ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

# SLOVENIAN VETERINARY RESEARCH

## SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2014; 51 (1): 1-51

Volume 51 | 1

Slov Vet Res • Ljubljana • 2014 • Volume 51 • Number 1 • 1-51

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

# SLOVENIAN VETERINARY RESEARCH

## SLOVENSKI VETERINARSKI ZBORNIK

Volume
51 | 1

Slov Vet Res • Ljubljana • 2014 • Volume 51 • Number 1 • 1-51

The Scientific Journal of the Veterinary Faculty University of Ljubljana

**SLOVENIAN VETERINARY RESEARCH**
**SLOVENSKI VETERINARSKI ZBORNIK**

Previously: RESEARCH REPORTS OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA
Prej: ZBORNIK VETERINARSKE FAKULTETE UNIVERZA V LJUBLJANI

4 issues per year / izhaja štirikrat letno



Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 129, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

Sponsored by the Slovenian Book Agency
Sofinancira: Javna agencija za knjigo Republike Slovenije

ISSN 1580-4003
Printed by / tisk: DZS, d.d., Ljubljana
Indexed in / indeksirano v: Agris, Biomedicina Slovenica, CAB Abstracts, IVSI
Urlich's International Periodicals Directory, Science Citation Index Expanded,
Journal Citation Reports/Science Edition
http://www.slovetres.si/

**SLOVENIAN VETERINARY RESEARCH**
**SLOVENSKI VETERINARSKI ZBORNIK**

**Slov Vet Res 2014; 51 (1)**

**Original Scientific Articles**

Slov Vet Res 2014; 51 (1): 5-10
UDC 636.594.09:616.995.1

**Original Scientific Article**

# THE FIRST DETECTION OF *Acuaria spinosa* IN PHEASANTS

Ivan Pavlović[1], Iulia Floristean[2], Viorel Floristean[3], Snežana Ivanović[1], Zoran Kulišić[4], Živka Ilić[1], Dubravka Jovičić[5]

[1]Scientific Veterinary institute of Serbia, V. Toze 14; [4]Faculty of Veterinary Medicine Belgrade; [5]Faculty Futura, University Singidunum, 11000 Belgrade, Serbia, [2]Directia Veterinara si pentru Siguranta Alimentelor Iasi; [3]Faculty of Veterinary Medicine Iasi, Iași, România

*Corresponding author, E-mail: dr_ivanp@yahoo.com

**Summary:** Parasitoses caused by the *Acuariidae* helminths sporadically occur in both free-living and artificially bred pheasant populations (*Phasianus colchicus* L.). In order to verify this situation, an investigation with the aim of detecting the *Acuaria species*. infection on seven pheasant farms in Serbia and Romania, was carried out. A total of 127 adult birds were examined. The collected nematodes were identified by their morphometric characteristics. The three different *Acuaria* species were identified including: *Cheilospirura (Acuaria) hamulosa, Dyspharinx (Acuaria) spiralis* and *Acuaria spinosa*. The parasites identified as the *Acuaria spinosa* species had equal lips and furrow cuticula. Both parasites had four spiny cordons 'which did not exceed a third of the anterior of the esophagus. The tail was short and rounded. Eggs were ellipsoid, thick shelled, embryonated, 0.039-0.41 µm long by 0.025-0.027 µm wide.
Furthermore, thefirst parasite's body length was 32 µm, with a width of 0.336 µm (maximal width at the middle of the body). Four spiny cordons were 0.797 µm long, and the cylindrical pharings were 0.018 µm long. The vulva lies just posterior to the middle of the body, 3.56 µm from the anterior end, 51 % of the total body length (TBL) from the anterior end. The anus lies at 0.25 µm from the posterior end. The body length of the second parasite was 34 µm with a width of 0.367 µm. Spiny cordons 'which did not exceed a third of the anterior of the oesophagus were 0.809 µm long, and the cylindrical pharings were 0.021 µm long. The vulva lies just posterior to the middle of the body, 5.64 µm from the anterior end, 57 % of the total body length (TBL) from the anterior end. The anus lies at 0.28 µm from the posterior end.
According to the authors knowledge this is the first detection of the *Acuaria spinosa* in pheasants (*Phasianus colchicus* L.).

**Key words:** *Acuariidae; Phasianus colchicus; Acuaria spinosa*

## Introduction

Parasitoses caused by nematodes produceshealth problems in both the free-living and artificially bred pheasant populations. The nematode infection is the most frequent infection transmitted through an intermediate host in the pheasant population. There are several papers regarding helminthoses in freeliving and farm bred pheasants. Most of these investigations were performed in Europe, namely in Czechoslovakia, Lithuania, Poland, Greece, Germany and Italy (2, 8, 10, 12, 17, 25).

In the West Balkan countries, numerous investigations were conducted in Romania (5, 14) and Serbia (20, 22, 23, 24). During those examinations parasitoses caused by the *Acuariidae* helminthes occurred sporadically, in both free-living and farm bred populations of pheasants (9, 16, 29). Therefore, this investigation was carried out on seven pheasant farms in order to establish the helminth fauna with an emphasis on the presence of the *Acuaruidae* species in the artificially bred pheasant population in Serbia and Romania.

Received: 27 June 2012
Accepted for publication: 23 August 2013

## Material and methods

The investigation was carried out on seven pheasant farms for hunting/restocking purposes including four farms in Serbia and three farms in Romania. The helminthes were collected from adult pheasants that had died of natural causes by parasitological necropsy. A total of 127 adult birds were examined. The collected nematodes were fixed in glacial acetic acid and preserved in 70 % ethanol. For further examination, parasites were clarified in lactophenol and nematodes were identified by their morphometric characteristics described by Skrjabin et al. (26) and Chabauda (4). All the laboratory techniques used were described by Šibalić and Cvetković (28).

Gizzards were dissected in a 0.85 % NaCl solution and the cuticle was removed for helminth investigation. Fragments of the parasitized gizzards were removed and immediately fixed in formalin. The material was then processed for paraffin embedding. Five micrometres thick sections were stained with hematoxylin and eosin.

Micrographs were obtained using a Carl Zeiss bright field microscope, by means of Practica PLC3 and a Kodachrome 64 film.

## Results

Infection with nematodes was found in 37.79 % (48/127) cases. Polyparasitism involving two species was detected in 28.34 % (36/127) of pheasants (13,21).

A total of 14 of 127 (11 %) examined pheasants were found to have been infected with the *Acuariidae* sp. During necropsy, specimens were found lying freely under the gizzard cuticle, partially or fully burrowed in the walls of the organ, mainly in the region of the caudal blind sac. There, specific macroscopic lesions were observed as present in the muscular portion of the stomach and included sclerotic and nodular lesions accompanied by a fine-whitish granular residuum, located underneath the corneous lining of the gizzard, at the points of nematodes implantation (15). Three different species were collected and identified: *Cheilospirura hamulosa, Dyspharinx spiralis*, and *Acuaria spinos*, which was found in onlyt one bird examined, which was represented by two parasites. Furthermore, it was found that *C. hamulosa* and *D. spiralis* infections occurred in both countries (on one farm in Serbia and two in Romania), while A. spinosa infection occured only in Romania. *Acuaria spinosa* was found in only one bird, and two worms were found.

The parasites identified as the Acuaria spinosa species had equal lips and furrow cuticula. Both parasites had four spiny cordons 'which did not exceed a third of the anterior of the esophagus. The tail was short and rounded. Eggs were ellipsoid, thick shelled, embryonated, and 0.039-0.41 μm long by 0.025-0.027 μm wide.

Furthermore, the first parasite's body length was 32 μm, the width was 0.336 μm (maximal width at the middle of the body). Four spiny cordons were 0.797 μm long, and cylindrical pharings were 0.018 μm long (Fig. 1). The vulva lies just posterior to the middle of the body, 3.56 μm from the anterior end, 51 % of the total body length (TBL) from the anterior end. The anus lies at 0.25 μm from the posterior end.

The second parasite's body length was 34 μm, and the width was 0.367 μm. Spiny cordons did not exceed exceed a third of the anterior of the esophagus. Spiny cordons were 0.809mm long, and the cylindrical pharynx was 0.021 μm long (Fig. 2). The vulva lies just posterior to the middle of the body, 5.64 μm from the anterior end, 57 % of the total body length from the anterior end. The anus lies at 0.28mm from the posterior end.

The histological examination revealed traumatic lesions in hemorrhagic and necrotic routes and tunnels generated by cuticular cordons and spines of the adult nematodes, surrounded by a conjunctive reaction (Fig. 3). Nematodes were observed in tunnels with fibrous walls, impregnated with various mesenchimal cells, predominant lymphocytes and eosinophils. The muscular wall of the stomach was almost completely replaced by conjunctive tissue, in which diffuse lymphoid infiltration and lymphoid nodules, as well as parasitic granuloma were observed.

## Discussion

*Acuaria spinosa* belongs to the *Acuariidae family*, the *Acuariinae subfamily*, and genus Acuaria. When first described in *Bonasa umbellus, the* species was named *Cheilospirura spinosa* (6, 7). In a later comparison withs nematode from the Acuariinea family it was concluded by Boughton (3) that they were the same species,

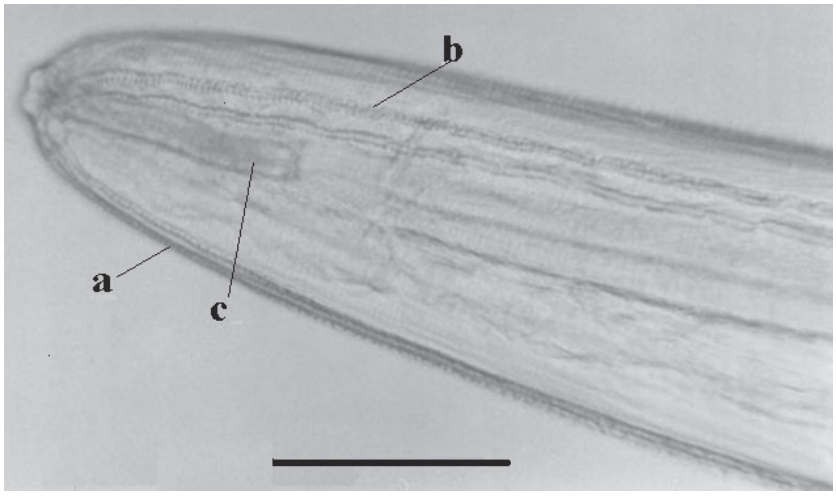


**Figure 1:** Anterior portion of a second sample of *Acuaria spinosa* ventral view, a) furrow cuticula, b) spiny cordons; c) pharings; Bar = 0.1 mm (Bar of 1 is common to 2 and 3)

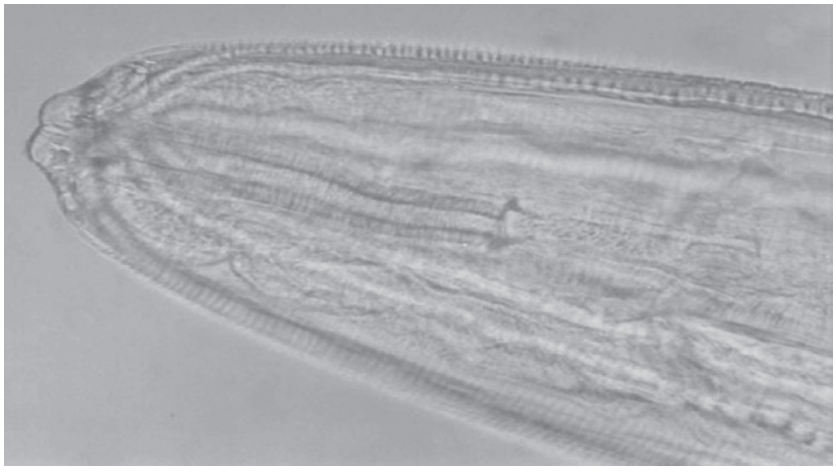

**Figure 2:** Anterior portion of a second sample of *Acuaria spinosa*, ventral view

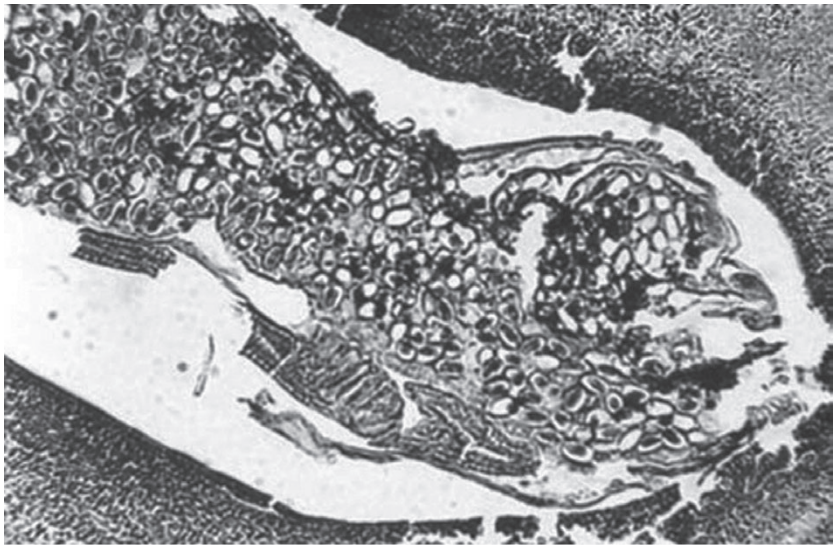

**Figure 3:** Perforated sites in the submucosa caused by *A. spinosa*

and that nematodes belong to the Acuaria genus. Finally, a complete description of parasites and their development was presented by Skrjabin et al. (25). According to Skrjabin et al., parasites measured 37-40 μm long and 0.31-0.34 μm wide and possessed two equal lips and furrow cuticula. These measurements coincide with the findings of this study (parasites body lengths were 32-34 μm and widths were 0.336- 0.367 μm; four spiny cordons were 0.797- 0.809 μm long ).

Grasshoppers (*Melanopus femurrubrum* and *M. differentialis*) serve as intermediate hosts for Acuaria spinosa. In these hosts the infective larvae develop and the final host acquire the infection by ingesting grasshoppers (7, 26).

*Acuaria spinosa* (syn. *Cheilospirura spinosa*) was first described as a species of nematode found in the gizzard of ruffed grouse in Michigan (USA) (27). Cram (6) detected *A.spinosa* in the horny lining of the gizzard of *Bonasa umbellus*, *Colinus virginianus* and *Pediocetes* (T*ympanuchus*) *phasianellus campostris*. Later, *A. spinosa* was found in *Alectoris graeca* (1) and in *Tympanuchus cupido* (9). The results of this study conclude that this is the first occurrence of *Acuaria spinosa* in ring necked pheasants (*Phasianus colchicus* L.).

The helminthes from the Acuariidae family sporadically occur in pheasants, as first reported in the USA by Cram (6). Later examination pointed to the factthat Acuariidae can be found in free living and farm bred pheasants in numerous countries in Europe (10, 11, 12, 13). During those examinations the following species were found: *C. hamulosa,* followed by *A. spiralis* and *A. gruveli.* In Serbia and Romania *C. hamulosa* was the most frequent Acuariida species (13, 14, 19, 20, 21).

In the infected birds, parasites of the Acuaridae genus caused severe gross lesions in the gizzard such as hemorrhages, ulcers and thickening of the mucosa and cuticle, and single yellowish nodules on the caudoventral muscle (16). Histological examination of the infected gizzards revealed discrete and coalescing nodular and cystic lesions in the mucosa and musculature which contained sections of the parasite. Cellular reaction in the lesions was characterised by a large number of lymphocytes, monocytes, plasma cells, heterophils, and, in some of the sections, a severe eosinophilic reaction. The mucosa and submucosa showed markedly thickened and diffuse mononuclear infiltration as well as reactive lymphoid nodules (15). During examinations it was found that pathological changes caused by *A.spinosa* were similar to cases of infection with other Acuaria species.

The presence of *Acuaria spinosa* in pheasants or other birds, except *Bonasa umbellus, Colinus virginianus, Pediocetes* (*Tympanuchu*) *phasianellus, Alectoris graeca* and *Tympanuchus cupido* was not reported in available literature. Furthermore, the presence of adult nematodes in gizzard suggests the possibility of adaptation of *Acuaria spinosa* to parasitism in a new host – pheasants. This is not unique, because *Brachylaemus fuscatus* and *Plagorchis megalorchis* were found in pheasants in Scotland (24). *Zygocotyle lunata* was found in 1.6 % of pheasants in Nebraska (11). Infection with *Ornithostrongylus quadriradiatus* in pheasants was found in the Belgrade area (18). During 2002 the first occurrence of *Thominx cyanopicae was established* in the new host – pheasants and their full adaptation to it, confirmed by histological aspects which revealed the pathological effect of parasites (14).

The first occurrence of *Acuaria spinosa* in pheasants has an epidemiological importance and indicates the necessity for continued parasitological examination of this bird species.

## References

1. Akhumyan KS, Khanbegyan RA. The helmint fauna of wild galliforms in Armenia (*Coturnix coturnix, Alectoris graeca, Perdix perdix, Lyrulus mlokosiewiczi* and *Tetraogallus caspius*). Zool Sb Akad Nauk Armyanskoi SSR, Inst Zool 1982; 18: 9–45.

2. Arnasteikskene T, Kazlauskas Yu, Kadite B. Parasitic fauna of pheasants (*Phasianus colchicus* L.) in the Lithuanian SSR. Acta Parasitol Lith 1970; 10: 95–101.

3. Boughton B. *Cheilospirura spin*osa (Cram,1927). Univ Minn Agr Exp Stat, 1937: 21–2. Techn Bull 121.

4. Chabauda G. Keys to the genera of the order Spirurida. Part 2. Spiruroidea, Habronematoidea and Acuarioidea. In: Anderson RC, Chabaud Willmott AS, eds. CIH keys to the nematode parasites of vertebrates. No. 3. Farnham Royal : Commonwealth Agricultural Bureaux,, 1975: 29–58.

5. Cosoroabă J, Ciolofan I. Controlul capilariozei si singamozei in crescatoriile de fazani si potârnichi. Lucr Sti Med Vet 1985; 27: 69–73.

6. Cram EB. Bird parasites of the nematodes

suborders Strongylata, Ascaridata and Spirurata. US Nat Mus Bull 1927; 140: 334–61.

7. Cram EB. Developmental stages of some Nematodes of the Spiruroidea parasitic in poultry and game birds. US Dept Agriculture, Tech Bull 1931: 227–8.

8. Gassal S, Schmäschke R. The helminth and coccidial fauna of pheasants (*Phasianus 1colchicus)* in view of the specific environmental conditions in pheasantries and in the wild. Berl Munch Tierarztl Wochenschr 2006; 119: 295–302.

9. Gibson DI, Bray RA, Harris EA (Compilers) Host-parasite database of the Natural history museum, London 2005. http://www.nhm.ac.uk/research-curation/scientific-resources/taxonomy-systematics/host-parasites/index.html (Dec. 2013)

10.Githkopoulos PR. Helminths in pheasants of Greek phesanteries. Hellenike Kten 1984; 27: 68–76.

11. Greiner EC. Parasites of Nebraska pheasants, 63 Phasianus colchicus. J Wildl Dis 1972; 8: 43–5.

12. Hospes R. Parasitosen des Jagdfasans. PhD dissertation. Giessen: Universiy of Giessen, 1996.

13. Floristean I. Examination of etiology, morphopathology and clinic of endoparasites of pheasants artificially raised. PhD dissertation. Iasi, Romania : University of Iaşi, 2003: 225 str.

14. Floristean I, Pavlović I. The first occurence of *Thominx cyanopicae* (Lopez Neyra, 1947) in pheasants (*Phasianus colchicus* L.). Acta Vet Beograd 2003; 53: 183–90.

15.Floristean I, Cotofan O, Merticariu S, Pavlović I. Etiological and morphopathological investigations in acuariosis in breeding pheasants. Lucr Sti Med Vet 2003; 46:141–4.

16. Menezes RC, Tortelly R, Gomes DC, Pinto RM. Pathology and frequency of *Cheilospirura hamulosa* (Nematoda, Acuarioidea) in Galliformes hosts from backyard flocks. Avian Pathol 2003; 32:151–6.

17. Okulewitz A, Modrezejavska M. Helminth fauna of pheasants (*Phasianus colchicus* L.) from the environs of Wroclaw in the autumn/summer period. Wiad Parazitol 1980; 26: 73–5.

18. Pavlović I, Hudina V, Kerš V, Blažin V, Čupić V. Helminthological fauna of pheasants in Belgrade zoo in period March-August 1988. Vet Glasn 1990; 44: 467–71.

19. Pavlović I. Ecto and endoparasites of pheasants artificially raised and measure to its control. MSc thesis. Belgrade, Serbia : University of Belgrade, 1991: 76 str.

20. Pavlović I, Kerš-Pavlović V, Jordanović B, Hudina V. Endoparasites of pheasants artificially raised. Lucr Sti Med Vet 1992; 26:104–7.

21. Pavlović I, Jakić-Dimić D, Kulišić Z, Florestean I. Most frequent nematode parasites of artificially raised pheasants (*Phasianus colchicus* L.) and measures for their control. Acta Vet Beograd 2003; 53: 393-8.

22. Pavlović I, Dačić M, Stokić-Nikolić S, Rajković M, Ivanović S, Đorđević M. Nematode parasites of pheasants (*Phasianus colchicus* L.) artificially raised in Serbia. Lucr Sti Ser D Zoot Anim Sci 2009; 52: 412–15.

23. Pavlović I, Đorđević M, Kulišić Z. Endoparasites of farm-reared pheasants (*Phasianus colchicus* L.) in Serbia. In: Modern aspects of sustainable management of game population. International symposium on hunting, Zemun-Belgrade, Serbia, 2012: 125–9.

