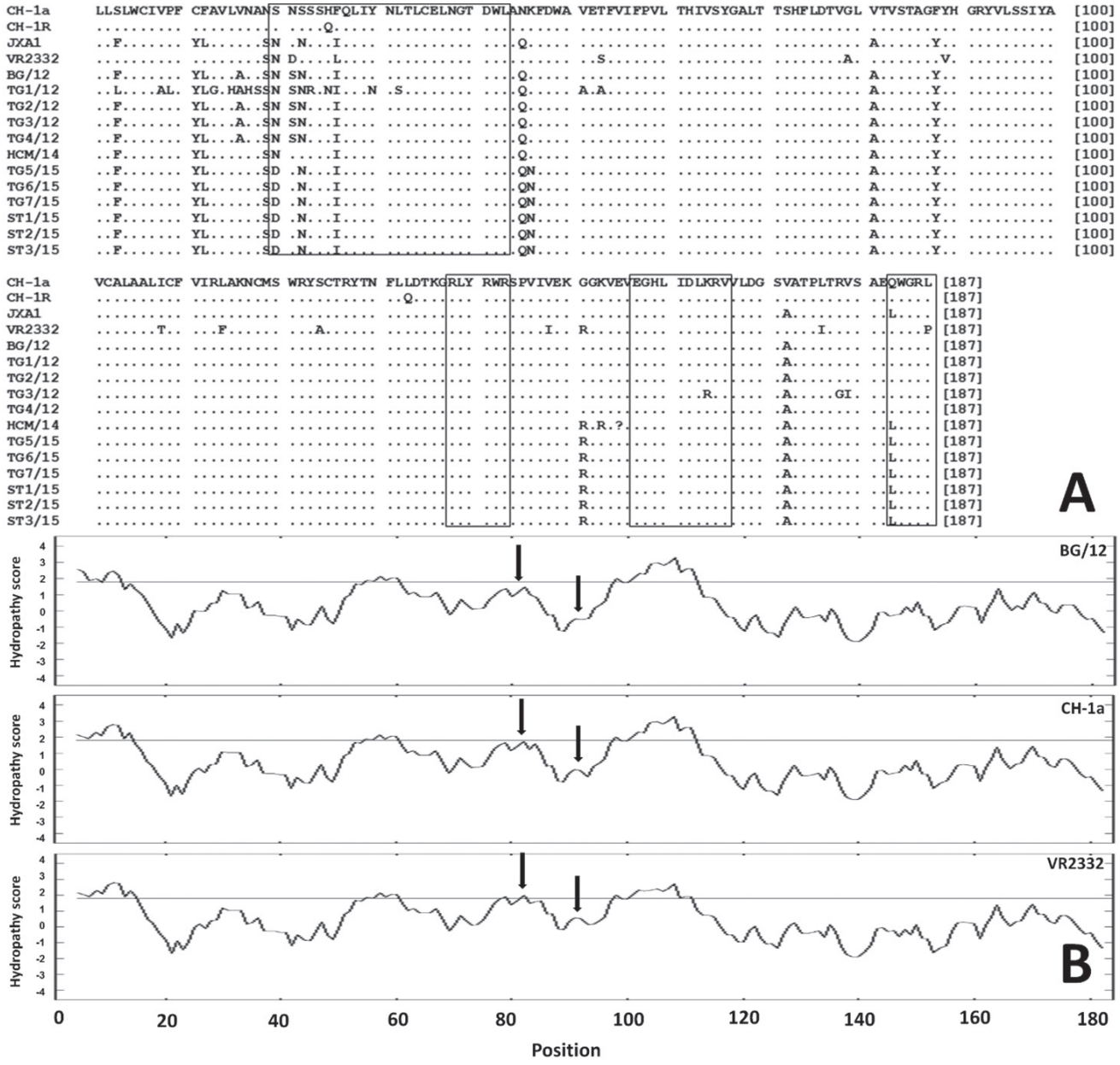

Figure 3: The variable positions of deduced amino acid sequences from PRRSV strains. A. Alignment of the deduced amino acid sequences of glycoprotein GP5 of 12 Vietnamese strains in comparison with strains CH-1a and VR2332. Dots indicate identical amino acids, and deletions are indicated by dashes (–). Black box indicates epitope. B. Hydrophobicity plots of ORF5 generated using the Kyte and Doolittle method. Major areas of difference are indicated by black arrows