24. Rayski C. An outbreak of helminthosis in pheasants chicks due to *Plagiorchis megalorchis* (Ress, 1952) with same critical remarks on P. (M) larcola Skrjabin 1924. Parasitology 1964; 54: 391–6.

25. Santilli F, Bagliacca M. Occurrence of eggs and oocysts of intestinal parasites of pheasant (Phasianus colchicus) in Tuscany (Italy) Eur J Wildlife Res 2012; 58: 369–72.

26. Skrjabin, KI, Soboleva NDV, Ivashkin M. Principles of nematology Spirurata of animals and man and the diseases caused by them. Part 3. Moscow: Izdatel'stvo Academii Nauk SSSR,1965: 131–2.

27. Stafseth HJ, Kotlan A. Report of investigations on an alleged epizootic of ruffed grouse in Michigan-America. J am Vet Med Assoc 1925; 67: 260–7.

28. Šibalić S, Cvetković Lj. Osnovi dijagnostike parazitskih bolesti Beograd: OZID, 1980: 36–7

29. Tampieri MP, Galuppi R, Rugna G. Survey on helminthofauna in pheasants from Eastern Europe. Parassitologia 2005; 47: 241–5.

## PRVO ODKRITJE *Acuaria spinosa* PRI FAZANIH

I. Pavlović, I. Floristean, V. Floristean, S. Ivanovic, Z. Kulišić, Ž. Ilić, D. Jovičić

**Povzetek:** Parazitoze, ki jih povzročajo črevesne gliste iz družine Acuariidae, naj bi se občasno pojavljale pri prosto živečih in gojenih populacijah fazanov. Opravili smo raziskavo, s katero smo želeli odkriti okužbo z *Acuaria species* na sedmih farmah fazanov v Srbiji in Romuniji. Skupno je bilo preiskanih 127 odraslih ptic. Zbrani nematodi so bili določeni glede na njihove morfološke lastnosti. Identificirali smo tri različne vrste *Acuaria: Cheilospirura (Acuaria) hamulosa, Dyspharinx (Acuaria) spiralis* in *Acuaria spinosa*. Zajedavci, ki so bili identificirani kot *Acuaria spinosa*, so imeli enake ustnice in nagubano kutikulo. Obe vrsti zajedavca sta imeli po 4 trnaste kordone, ki niso presegali tretjine sprednjega dela požiralnika. Njihov rep je bil kratek in zaokrožen. Jajčeca so bila embrionirana, elipsoidne oblike z debelo lupino, dolga 0,039-0,41 µm in široka 0,025-0,027 µm.
Telesna dolžina prvega zajedavca je bila 32 µm s širino 0,336 µm (maksimalna širina na sredini telesa). Štirje trnasti kordoni so bili dolgi po 0,797 µm, valjasto žrelo pa 0,018 µm. Vulva je ležala posteriorno na sredini telesa, oddaljena 3,56 µm od sprednjega konca in 51-odstotkov skupne dolžine telesa od anteriornega dela. Anus je ležal 0,25 µm od posteriornega dela. Telesna dolžina drugega parazita je bila 34 µm, širina pa 0,367 µm. Trnasti kordoni ki niso presegli tretjine anteriornega dela požiralnika, so bili dolgi 0,809 µm, valjasto žrelo pa 0,021 µm. Vulva je ležala posteriorno od sredine telesa, oddaljena 5,64 µm od anteriornega konca in 57-odstotkov skupne dolžine telesa od anteriornega dela. Anus je ležal 0,28 µm od posteriornega konca.
Po naših podatkih je to prvi primer odkritja *Acuaria spinosa* pri fazanih (*Phasianus colchicus* L.).

**Ključne besede:** *Acuariidae; Phasianus colchicus; Acuaria spinosa*

Slov Vet Res 2014; 51 (1): 11-9
UDC 636.3.09:611.013.8:611.018:611.068

**Original Scientific Article**

# MORPHOLOGICAL ADAPTATIONS FOR HISTOTROPHIC NUTRITION IN THE PLACENTA OF WEST AFRICAN DWARF GOATS

Udensi M. Igwebuike*, Daniel N. Ezeasor

Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Nigeria Nsukka, Nigeria

*Corresponding author, E-mail: udensi.igwebuike@unn.edu.ng,

**Summary:** Gravid uteri harvested from 11 pregnant West African Dwarf goats were used to study morphological adaptations for secretion and absorption of histotroph in interplacentomal areas of the goat placenta. The results showed numerous glandular acini in the richly vascularized sub-epithelial connective tissue of the endometrium. Secretory cells of the glandular acini exhibited cytoplasmic organelles for synthesis and packaging of secretory products, as well as many membrane-bound secretory vesicles. At the foeto-maternal interface, microvillar interdigitations occurred between uterine epithelial cells and foetal trophoblasts. In addition, areolae were observed near the openings of uterine glands onto the endometrial surface. Trophoblast papillae, which are evaginations of the foetal chorioallantoic membrane, extended into the areolar cavity and lumina of uterine gland openings. The areolae are specialized sites for storage and absorption of uterine gland secretions, while trophoblast papillae may correspond to areas for substantial absorption of uterine gland secretions by foetal trophoblasts. Areolae and trophoblast papillae persisted up to day 90 of gestation in the West African Dwarf goat. This suggests that uterine gland secretions are required by the conceptus even after implantation and establishment of haemotrophic nutrition in the goat. Thus, this study has provided morphological evidence for substantial production and transfer of uterine gland secretions from the dam to the foetus in interplacentomal areas of the placenta of West African Dwarf goats.

**Key words:** uterine gland; foetal trophoblast papillae; areolae; interplacentomal area; placenta

## Introduction

Ruminant placenta is classified as cotyledonary on the basis of its gross anatomical features. It exhibits discrete areas of attachment, the placentomes, which are separated by interplacentomal areas (1, 2). Placentomes are formed by interdigitation of long, profusely branched cotyledonary villi of the chorioallantois with deep caruncular crypts of the endometrium (3).

Received: 17 October 2012
Accepted for publication: 13 September 2013

Whereas placentomes are sites for haemotrophic exchange of nutrients and metabolites between the foetus and the dam, interplacentomal areas of the endometrium have been shown to contain large numbers of branched coiled uterine glands (4, 5). Endometrial glands synthesize and secrete or transport a variety of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins and other substances, collectively referred to as histotroph (4).

Studies conducted on uterine gland knockout ewes strongly suggest that uterine glands and their secretions are essential for peri-implantation

conceptus growth and survival (6, 7, 8, 9). Embryo transfer experiments in uterine gland knockout ewes revealed that endometrial gland secretions begin to impact conceptus growth and development on day 11 of pregnancy (10). Available evidence supports the idea that secretions of endometrial glands are the primary regulators of conceptus survival, development, production of pregnancy recognition signals, implantation and placentation (5, 11, 12, 13). Indeed, uterine secretions contain proteins such as osteopontin, an acidic component of the extracellular matrix, which binds to integrin receptors expressed on endometrial luminal epithelium and conceptus trophoblasts, to stimulate changes in morphology of the trophoblasts and mediate adhesion between uterine luminal epithelium and foetal trophoblastic epithelium (14, 15).

Endometrial gland hyperplasia occurs between day 15 and 50 of gestation in ewes, followed by hypertrophy to increase surface area that allows for maximal production of their secretions after gestation day 60 (16). This suggests that foetal requirement for uterine gland secretions may not be restricted to the peri-implantation period in sheep. The objective of the present study is to investigate morphological adaptations that may enhance the secretion and absorption of histotroph in interplacentomal areas of the placenta of West African Dwarf goats, using light and transmission electron microscopic techniques.

## Materials and methods

### *Animals*

All procedures involving animals were conducted according to the guidelines for the protection of animal welfare at the University of Nigeria Nsukka.

The 11 female West African Dwarf goats used for this study were purchased from local markets in Nsukka Local Government Area, Enugu State, Nigeria. The animals were housed in goat-pens at the Animal House Unit of the Faculty of Veterinary Medicine, University of Nigeria Nsukka. Confirmation of pregnancy was by ultrasonography carried out at the Veterinary Teaching Hospital, University of Nigeria Nsukka. Stages of pregnancy were determined by recording the mating date and monitoring any return to oestrus. When does failed to return to service, the first day after the last mating was taken as the first day post coitum (dpc). Subsequent stages of pregnancy were determined from that date. The stages of pregnancy studied were 20, 45, 50, 75, 85, 90, 100, 110, 115, 130 and 140 days of gestation. Gravid uteri were harvested from the pregnant goats at slaughter immediately after exsanguination.

### *Histological preparations*

Each gravid uterus was incised and interplacentomal areas of the placenta were carefully cut free and fixed by immersion in 10% neutral-buffered formalin. The samples were dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin wax. 5-6 µm thick sections were cut and stained with haematoxylin and eosin (H&E) for light microscopy. Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic China Group Ltd.) attached to a Leica binocular microscope.

### *Transmission electron microscopy*

Samples of interplacentomal tissues for transmission electron microscopy were fixed by immersion in modified Karnovsky's mixture containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer at pH 7.4. The samples were further post-fixed in 1% osmium tetraoxide in Millionig's buffer. Dehydration of the tissue samples was accomplished in increasing concentrations of ethanol and propylene oxide. Thereafter, the samples were embedded in epoxy resin and cured overnight in an embedding oven at 65°C. Semithin sections (1 µm thick) were cut and stained with toluidine blue for light microscopy. Ultrathin sections (50-90 nm thick) were cut and stained with Reynold's lead citrate and saturated aqeous uranyl acetate. These were examined under the Philips CM 10 transmission electron microscope. Images were captured using an Olympus MegaView III digital camera (Olympus Corporation Japan) attached to the transmission electron microscope.

## Results

### *Light microscopic features*

Uterine epithelial lining of interplacentomal areas of the goat placenta consisted predominantly of mononucleated columnar epithelial cells, whose apical surfaces made contact with trophoblastic epithelium of the foetal chorioallantois at the foetomaternal interface. There was simple apposition between the uterine epithelium and the foetal trophoblastic epithelium (Figure 1). The richly vascularized sub-epithelial connective tissue of the uterine wall contained many glandular acini (Figures 1, 2). Each glandular acinus was made up of columnar secretory cells that rested on a basement membrane (Figures 2, 3). Blood capillaries abut and indent the basement membrane of the glandular epithelium (Figure 2). Cytoplasm of the secretory cell stained predominantly basophilic, and the round or oval nucleus was located towards the base of the cell, while secretory products accumulated in the apical cytoplasm (Figure 3). Secretory cells appeared to release their products into the lumen of the acinus by apocrine method of elaboration (Figure 4).

Uterine glands opened onto the surface of the endometrium. The apposed foetal trophoblastic epithelium draped over uterine gland openings to enclose spaces known as areolae at the foetomaternal interface (Figure 5). Trophoblast papillae were observed as evaginations of the foetal chorioallantois that extended into the areolar cavity and lumina of uterine gland openings. They were present at day 90 of gestation (Figure 6). The external surface of the trophoblast papilla was lined by trophoblasts, while its core consisted of foetal connective tissue.

### *Transmission electron microscopic features*

Interplacentomal uterine epithelium was composed of tall columnar cells that rested on a well-defined basal lamina and extended to the foetomaternal junction (Figure 7). Each cell possessed a single oval nucleus, and its cytoplasm showed abundant mitochondria and many cytoplasmic vacuoles (Figure 8). Adjoining uterine epithelial cells were bound together by apicolateral tight junctions, while the apical plasmalemma of these cells was modified into numerous microvilli for interdigitation with similar processes of the foetal trophoblastic epithelium (Figure 8).

Uterine glands of the goat placenta were made up of tall columnar or cuboidal secretory cells (Figure 9). Each secretory cell possessed a basally located oval nucleus that showed slight indentation. The nucleolus was prominent, and the outer membrane of the nuclear envelop was studded with polysomes. Major characteristics of the cytoplasm of the secretory cell were predominance of granular endoplasmic reticulum cisternae and accumulation of secretory vesicles in the apical cytoplasm of the cell (Figure 9).

## Discussion

Endometrial glands occur in interplacentomal areas of the placenta of West African Dwarf goats. This is similar to the reports of previous studies in other ruminant species (4, 5). Ultrastructural features of the glandular epithelium in the goat suggest that the secretory cells are actively engaged in protein synthesis. The abundant granular endoplasmic reticulum may be responsible for the production of numerous membrane-bound secretory vesicles present in the cytoplasm of the cell. Moreover, variation in the shape or height of the secretory cells from tall columnar to low cuboidal forms may be related to the mode of elaboration of the secretory products by apocrine method.

It is obvious from our study that secretions of endometrial glands are made available to the conceptus through openings of uterine glands onto the endometrial surface. A previous study in sheep revealed that during the pre-implantation period, from day 4 to 15 of gestation, conceptuses are free-floating in the uterine lumen where they are bathed in and are thought to be supported by uterine gland secretions (17). In addition to providing nutrition for the conceptus, uterine gland secretions contain growth factors and cytokines that promote cell division, proliferation, morphogenesis and differentiation (18). It has been reported that uterine gland knockout ewes are unable to support pregnancy up to Day 25 of gestation, because the uterine gland knockout phenotype, characterized by extreme reduction in or absence of endometrial glands constitutes a uterine lesion that compromises peri-implantation conceptus growth and survival (6, 7, 10).

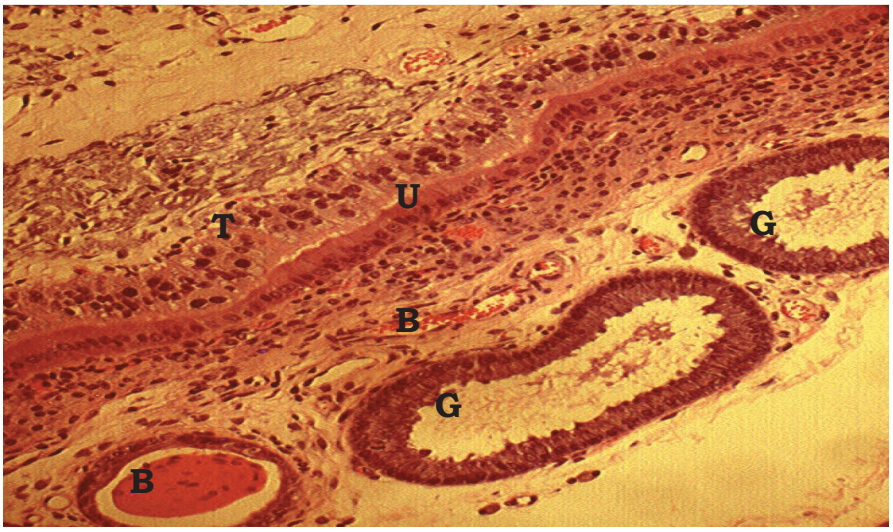


**Figure 1:** Interplacentomal area of the goat placenta showing simple apposition between foetal trophoblastic epithelium (T) and uterine epithelium (U). The uterine wall exhibited endometrial glands (G) and their associated blood vessel (B). H&E stain x100

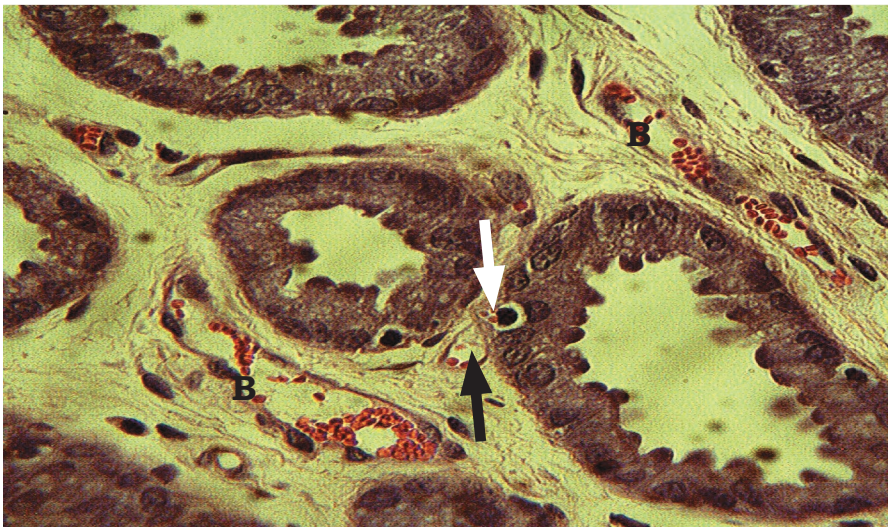


**Figure 2:** Photomicrograph showing blood vessels (B) associated with the endometrial glands. The blood capillary may abut (black arrow) or indent (white arrow) the basement membrane of the glandular acinus (arrows). H&E stain x400

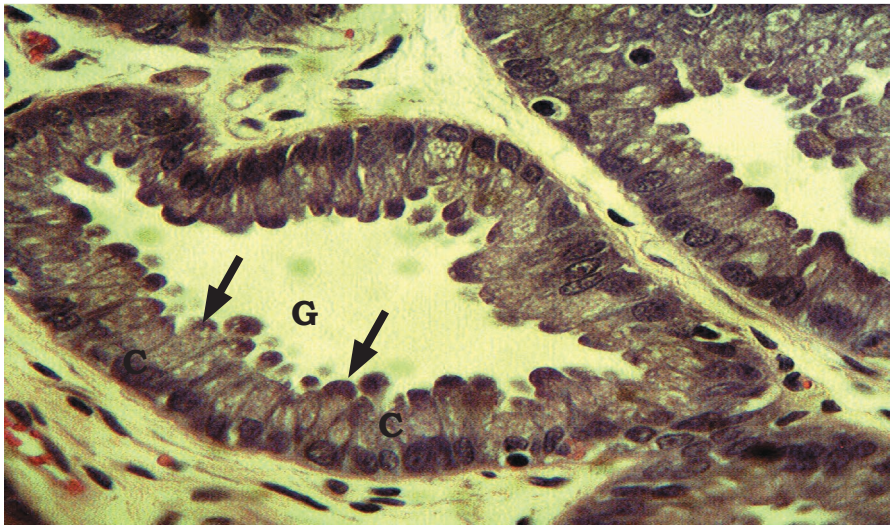


**Figure 3:** Uterine glandular acini (G) at day 85 of gestation showing columnar secretory cells. The secretory products accumulate in the apical cytoplasm (arrows) of the cells. H&E stain x400

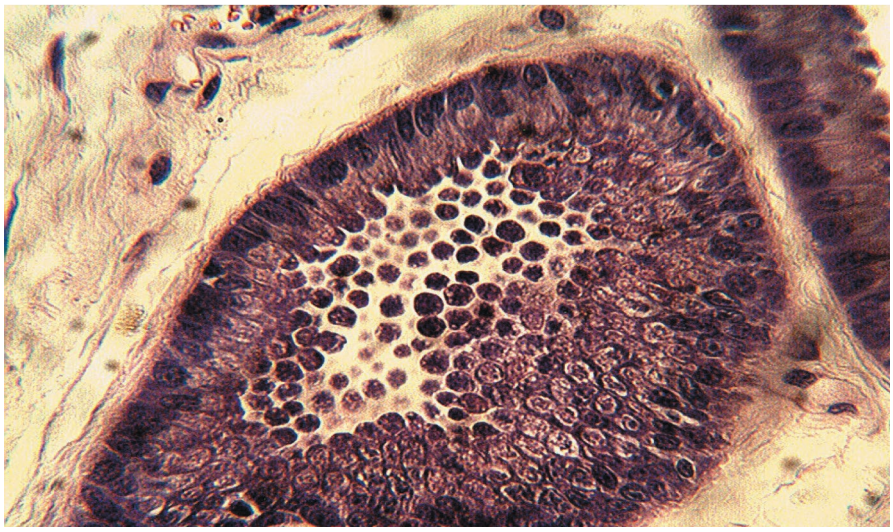

**Figure 4:** Uterine glandular acinus at day 140 of gestation. The cells elaborate their secretory products by apocrine method. H&E stain x400

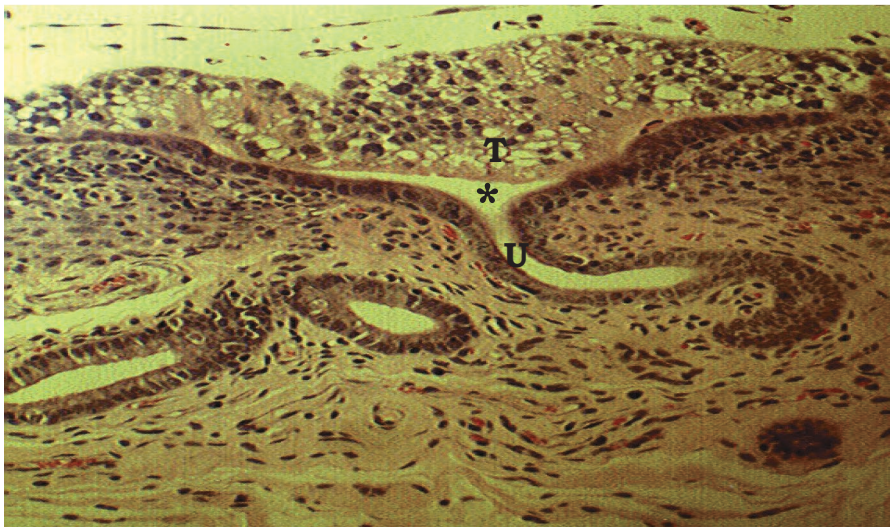


**Figure 5:** Photomicrograph of the interplacentomal area of a goat placenta at day 20 of gestation showing that the trophoblastic epithelium (T) drapes over the opening of uterine glands (U) resulting in the areola (asterisk). H&E stain x100

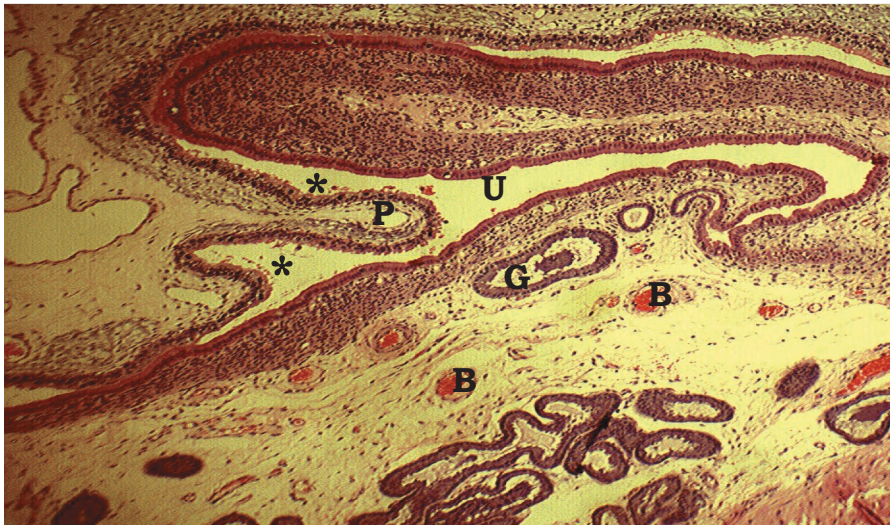


**Figure 6:** Photomicrograph of the interplacentomal area of a goat placenta at day 90 of gestation. A trophoblast papilla (P) extends into the areolar cavity (asterisk) and into the opening of uterine glands (U). Note glandular acini (G) and blood vessels (B). H&E stain x40

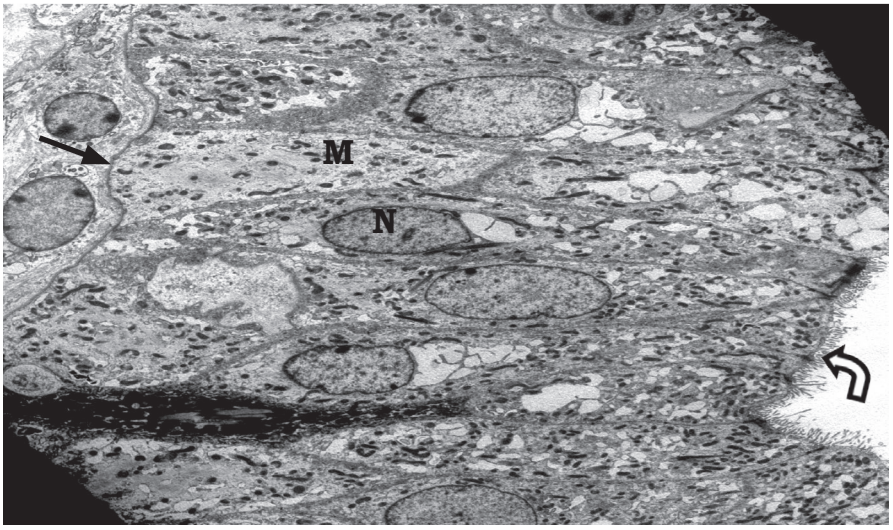


**Figure 7:** Electron micrograph of the interplacentomal uterine epithelium at day 110 of gestation showing tall columnar cells (M) that rest on a basal lamina (arrow). The apical cell membrane is modified into microvilli (curved arrow). Note the nucleus (N). TEM x1450

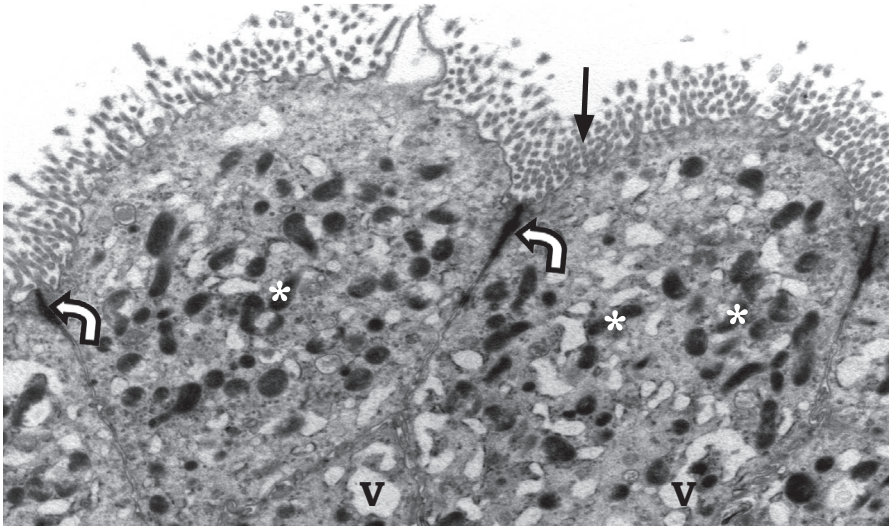


**Figure 8:** Apicolateral tight junctions (curved arrows) occur between adjoining uterine epithelial cells. Note numerous mitochondria (asterisks) and vacuoles (v) in their cytoplasm, as well as microvillar modification of the apical cell membrane (black arrow). TEM x5800

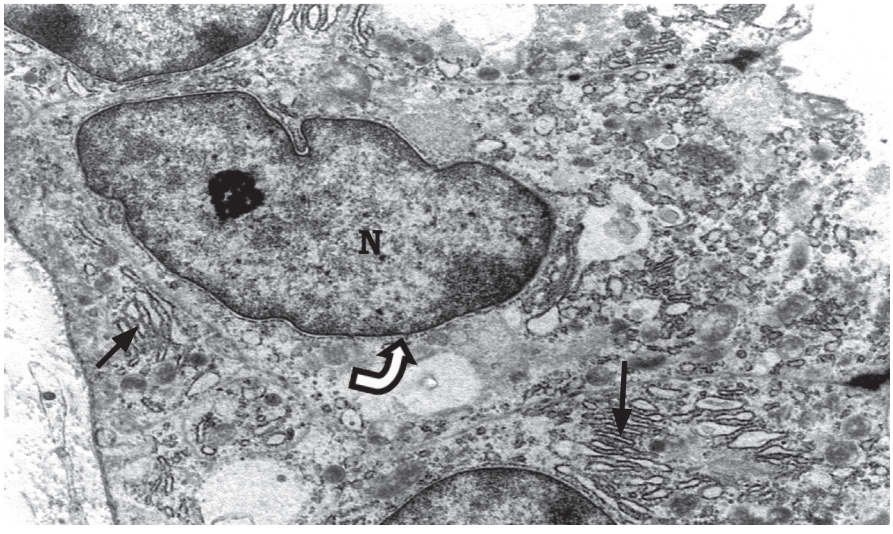


**Figure 9:** Micrograph of the uterine glandular secretory cells showing oval nucleus (N) with a slight indentation and a prominent nucleolus. The cytoplasm contains many cisternae of granular endoplasmic reticulum (arrows). The outer membrane of the nuclear envelop (curved arrow) is studded with ribosomes TEM x5800

One of the morphological features observed in this study is association of several blood vessels with endometrial glandular acini. This may facilitate exposure of the glandular epithelium to the influence of hormones and other substances present in maternal blood stream. This suggestion is supported by reports that the ruminant endometrium is exposed sequentially to oestrogen, progesterone, interferon-tau, placental lactogen and placental growth hormone during pregnancy (19, 20). These hormones constitute a servomechanism that activates and maintains remodeling of the endometrium, as well as endometrial gland morphogenesis and differentiated secretory function. In particular, placental lactogen, which is produced by trophoblast binucleate cells of ruminant conceptuses (21, 22), was detected in ovine maternal serum from day 50 of gestation, with peak levels occurring between 120 and 130 days of pregnancy (21, 23). Temporal changes in conceptus production of placental lactogen are correlated with endometrial gland morphogenesis and increased production of uterine milk protein and osteopontin by the glandular epithelium (14, 16, 24, 25).

Areolae occurred near the openings of uterine glands in the placenta of West African Dwarf goats during the peri-implantation period and up to the second trimester of pregnancy. Placental areolae have been demonstrated in sheep and pigs as specialized sites for storage and absorption of uterine gland secretions by the conceptus (1, 13). In addition, absorption of uterine gland secretions by the conceptus may be enhanced by the microvillar interdigitations between uterine and trophoblastic epithelial layers. The microvillar processes make for increase in surface area between maternal and foetal components of the placenta. Furthermore, trophoblast papillae may correspond to areas for substantial absorption of uterine gland secretions by the conceptus. Although previous studies showed that trophoblast papillae are temporary structures that disappeared by day 20 of pregnancy in sheep placenta (26) or occurred between day 14 and day 23 of gestation in goat placenta (27), our results indicate that trophoblast papillae are present in West African Dwarf goats at day 90 of gestation. The persistence of trophoblast papillae up to the second trimester of pregnancy in West African Dwarf goats may be proof that uterine gland secretions are required by the conceptus even after implantation and establishment of haemotrophic nutrition in the goat. It suggests that histotrophic nutrition complements haemotrophic nutrition and influences conceptus development, and growth of the foetus and placenta. Indeed, components of histotroph, the insulin-like growth factors (IGF-1 and IGF-2) have been shown to modulate foetal and placental growth (28, 29).

In conclusion, our study has provided clear morphological evidence for substantial production and transfer of uterine gland secretions from the dam to the conceptus in interplacentomal areas of the placenta of West African Dwarf goats. It supports the idea that conceptus requirement for histotroph remains critical even after implantation and establishment of haemotrophic nutrition in the goat.

## Acknowledgements

An aspect of this study was funded by the Education Trust Fund (ETF) of Nigeria through the ETF-AST&D award 2009 – 2010. The technical assistance of Ms. Lizette du Pleisis and Ms. Anna van Wilpe is gratefully acknowledged.

## References

1. McGeady TA, Quinn PJ, Fitzpatrick ES, Ryan MT, Cahalan S. Veterinary embryology. Oxford : Blackwell Publishing, 2006.

2. Igwebuike UM, Ezeasor DN. The morphology of placentomes and formation of chorionic villous trees in West African Dwarf goats (*Capra hircus*). Vet Arh 2013; 83: 313–21.

3. Davis CJ, Fisher PJ, Schlafer DH. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. Placenta 2000; 21: 194–202.

4. Bazer FW. Uterine protein secretions: relationship to development of the conceptus. J Anim Sci 1975; 41: 1376–82.

5. Gray CA, Bartol FF, Tarleton BJ, et al. Developmental biology of uterine glands. Biol Reprod 2001; 62: 1311–23.

6. Gray CA, Bartol FF, Taylor KM, et al. Ovine uterine gland knockout model: effects of gland ablation on the estrous cycle. Biol Reprod 2000; 62: 448–56.

7. Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE. Evidence that an absence of endometrial gland secretions in uterine gland

knockout (UGKO) ewes compromises conceptus survival and elongation. Reproduction 2002; 127: 289–300.

8. Johnson GA, Bazer FW, Jaeger LA, et al. Muc-1, integrin and osteopontin expression during the implantation cascade in sheep. Biol Reprod 2001; 65: 820–8.

9. Burghardt RC, Johnson GA, Jaeger LA, et al. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. Cells Tissues Organs 2002; 171: 202–17.

10. Gray CA, Taylor KM, Ramsey WS, et al. Endometrial glands are required for pre-implantation conceptus elongation and survival. Biol Reprod 2001; 64: 1608–13.

11. Bartol FF, Wiley AA, Floyd JG, et al. Uterine differentiation as a foundation for subsequent fertility. J Reprod Fertil 1999; 54: 287–302.

12. Carson DD, Bagchi I, Dey SK, et al. Embryo implantation. Dev Biol 2000; 223: 217–37.

13. Spencer TE, Bazer FW. Uterine and placental factors regulating conceptus growth in domestic animals. J Anim Sci 2004; 82(E-Suppl): e 4-13. http://www.journalofanimalscience.org/content/82/13_suppl/E4.long (Sept. 2013)

14. Johnson GA, Burghardt RC, Spencer TE, Newton GCR, Ott TL, Bazer FW. Ovine osteopontin II: osteopontin and alpha (v) beta (3) integrin expression in the uterus and conceptus during the pre-implantation period. Biol Reprod 1999; 61: 892–9.

15. Johnson G, Dunlap K, Erikson D, et al. The sheep conceptus differentially regulates uterine secreted phosphoprotein-1 that correlates with increased histotrophic and hematotrophic support of pregnancy. Biol Reprod 2007; 77: 103.

16. Stewart MD, Johnson GA, Burghardt RC, et al. Prolactin receptor and uterine milk protein expression in the ovine uterus. Biol Reprod 2000; 62: 1779–89.

17. Heap RB, Flint AP, Gadsby JG. Role of embryonic signals in the establishment of pregnancy. Br Med Bull 1979; 35: 129–35.

18. Martal J, Chene N, Camous S, et al. Recent developments and potentialities for reducing embryo mortality in ruminants: the role of IFN-tau and other cytokines in early pregnancy. Reprod Fertil Dev 1997; 9: 355–80.

19. Spencer TE, Johnson GA, Burghardt RC, Bazer FW. Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biol Reprod 2004; 71: 2–10.

20. Noel S, Herman A, Johnson GA, et al Ovine placental lactogen specifically binds to endometrial glands of the ovine uterus. Biol Reprod 2003; 68: 772–80.

21. Anthony RV, Liang R, Kayl EP, Pratt SL. The growth hormone/prolactin gene family in ruminant placentae. J Reprod Fertil Suppl 1995; 49: 83–95.

22. Igwebuike UM. Trophoblast cells of ruminant placentas: a minireview. Anim Reprod Sci 2006; 93: 185–98.

23. Martial J, Djiane J. The production of chorionic somatomammotrophin in sheep placenta. Biochem Biophys Res Commun 1975; 65: 770–8.

24. Johnson GA, Spencer TE, Burghardt RC, Bazer FW. Ovine osteopontin I: cloning and expression of mRNA in the uterus during the peri-implantation period. Biol Reprod 1999; 61: 884–91.

25. Johnson GA, Burghardt RC, Joyce MM, et al. Osteopontin is synthesized by uterine glands and a 45-kDa cleavage fragment is localized at the uterine-placental interface throughout ovine pregnancy. Biol Reprod 2003; 69: 92–8.

26. Wooding FBP, Staples LD, Peacock MA. Structure of trophoblast papillae on the sheep conceptus at implantation. J Anat 1982; 134: 507–16.

27. Wango EO, Wooding FBP, Heap RB. The role of trophoblastic binucleate cells in implantation in the goat: a morphological study. J Anat 1990; 171: 241–57.

28. Baker J, Liu J-P, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and post-natal growth. Cell 1993; 75: 73–82.

29. Igwebuike UM. Impact of maternal nutrition on ovine foetoplacental development: a review of the role of insulin-like growth factors. Anim Reprod Sci 2010; 121: 189–96.

## MORFOLOŠKE PRILAGODITVE HISTOTROFIČNE PREHRANE V PLACENTI ZAHODNOAFRIŠKIH PRITLIKAVIH KOZ

U. M. Igwebuike, D. N. Ezeasor

**Povzetek:** Za raziskavo morfoloških prilagoditev za izločanje in vsrkavanje histotrofe na področjih med placentomi v placentah koz so bile uporabljene maternice 11 brejih zahodnoafriških pritlikavih koz. Rezultati so pokazali številne žlezne acinuse v subepitelijskih področjih endometrija, ki so bili bogato ožiljeni. Sekretorne celice žleznih acinusov so v citoplazmi celic vsebovale organele, potrebne za tvorbo in združevanje izločevalnih produktov, kakor tudi veliko mešičkov, povezanih z membranami. Na področju, kjer se stikata placenta matere in placenta zarodka/plodu, so bile opažene interdigitacije mikrovilov. Areole so bile opažene na področju prehoda maternične žlez v področje endometrija. Papile trofoblasta, ki so invaginacije plodove horioalantoisne membrane, so se razširile v notranje področje areol in področja lumnov izvodil maternične žlez. Areole so specializirana mesta za shranjevanje in vsrkavanje izločkov maternične žlez, medtem ko papile trofoblastov lahko ustrezajo področjem močnega vsrkavanja izločkov maternične žlez, ki jih vsrkavajo plodovi trofoblasti. Areole in papile trofoblastov so bile pri zahodnoafriških pritlikavih kozah prisotne od 90. dneva brejosti naprej. Ta podatek kaže, da so izločki maternične žlez pomembni za vzdrževanje brejosti tudi po vsaditvi zarodka in vzpostavitvi hemotrofične prehrane pri kozah. Študija je priskrbela morfološke dokaze za močno produkcijo in prenos izločkov maternične žlez od matere do zarodka na področjih med plancentomi v placenti zahodnoafriških pritlikavih koz.

**Ključne besede:** uterine žleze; papile fetalnega trofoblasta; areole; področja med plancetomi; placenta

Slov Vet Res 2014; 51 (1): 21-8
UDC 636.09:616.98-097.3:57.083.3:599.731.1(497.4)

**Original Scientific Article**

# PREVALENCE OF ANTIBODIES AGAINST SELECTED PATHOGENS IN WILD BOARS *(SUS SCROFA)* IN SLOVENIA

Marina Štukelj[1], Ivan Toplak[2], Gorazd Vengušt[3]*

[1]Institute for the Health Care of Pigs, [2]National Veterinary Institute, Institute for Microbiology and Parasitology, [3]National Veterinary Institute, Institute for Breeding and Health Care of Wild Animals, Fish and Bees, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: gorazd.vengust@vf.uni-lj.si

**Summary:** One hundred eighty-four blood samples were collected from wild boars (*Sus scrofa*) shot in Slovenia during the hunting season 2010/2011. Samples were tested by enzyme immunoassays for antibodies against four viral and two bacterial diseases. 83 samples (45.1%) had antibodies against Aujeszky's disease virus (ADV), 165 (89.7%) against porcine circovirus type 2 (PCV2), 29 (15.8%) against *Mycoplasma hyopneumoniae* (Mhyo), 52 (28.3%) against *Actinobacillus pleuropneumoniae* (APP). Antibodies against classical swine fever virus (CSFV) and against porcine reproductive and respiratory syndrome virus were not detected (PRRSV).

**Key words:** bacterial infection; serologic survey; Slovenia; viral infections; wild boar

## Introduction

In Slovenia wild boars are present all over the country; however, the highest density can be found in the southwest part of the country (1). The wild boar is one of the most important big game species in Slovenia with a hunting bag of around 8.000 pigs per year. A drastic increase in the population density of wild boars in Slovenia occurred during the last decade despite the hunting-related reduction of their population. Since 2005 the hunting bag of wild boar has increased from 6.892 to 8.742 in 2010 (2).

The highest density of the domestic pig population is located in the eastern part of the country (Fig. 1). Moreover, the majority of the Slovenian pig industry involved traditional small pig farms (17494) with less than 20 pigs; 761 farms with 21 to 50 pigs; 330 farms with 51 to 100 pigs; 246 farms with 101 to 200 pigs; 151 farms with 201 to 500 pigs; 40 farms with 501 to 1000 pigs; and only 13 farms with more than 1001 pigs. From a sum total of 312.373 pigs in Slovenia, 31.309 are breeding sows; 996 are boars; and 280.068 are primarily fatteners (data from National animal

Received: 1 February 2013
Accepted for publication: 23 July 2013

registry; owner Ministry of Agriculture and the Environment). Domestic pigs live mostly without any close contact with the wild boar population. Nevertheless, these "backyard" pig operations might be potential points for the introduction and spread of diseases from wild boars to domestic pigs.

In recent years there has been a growing interest in the role of wild boar populations within the epidemiology of important infectious diseases of swine. This interest occurs due to an increase in the wild boar population density worldwide, leading to a higher probability of disease transmission (3). Wildlife can act as a reservoir for pathogens shared with their related domestic species, being able to transmit and maintain them even without the presence of the domestic reservoir (4). Many pathogens are shared by wild boars and domestic swine such as classical swine fever (CSF) (5), Aujeszky's disease (AD) (6), porcine circovirus type 2 (PCV2) (3), porcine reproductive and respiratory syndrome (PRRS) (7), *Mycoplasma hyopneumoniae* (Mhyo) (8) and *Actinobacillus pleuropneumoniae* (APP) (9).