Sequence analysis

RT-PCR products were purified and used as sequencing templates. The nucleotide sequences were directly sequenced (Macrogen, Seoul, Korea). The sequence trimming was used to remove misleading data from the ends of sequencing fragments. After trimming, the size of the ORF5 sequences was 561 bp (deduced amino acid sequences are 187 aa). The comparisons of the ORF5 sequences were analysed for 12 Vietnamese PRRSV strains and other strains from Genbank. The sequences alignment was performed with CLUSTAL W (14). The Tamura & Nei model was used as a genetic distance model. A neighbour-joining method was applied for phylogenetic construction (15). Bootstrap analysis (using 1000 replications) was used to assess the confidence in

branching order. The DNA sequences of ORF5 were translated into amino acid sequences to investigate genetic variation in the amino acid level. The amino acid sequences of CH-1a, CH-1R, JXA1, and VR2332 were used as reference PRRSV strains. The hydrophobicity plots of GP5 were generated using the Kyte and Doolittle method (16).

Results

The alignment of deduced amino acid sequences indicated that the identity between Vietnamese strains with CH-1a, JXA1, and VR2332 strains were 87–93%, 91–98%, and 83–89%, respectively. The genetic distance between Vietnamese strains with type II strains (0.114) was lower than type I strains (0.477 - 0.547) (Table 2).

A phylogenetic tree was constructed based on the nucleotide sequences from the ORF5 region of 12 Vietnamese strains and other type I and type II strains. All Vietnamese PRRSV strains were located in the type II group. These strains were also separated into two clusters. Cluster 1 included strains BG/12, TG1/12, TG2/12, TG3/12, and TG4/12 with 98% bootstrap value, the other strains HCM/12, TG5/15, TG6/15, TG7/15, ST1/15, ST2/15, and ST3/15 were belonged to cluster 2. The genetic divergence between Vietnamese strain cluster 1 and Vietnamese strain cluster 2 is 0.03±0.005.

In this study, two N-linked glycosylation sites (N31 and N38) were conserved in all Vietnamese PRRSV strains (Figure 3). The mutation of the N21 glycosylation site (N→S) was observed in all Vietnamese strains of cluster 1. The core sequence of the neutralizing epitopes (H25, Q27, I29, Y30 and N31) of GP5 was presented in Vietnamese strains except the TG1/12 strain. This strain revealed two mutations at position 25 (H→N) and position 30 (Y → N). The C terminus of GP5 protein contains three minimal epitopes including RLYRWR (aa 138 → aa 143), EGHLIDLKRV (aa 157 → aa 166), and QWGRL (aa 183 → aa 187) (17). These regions are highly conserved in Vietnamese strains. The mutation at position 183 (Q→L) occurred in all Vietnamese strains from cluster 2.

The Figure 3 demonstrated the GP5 hydrophobicity plots of strains BG/12, CH-1a, and VR2332. The strain BG/12 had a profile similar to strains CH-1a and VR2332. However, the hydrophobicity plots also revealed two distinct positions of the profile of strain BG/12 from strains CH-1a and VR2332. One of the differences was located between amino acids 75 and 85, where a hydrophilic peak was absent. Another variable area was hydrophilic peak loss at position amino acid 91. Each of two differences resulted from a single amino acid substitution, V to A (amino acid 81) and F to Y (amino acid 88), respectively. There were 2 amino acid substitutions in Vietnamese strains from cluster 1 (V → A at position 16, N → S at position 21). Two other amino acid substitutions were observed in Vietnamese strains from cluster 2 (G → R at position 151, and Q → L at position 183).