CSF is caused by an infection with CSF (hog cholera) virus (CSFV). CSF is a disease listed by The World Organisation for Animal Health (OIE) (10). Under natural conditions, the infection occurs in domestic pigs and wild boar causing

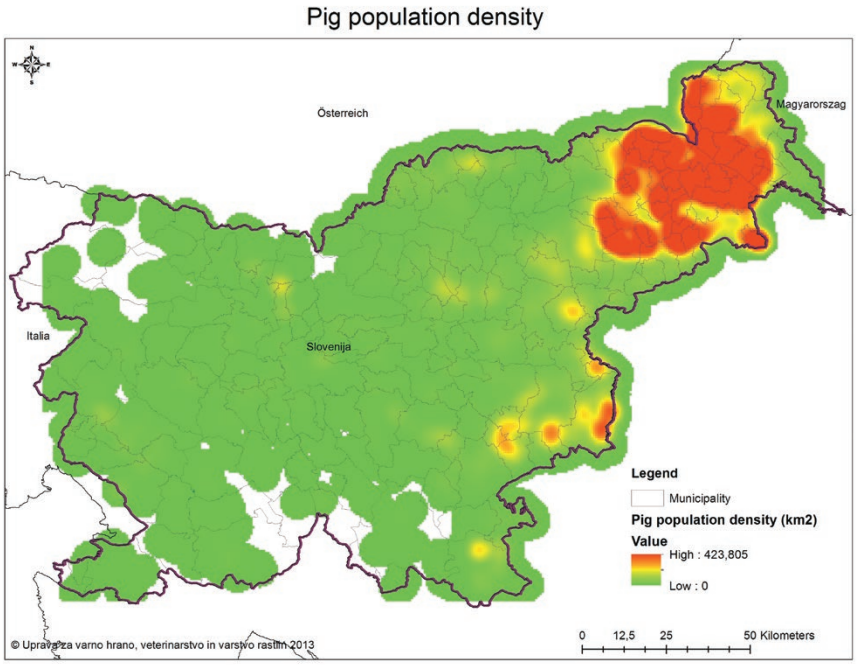


**Figure 1:** Domestic pig density in Slovenia per km$^2$ (National animal registry; owner Ministry of Agriculture and the Environment)

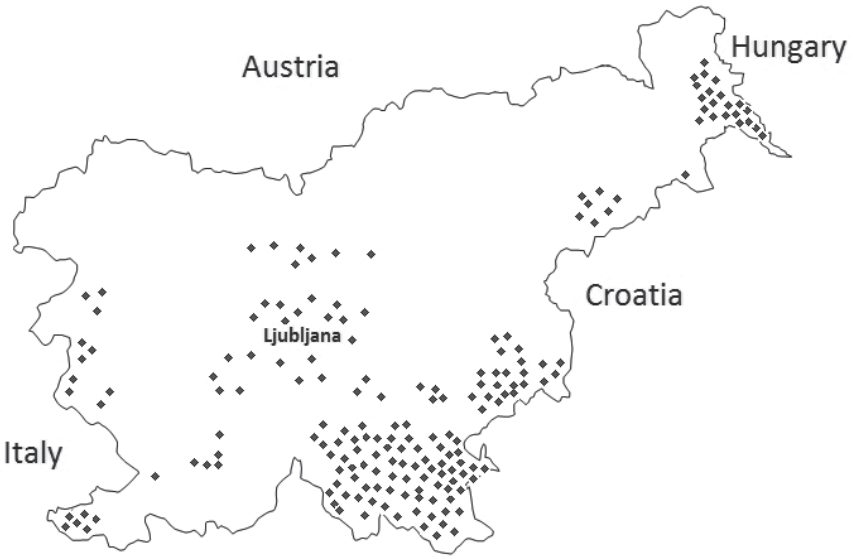


**Figure 2:** Sampling locations (squares) of wild boar (*Sus scrofa*) in Slovenia. Blood samples were collected from 184 shot wild boars throughout the country during the hunting season 2010/2011

major economic losses especially in countries with an industrialized pig production (5).

AD or pseudorabies virus is worldwide distributed swine alphaherpes virus that infects wild and domestic swine as natural host (14). AD virus (ADV) also infects a wide range of other hosts except humans and primates. Mammals other than swine are considered dead-end hosts because infection is fatal before virus excretion. ADV has the ability of establishing a lifelong latent infection in neuronal and non-neuronal cells in swine (6). This particularity of herpes viruses can lead to virus persistence at herd level due to the reactivation of latent infections and consequent virus excretion. This feature remains one of the most important issues regarding ADV epidemiology in the domestic pig and wild boars (15).

PCV2 is a member of the *Circoviridae* family. The virus is ubiquitous in domestic swine population with antibody prevalence reaching almost 100% (3). PCV2 causes postweaning multisystemic wasting syndrome (PMWS) in domestic pig and other diseases and conditions referred to as porcine circovirus diseases (16). Due to relatively unspecific clinical signs, the establishment of a final diagnosis of PMWS is based on three different criteria: clinical signs, the presence of very specific lesions in lymphoid tissues and the presence of PCV2 in these tissues (17, 18). PRRS is one of the economically most important diseases in domestic swine (19). Porcine reproductive and respiratory syndrome virus (PRRSV) is an *Arterivirus* of swine, spread quickly and became enzootic in the pig population in most countries all over the world (20). Rates of spread and infection are advanced in areas with high herd and population densities (21). Aerosol and insects are able to bridge up to 3km (22). Mhyo is the principal etiological agent responsible for enzootic pneumonia in pigs. The clinical outcome of Mhyo infection depends on environment and management conditions and the production system in operation (23). APP is an important pathogen of the porcine respiratory tract and is considered an obligate parasite of the respiratory tract (24). There are no other natural hosts. Two biotypes and several serovars exist. All serovars are capable of causing disease (9). Despite the enormous role of APP distribution in domestic swine production, a very few published data about prevalence and distribution of APP infections in wild boars are available.

The aim of the study was to estimate the prevalence of selected pathogens in wild boar population in Slovenia and estimating the risk of infection for domestic swine.

## Material and methods

Blood samples were collected from 184 shot wild boars throughout the country (Fig. 2) during the hunting season 2010/2011.

Immediately after shoot blood was collected from the animal into sterile serum separation tubes (Vacuette; Greiner Bio-one, Kremsmunster, Austria) and sent to the laboratory. Serum was obtained by centrifugation (at 1300 x g at 4°C for 10 minutes) and frozen at -20°C until analysed.

For all serology the Enzyme-Linked ImmunoSorbent Assay (ELISA) from different manufacturers were used (Table 1). Tests were carried out in accordance with the manufacturer's manual. The results were expressed as positive or negative based on producer's recommended cut off value.

For statistical purposes, the data were divided into age groups (young (≤ 1 yr) and old (≥1 yr)). Statistical analysis for potential age effects on antibody prevalence was performed by $x^2$.

**Table 1:** Employed ELISAs for selected pathogens

| Pathogen | ELISA | Sensitivity | Specificity |
|---|---|---|---|
| CSFV | CHEKIT-CSF-SERO, Idexx Laboratories, former Dr. Bommeli | 93-98% | 99% |
| ADV | Pseudorabies/Aujeszky disease virus gB PRV-gB-Ab (Svanova) | 99.6% | 99.3% |
| PCV2 | Porcine Circo Virus type 2 antibody test kit (BioChek) | 85% | 95,6% |
| PRRS | HerdChek PRRS X3 antibody test kit (IDEXX) | 98.9% | 99.9% |
| Mhyo | Mycoplasma hyopneumoniae antibody test kit (BioChek) | 85% | 99% |
| APP | APP-ApxIV antibody test kit (IDEXX) | 95% | 99% |

**Table 2:** Prevalence of antibodies against selected pathogens in wild boars (*Sus scrofa*) in Slovenia (n=184)

| Pathogen | No. of positive | P (%) | 95% CI |
|---|---|---|---|
| CSFV | 0 | 0 | 0-2 |
| ADV | 83 | 45.1 | 37.7-52.6 |
| PCV2 | 165 | 89.7 | 84.2-93.6 |
| PRRSV | 0 | 0 | 0-2 |
| Mhyo | 29 | 15.8 | 10.8-21.9 |
| APP | 52 | 28.3 | 21.8-35.4 |

P: prevalence; CI: confidence interval

## Results

In total of 184 sera examined, in 83 (45.1%) sera we detected antibodies against ADV, in 165 (89.7%) sera antibodies against PCV2, in 29 (15.8%) sera antibodies against Mhyo and in 52 (28.3%) sera antibodies against APP. Antibodies against CSFV and PRRSV were not detected. The results are summarised in table 2. Statistically significant differences were noted regarding seroprevalence to ADV across age ($x^2 = 12.67$, df = 1, $P<0.001$), with more positive adults (85%) than juveniles (40%). We did not find statistically significant age-related differences for PCV2, Mhyo and APP.

## Discussion

CSFV has no known zoonotic potential; however, its presence in the region has serious economic implications for domestic pig farming and hunting tourism. It is a serious and contagious viral disease affecting pigs and wild boars that can be directly or indirectly transmitted from infected wild boar to domestic pigs. In this study no antibodies to CSF were detected in tested wild boar. The last outbreak of CSF in wild boar were reported in 1965 (12) and domestic swine in 1996 (11), respectively. Reports on CSF outbreaks in domestic pig in years 2006-2008 (25) and wild boar population in years 2009-2010 in Croatia (26) remind us about constant risk for introduction of CSF in Slovenia from Balkan area therefore, constant monitoring is an important part of control of CSF in wild boar (13) to remain free of CSF.

In many parts of the world, efforts are being carried out to control ADV in domestic pigs. In Europe, most countries have implemented strict national eradication programs based on initial large scale vaccination of pigs with attenuated glycoprotein E (gE)-deleted vaccines. In countries that have reached the AD-free status, vaccination against ADV is forbidden (27). Slovenia is country officially free of AD from year 2010 in domestic pigs (28). The prevalence of antibodies against ADV found in the present study was 45.1%, which is higher than seroprevalence (31%) previously reported by Vengust et al. (29) in Slovenia. The increase of the population density of the wild boar in recent years could be the reason for higher prevalence; however, more studies are needed to determine the main cause. Lower seroprevalence was also reported from Germany (40%) (30), Italy (30.7%) (31, 32), Poland (11%) (33), France (3.5%) (34), Croatia (38.5%) (35) and Switzerland (2.8%) (36), whereas higher seroprevalence was established in Spain (49%) (27). Our data indicate that the risk of infection increases with age, and this is consistent with results from other studies (37, 38). Sample sizes per individual site were small, especially in east region (Goriško). The limited sample size means that results, particularly regarding areas, need to be taken with caution. However, wild boar AD antibodies seroprevalence in this study and study conducted in 2006 in the same region, indicate that AD remains endemic at low prevalences in the east Slovenia wild boar populations. Domestic pigs in Slovenia are free of AD. There is an evidence that ADV infection in domestic pigs and wild swine represents epidemiologically distinct infection cycles (32, 39) and experimental studies suggest that there is no possibility that infected wild swine can shed virus in sufficient amounts to trigger infection (40, 41). Taken together, these findings indicate that the virus isolated from wild boar is highly adapted to the population. Furthermore, the molecular

biological characterization of the wild boar ADV clearly supports the hypothesis that the infectious cycle within the wild boar population in eastern Germany is independent of that in domestic pigs (40). We can speculate that this observation can be the reason why our domestic pigs remain free of ADV.

It is known from serological surveys that PCV2 is ubiquitous with serological prevalence close to 100% in finishing pigs worldwide (17, 42). According to the Golinar-Oven (43) all tested domestic pigs in Slovenia were also positive. Thus, considering the ubiquitous distribution of PCV2 among domestic pig populations, and the known risk factors for PMWS in domestic pigs such as poor hygiene and crowding (18), we expected that the seroprevalence to PCV2 will be much lower in wild boar populations. In contrast the prevalence of antibodies against PCV2 in the present study was 89.7% higher than that reported by Ruiz-Fons et al. (44) (between 20 and 48%) in the wild boar population of Europe. Roic et al. (37) reported very low seroprevalence (15.1%) in Croatia. In Slovenia the prevalence is slightly lower in wild boars than in domestic pigs; but the fact that factors such as living conditions, age of infection, extent of PCV2 shedding, early weaning, and vaccinations (45), are the factors that may enhance the spread of the virus in commercial swine, may not be applicable to wild boar populations and may not be the key issues of transmission of the virus. Also the transmission between domestic pigs and wild boars is quite difficult while the high density of domestic pigs takes place in the eastern part of the country and the majority of wild boars live in the southwestern part of Slovenia. On the other hand, Toplak et al. (46) reported that the high diversity of strains of PCV2 detected in wild boars could be evidence for the persistence of PCV2 infection for a longer period and for the introduction of the virus from different sources. Furthermore, the population density, the infection pressure and the specificity of the biotope where the wild boar families live could play an important role in the effective transmission of virus between the boar and domestic pigs in the area. The results of sequencing analysis in Slovenia and Serbia, showing identical or very similar sequences of PCV2 strains in wild boar and domestic pigs, confirm this last possibility (47).

In the present study there was no detection of antibodies against PRRSV in wild boar population in Slovenia which concur with the previous report on wild boar by Vengust et al. (29). Contrary antibodies against PRRSV have been found in Germany (0.4%) (48), France (1.3%) (36), Croatia (6.3%) (37) Italy (37.7%) (33) and the United States of America (USA) 1.7% (49). The detected prevalence of antibodies in 2010 against PRRSV in Slovenia from farms with more than 30 breeding sows was 48% (50). The sequencing results of 258 nucleotides in ORF7 from 30 representative herds with PRRSV-positive samples revealed the circulation of six genetically different strains of PRRSV, all belonging to the EU subtype 1 (51). The negative result of PRRSV observed in this study was in agreement with previous publications and suggests that the wild boars may be a low-risk reservoir for the transmission of the virus to domestic pigs (6, 45).

Prevalence of Mhyo obtained in this study (15.8%) was lower to that obtained previously in Slovenia (21%) (29) and lower to that observed in France (58%) (52) and the USA (32%) (53). However, the lack of consistent gross lesions compatible with enzootic pneumonia (EP) in studied animals shows that the effect of Mhyo probably remains subclinical in this species (8). Examination of the 36 sera from domestic swine in Slovenia has revealed antibodies against Mhyo in 83.3% (43). Based on the relatively high prevalence of Mhyo in conventional pig farms (43), the fact that the domestic pig could be the real reservoir for the wild boar should not be ruled out. The wild boar may only represent a potential Mhyo threat for Mhyo free farms (8).

Prevalence of APP seropositive pigs obtained in this study (28.3%) was lower to that obtained previously in Slovenia (52%) (29). A similar prevalence was reported by Reiner et al. (9) in Germany where more than one-third of the tested animals were infected. APP is a highly contagious and economically significant respiratory disease in domestic swine with significant negative impact on the pig production because of increased medication and decreased weight gain. Serological evidence for APP infection has recently been reported in domestic swine in Slovenia, with a seroprevalence rate of 100% (43). APP is the airborne disease and it seems that there could be some correlation between APP in wild boars and domestic pigs; however, this still need to be evaluated.

Detection of antibodies against ADV, PCV2, Mhyo and APP in the wild boar of the present study

supports the hypothesis that these animals may be reservoirs of swine diseases for domestic swine. It is very difficult to conclude if there is any association between the infectious agent that appear in both domestic and wild boar populations. In the case of ADV we assume that transmission between populations does not occur although the positive wild boars origin from all parts of Slovenia. PRRS is widely disseminated in domestic pig population and is not present in wild boar population. These data suggest that there is no transmission of diseases from wild boars to the domestic swine or vice versa in pig production system used in Slovenia. With outdoor raised population of domestic pigs the possibility of disease transmission from wild boars to domestic pigs will increase. Permanent control of wild and domestic swine populations may be important measure on national level for minimizing the spread and transmission of diseases among these two populations.

## Acknowledgements

The authors thank the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection for funding the sampling and testing for CSF and AD. Samples were collected as part of a monitoring programme of classical swine fever in wild boar coordinated by the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection. The authors acknowledge BioChek, Inc. for donating diagnostic kits for PCV2 and Mhyo. Last but not least, we express our gratitude to associate professor Zdravko Valenčak, the director of the Institute for Health Care of Pigs, for his proof reading and valuable scientific recommendations and Demará Brake Ivanič for review of English.

## References

1. Stergar M, Jonozovič M, Jerina K. Distribution and relative densities of autochthonous ungulates in Slovenia. Gozd Vest 2009; 67(9): 367–80.

2. Odstrel divjadi. Stat Letop 2011;W 50: 327.

3. Fabisiak M, Szczotka A, Podgorska K, Stadejek T. Prevalence of infection and genetic diversity of porcine circovirus type 2 (PCV2) in wild boar (*Sus scrofa*) in Poland. J Wild Dis 2012; 48(3): 612–8.

4. Gortazar C, Ferroglio E, Hofle U, Frolich K, Vicente J. Diseases shared between wildlife and livestock: an European perspective. Eur J Wild Res 2007; 53: 241–56.

5. Artois M, Depner KR, Guberti V, Hars J, Rossi S, Rutili D. Classical swine fever (hog cholera) in wild boar in Europe. Rev Sci Tech OIE 2002; 21(2): 287–303.

6. Ruiz-Fons F, Vidal D, Höfle U, Vincite J, Gortazar C. Aujeszky's disease virus infection patterns in European wild boar. Vet Microbiol 2007; 120: 241–50.

7. Reiner G, Fresen C, Bronnert S, Willems H. Porcine reproductive and respiratory syndrome virus (PRRSV) infection in wild boars. Vet Microbiol 2009; 136: 250–8.

8. Sibila M, Mentaberre G, Boadella M et al. Serological, pathological and polymerase chain reaction studies on *Mycoplasma hyopneumoniae* infection in the wild boar. Vet Microbiol 2010; 144: 214–8.

9. Reiner G, Fresen C, Bronnert S, Haack I, Willems H. Prevalence of *Actinobacillus pleuropneumoniae* ifection in hunted wild boars (*Sus scrofa*) in Germany. J Wild Dis 2010; 46(2): 551–5.

10. OIE. Notification of diseases and epidemiological information. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals. Paris: World Organisation for Animal Health (OIE), 2011: Chapter 1.1.

11. Kovač Z, ed. Animal health status in Slovenia in 1998. Ljubljana : Veterinary Administration of the Republic of Slovenia, 1999: 34 str.

12. Valentinčič S, ed. Bolezni divjadi. Ljubljana : Lovska zveza Slovenije, 1981: 281 str. (Zlatorogova knjižnica, 12)

13. Toplak I, Maurer Wernig J, Vengušt G, et al. Control of classical swine fever (CSF) in Slovenia. In: Proceedings of the International Conference Prevention of Classical Swine Fever in the Border Region Croatia - Serbia (STOP - CSF). Novi Sad: Scientific Veterinary Institute of Serbia, 2012: 60–70.

14. Kluge JP, Beran GW, Hill HT, Platt KB. Pseudorabies (Aujeszky's disease). In: Straw BE, D'Allaire S, eds. Diseases of swine. Ames: Blackwell Science, 1999: 233–46.

15. Pejsak ZK, Truszczynski MJ. 2006. Aujeszky's disease (Pseudorabies). In: Straw BE, D'Allaire S, eds. Diseases of swine. Ames: Blackwell Science, 2006: 419–33.

16. Allan G, Meehan B, Todd D, et al. Novel porcine circoviruses from pigs with wasting

disease syndromes. Vet Rec 1998: 142; 467–8.

17. Segales J. Update on postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome diagnostics. J Swine Health Prod 2002; 10: 277–81.

18. Segales J, Domingo M. Postweaning multisystemic wasting syndrome (PMWS) in pigs: a review. Vet Q 2002; 24: 109–24.

19. Cho JG, Dee SA. Porcine reproductive and respiratory syndrome virus. Theriogenology 2006; 66: 655–62.

20. Zimmerman JJ, Benfield DA, Murtaugh MP, Osorio F, Stevenson GW, Torremorell M. Porcine reproductive and respiratory syndrome virus (Porcine arterivirus). In: Straw BE, D'Allaire S, eds. Diseases of swine. Ames : Blackwell Science, 2006: 384–417.

21. Mortensen S, Stryhn H, Søgaard R, et al. Risk factors for infection of sow herds with porcine reproductive and respiratory syndrome (PRRS) virus. Prev Vet Med 2002; 53: 82–101.

22. Dee SA, Deen J, Otake S, Pijoan C. An experimental model to evaluate the role of transport vehicles as a source of transmission of porcine reproductive and respiratory syndrome virus to susceptible pigs. Can J Vet Res 2004; 68:128–33.

23. Sibila M, Pieters M, Molitor T, Maes D, Haesebrouck F, Segales J. Current perspectives on the diagnosis and epidemiology of *Mycoplasma hyopneumoniae* infection. Vet J 2009; 181: 221–31.

24. Gottschalk M, Taylor DT. *Actinobacillus pleuropneumoniae*. In: Straw BE, D'Allaire S, eds. Diseases of swine. Ames: Blackwell Science, 2006: 563–76.

25. Pavlak M, Vrkić V, Cvitkovic D, Šeparović A, Gašpar A, Tadić M. Some epidemiological aspects of classical fever in Croatia. Vet Arh 2011; 81(1): 51–66.

26. Jemeršić L, Keros T, Roić B, Balatinec J, Terzić S, Brnić D. Classical swine fever seroprevalence results in wild boars in year one (2009) and two (2010) after an outbreak. In: 6th International Symposium on Emerging and Re-emerging Pig Diseases. Barcelona: Centre de Recerca en Sanitat Animal (CReSA), 2011: 294.

27. Boadella M, Gortazar C, Vicente J, Ruiz-Fons F. Wild boar: an increasing concern of Aujeszky's disease control in pigs? BMC Vet Res 2012; 8(7): e 1–7. http://www.biomedcentral.com/1746-6148/8/7

28. Commission Decision of 6 August 2010. Amending Annexes I and II to Decision 2008/185/EC as regards the inclusion of Slovenia in the list of Member States free of Aujeszky's disease and of Poland and regions of Spain in the list of Member States where an approved national control programme for that disease is in place. Off J L 2010; 208 (7.8.2010): 5–8.

29. Vengust G, Valencak Z, Bidovec A. A serological survey of selected pathogens in wild boar in Slovenia. J Vet Med 2006; 53: 24–7.

30. Pannwitz G, Freuling C, Denzin N et al. A long-term serological survey on Aujeszky's disease virus infection in wild boar in East Germany. Epidemiol Infect 2011; 140(2): 348–58.

31. Montagnaro S, Sasso S, De Martino L, et al. Prevalence of antibodies to selected viral and bacterial pathogens in wild boar (*Sus scrofa*) in Campania Region, Italy. J Wild Dis 2010; 46: 316–9.

32. Lari A, Lorenzi D, Nigrelli D, Brocchi E, Faccini S, Poli A. Pseudorabies virus in European wild boar from Central Italy. J Wild Dis 2006; 42: 319–24.

33. Szweda W, Lipowski A, Ciecierski H, Zalewski K, Pirus T. European wild boar (*Sus scrofa*) as reservoir of Herpesvirus suis 1. J Vet Med 1998; 54: 541–4.

34. Albina E, Mesplede A, Chenut G, et al. A serological survey on classical swine fever (CSF), Aujeszky's disease (AD), and porcine reproductive and respiratory syndrome (PRRS) virus infection in French wild boars from 1991 to 1998. Vet Microbiol 2000; 77: 43–57.

35. Roic B, Jemersic L, Terzic S, Keros T, Balatinec J, Florijancic T. Prevalence of antibodies to selected viral pathogens in wild boars (*Sus scrofa*) in Croatia in 2005-06 and 2009-10. J Wild Dis 2012; 48(1): 131–7.

36. Köppel C, Knopf L, Ryser MP, Miserez R, Thür B, Stärk KDC. Serosurveillance for selected infectious disease agents in wild boars (*Sus scrofa*) and outdoor pigs in Swizerland. Eur J Wild Res 2007; 53: 212–20.

37. Lutz W, Junghans D, Schmitz D, Müller T. A long-term survey of pseudorabies virus infections in European wild boar of western Germany. Z Jagdwiss 2003; 49: 130–40.

38. Vicente J, Ruiz-Fons F, Vidal D, et al. Serosurvey of Aujeszky's disease virus infection in European wild boar in Spain. Vet Rec 2005; 156: 408–12.

39. Müller T, Teuffert J, Ziedler K, et al. Pseudorabies in European wild boar from eastern Ger-

many. J Wild Dis 1998; 34: 251–8.

40. Müller T, Klupp B, Zellmer R, et al. Characterisationof pseudorabies virus isolated from wild boar (*Sus scrofa*). Vet Rec 1989; 143: 337–40.

41. Hahn EC, Page GR, Hahn PS, et al. Mechanism of transmission of Aujeszky's disease virus originating from feral swine in the USA. Vet Microbiol 1997; 55: 123–30.

42. Rosell C, Blanchard P, Larour G, et al. Post-weaning multisystemic wasting syndrome (PMWS) in France: serological profiles of affected versus non-infected herds and preliminary analytical epidemiology. Pig J 2002; 50: 124–34.

43. Golinar-Oven I. Establishment and application of serum profile for control measures against selected pathogens of porcine respiratory disease complex on large pig farm: dissertation. Ljubljana : Veterinarska fakulteta, Univerza v Ljubljani, 2011.

44. Ruiz-Fons F, Segales J, Gortazar C. A review of viral diseases of the European wild boar: effects of population dynamics and reservoir role. Vet J 2008; 176: 158–69.

45. Vicente J, Leon-Vizcaino L, Gortazar C, Jose Cubero M, Gonzalez M, Martin-Atance P. Antibodies to selected viral and bacterial pathogens in European wild boars from south-central Spain. J Wild Dis 2002; 38: 649–52.

46. Toplak I, Grom J, Hostnik P, Barlic-Maganja D. Phylogenetic analysis of type 2 porcine circoviruses identified in wild boar in Slovenia. Vet Rec 2004; 155: 178–80.

47. Toplak I, Lazić S, Lupulović D, et al. Study of the genetic variability of porcine circovirus type 2 detected in Serbia and Slovenia. Acta Vet Hung 2012; 60(3): 409–20.

48. Oslage U, Dahle J, Müller T, Kramer M, Beier D, Liess B. Prevalence of antibodies against the viruses of European swine fever, Aujeszky's disease and “porcine reproductive and respiratory syndrome” in wild boars in the federal states Sachen-Anhalt and Brandenburg. Deutsch Tierarztl Wochenschr 1994; 101: 33–8.

49. Saliki JT, Rodgers SJ, Eskew G. Serosurvey of selected viral and bacterial diseases in wild swine from Oklahoma. J Wild Dis 1998; 34: 834–8.

50. Toplak I, Štukelj M, Zabavnik-Piano J, Hostnik P, Grom J, Valenčak Z. Research of Porcine reproductive and respiratory syndrome virus (PRRSV) in Slovenia in 2010. Ljubljana: Veterinary Faculty, National Veterinary Institute, 2010: 40 str.

51. Toplak I, Rihtarič D, Hostnik P, Grom J, Štukelj M, Valenčak Z. Identification of a genetically diverse sequence of porcine reproductive and respiratory syndrome virus in Slovenia and the impact on the sensitivity of four molecular tests. J Virol Methods 2012; 179: 51–6.

52. Marois C, Tocqueville V, Le Potier MF, Hars J, Kobisch M. Detection of *Mycoplasma hyopneumoniae* in French wild boars. In: 19th International Pig Veterinary Society. Copenhagen: Kjellerup, 2000: 213.

53. Baker SR, O'Neill KM, Gramer MR, Dee SA. Estimates of the seroprevalence of production-limiting diseases in wild pigs. Vet Rec 2011; 168: 564.

## POJAVNOST PROTITELES PROTI NEKATERIH POVZROČITELJEM BOLEZNI PRI DIVJIH PRAŠIČIH V SLOVENIJI

M. Štukelj, I. Toplak, G. Vengušt

**Povzetek:** Odvzetih je bilo184 krvnih vzorcev divjih prašičev (*Sus scrofa*), odstreljenih v lovski sezoni 2010/2011 v Sloveniji. Vzorce smo testirali s testom ELISA za dokaz protiteles proti štirim virusnim in dvema bakterijskima boleznima prašičev. Pri 83 vzorcih (45.1 %) smo potrdili protitelesa proti bolezni Aujeszkega, pri 165 (89.7 %) vzorcih smo potrdili protitelesa proti prašičjemu cirkovirusu tipa 2, pri 29 (15.8 %) vzorcih protitelesa proti *Mycoplasma hyopneumoniae* in pri 52 (28.3 %) vzorcih protitelesa proti *Actinobacillus pleuropneumoniae*. Protiteles proti klasični prašičji kugi in protiteles proti prašičjem reprodukcijskem in respiratornem sindromu pri testiranih divjih prašičih nismo potrdili.

**Ključne besede**: bakterijske infekcije; serologija; Slovenija; virusne infekcije; divji prašiči

Slov Vet Res 2014; 51 (1): 29-34
UDC 57.08:612.44:572.7:599.323.4:636.028

**Original Scientific Article**

# THYROID HORMONES LEVELS AND MORPHOMETRIC SPECIFICS OF THYROID GLAND IN ApoE DEFICIENT (ApoE KO) MICE

Toni Tripunoski[1], Jasmina Dimitrova-Shumkovska[2], Trpe Ristoski[3], Irena Petrova[4], Sasho Panov[2], Ana Ugrinska[1], Daniela PopGjorceva[1]

[1]Institute of Pathophysiology and Nuclear Medicine, Medical Faculty, [2]Institute of Biology, Faculty of Natural Sciences and Mathematics, [3]Department of Pathology, Faculty of Veterinary Medicine, [4]Institute of Medical Histology and Embryology, Medical Faculty, Ss. Cyril and Methodius University- Skopje, Republic of Macedonia

*Corresponding author, E-mail: tonitr@yahoo.com

**Summary:** The specificity of biological features in strains used as animal models is of particular importance of the interpretation of experimental data. In this paper we examine thyroid hormones levels as well as diameter of thyroid follicles and the follicular epithelium height in ApoE-knockout (ApoE KO) mice, compared to the wild type (WT) - C57BL/6 strain as a control group. ApoE lipoprotein deficiency in this strain leads to development of severe hypercholesterolemia and atherosclerotic lesions in the blood vessels, even when mice are fed regular mouse chow. The results of our study revealed difference in $FT_3$ plasma level between the two strains of mice with no significant difference in $FT_4$ level. The morphometric analysis showed a significantly higher follicular epithelium in ApoE KO mice compared to the wild strain, which is an indication of greater TSH stimulation in ApoE KO strain. Regarding the diameter of thyroid follicles, our results corroborate no significant differences between the genetically modified animal strains, which are consistent with previous data from other studies that found no correlation of the above mentioned parameter with thyroid status of animals. The difference in thyroid function between the two strains of experimental animals could be due to altered peripheral metabolism of thyroid hormones, a consequence of the altered liver physiology in ApoE KO mice.

**Key words:** ApoE KO mice; thyroid gland; morphometry

## Introduction

ApoE-knockout mice (apoE KO) have been extensively used to study the relation of hypercholesterolemia and lipoprotein oxidation to atherogenesis (1, 2, 3). Apolipoprotein E exerts several functions regarding lipid and cholesterol transport and metabolism: 1) apoE functions as an important carrier protein in the redistribution of lipids among cells (by incorporation into HDL (as HDL-E); 2) it plays a prominent role in the transport of cholesterol (by incorporating into intestinally synthesized chylomicrons); 3) it takes part in metabolism of plasma cholesterol and triglyceride (by interaction with the LDLR and the receptor binding of apoE lipoproteins (4, 5).

The lack of ApoE lipoprotein caused by the targeted deletion of the Apo E gene in this strain leads to the development of severe hypercholesterolemia and atherosclerotic lesions in the blood vessels, even when mice are fed regular mouse chow (6, 7). The development and phenotype appearance of the lesions are quite

Received: 27 February 2013
Accepted for publication: 21 October 2013

similar to those in human atherosclerosis (8).

Thyroid hormones have important role in the lipid metabolism and in the pathophysiology of atherosclerosis. There are many publications that report elevated levels of cholesterol and other serum lipids in hypothyroidism, which increase the risk of development of atherosclerosis (9, 10). On the other hand it has been reported that ApoE lipoprotein has a role in the intracellular entry of thyroid hormones in peripheral tissues (11, 12). Having in mind these interactions between the thyroid hormones and lipids, it is very important to establish the connection between thyroid function and the thyroid morphometric indices in the strains used as animal model in the research on atherosclerosis.

The goal of this study was to examine the thyroid function and morphometric specifics of the thyroid tissue in ApoE KO mice compared to the wild type (WT) - C57BL/6 mice.

## Materials and methods

For the present study, 16 weeks old apoE KO mice (B6.129P2-apoE$^{tm1}$ N11) in comparison to C57BL/6 background mice (i.e. wild type, WT), ten animals per group, were used. After overnight fast of 12 hours, animals were anesthetized under ketamine/xylazine anesthesia (90 mg/kg i.p. and 10 mg/kg, i.p. respectively). Blood samples were obtained by cardiac puncture, collected into tubes and centrifuged at 1450 × g at 4°C for 10 minutes. Lipoprotein levels in circulating blood were determined by enzyme-colorimetric test (Human diagnostics, Germany), and analyzed by Olympus, AU 400 – system. The serum levels of free $T_4$ ($FT_4$) and free $T_3$ ($FT_3$) were determinated using immunoradiometric assay according to the manufacturer's protocol (DYNO test; Brahms Diagnostics GmbH, Henningsdorf/Berlin, Germany) using the gamma counter (PC-RIA MAS STRATEC). Formalin-fixed and paraffin embedded thyroid glands were used for histomorphometric analysis. Histological sections were stained with hematoxylin/eosin (H&E) technique and morphometric features were analyzed by light microscope connected to a video camera (Nicon-Eclipse E600, Program Lucia 4.21). The measurements were made in thirty follicles, at five random different points. Follicular epithelium height and follicular diameter from peripheral and from the central parts of the thyroid lobes were measured with the Weibel's multipurpose test system $M_{42}$ (Wild, Switzerland) (13).

Statistical data processing was performed using the software system STATISTICA for Windows XP Professional. Group results are presented as means ± standard deviation (SD). The statistical significance of the differences between the means of the experimental groups was tested by the Student's *t*-test for unpaired samples. The differences were considered as statistically significant when $p<0.05$.

All procedures with the animals were in accordance with Local Animal Care Committee in conformity with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123, Appendix A). The mice were housed in polycarbonate cages in a pathogen – free facility set on a 12h light-dark cycle and given *ad libitum* access to water and standard laboratory feed. Prior to the experimental procedures, the rats were fed a commercial standard pellet feed (Filpaso, 52.11, Skopje, Republic of Macedonia), named "standard feed" hereafter.

## Results

The plasma levels of cholesterol, HDL and triacylglycerol (TAG) in ApoE KO mice were significantly higher compared to WT strain (Table 1). The 16 weeks old Apo E KO mice had increased cholesterol levels for 5 fold higher vs their wild counterparts (67.7 ± 23.3 mg/dL vs 383.7 ± 47.3 mg/dL, $p<0.001$).

In the present study, the level of $FT_4$ was in the range 19.78 ± 0.99 pmol/L in ApoE KO mice and 19.74 ± 0.75 pmol/L in WT. There was no significant difference between the two strains (table 2). The range of $FT_3$ in ApoE KO mice was 2.03 ± 0.38 pmol/L and at WT the range was 2.51 ± 0.31 pmol/L. Regarding FT3, we observed significantly reduced difference in ApoE KO mice vs WT mice, $p< 0.05$ (Table 1).

According to the morphometric analysis, the follicular epithelium height in ApoE KO mice was higher compared to those of the wild strain, but there was no difference with regard to the follicular diameter between the two strains. The increased height of the follicular epithelium in ApoE mice is shown in Figure 1.

**Table 1:** Plasma lipoprotein and thyroid hormone levels in ApoE KO mice and their WT counterparts

| Strain | Plasma lipoprotein levels (mg/dL) | | | Thyroid hormone level (pmol/L) | |
|---|---|---|---|---|---|
| | Chol | TAG | HDL | $FT_4$ | $FT_3$ |
| ApoE KO | 383.7+47.3*** | 117.7+24.5* | 67.0+16.3* | 19.78 ± 0.99 | 2.03 ± 0.38* |
| WT | 67.7+23.3 | 71.0+18.4 | 33.2+5.1 | 19.74 ± 0.75 | 2.51 ± 0.31 |

* $p < 0.05$; *** $p < 0.001$

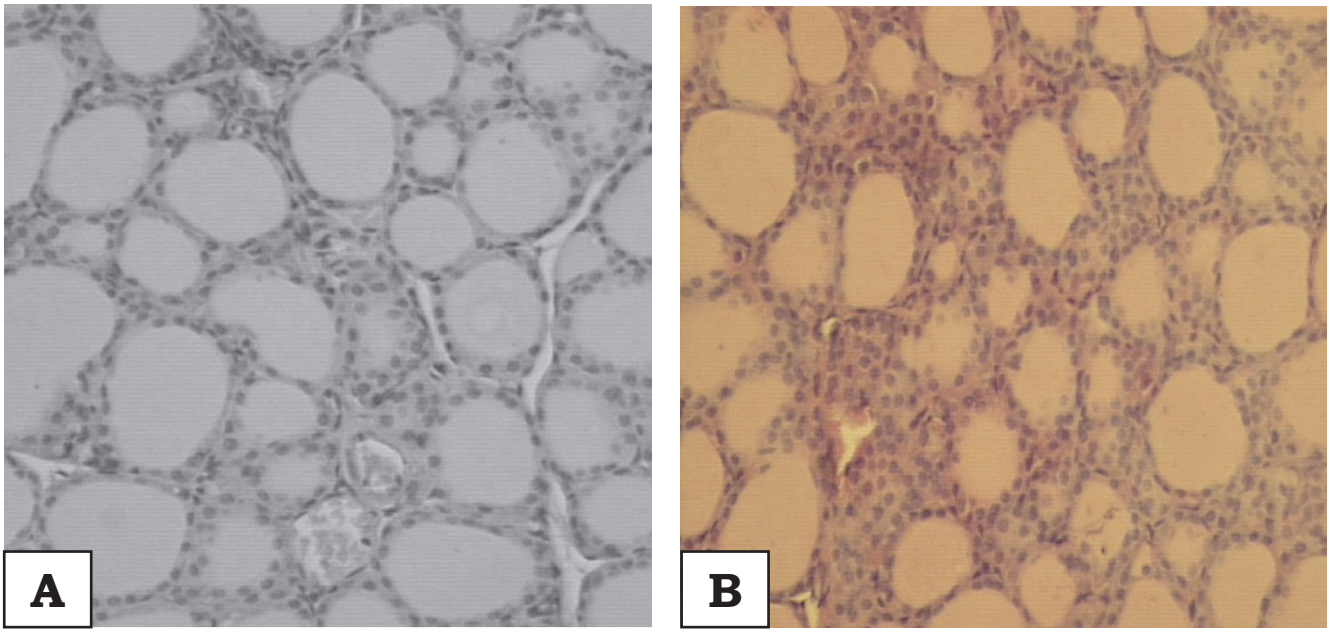


**Figure 1:** Thyroid gland morphology in WT (A) and ApoE KO (B) mice (H&E x 20). Histological section of thyroid tissue characterized by the difference in height of the thyroid epithelium between the two groups with no significant difference in the size and shape of the thyroid follicles

**Table 2:** Follicles diameter and epithelial cells height in ApoE KO mice and their WT counterparts

| Follicles diameter and epithelial cells height (μm) | | |
|---|---|---|
| | WT | ApoE KO |
| Peripheral epithelial cells height | 6.5 ± 1.91 (N=9) | 9.9 ± 2.1*** (N=8) |
| Peripheral follicles diameter | 110.3 ± 27.4 (N=8) | 108.95 ± 20.47 (N=8) |
| Central epithelial cells height | 6.85 ± 1.31 (N=8) | 10.3 ± 1.95*** (N=10) |
| Central follicles diameter | 64.7 ± 16.0 (N=9 | 68.45 ± 17.57 (N=7) |

*** $p < 0.001$

The mean height of the peripheral epithelium in ApoE KO was 9.9 ± 2.5 μm compared to 6.5 ± 1.9 μm in WT mice (Table 3). The difference was even more noticeable in the central epithelial cells in which the mean height in ApoE KO was 10.3 ± 1.9μm compared to 6.8 ± 1.3 μm in WT mice (Table 2).

## Discussion

We compared some morphometric characteristics of the thyroid gland between ApoE KO and wild type - C57BL/6 mice, as an indicator of thyroid status for the both strains (14, 15). The results of biochemical analysis showed significantly higher plasma concentrations of cholesterol and TAG in the ApoE KO strain (Table 1) mainly due to the impaired reverse cholesterol transport (16). According to the statistical analysis, the existence of significant difference in the height of the follicular epithelium compared to vehicle control was very intriguing. Namely, the values for the height of thyroid epithelium were significantly higher in ApoE KO compared to wild strain mice, both in peripheral and in central follicles of the thyroid lobes (Table 2). Regarding the diameter of thyroid follicles, no significant difference between the two experimental groups was found (Table 2). These features, greater than usual, are a typical characteristic of TSH stimulation of the thyroid tissue. In experimental hypothyroidism, there is always a negative correlation between the level of thyroid hormones ($T_4$ and $T_3$) and the follicular epithelium height, while no correlation with the diameter of thyroid follicles is found (17, 18).

Our results also showed lower $FT_3$ levels in ApoE KO mice, indicating a lower peripheral conversion rate of $FT_4$ to $FT_3$. According to our knowledge, no data concerning this issue are published in the literature up to date. On the other hand the morphometric indices in our study suggest increased TSH stimulation that is due to the lower levels of $FT_3$ in ApoE KO mice.

There are several possible explanations for the differences in thyroid morphology and function between the two strains. The altered thyroid function in ApoE KO mice could be due to accumulation of oxidative damage and functional disorder of the liver, typical for an ApoE KO strain, fed a standard animal diet (19). The liver has a very important role in the thyroid hormone metabolism. The impaired conversion of $T_4$ to $T_3$, a consequence of lower activity of hepatic *5' deiodinase,* leads to reduction of the physiologically active $T_3$ and consequently increases the physiologically inert reverse triiodothyronine ($rT_3$). Patients with chronic liver disease have marked elevation in $rT_3$ accompanied by reduced $T_3$, as well as increasing TSH levels (20). This type of altered thyroid function could be a serious basis for an explanation of our findings.

The absence of ApoE lipoprotein in the ApoE KO strain also might play a role in the altered thyroid metabolism in these mice, considering the proposed role of this lipoprotein in the intracellular transport of thyroxin. Such claims are based on previous studies that showed the existence of a thyroid hormone binding domain in the ApoE molecule, which could be involved in the intracellular transport of thyroxin (21). The authors of the study assumed that this could facilitate the entry of thyroid hormones into cells through ApoB/ApoE receptors, widely distributed in tissues (22).

The lower intracellular internalization of the thyroxin that leads to mental retardation due to ApoE polymorphism was also proposed by Wang et al. (12). This study has shown link between Apoe polymorphism and the incidence of mental retardation in the population of iodine deficient regions of China. The mental retardation was more frequent between individuals with ApoE4 isoform (12). Based on these facts, the complete absence of ApoE lipoprotein in ApoE KO mice should have significant implications on intracellular entry of thyroid hormones in peripheral tissues. The setting resembles the syndrome of thyroid hormone peripheral resistance with higher levels of $FT_4$ and normal or slightly elevated TSH (23). This kind of changes could induce morphometric changes similar to the ones reported in our study.

## Conclusion

The results of our study revealed difference in the height of the thyrocytes and levels of $FT_3$ between the two strains of mice. The possible explanation of these results is the altered liver function in ApoE KO mice and subsequently altered peripheral metabolism of thyroid hormones or the role of the ApoE lipoprotein in the intracellular internalization of the thyroid hormones. Having in mind the role of thyroid hormones in the genesis of atherosclerosis, the establishment of normal thyroid hormone range in ApoE KO mice is very important when developing animal models for the research on atherosclerosis. This would be particularly important for studying concomitant conditions with impaired thyroid function, when ApoE KO strains are used as animal models.

## References


1. Hoen PA, Van der Lans CA, Van Eck M, Bijsterbosch MK, Van Berkel TJ, Twisk J. Aorta of ApoE-deficient mice responds to atherogenic stimuli by a prelesional increase and subsequent decrease in the expression of antioxidant enzymes. Circ Res 2003; 93(3): 262–9.