Discussion

In this study, the genetic variation of the Vietnamese PRRSV strains using ORF5 sequences were assessed. The phylogenetic analysis indicated that all Vietnamese strains belong to type II. The previous study showed that some Vietnamese PRRSV strains, collected from Northern areas (Ha Noi City, Quang Ninh Province, Nghe An Province) or Southern areas (Dong Nai Province, Tay Ninh Province) belonged to type II (18). Another study demonstrated that the Vietnamese PRRSV strains collected from 2008 to 2012 in Northern areas (Dien Bien Province) and Southern areas (Can Tho City, Dong Thap Province, Dong Nai Province) also belonged to type II (19). These results revealed the large distribution PRRSV strains of type II in Vietnam. The Vietnamese cluster 2 strains showed 4 different variable positions from Vietnamese cluster 1 strains (A → V at position 16, S → N at position 21, G → R at position 151, and L → Q at position 183). The PRRS virus could be transmitted between farms from various areas. The shipment of semen for artificial insemination may be an important mode of transmission of PRRSV between farms (20). Moreover, PRRSV-infected waterfowl carry and shed live infectious virus, implying that PRRSV may travel between farms in animal vectors (21, 22). It also has been suggested that airborne transmission is important for the spread of PRRSV between nearby farms (23). In Vietnam, households accounted for about 90% of pig stocks (24), and pigs were transported by personal vehicles or trucks between different regions and from Northern areas to Southern areas for consumption. Thus, this transport caused the PRRSV transmission between different areas.

Nucleotide sequence analysis revealed 88 to 99% aa identity among strains from the same continent, and only 52 to 55% aa identity between type I and type II strains (25). Vietnam and China are located in the same continent. Thus, most of the amino acid substitutions observed amongst strains are clustered in a hypervariable region (between aa 26 and 39) adjacent to the amino-terminal signal sequence, which also involves N-linked glycosylation sites varying from none to three (25). In this study, five amino acid substitutions were found in the variable region in Vietnamese strains of cluster 1 and CH-1a, CH-1R. One of the above amino acid substitutions is located at position 21 of GP5 protein, which is an N-linked glycosylation site (N → S). These results revealed that the Vietnamese PRRSV strains and Chinese PRRSV strain have a close genetic relationship.

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RAZNOLIKOST GENA ORF5 V SEVIH VIRUSA VIETNAMSKEGA PRAŠIČJEGA RESPIRATORNEGA IN REPRODUKTIVNEGA SINDROMA

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Povzetek: Prašičji respiratorni in reproduktivni sindrom je huda bolezen, ki povzroča velike izgube v gospodarstvu in vpliva na razvoj kmetijstva. V raziskavi smo želeli oceniti gensko raznolikost gena ORF5 v 12 sevih virusa vietnamskega prašičjega respiratornega in reproduktivnega sindroma (PRRSV). Filogenetska analiza baznih zaporedij ORF5 vietnamskih in drugih sevov je pokazala, da vietnamski sevi pripadajo tipu II. Tudi vietnamski sevi so bili ločeni v dve skupini. Pet sevov (BG/12, TG/12, TG2/12, TG3/12 in TG /12) je bilo združenih v skupino 1 z 98-odstotno vrednostjo bootstrap, medtem ko je bilo ostalih sedem sevov (HCM/14, TG5/15, TG6/15, TG7/15, ST1/15, ST2/15 in ST3/15) uvrščenih v drugo skupino. Poravnava zaporedij aminokislin je pokazala, da je podobnost med vietnamskimi sevi in sevi CH-1a 87–93-odstotna, JXA1 91–98-odstotna in VR2332 83–89-odstotna. Pri petih vietnamskih sevih iz skupine 1 smo opazili mutacijo mesta z glikozilacijo N21 (N S) zaporedja GP5. Glavno zaporedje nevtralizacijskih epitopov (vključno s petimi položaji pri H25, Q27, I29, Y30 in N31) v GP5 je bilo opaženo pri vseh vietnamskih sevih, razen v sevu TG1/12. Grafikoni hidrofobnosti GP5 so pokazali dva različna položaja BG/12 iz CH-1a in VR2332 sevov. Prva razlika je bila odsotnost hidrofilnega vrha pri položaju aminokislin 85 do 95. V tem področju so imeli sevi CH-1a in VR2332 tri hidrofilne vrhove. Druga razlika je bila izguba drugega hidrofilnega vrha na položaju aminokisline 100.