2. Yang H, Roberts JL, Shi MJ, et al. Retardation of atherosclerosis by overexpression of catalase or both Cu/Zn-superoxide dismutase and catalase in mice lacking apoliporotein E. Circ Res 2004; 95 1075–81.

3. Kunitomo M, Yamaguchi Y, Kagota S, Yoshikawa N, Nakamura K, Shinozuka K. Biochemical evidence of atherosclerosis progression mediated by increased oxidative stress in apolipoprotein E-deficient spontaneously hyperlipidemic mice exposed to chronic cigarette smoke. J Pharmacol Sci 2009; 110: 354–61.

4. Quinn CM, Kågedal K, Terman A, Stroikin U, Brunk UT, JessupW, Garner B. Induction of fibroblast apolipoprotein E expression during apoptosis, starvation-induced growth arrest and mitosis. Biochem J 2004; 378: 753–61.

5. Elliott DA, Kim WS, Jans DA, Garner B. Apoptosis induces neuronal apolipoprotein-E synthesis and localization in apoptotic bodies. Neurosci Lett 2007; 416: 206–10.

6. Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc Natl Acad Sci USA 1992; 89: 4471–5.

7. Imaizumi K. Diet and atherosclerosis in apolipoprotein E-deficient mice. Biosci Biotechnol Biochem 2011; 75(6): 1023–35.

8. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop of all phases of atherosclerosis throughout the arterial tree. Atheroscler Thromb 1994; 14: 133–40.

9. Tancevski I, Wehinger A, Demetz E, et al. Reduced plasma HDL cholesterol in hyperthyroid mice coincides with decreased hepatic ABCA1 expression. Endocrinology 2008; 149(7): 3708–12.

10. Ichiki T. Thyroid hormone and atherosclerosis. Vascul Pharmacol 2010; 52(3/4): 151–6.

11. Krul ES, Tikkanen MJ, Schonfeld G. Heterogeneity of apolipoprotein E epitope expression on human lipoproteins:importance for apolipoprotein E function. J Lipid Res 1988; 29: 1309–25.

12. Wang HY, Zhang FC, Gao JJ, et al. Apolipoprotein E is a genetic risk factor for fetal iodine deficiency disorder in China. Molecular Psychiatry 2000; 5: 363–8.

13. Weibel ER. Stereological methods. I. Practical methods for biological morphometry. New York: Academic Press, 1979: 1–415.

14. Botts S, Jokinen MP, Isaacs KR, Meuten DJ, Tanaka N. Proliferative lesions of the thyroid and parathyroid glands. In: Guides for toxicologic pathology. Washington: STP/ARP/AFIP, 1991: 1-12

15. Ferreira E, Silva AE, Serakides R, Gomes AES, Cassali GD. Model of induction of thyroid dysfunctions in adult female mice. Arq Bras Med Vet Zootec 2007; 59(5): 1245–9

16. Davignon J. Apolipoprotein E and atherosclerosis: beyond lipid effect. Arterioscler Thromb Vasc Biol 2005; 25: 267–9.

17. Serakides R, Nunes VA, Santos RL, Cassali GD, Costa Neto PP. Histomorphometry and qantification of nucleolar organizer regions in bovine thyroid containing methylthiouracil residues. Vet Pathol 1999; 36: 574–82.

18. Altholtz LY, La Perle KM, Quimby FW. Dose-dependant hypothyroidism in mice induced by commercial trimethoprim-sulfamethoxazole rodent feed. Comparative Medicine 2006; 56(5): 395–401.

19. Folkmann JK., Loft S, Moller P. Oxidatively damaged DNA in aging dyslipidemic ApoE KO and wild-type mice. Mutagenesis 2007; 22(2): 105–10.

20. Malik R, Hodgson H. The relationship between thyroid gland and the liver. Q J Med 2002; 95: 559–69.

21. Benvenga S, Cahnmann HJ, Rader D, Kindt M, Robbins J. Thyroxine binding to the apolipoproteins of high density lipoproteins HDL2 and HDL3. Endocrinology 1992; 131(6): 2805–11.

22. Benvenga S, Cahnmann HJ, Robbins J. Characterization of thyroid hormone binding to apolipoprotein-E: localization of the binding site in the exon 3-coded domain. Endocrinology 1993; 133(3): 1300–5.

23. Agrawal NK, Goyal R, Rastogi A, Naik D, Singh SK. Thyroid hormone resistance. Postgrad Med J 2008; 84 (995): 473–7.

# RAVEN ŠČITNIČNIH HORMONOV IN MORFOLOŠKE POSEBNOSTI ŠČITNICE PRI MIŠIH BREZ GENA ZA APOE (ApoE KO) TER KONTROLNIH MIŠIH DIVJEGA TIPA

T. Tripunoski, J. Dimitrova-Shumkovska, T. Ristoski, I. Petrova, S. Panov, A. Ugrinska, D. PopGjorceva

**Povzetek:** Poznavanje specifičnih bioloških lastnostih pri sevih, ki se uporabljajo kot živalski modeli, je pomembno za pravilno interpretacijo rezultatov raziskav. V članku so opisane raziskave delovanja ščitnice (raven ščitničnih hormonov v krvi, premer ščitničnih foliklov ter višina folikularnega epitelija) pri miših z izbitim genom ApoE in primerjava teh vrednosti z vrednostmi pri miših divjega tipa seva C57BL/6. Pomanjkanje lipoproteina ApoE pri tem sevu miši povzroči močno hiperholesterolemijo in aterosklerotičnih poškodbe v krvnih žilah, tudi kadar se miši hranijo z navadno krmo. Rezultati raziskave so pokazali razlike v ravni prostega $T_3$ v krvni plazmi med obema skupinama miši, ni pa bilo statistično značilnih razlik v ravni prostega $T_4$. Morfometrične analize so pokazale statistično značilno višji folikularni epitelij pri miših z izbitim genom ApoE v primerjavi z divjim tipom miši, kar kaže na večje spodbujanje ščitnice s TSH pri miših z izbitim genom ApoE v primerjavi z divjim tipom miši. Pri premeru ščitničnih foliklov niso bile ugotovljene statistično značilne razlike med skupinama miši, kar je skladno s prejšnjimi podatki iz drugih študij, pri katerih niso našli povezanosti med omenjenim parametrom in statusom ščitnice pri živalih. Razlika v delovanju ščitnice pri obeh skupinah miši je morda posledica sprememb v periferni presnovi ščitničnih hormonov zaradi spremenjenega delovanja jeter pri miših brez gena ApoE.

**Ključne besede:** ApoE KO miši; ščitnica; morfometrija

Slov Vet Res 2014; 51 (1): 35-42
UDC 636.3.082.453.5:612.616.2:57.086.13

**Original Scientific Article**

# THE EFFECT OF Equex STM® IN FREEZING MEDIA ON POST THAW MOTILITY, VIABILITY AND DNA INTEGRITY OF FROZEN - THAWED RAM SPERMATOZOA

Nataša Šterbenc[1], Marjan Kosec[1], Heinrich Bollwein[2,3], Primož Klinc[1]

[1]Clinic for Reproduction and Equine Medicine, Vet Faculty, University of Ljubljana, Ljubljana, Slovenia, [2]Clinic for cattle, University of Veterinary Medicine Hannover, Hannover, Germany, [3]Clinic for Reproduction Medicine, University of Zürich, Switzerland

*Corresponding author, E-mail: natasa.sterbenc@vf.uni-lj.si

**Summary:** In this study we investigated the effect of Equex STM® on quality and in-vitro survival of ram spermatozoa frozen in Tris egg yolk based extender. Ejaculates from 6 crossbreed rams were frozen according to the standard procedure after two step dilution with Tris-egg yolk extender (1). The second extender, added to the semen at 5° C, contained 14 % of glycerol and was supplemented with detergent 0.75 % Equex STM® (group OEP) or contained no detergent (control group). After thawing the samples were incubated in a water bath at 37° C and analysis were performed 10 minutes, 6, 12 and 24 hours later. Motility and the viability (Viadent®) of the semen were analysed with Hamilton Thorne Biosciences, Version 12.3 and membrane integrity with HOS (hypoosmotic swelling test). DNA fragmentation (DFI %) of F/T spermatozoa was analyzed 10 minutes and 3 hours after thawing using sperm chromatin structure assay (SCSA™). The sperm membrane integrity was analysed 15 minutes and 3 hours after thawing by Sybr-14/PI test. Percentage of motile spermatozoa was significantly higher in OEP group in comparison to control group at 0, 6, 12 and 24h ($P < 0.001$). Viability of spermatozoa was significantly higher ($P < 0.001$) in OEP compared to control group in all analysed times after thawing. Percentage of HOS positive spermatozoa was significantly higher in OEP compared to control group respectively for 0 ($P = 0.001$), 6 ($P =< 0.001$), 12 and 24h ($P = 0.002$) after thawing.

**Key words:** Semen; ram; Equex STM®; flow cytometry; Viadent®; SCSA™; Sybr-14/PI

## Introduction

Freezing of the semen in liquid nitrogen enable long term storage of fertile spermatozoa from different animal species (2, 3, 4, 5). The process of cryopreservation involves different steps which are harmful to spermatozoa and consequently reduce their quality and fertility. Another reason for reduced fertility in sheep inseminated intra-cervical with frozen-thawed semen is the anatomical structure of the ewe's cervix and passage of viable spermatozoa through the cervix. Polyunsaturated fatty acids in the membranes of spermatozoa, which are exposed to lipid peroxidation during freezing and thawing process are believed to be one of the main reasons for reduced fertility (6). Deep freezing of spermatozoa increases the concentration of reactive oxygen species (ROS) in the semen from various species (7, 8, 9). ROS represent a wide variety of different free radicals. Among the most common forms of ROS, which affect the viability and functionality of spermatozoa, include hydroxyl radicals (OH •), superoxide radicals (O2 •), hydrogen

Received: 26 June 2013
Accepted for publication: 25 September 2013

peroxide (H2O2), peroxide radicals (ROO •), hypochlorite radicals, etc.(10, 11). ROS inactivate various proteins and promote the peroxidation of unsaturated fatty acids in cell membranes (12). Sperm membrane is very susceptible to lipid peroxidation because of its high content of unsaturated fatty acids (13). Peroxidation of unsaturated fatty acids leads to loss of integrity and consequently functions of the spermatozoa membrane**.** Freezing and oxidative stress cause decrease of spermatozoa motility, fertilizing ability (14) and also effects the stability of DNA (15).

However, minor concentrations of ROS have a positive effect on some of the vital function of spermatozoa. They help to regulate the function of spermatozoa, for example hydrogen peroxide in small quantities stimulates capacitation of spermatozoa, their hiperactivation, acrosomal reaction and fertilization (16, 17). Dead and damaged spermatozoa represent a source of ROS which have a detrimental effect on motility and viability of spermatozoa (18), adverse affect on the integrity of the spermatozoa membrane (19) and on the integrity of the DNA (20).

Natural mechanisms which protect spermatozoa against lipid peroxidation include various antioxidants and enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, etc. These antioxidants are very important for the protection of spermatozoa in different animal species (21, 22, 23, 24). Addition of detergent to the extender for freezing represents another mechanism for membrane protection during freezing. Cryopreservation process induces serious detrimental changes in sperm function. The cell and acrosomal membranes of spermatozoa are considered to be the primary site of these modifications due to thermal, mechanical, chemical and osmotic stresses and are critical for semen survival. Equex STM® improves the post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell membranes, particularly acrosomal membranes, and to protect spermatozoa against the toxic effects during the freeze-thaw process (25, 26). It is well known that small amount of detergent added to the extender that contains egg yolk, have a positive effect on membrane stability during freezing /thawing process. Equex STM® added to extender for freezing the dog semen protect spermatozoa from damage incurred during the deep freezing process (27).

## Materials and methods

### *Semen Collection, Processing and Sperm Cryopreservation*

The animals were housed on the Clinic for Reproduction and Horses, Vet Faculty, University of Ljubljana, Slovenia. Semen (6 ejaculates per ram) was collected with electro–ejaculation from 6 cross-breed rams. Immediately after collection, the ejaculates were transferred into tube and kept in a water bath at 27° C until the analysis and further processing.

Analysis of the semen concentration and motility was performed before further processing.

Semen concentration was measured with spectrophotometer (photometer SDM 5, Mini Tüb) and motility was analysed with phase contrast microscope (Olympus BX 40). After analysis, the ejaculates were diluted and frozen according to two step procedure with modified Tris egg yolk extender: Tris 263 mM, citric acid 85 mM, fructose 73 mM, egg yolk 20 %, 340 mOsm, pH 7.0 (Merck, Darmstadt, Germany). Each ejaculate was divided into two parts. Aliquots (200µL) of fresh semen were diluted with 1800µL of extender I warmed to 27° C. Diluted samples were placed in 90 ml water bath, which enable slowly cooling (2h) to +5° C. After cooling to +5° C, 2000 µL of the second extender was added to the semen. It either contained no Equex STM® (control group) or was supplemented with Equex STM® (0.38 % final Equex STM® concentration; Nova Chemical Sales Inc., Scituate, USA). Both extenders also contained 14 % of glycerol (Kemika, Zagreb, Croatia). Diluted samples were aspirated into 0.5ml straws and frozen in nitrogen vapour, 4 cm above the liquid nitrogen. Frozen samples were kept in liquid nitrogen for at least two months before thawing and analysis. Frozen straws were thawed in water bath at 37° C for 17 second.

### *Analysis of Motility and Viability (Viadent®)*

Semen motility and viability was analysed after incubation of the samples in water bath at 37° C for 10 minutes, 6, 12 and 24 hours. Analysis was performed with a computer assisted analyzer (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) in a counting chamber (Makler counting chamber®). Five automatic selected

fields were analysed per sample. Semen viability was analysed with Viadent® (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) assay according to the directions of the manufacturer.

*Chromatin Structure Assay (SCSA™)*

The SCSA is an acridine orange (AO) staining technique which uses a metachromatic dye, AO (chromatographically purified No 04539, Polysciences Inc., *Eppelheim, Warrington, PA, USA*) to evaluate the ratio of single- (abnormal) and double-stranded (native) DNA present in individual spermatozoa. Abnormal chromatin structure was defined as the susceptibility of spermatozoa DNA to undergo acid-induced denaturation in situ. Following the exposure of the prepared DNA to AO, the degree of chromatin integrity (percentage of DNA fragmentation index ( % DFI)) was analysed by flow cytometric measurement of the metachromatic shift from green (stable, double-stranded DNA) to red (denaturated, single-stranded DNA) AO fluorescence emitted by each individual spermatozoa. The SCSA™ procedure was performed 10 minutes and 3 hours after thawing using the flow cytometry (EPICS XL – MCL, Beckman Coulter). Samples were extended immediately after thawing (0 h) to a final concentration of $2x10^6$ spermatozoa/mL using TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1mM EDTA, pH 7.4). Two aliquots of each sample were evaluated for their DNA fragmentation, using the SCSA™ as previously described by Evenson and Jost (28, 29). Acid-induced denaturation of DNA in situ was achieved by adding 0.4 mL of acid-detergent solution (0.1 % (v/v) Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.2) to 200 µL of extended semen sample. After 30 s, semen was stained by adding 1.2 mL of AO staining solution containing 6µg purified AO per mL of buffer (0.1 M citric acid, 0.2 M $Na_2HPO_4$, 1 mM EDTA, 0.15 M NaCl, pH 6.0). Stained semen was incubated on ice for 3 min before flow cytrometric analysis.

*SYBR-14/PI test*

A combination of stains, one specific for live spermatozoa - SYBR-14 and the other specific for spermatozoa that lost membrane integrity -propidium iodide (Invitrogen™, Molecular Probes Inc., Eugene, OR, USA) were used to determine the proportion of live spermatozoa. SYBR 14 is a membrane permeant stain, fluorescenting bright green, which binds to DNA of all spermatozoa. While PI is a red fluorescence stain and binds to DNA in spermatozoa with damaged membranes (30). A third population of spermatozoa is moribund and stains with both red and green (doubly-stained). The SYBR 14/PI procedure was performed 15 minutes and 3 hours after thawing using flow cytometer (EPICS XL – MCL, Beckman Coulter). For analysis, five microliters of 10 µM SYBR 14 in DMSO and 3µl PI were added to 500µl of semen samples diluted to the concentration of $5\times10^6$ spermatozoa mL with Tyrode's salt solution (Sigma – Aldrich Chemical Co., St. Louis, USA). Samples were analysed with the flow cytometer following 15 minutes incubation on 37°.

*Hypoosmotic swelling test (HOS)*

The HOS test was used as an assay to evaluate the functional activity of the spermatozoa membrane. The procedure used was that described by Jeyendran et al. (31), adapted for ram semen by Garcia Artiga (32). An aliquot of 50µL of frozen-thawed semen was diluted in 500 µL of hypoosmotic solution and incubated at 37° C for 30 min. A total of 200 spermatozoa were counted. Percentage of spermatozoa population with swollen and/or coiled tail was scored under a phase contrast microscope (400x magnifications).

*Statistical analyses*

All statistical analyses were performed using Sigma Stat Version 3.1 (Systat Software Inc., Chicago, IL, USA). In results average values are expressed as mean ± SD. Differences between the two extenders at 0, 6, 12 and 24 hours were analysed using one way analysis of variance (HOS, progressive motility at T0) or Kruskal-Wallis test (motility, progressive motility at T6, T12 and T24, viability, PMI and DFI), depending on the distribution of the data. All values of $P < 0.05$ were considered significant.

## Results

Percentage of motile spermatozoa was significantly higher in OEP group in comparison to control group (78.4 ± 15.7 vs 43.3 ± 23.9; 66.5 ± 16.9 vs. 15.7 ± 20.6; 46.8 ± 26.3 vs. 7.0 ± 16.6

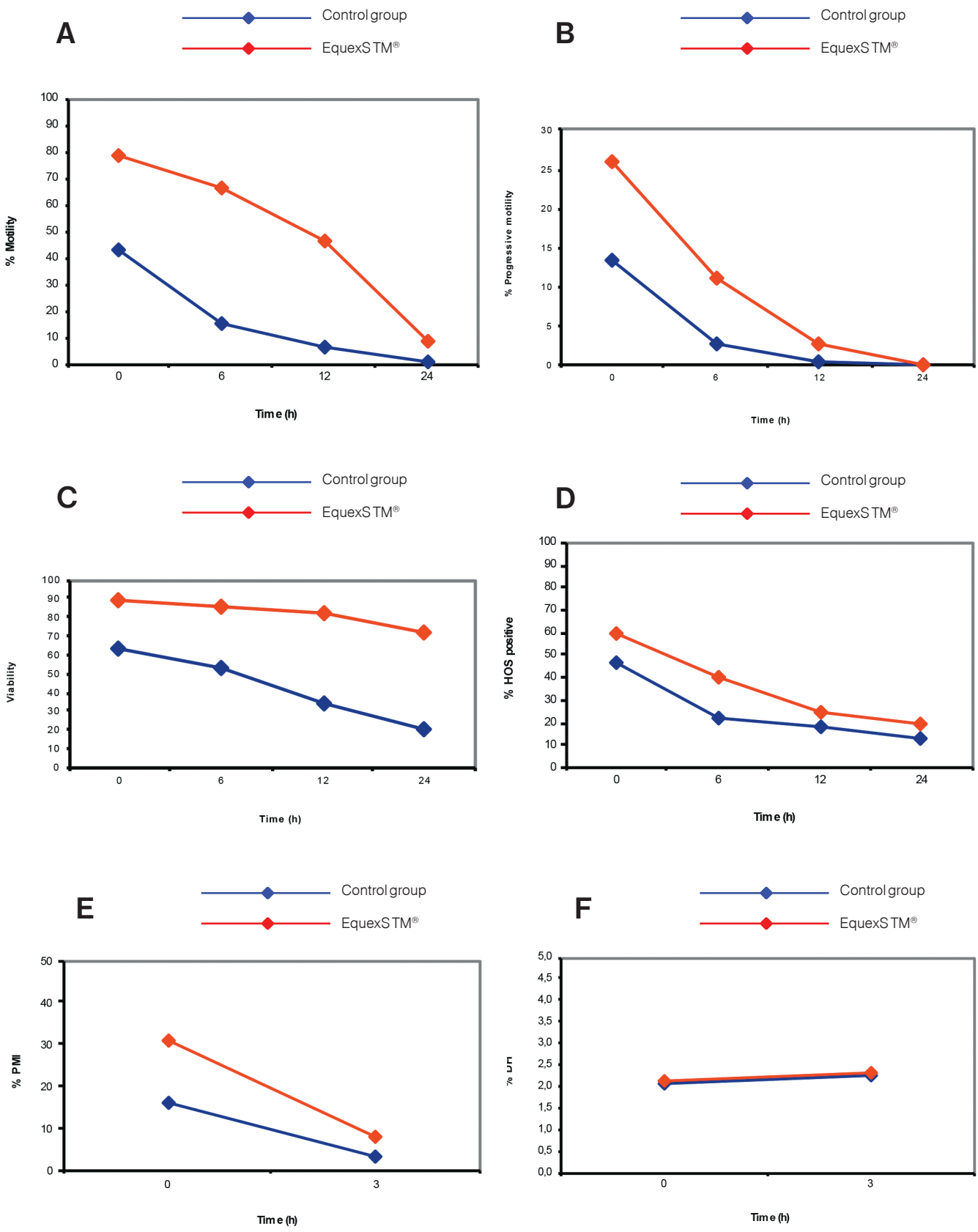


**Figure1:** Changes in the sperm data after thawing: (A) motility (a:b = p =< 0.001), (B) progressive motility (a:b = p < 0.05), (C) viability (a:b = p =< 0.001), (D) HOS positive (a:b = p < 0.05), (E) membrane integrity (a:b = p =< 0.001) and (F) DNA integrity (a:b = p>0.05)

and 9.3 ± 11.4 vs. 0.8 ± 3.5) respectively for 0, 6, 12 and 24h after F/T (P =< 0.001). Analysis of progressive motility also revealed significantly higher percentage in OEP compared to control group (26.1 ± 9.2 vs. 13.6 ± 9.2; 11.3 ± 6.8 vs. 2.8 ± 6.3; 2.8 ± 2.9 vs. 0.3 ± 0.8; 0.2 ± 0.4 vs. 0.0 ± 0.2) respectively for 0 (P = 0.025), 6, 12 and 24h after F/T (P =< 0.001). Analysis of viability also revealed significantly higher (P =< 0.001) percentage of viable spermatozoa in OEP compared to control group (88.4 ± 8.3 vs. 63.5 ± 17.7; 85.1 ± 11.8 vs. 52.9 ± 21.2; 83.1 ± 14.0 vs. 33.6 ± 30.3; 72.5 ± 23.1 vs. 21.1 ± 26.1) respectively for all times. Percentage of HOS positive spermatozoa was also significantly higher in OEP compared to control group respectively (60.0 ± 16.2 vs. 46.8 ± 16.9; 40.8 ± 12.5 vs. 22.5 ± 11.5; 25.1 ± 9.0 vs. 17.9 ± 9.4; 19.4 ± 7.8 vs. 13.5 ± 7.6) for 0 (P = 0.001), 6 (P =< 0.001), 12 and 24h after F/T (P = 0.002). Percentage of plasma membrane integrity (PMI) was significantly higher in OEP compared to control group respectively (30.6±14.2 % vs. 16.1±13.3 %; 7.8±3.3 vs. 3.2±4.1 %) for 0 and 3h after F/T (P =< 0.001). Analysis of SCSA™ revealed no difference (2.1±1.3 % vs. 2.0±1.3 %; 2.3±1.1 vs. 2.3±1.2 %) in DFI %-values between OEP group compared to control group respectively for 0 (P = 0.599) and 3h (P = 0.760) after thawing.