Ključne besede: genotip; ORF5; filogenetika; PRRSV; Vietnam

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Original Research Article

GUINEA PIG FETUS DOES NOT CHANGE ITS PRESENTATION DURING SECOND HALF OF GESTATION

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Abstract: Objectives: The aims of this paper are: 1. To investigate spontaneous changes in fetal presentation during the second half of gestation in guinea pig fetus, 2. to observe provoked changes of fetal presentation, by passive positioning fetus into an upside-down position and "sitting" position. Eight fetuses from singleton pregnancies were included. Experimental design: Ultrasound examinations (Toshiba Nemio SSA-550A apparatus) were started on the 26th day of gestation (GD). Each fetus was examined 2-3 times in each 5-day interval until the end of gestation. First the spontaneous orientation of fetus was determined by tracking along the longitudinal and transversal axes of the fetus with the ultrasound probe. Then the fetus was brought into a head-down position relative to gravity for 5 minutes. The examination was repeated with the fetus in the head-up position. Results: In the period from the 26th – 30th GD in 7 of 21 observations spontaneous changes of presentation were observed. From the 31st GD all 133 observations were negative regarding spontaneous changes of presentation and situs. The chi-squared test showed that difference in spontaneous changes of presentation and situs before versus after 30th day of gestation had a statistical significance ($\chi^2 = 25.16$ $p < 0.05$). Frequency of ultrasound examinations before and after 30th day of gestation was not statistically significant (Fisher exact chi square $p = 0.46$, $p > 0.05$). Fetuses were brought into a head-down and head-up 154 times. In none of these attempts did a fetus change presentation during the five minutes of observation. Conclusion: Guinea pig fetuses do not spontaneously or at provocation change presentation after 30th GD.

Key words: fetal presentation; gestation; guinea pig; ultrasound

Introduction

Etiology of fetal presentation in mammals has not been fully elucidated yet (1,2). Adequate presentation at birth is significant since anterior presentation in herd animals enables physiological delivery. Posterior presentation is accompanied by an inadequate dilatation of the birth channel, and consequently, a difficult delivery (3). In a previous paper, it was postulated that fetal presentation is a consequence of postural development (1). In herd animals, locomotor-postural development occurs during the second half of gestation. In the anterior presentation, the cranial part of the body is above the caudal part. The hind legs are the source of the main propulsive force. When the fetus has its hind legs below its cranial part, it has an optimal mobility. In up-side down position the fetus has more difficulties to move (1).

Guinea pig are precocial rodents that have full locomotion at birth. In previous studies with guinea pigs it was shown that a reflex of turning from supination to pronation can be induced in the

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guinea pig fetus and that its intrauterine behavior may be gravity-dependent (4). For guinea pigs, there is no data about spontaneous or provoked changes in fetal presentation during gestation.

The aim of this paper is to investigate spontaneous changes in fetal presentation and situs during the second half of gestation. In addition, a secondary aim is to determine the existence of active turning of the fetus through 180 degrees, from its passive positioning into an upside-down position. It is assumed that in the second half of gestation a fetus that is passively positioned in an upside-down position will turn around though 180 degrees.

Material and method

Experimental animals

The study subjects were albino guinea pigs (*Cavia porcellus*) obtained from the Department of Biochemistry, Faculty of Medicine, Clinical Center of Vojvodina, Novi Sad. The experiments with animals were approved by the Ethics Committee of the University of Novi Sad No 04-29/62). The guinea pigs were kept in 400Wx1000Lx300H mm plastic containers in a harem system: two females and one male. Pregnant females were moved to 300Wx300Lx300Hmm plastic containers after the 60th day of gestation, where they were kept separate until delivery, and afterwards for the first 15 days with the offspring. The animals had a standard commercial pellet diet and *ad libitum* water enriched with vitamin C (30 mg/100 ml water). Artificial cycles with 12 hours of light (08:00–20:00) and 12 hours of dark were provided. The room temperature was maintained at 22 ± 2°C. The air was recirculated 10 times per hour.

Inspection of vaginal introitus was performed daily, and the day of vaginal membrane perforation was taken as the first day of gestation. Individual guinea pigs were identified by yellow patterns on their backs. Eight pregnant guinea pigs with one fetus were included. The number of fetuses was determined by ultrasound examination. Pregnant females were shaved before examination; the shaving of the abdominal region was made during a short-term inhalatory ether narcosis on the 25th day of gestation.

Ultrasound examination

Ultrasound examinations were started on the 26th day of gestation, until when the first movements of the guinea pig fetus usually occurs (5). During gestation that usually lasts for 66 days each fetus was examined 2-3 times in each 5-day interval (gestation days 26-30, 31-35, 36-40, 41-45, 46-50, 51-55, 56-60, ≥61). Immediately before the examination the pregnant guinea pigs were supported in a supine position on a 15x30cm board using plastic strips with clasps, fastened over the thoracic area and both hind legs. The strips were pulled through holes in the board near the body of the animal and fastened on the other side of the board.

Ultrasound examinations were performed with a Toshiba Nemio SSA-550A apparatus with a 6-11 Hz linear probe. Pregnant females were brought into supination with the board on which they were fastened to. The orientation of fetus was determined by tracking along the longitudinal and transversal axes of the fetus with the ultrasound probe. The position of the fetus was determined on the basis of the positions of its head, spine, heart, forelimbs and hind legs (Image 1). Then the board was rotated until the fetus was brought into a head-down position relative to gravity and any changes in fetal presentation were observed for 5 minutes. The same subjects were used as controls. After a 2-minute pause, the examination was repeated with the fetus in the head-up position.

Statistical analysis

Statistical tests were completed using the SPSS software (version 21, 2012, IBM, Armonk, NY, USA). Results with a p - value of $p<0.05$ was accepted to be statistically significant.

Results

Spontaneous changes of presentation

Table 1 - summarizes data related to fetal presentation and situs at the beginning of ultrasound examination, with pregnant females in supination. A transversal lie with the head on the right side was the most frequent situs. At the last examination before delivery there was one anterior and one posterior presentation of the

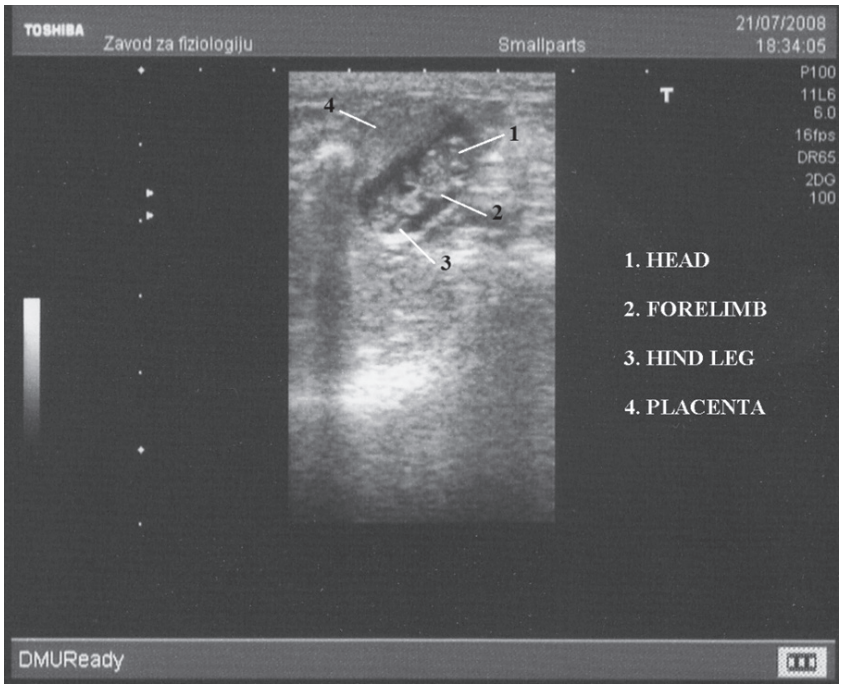

Figure 1: 29-days old guinea pig fetus

Table 1: Fetal presentation and situs at the beginning of ultrasound examination, with pregnant females in supination