## Discussion

In present study we analysed the effect of Equex STM® on freezability of ram semen. With great certainty we found that addition of 0.38 % detergent into egg yolk based extender has a beneficial effect on the quality of F/T ram semen. This improvement was seen as higher motility, plasma membrane integrity and survival rate of spermatozoa in F/T samples after addition of specific detergent. The results of this study demonstrate that the addition of Equex STM® in the freezing extender protect spermatozoa during freezing and thawing process. These results are in agreement with the study of Akourki et al. (33). Although in our study the initial motility after thawing was higher in OEP group in comparison to the results of Akourki et al. (33). Further analysis after thawing and incubation on 37° C for 24 hours also revealed beneficial effect of Equex STM® on motility, progressive motility, viability, HOS and PMI. In assessing semen quality, animal and human fertility was developed the SCSA test which measure semen DNA integrity. SCSA data on thousands of semen samples from bulls, stallions, boars and exotic cats show the clinical value of this assay for animal fertility assessment. SCSA can utilize a fresh or frozen-thawed semen sample and using the features of flow cytometry, collect and analyse data on 5000 or more cells within a few min of time to evaluate semen quality and further define the relationship of semen quality to fertility (34).

However, we could not find any positive effect on DNA integrity. Some previous studies have shown the negative correlation between the percentage of spermatozoa with denaturated DNA and fertilizing capacity of spermatozoa (35, 36). In our previous research we compared extenders with two different antioxidants and there was no significant difference of DFI between groups which contained antioxidants and control group (37). In present research integrity of DNA was in comparison to Bucak et al. (38), relatively high in all samples and we could not find any effect of detergent on this parameter. Results of DNA integrity and results for other parameters (motility, progressive motility, viability, PMI and HOS) suggest that even a standard protocol used in our study enabled high quality freezing of ram spermatozoa and that the detergent additionally improved the freezing capacity of ram spermatozoa.

These results suggest that spermatozoa frozen in the presence of Equex STM® also have a better fertility compared to the samples that were frozen without the addition of detergent. Pursel et al. (39) found a positive correlation between percentages of motile spermatozoa after thawing and their fertilizing capacity. Intravaginal insemination of bitch with frozen canine semen supplemented with detergent resulted in an overall pregnancy rate, similar to that obtained after natural mating (40). The addition of Equex STM® to the freezing extender had a positive effect on the motility, PMI immediately after freezing-thawing in different species (25, 26, 39, 41). Active compound in Equex STM® is sodium dodecyl sulphate (SDS), a water-soluble anionic detergent that solubilizes active molecules but have a toxic effect on spermatozoa membranes (26, 39, 42). The detergent functions are through the modification of egg-yolk components, this increase sperm membrane permeability and reduce osmotic stress during the freezing–thawing process (26, 39). The cryoprotective effect of Equex

STM® is only seen in the presence of egg yolk and that indicate to be exerted by modification of egg yolk lipoproteins (26, 43). Increased stability of spermatozoa plasma membrane integrity was also seen in our study based on Tris egg yolk extender supplemented with Equex STM®. This positive effect of Equex STM® on post-thaw quality of spermatozoa was found in studies from different animal species, i.e. dog (27, 40, 43), bull (26) , boar (39, 44, 45, 46), stallion (25) and cat (47).

In previous studies it was found that optimal concentration of detergent in freezing extender varied between 0,5 and 1 % for extenders containing 20 % egg yolk (39). Higher concentration of the detergent had a detrimental effect on membrane stability and quality of spermatozoa (26). Axner et al. (48) concluded that addition of Equex STM® to the freezing extender reduces acrosome damage but decreases spermatozoa longevity during post-thaw in vitro incubation of cat epidydimal spermatozoa. However, this result is contradictory with experiments in other species such as bull (26), dog (40) and ram (33). The benefficial effects of Equex STM® on motility, progressive motility, viability and acrosome integrity on buck spermatozoa were found especially during the first two hours after thawing. The long incubation of buck spermatozoa in detergent had an adverse effect during the three hours incubation on 37° C (49). Detergent has also a beneficial effect on post thaw motility of alpaca spermatozoa, while acrosome integrity was unaffected (50). Similar results for motility were also found in our study.

In conclusion, this data clearly indicate that the addition of Equex STM® to the modified Tris egg yolk extender used for freezing of ram spermatozoa significantly improves post-thaw semen quality.

Further studies are necessary to verify, if addition of detergent to the egg yolk based extenders used for freezing of ram semen, would have the same positive effect on fertility of frozen-thawed ram semen.

## Acknowledgements

The authors are thankful to Drago Kompan, Dušan Birtič and Domen Drašler (Department of Animal Science, Biotechnical faculty, University of Ljubljana) for the animals used in this study, as well as to Christel Hettel, technical assistant in the Sperm Laboratory, Clinic for Cattle, University of Veterinary Medicine Hannover, for her friendly support.

## Reference

1. Gil J, Soderquist L, Rodriguez-Martinez H. Influence of centrifugation and different extenders on post-thaw sperm quality of ram semen. Theriogenology 2000; 54: 93–108.

2. Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000; 60/61: 481–92.

3. Blottner S, Warnke C, Tuchscherer A, et al. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim Reprod Sci 2001; 65: 75–88.

4. Salamon S, Maxwell WM. Storage of ram semen. Anim Reprod Sci 2000; 62: 77–111.

5. Roca J, Hernandez M, Carvajal G, et al. Factors influencing boar sperm cryosurvival. J Anim Sci 2006; 84: 2692–9.

6. Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl 1992; 13: 232–41.

7. Bilodeau JF, Chatterjee S, Sirard MA, et al. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev 2000; 55: 282–8.

8. Ball BA, Vo AT, Baumber J. Generation of reactive oxygen species by equine spermatozoa. Am J Vet Res 2001; 62: 508–15.

9. Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol Reprod Dev 2001; 59: 451–8.

10. Halliwell B, Gutteridge JM. The chemistry of oxygen radicals and other derived species. Oxford: Clarendon Press, 1989: 22–85.

11. Makker K, Agarwal A, Sharma R. Oxidative stress & male infertility. Indian J Med Res 2009; 129: 357–67.

12. Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. Fertil Steril 1979; 31: 531–7.

13. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 1989; 41: 183–97.

14. Aitken RJ, Sawyer D. The human

spermatozoon--not waving but drowning. Adv Exp Med Biol 2003; 518: 85–98.

15. Peris SI, Morrier A, Dufour M, et al. Cryopreservation of ram semen facilitates sperm DNA damage: relationship between sperm andrological parameters and the sperm chromatin structure assay. J Androl 2004; 25: 224–33.

16. de Lamirande E, Gagnon C. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int J Androl 1993; 16: 21–5.

17. Griveau JF, Le Lannou D. Reactive oxygen species and human spermatozoa: physiology and pathology. Int J Androl 1997; 20: 61–9.

18. Perez-Crespo M, Moreira P, Pintado B, et al. Factors from damaged sperm affect its DNA integrity and its ability to promote embryo implantation in mice. J Androl 2008; 29: 47–54.

19. Lenzi A, Gandini L, Lombardo F, et al. Polyunsaturated fatty acids of germ cell membranes, glutathione and blutathione-dependent enzyme-PHGPx: from basic to clinic. Contraception 2002; 65: 301–4.

20. Baumber J, Ball BA, Linfor JJ, et al. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl 2003; 24: 621–8.

21. Abu-Erreish G, Magnes L, Li TK. Isolation and properties of superoxide dismutase from ram spermatozoa and erythrocytes. Biol Reprod 1978; 18: 554–60.

22. Sarlos P, Molnar A, Kokai M, et al. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. Acta Vet Hung 2002; 50: 235–45.

23. Aitken RJ, Baker MA. Oxidative stress and male reproductive biology. Reprod Fertil Dev 2004; 16: 581–8.

24. Sikka SC. Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. J Androl 2004; 25: 5–18.

25. Martin JC, Klug E, Gunzel AR. Centrifugation of stallion semen and its storage in large volume straws. J Reprod Fertil Suppl 1979; 27: 47–51.

26. Arriola J, Foote RH. Glycerolation and thawing effects on bull spermatozoa frozen in detergent-treated egg yolk and whole egg extenders. J Dairy Sci 1987; 70: 1664–70.

27. Ponglowhapan S, Chatdarong K. Effects of Equex STM Paste on the quality of frozen-thawed epididymal dog spermatozoa. Theriogenology 2008; 69: 666–72.

28. Evenson DP, Baer RK, Jost LK. Long-term effects of triethylenemelamine exposure on mouse testis cells and sperm chromatin structure assayed by flow cytometry. Environ Mol Mutagen 1989; 14: 79–89.

29. Evenson DP, Thompson L, Jost L. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. Theriogenology 1994; 41: 637–51.

30. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 1995; 53: 276–84.

31. Jeyendran RS, Van der Ven HH, Zaneveld LJ. The hypoosmotic swelling test: an update. Arch Androl 1992; 29: 105–16.

32. Garcia-Artiga C. Test de endosmosis en ovino. In: 7th International Meeting on Animal Reproduction. Murcia, 1994: 77–81.

33. Akourki A, Gil L, Echegaray A, et al. Effect of the extender supplement Equex-STM on cryopreserved semen in the Assaf sheep. Cryo Lett 2004; 25: 147–54.

34. Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci 2000; 22: 169–89.

35. Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol Reprod 1987; 36: 915–25.

36. Morrell JM, Johannisson A, Dalin AM, et al. Sperm morphology and chromatin integrity in Swedish warmblood stallions and their relationship to pregnancy rates. Acta Vet Scand 2008; 50: e 2. (8 str.) http://www.actavetscand.com/content/50/1/2 (16. Jan. 2014)

37. Šterbenc N, Bollwein H, Klinc P. The effect of antioxidants (sodium pyruvate and catalase) on post thaw motility, viability and DNA integrity of ram spermatozoa. C R Acad Bulg Sci 2009; 62: 615–20.

38. Bucak MN, Keskin N, Taspinar M, et al. Raffinose and hypotaurine improve the post-thawed Merino ram sperm parameters. Cryobiology 2013; 67: 34–9.

39. Pursel VG, Schulman LL, Johnson LA. Effect of Orvus ES Paste on acrosome morphology, motility and fertilizing capacity of frozen-thawed boar sperm. J Anim Sci 1978; 47: 198–202.

40. Rota A, Strom B, Linde-Forsberg C, et al. Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro

incubation at 38 degrees C. Theriogenology 1997; 47: 1093–101.

41. Cheng FP, Wu JT, Chan JP, et al. The effect of different extenders on post-thaw sperm survival, acrosomal integrity and longevity in cryopreserved semen of Formosan Sika deer and Formosan Sambar deer. Theriogenology 2004; 61: 1605–16.

42. Helenius A, Simons K. Solubilization of membranes by detergents. Biochim Biophys Acta 1975; 415: 29–79.

43. Pena A, Linde-Forsberg C. Effects of Equex, one- or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 2000; 54: 859–75.

44. Wu TW, Cheng FP, Chen IH, et al. The combinatorial effect of different Equex STM paste concentrations, cryoprotectants and the straw-freezing methods on the post-thaw boar semen quality. Reprod Domest Anim 2013; 48: 53–8.

45. Buranaamnuay K, Tummaruk P, Singlor J, et al. Effects of straw volume and Equex-STM on boar sperm quality after cryopreservation. Reprod Dom Anim 2009; 44: 69–73.

46. Abaigar T, Holt WV, Harrison RA, et al. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod 1999; 60: 32–41.

47. Zambelli D, Iacono E, Raccagni R, et al. Quality and fertilizing ability of electroejaculated cat spermatozoa frozen with or without Equex STM Paste. Theriogenology 2010; 73: 886–92.

48. Axner E, Hermansson U, Linde-Forsberg C. The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim Reprod Sci 2004; 84: 179–91.

49. Anakkul N, Suwimonteerabutr J, Singlor J, et al. Effect of Equex STM paste on the quality and motility characteristics of post thawed cryopreserved goat semen. Thai J Vet Med 2011; 41: 345–51.

50. Morton KM, Evans G, Maxwell WM. Effect of glycerol concentration, Equex STM supplementation and liquid storage prior to freezing on the motility and acrosome integrity of frozen-thawed epididymal alpaca (*Vicugna pacos*) sperm. Theriogenology 2010; 74: 311–6.

## VPLIV DETERGENTA EQUEX STM® NA GIBLJIVOST, VITALNOST IN INTEGRITETO DNK ZAMRZNJENIH/ODMRZNJENIH OVNOVIH SEMENČIC

N. Šterbenc, M. Kosec, H. Bollwein, P. Klinc

**Povzetek:** V raziskavi smo preučevali vpliv Equex STM® na kakovost in preživetje ovnovih semenčic, zamrznjenih v razredčevalcu z dodanim jajčnim rumenjakom. Ejakulati 6 ovnov so bili zamrznjeni po standardnem dvostopenjskem postopku z razredčevalcem Tris z dodatkom jajčnega rumenjaka (1). Drugi razredčevalec je bil dodan semenu pri 5 °C in je dodatno vseboval le glicerol (kontrolna skupina) ali glicerol in Equex STM® (skupina OEP). Po odmrzovanju smo vzorce inkubirali v vodni kopeli pri 37 °C. Analize smo opravili po 10 minutah ter 6, 12 in 24 urah. Analiza vzorcev na gibljivost in test na preživitveno sposobnost semenčic (Viadent®) sta bila opravljena z računalniško analizo semena (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA), integriteta membrane semenčic pa s hipoozmotskim testom (HOS). Test integritete DNK semenčic (SCSA™) je bil opravljen z uporabo pretočne citometrije v času 10 minut in 3 ur z določanjem DNK fragmentacijskega indeksa (DFI%). Test integritete membrane semenčic (Sybr-14/PI) je bil opravljen z uporabo pretočne citometrije v času 15 minut in 3 ur po tajanju. Odstotek gibljivih semenčic je bil pri OEP v primerjavi s kontrolno skupino 0, 6, 12 in 24 ur po tajanju statistično značilno višji ($P<0.001$). Analiza preživitvene sposobnosti semenčic je prav tako pokazala statistično značilno višji odstotek ($P<0.001$) vitalnih semenčic pri OEP v primerjavi s kontrolno skupino. Odstotek pozitivnih semenčic pri testu HOS je bil tudi statistično značilno višji pri OEP v primerjavi s kontrolno skupino, in sicer 0 ($P=0.001$), 6 ($P=<0.001$), 12 in 24 ur ($P=0.002$) po tajanju.

**Ključne besede:** seme; oven; Equex STM®; pretočna citometrija; Viadent®; SCSA™; Sybr-14/PI

Slov Vet Res 2014; 51 (1): 43-51
UDC 636.2.09:616.9-079:578:57.083.2

**Original Scientific Article**

# FIRST DETECTION OF SCHMALLENBERG VIRUS INFECTIONS IN SLOVENIA, 2012

Ivan Toplak*, Vasilij Cociancich, Danijela Rihtarič, Polona Juntes, Tomislav Paller

National Veterinary Institute, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: ivan.toplak@vf.uni-lj.si

**Summary:** In late summer 2011, Germany and the Netherlands reported the first cases of acute infection in cattle caused by a novel *Orthobunyavirus*, named the Schmallenberg virus (SBV). The first malformations due to SBV were observed in December 2011 in the Netherlands, Belgium and Germany. SBV was first identified in Slovenia in a flock of 23 sheep where nine aborted foetuses with malformations were found on a farm. Viral nucleic acid of SBV was detected by real-time polymerase chain reaction (RT-qPCR) from the brain and spleen samples with a protocol developed by the Friedrich-Loeffler Institute (FLI), Germany. Between January and April 2013 a total of 77 malformed calves were tested and 25 calves identified as SBV positive by RT-qPCR. The majority of malformed animals had one or more of the following pathological lesions: arthrogryposis, brachygnathia, torticollis, scoliosis, hydranencephaly and brain and spinal cord malformations. Additionally, two archive samples collected in September 2012 were identified as SBV positive, confirming that SBV infection was already present in Slovenia in 2012. The sequencing analysis of the partial L-segment confirmed that the strain detected in Slovenia was 100% identical to the Schmallenberg virus isolate Germany (JX853179), identified in 2011. SBV-positive herds have been located throughout Slovenia.

**Key words:** first report; Schmallenberg virus; diagnosis; Slovenia

## Introduction

The Schmallenberg virus (SBV) is the name of a new virus that recently emerged in Europe (1). SBV has a high homology to the genus *Orthobunyavirus* of the Simbu serogroup (1, 2). It was first detected in late summer 2011 in Germany and the Netherlands in cattle exhibiting milk drop, fever and diarrhoea. Later, the infection was associated with congenital malformations in lambs, calves and goat kids, as a result of infection from the mother during the vulnerable early stages of gestation (3, 4). The results of full-genome and serologic investigations indicated that SBV belongs to the species Sathuperi virus and is a possible ancestor of the reassortant Shamonda virus (5).

Since the first report, SBV has continued to spread to neighbouring countries and, after more than a year, has become endemic in north-western parts of Europe (1, 3, 6, 7). Data from the Netherlands and Belgium indicate that the virus spreads very rapidly, reaching a seroprevalence of

Received: 25 July 2013
Accepted for publication: 23 December 2013

80-90% within a few months (2, 4, 8). The collected data from 15 European countries covering 6,000 infected herds become available at the end of 2012 (6). After detection of SBV infection in eight countries (Belgium, France, Italy, Germany, the Netherlands, Luxembourg, Spain and the United Kingdom) at the beginning of 2012, SBV infections were identified in Denmark, Finland, Poland, Sweden, Switzerland, Ireland, Norway, Estonia and Austria in summer and autumn 2012 (6). Transmission of SBV into Polish and Estonian cattle herds provide evidence of recent spreading of infection to the eastern part of Europe (7, 9). Infection of a herd is confirmed by virus detection or detection of SBV antibodies (1, 10).

The detection of SBV in *Culicoides* (*C. obsoletus complex*, *C. dewulfi* and *C. chiopterus*) during the summer and early autumn in Belgium, Denmark and Poland by real-time polymerase chain reaction (RT-qPCR) strongly indicates that these species are relevant vectors for SBV and are probably involved in the rapid transmission of the disease (9, 11, 12). Comparative analysis of different organs and tissues suggests that the cerebrum, cerebellum and brain stem are the most appropriate tissues for SBV detection by RT-qPCR (13, 14). RT-qPCR for the detection of the SBV genome in the L-segment was developed and provided by the Friedrich-Loeffler Institute (FLI), Germany. This method was implemented at the National Veterinary Institute in Ljubljana in Slovenia in the beginning of 2012. The aims of this study are to provide the results of SBV monitoring from clinical samples of malformed foetuses in Slovenia, and to present the regional distribution of identified SBV-positive herds.

## Materials and methods

In total, five malformed lambs and 77 malformed calves originating from 73 different herds were included in the study. Aborted and/or stillborn calves and lambs showing one or more of the malformations (arthrogryposis, torticollis, scoliosis, brachygnathia, hydranencephaly and hypoplasia of the cerebrum) and therefore considered as SBV suspected, were collected from January to April 2013 by famers and veterinarians. Suspect cases were sent to pathology for necropsy at the National Veterinary Institute. Samples were collected and tested in the frame of official surveillance activities for SBV. Additionally, nine aborted and/or stillborn calves (non-malformed) stored as archive tissue samples in laboratory (collected in 2012) and 135 randomly selected serum samples obtained from clinically healthy cattle, collected between August and November 2012 were tested for SBV by RT-qPCR. From the 82 malformed animals showing arthrogryposis-hydranencephaly syndrome and nine archive, non-malformed animals, tissue samples of the spleen and brain (cerebral cortex, brain stem and cerebellum) were collected and tested by RT-qPCR.