Days of gestation	Longitudinal lie No (%)		Transverse lie No (%)				Total No (%)
	AP	PP	HL	HR	SP	USDP	
26-30	5 (23.80%)	7 (33.33%)	3 (14.28%)	6 (28.56%)	0	0	21
31-35	4 (17.39%)	6 (26.08%)	3 (13.04%)	8 (34.74%)	2 (8.69%)	0	23
36-40	6 (28.57%)	5 (23.80%)	4 (19.04%)	6 (28.57%)	0	0	21
41-45	5 (31.25%)	4 (25%)	1 (6.25%)	6 (37.50%)	0	0	16
46-50	4 (23.52%)	4 (23.52%)	3 (17.64%)	6 (35.29%)	0	0	17
51-55	3 (16.66%)	2 (11.11%)	5 (27.77%)	8 (44.44%)	0	0	18
56-60	5 (26.31%)	3 (15.78%)	2 (10.52%)	9 (47.36%)	0	0	19
≥ 61	2 (10.52%)	3(15.78%)	4 (21.04%)	10 (52.63%)	0	0	19

fetus, as well as six transverse lies; in 4 cases with the head on the right side and in two cases with the head on the left side.

In the period from the 26th to the 30th day of gestation a total of seven changes in presentation and situs (in five fetuses) were observed, that indicate turning around the longitudinal axis of the body for 180°. From the 31st day of gestation none of the fetuses in our sample changed their body orientation by 180°. They oscillate around one position for 45° during whole second period of gestation. This means that no fetus made a spontaneous turn around its longitudinal axis. The chi-squared test showed that difference in changes of presentation and situs before vs after 30th day of gestation had a statistical significance of x^2 = 25.16 p <0.05. The strength of these association is very strong (phi = - 0.77, Cramer's V = 0.77, p < 0.001). Frequency of ultrasound examinations before and after 30th day of gestation was not statistically significant (Fisher exact chi square p = 0.46, p > 0.05). Oscillations around one position by 45° were present in all experimental animals during the whole examined period. There is no

statistically significant difference in frequency of oscillations around one position for 45° between each examined period of pregnancy (Fisher exact chi square p = 0.46, p > 0.05).

Provoked changes in presentation

Fetuses were brought into a head-down position for a total of 154 times and in none of these attempts did a fetus through 180° degrees during the five minutes of observation. The fetuses did not change their presentation. The same results were obtained when fetuses were positioned in a head-up position after a 2-minute pause.

Discussion

The results of this study indicate that there are two periods of gestation in the guinea pig with regard to its situs and presentation. After the occurrence of the first movements on gestation days 25 and 26, the fetus is capable of passive or active turning around its longitudinal axis for 180° degrees only for a few days. After that, throughout the second half of gestation, its intrauterine position is fixed and it is no longer capable of turning around its longer axis. This finding is contrary to other mammalian species in which postural development occurs prenatally. The incidence of different presentations and lies during gestations with one fetus in human species (6,7) and herd precocial mammals (8-10) indicates the existence of three stages. The beginning of the first stage is characterized by equal proportions of longitudinal and transverse lies with equal proportions of breech and cephalic presentations within the longitudinal lie. During this stage, there is an increasing incidence of longitudinal lie with a proportional decrease in transverse lie. In the second stage, which occurs in the second half of gestation, a transverse lie is almost completely absent, whereas in the longitudinal lie there is an increasing incidence of anterior (cephalic) presentation and a proportional decrease in posterior (breech) presentation. By the end of this stage, around 95% of fetuses are in a longitudinal lie with cephalic presentation. In the third stage, during the last weeks of gestation, there is a further mild increase in the incidence of longitudinal lie with cephalic presentation (2,11). Published data show that in humans and herd mammals there is spontaneous turning around the longer axis in the second half of gestation (2,9). Studies with exteriorization of guinea pig fetuses have shown that turning around the shorter axis occurs when gestation is terminated around the 60th day. Taking an upright position and maintaining balance occurs around the 63rd day of gestation (12). In a study dealing with intrauterine induction of righting reflex from supination to pronation, turning to one side was present already around the 40th day of gestation, and it was the most frequent after the 60th day of gestation (4). Despite the presence of postural reactions in the guinea pig fetus in the second half of gestation and its ability to turn around the shorter body axis, turning around the longer body axis could not be induced by positioning the fetus in an upside-down position. In a sheep fetus, in the period of an exclusive increase in the incidence of anterior presentation, it is possible to induce the fetus to turn around its longer axis by positioning it in a head-down position (2). Therefore, it is likely that the turning around the longitudinal body axis in the guinea pig fetus is not possible due to physical characteristics i.e. lack of space. The guinea pig fetus is therefore not a suitable and adequate experimental model for confirmation of the concept that presentation of the human and herd mammals fetuses is influenced by their intrauterine postural development.

A limitation of the present study is that it was not able to register changes in fetal position greater than 180° degrees. A 270° degree turn would have been registered as a 90° degree turn. The absence of erratic changes in fetal position indicates that this problem does not affect the results of the study. Oscillations of 45° from the basic position are probably caused by changes in the position of the internal organs.

In order to confirm or discard the hypothesis on the influence of postural development on presentation of the fetus, it is necessary to advance the experimental model of the sheep fetus. A problem with the sheep fetus as an experimental model is that the fetus assumes a transverse lie when the pregnant female is positioned into a sitting position. This manipulation with the pregnant female is performed in order to provoke a fetus in anterior presentation to assume a head-down position and turn for 180° degrees. In order to overcome this problem, investigators used external pressure with their palms on the

abdominal walls of pregnant females (2). However, this is an inappropriate method because of external, physical stimulation of fetal movements. It is necessary to surgically fix the horn of the uterus (hornopexia) to accomplish stable longitudinal situs of the fetus prior to studying its reaction to changes of gravity vector in the intrauterine environment.

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PLODOVI MORSKIH PRAŠIČKOV NE SPREMENIJO LEGE V DRUGI POLOVICI BREJOSTI

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Povzetek: Namen prispevka je: 1. raziskati spontane spremembe v legi plodu v drugi polovici brejosti pri plodovih morskih prašičkov; 2. opazovati vzpodbujene spremembe lege plodov v položaj od zgoraj-navzdol in v »položaju sedenja«.V raziskavo je bilo vključenih osem plodov iz ene brejosti. Z ultrazvočnimi pregledi (aparat Toshiba Nemio SSA-550A) smo pričeli 26. dan brejosti. Vsak plod je bil pregledan 2-3-krat v 5-dnevnih razmikih do konca brejosti. Najprej je bila ugotovljena spontana orientacija plodu z določanjem njegove vzdolžne in prečne osi z ultrazvočno sondo. Potem je bil plod za 5 minut obrnjen v položaj z glavo navzdol, sorazmerno s težo. Pregled plodu je bil ponovljen v položaju z glavo navzgor. V obdobju od 26. do 30. dneva brejosti smo v 7 od 21 opazovanj opazili spontane spremembe lege plodu. Od 31. dneva brejosti dalje pri vseh 133 opazovanjih ni bilo opaziti spontanih sprememb lege plodu. Analiza Hi-kvadrat je pokazala, da je razlika v spontanih spremembah lege plodu pred in po 30. dnevu brejosti statistično značilna ($\chi^2 = 25{,}16$ $p < 0{,}05$). Pogostnost ultrazvočnih preiskav pred in po 30. dnevu brejosti ni bila statistično značilna (Fisherjev natančni Hi-kvadrat test $p = 0{,}46$). Plodovi so bili postavljeni v položaj z glavo navzdol in navzgor 154-krat. V nobenem od teh poskusov ni prišlo do spontane spremembe lege plodu med petminutnim opazovanjem. Ti rezultati torej kažejo, da plodovi morskih prašičkov ne spremenijo lege spontano ali po provokaciji po 30. dnevu brejosti.

Ključne besede: sprememba lege plodu; brejost; morski prašiček; ultrazvok

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