In the laboratory for virology, a total of 2 g of different tissues (spleen, cerebral cortex, brain stem and cerebellum) were homogenized as a pool in 10 ml of RPMI-1640 (Gibco, Invitrogen Corporation, USA) with a homogenizer (IUL Instruments, Germany). After homogenization, the suspension samples were centrifuged for 15 minutes at 3,000rpm × g. Tissue supernatants were recovered and used for the extraction of total RNA. Total RNA was extracted from 140 µl of tissue supernatant/serum samples using the QIAamp® Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The previously designed primers and probes and protocol for the detection of the SBV genome (L-segment) by RT-qPCR were kindly provided by FLI, Germany (2). The forward primer sequence SBV-L1-11F was 5'-TTGCCGTTTGATTTTGAAGTTGTG-3' and the reverse primer sequence SBV-L1-155R was 5'-TCAGGGATCGCAAATTAAAGAACC-3'. The TaqMan probe sequence SBV-L1-36 (5'-TCATCCGTGCTGACCCTCTGCGAG-3') was labelled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5' end and with the black hole quencher dye (BHQ1) at the 3' end. The protocol was applied and adapted to our laboratory conditions as follows. Amplification was carried out in a single step with Superscript™ III Platinum® One–Step qRT-PCR kit with ROX (Invitrogen, USA). Briefly, the reaction was performed in 20 µl total volume, consisting of 1.5 µl water, 10 µl of 2 x reaction mix with ROX, 0.5 µl of 50 mM Mg $SO_4$, 1 µl of each primer with concentration 20 µM, 0.5 µl of 5 µM probe and 5 µl of extracted RNA. The protocol was optimized using a series of ten-fold RNA dilutions. RT-qPCR was performed on the Mx3005P system (Stratagene, USA) with the program including a reverse transcription step at 55 °C for ten minutes, followed by a denaturation step at 95 °C for ten minutes, and

45 cycles of 95 °C for 15 seconds, 56 °C for 20 seconds and 72 °C for 30 seconds. To ensure the accurate performance of the real-time PCR test, at least one positive control (SBV RNA provided by FLI, Germany; dilution of standard with a cycle threshold [Ct] of approximately 30) and one negative control (tissue sample of bovine origin from year 2010, free of SBV RNA) were included in each run.

The positive samples from the first three SBV-positive herds were directly sequenced from RT-PCR products in both directions using the Macrogen sequencing service (Macrogen, The Netherlands) and the RT-qPCR amplification primers to confirm the specificity of the RT-PCR assay. For each sample, 97 nucleotide long sequences of segment-L were aligned with the published data using BLAST (available at http://www.ncbi.nlm.-nih.gov/) at the National Centre for Biotechnology Information (NCBI).

## Results

Various degrees of deformities were observed in the five lambs and 77 calves at necropsy, namely arthrogryposis, torticollis, scoliosis, brachygnathia, hydranencephaly and hypoplasia of the cerebrum, cerebellum and spinal cord (Table 1, Figure 2-8). The first clinical case of SBV infections, confirmed by laboratory diagnosis in Slovenia, was identified on January 9, 2013 from a flock with 23 sheep, where nine aborted foetuses were found. Five foetuses were sent for necropsy and two (O-1a/2013 and O-1b/2013) were detected as SBV positive by RT-qPCR. By the end of April 2013, an additional 25 bovine herds were identified as SBV positive (Table 1). The distribution of positive herds on the map of Slovenia revealed that SBV infection was already present throughout the country (Figure 1). The detection of the highest number of positive herds in the central area is the result of intensive sampling in this region. RT-qPCR results showed that 27 (32.9%) of 82 examined malformed animals were interpreted as SBV-genome positive according to detected Ct values. Despite observed congenital malformations, 56 out of 82 tested malformed animals were identified as SBV negative.

Out of 144 tested archive samples (nine tissue samples of aborted and/or stillbirth calves and 135 serum samples), two samples (from herds B-1/2012 and B-2/2012) were identified as SBV positive. Both of these SBV-positive samples were collected on September 13, 2012, thus confirming the presence of infection already in 2012. The first SBV-positive sample from herd B-1/2012 originated from a foetus aborted in the fourth month of gestation; the farm was located in the municipality of Žiri, approximately 50 km north-west of Ljubljana (Figure 1). The detected Ct value in this foetus was 22.98, which was the lowest detected Ct value among the positive samples in this study. The second SBV-positive sample from

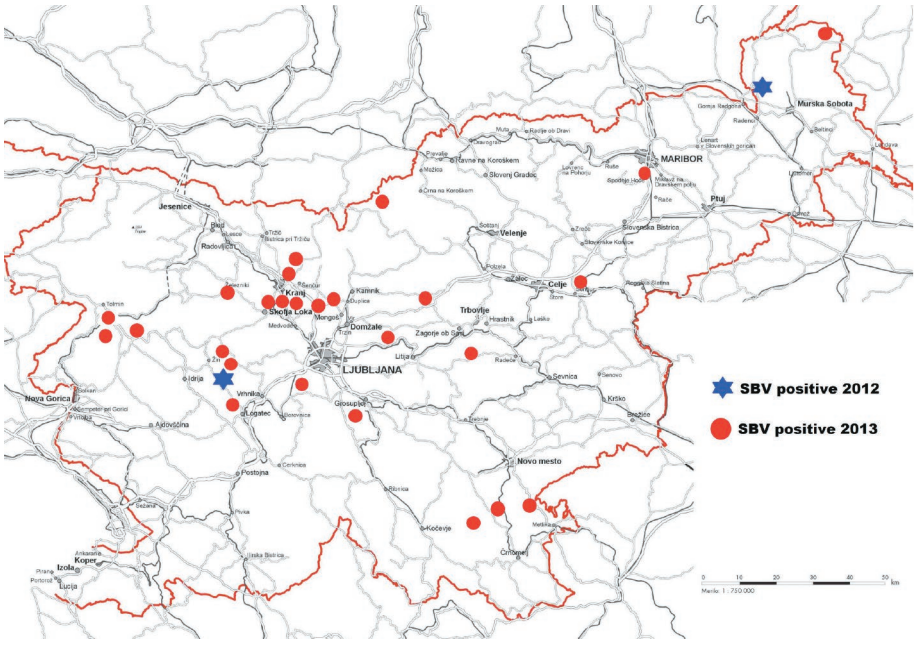


**Figure 1:** Location of 28 herds with SBV-genome positive results identified in Slovenia by end of April 2013 (blue stars for positive herds [B-1/2012 and B-2/2012] detected in September 2012, red dots for herds detected in 2013

**Table 1:** Total number of SBV-positive animals detected by RT-qPCR from September 2012 to end of April 2013 (samples, date of collection, municipality name, detected Ct value and description of observed malformations during necropsy)

| Herd number | Date of sampling | Municipality | Ct value | ART | BRA | TOR | SCO | HYD | HYP |
|---|---|---|---|---|---|---|---|---|---|
| *B-1/2012* | *13 September 2012* | *Žiri* | *22.98* | | | | | | |
| *B-2/2012* | *13 September 2012* | *Murska Sobota* | *29.60* | | | | | | |
| O-1a/2013 | 04 January 2013 | Semič | 26.48 | X | X | X | | | X |
| O-1b/2013 | 04 January 2013 | Semič | 27.07 | | X | X | | | X |
| B-2/2013 | 30 January 2013 | Kamnik | 36.67 | X | X | X | X | | X |
| B-3/2013 | 19 February 2013 | Komenda | 34.23 | X | | X | X | | X |
| B-4/2013 | 22 February 2013 | Gorenja vas-Poljane | 35.31 | X | | X | X | X | |
| B-5/2013 | 04 March 2013 | Dol pri Ljubljani | 38.58 | X | X | | X | | X |
| B-6/2013 | 03 March 2013 | Železniki | 37.95 | X | | X | | | |
| B-7/2013 | 07 March 2013 | Logatec | 33.93 | X | X | X | X | | |
| B-8/2013 | 07 March 2013 | Žiri | 39.03 | X | | | X | | |
| B-9/2013 | 05 March 2013 | Kranj | 29.64 | X | | X | X | X | X |
| B-10/2013 | 13 March 2013 | Kranj | 37.33 | X | | | X | | X |
| B-11/2013 | 18 March 2013 | Hodoš | 37.10 | | | X | | | |
| B-12/2013 | 19 March 2013 | Tolmin | 34.50 | X | | X | | | |
| B-13/2013 | 19 March 2013 | Nova Gorica | 34.89 | X | | X | X | | X |
| B-14/2013 | 18 March 2013 | Šentjur pri Celju | 36.55 | X | | | X | | X |
| B-15/2013 | 18 March 2013 | Preddvor | 37.53 | X | | X | X | | X |
| B-16/2013 | 26 March 2013 | Vodice | 29.80 | X | X | X | X | X | |
| B-17/2013 | 22 March 2013 | Semič | 35.45 | | X | X | | | X |
| B-18/2013 | 21 March 2013 | Kanal | 33.10 | X | | X | | | X |
| B-19/2013 | 02 April 2013 | Kočevje | 36.80 | X | X | | X | | |
| B-20/2013 | 02 April 2013 | Kranj | 37.16 | X | | X | X | | |
| B-21/2013 | 02 April 2013 | Kranj | 42,43 | X | | X | X | | X |
| B-22/2013 | 02 April 2013 | Litija | 34.88 | X | | X | | | X |
| B-23/2013 | 02 April 2013 | Brezovica | 36.88 | X | | | X | X | X |
| B-24/2013 | 05 April 2013 | Ivančna Gorica | 35.48 | X | X | X | | X | X |
| B-25/2013 | 21 March 2013 | Solčava | 37.79 | X | | X | X | | |
| B-26/2013 | 08 April 2013 | Hoče-Slivnica | 37.88 | X | | X | X | X | X |

Abbreviations: (herd numbers) B: bovine herd, O: ovine herd; ART: arthrogryposis; BRA: brachygnathia; TOR: torticollis; SCO: scoliosis; HYD: hydranencephaly/porencephaly; HYP: cerebellar hypoplasia

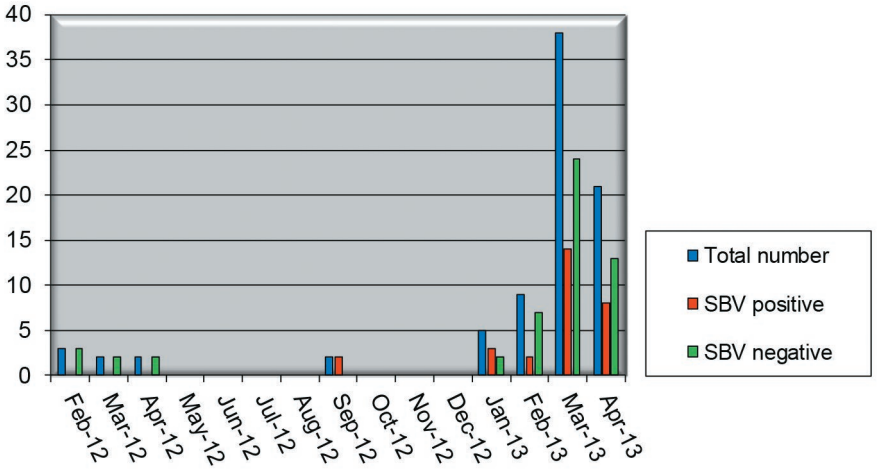


**Figure 2:** Number of tested malformed lambs and calves in Slovenia February 2012 to end of April 2013 (SBV-positive and -negative sample numbers by RT-qPCR, monthly

**Figure 3:** SBV-positive calf with severely deformed limbs (*arthrogryposis)*

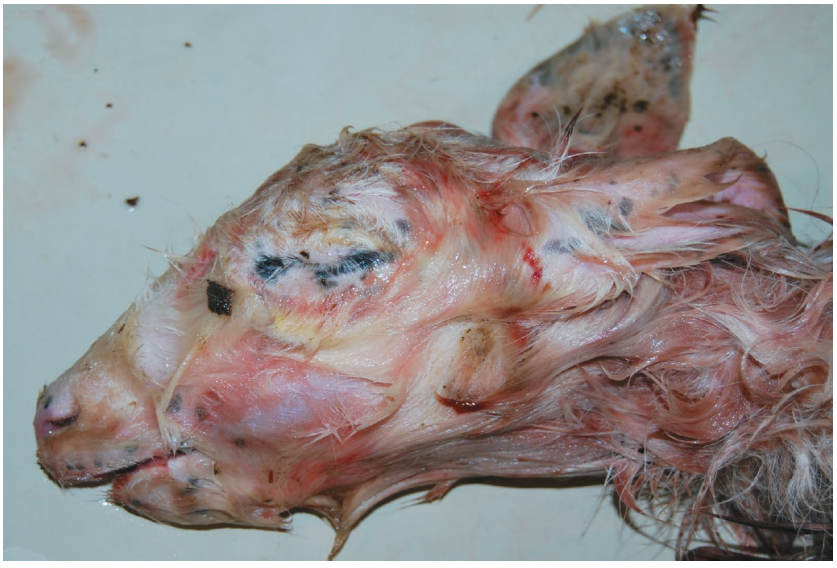

**Figure 4:** SBV-positive lamb with shortened lower jaw (*brachygnathia*)

**Figure 5:** SBV-positive calf with twisted neck (*torticollis*) and arthrogryposis of the limbs

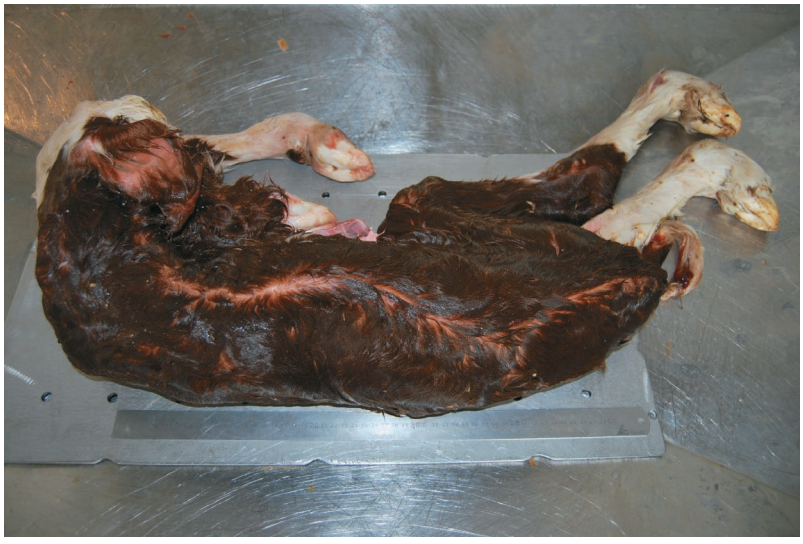

**Figure 6:** SBV-positive calf with lateral deviation of the spine (*scoliosis*), arthrogryposis and mild torticollis

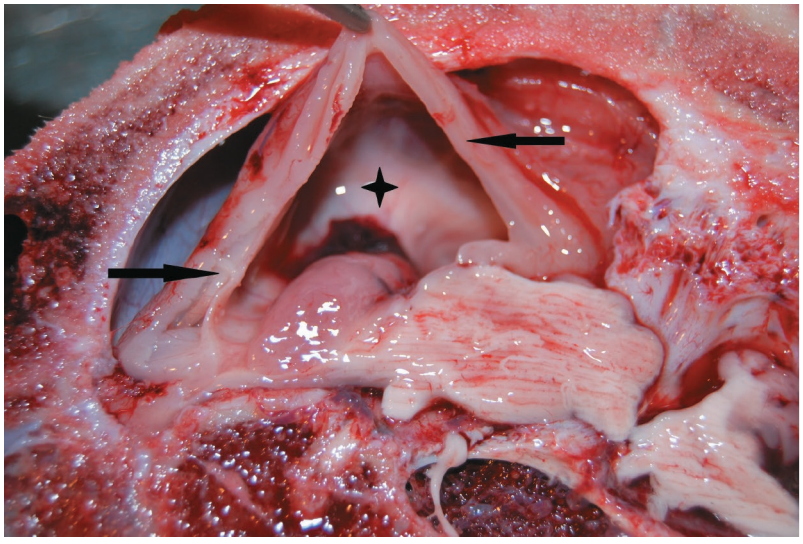

**Figure 7:** SBV-positive calf brain section; parenchyma of the cerebral hemisphere is very thin (arrows) and almost completely replaced with a fluid filled cyst (+) (*hydranencephaly*).

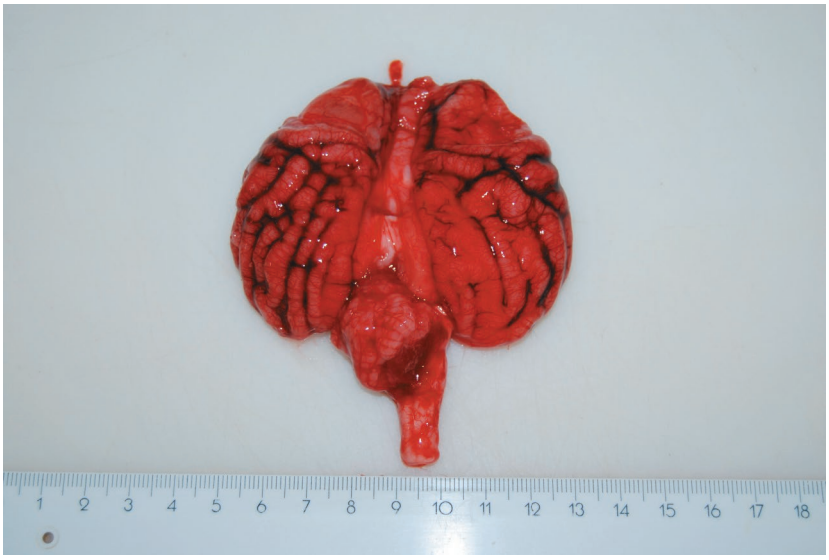


**Figure 8:** SBV-positive calf brain; greatly reduced size of cerebellum (*cerebellar hypoplasia*)

herd B-2/2012 was identified from a three-month old healthy calf located in a small village near the Austrian border in the municipality of Cankova, near Murska Sobota, approximately 220 km east from the location of the first SBV-positive herd (Figure 1). This observation confirmed that acute infection with SBV was already present in two separate locations in Slovenia in September 2012. The sequencing results confirmed that the detected strain in Slovenia shows a 100% identity in 97 determined nucleotides (partial L-segment, genome position 403-500) with the strain named Schmallenberg virus isolate Germany (GenBank Acc. No. JX853179), identified in Germany in 2011.

## Discussion

This study demonstrates the detection of the SBV genome from clinically affected ruminants in Slovenia for the first time. The first official detection of the SBV genome in two malformed lambs was on January 9, 2013 from an ovine flock located in Semič near the Croatian border in the southern part of Slovenia. Later, SBV was detected on 27 additional cattle farms throughout Slovenia. Malformations found in detected SBV-positive cases were similar to those described in lambs and calves in other European countries (Germany, France, and Belgium), and the causative agent was the same.

The rapid and wide expansion of SBV is probably the result of transmission by vectors in the southern direction during the summer months since the first SBV antibodies were detected in September 2012 in neighbouring country Austria (15). The preliminary study results for SBV antibodies showed that Slovenian cattle had been naïve until August 2012, but on August 29, 2012, the first SBV-antibody-positive herd was identified by enzyme-linked immunosorbent assay (ELISA) test (16). Previous reports showed that acute infection with SBV in adult animals causes mild or unspecific clinical signs in ruminants (1, 3). This explains why the first stage of the SBV infection in 2012 was not recognised by our farmers and veterinarians. However, with retrospective detection of the SBV genome from two archive samples collected in September 2012, it is confirmed here that infection started in Slovenia almost at the same time as it was detected in Austria. From January to the end of April 2013, numerous cases of devastating malformations in new-borns were observed and an increased number of samples from different locations were detected positive by RT-qPCR (Figure 1).

The pathological findings made following detection of malformed foetuses and the rapid spread of the disease through Slovenia is very similar to the findings and spread reported in Germany, the Netherlands, Belgium and France in autumn 2011 and the beginning of 2012 (3, 8, 13). The detection of SBV outbreaks in new areas of Europe in 2012, such as Sweden, Norway, Finland, Estonia, Switzerland, Poland, Austria and now Slovenia, suggests the tendency of the

infection to spread quickly to new areas with a naïve population. Positive samples were identified in this study by the RT-qPCR method, with a protocol targeting the L-segment of SBV from a pool of samples consisting of the spleen and several parts of the brain, recommended as the most suitable material (13, 14). The identification of 32.9% SBV-positive samples from the tested malformed animals is similar to that of previous reports, while in Belgium, it was between 28% and 44% (4, 14), and in the Netherlands, from 9% to 70% (9, 13, 17). Our results support the previous observations that the presence of SBV RNA can be detected only in certain percentage of the SBV-suspected new-borns, because of SBV clearance after infecting the foetus (14).

Some authors have suggested using an additional tests for antibody detection in malformed animals, such as the virus neutralization test (VNT) or ELISA test to increase the reliability of SBV diagnosis (10, 17). The VNT showed that 95% of the malformed lambs were positive for SBV, although this percentage was lower for malformed calves (13). This observation was tested in our laboratory on 17 malformed calves (collected in April and May 2013) with RT-qPCR (tissue suspension) and ELISA (serum or thoracic fluid) and 15 (88.3%) of 17 calves were SBV-antibody-positive in ELISA test (ID Screen Schmallenberg Virus indirect ELISA, ID.vet Innovative Diagnostics, France), while only 7 (41.1%) of them were detected SBV-genome-positive by RT-qPCR (unpublished data). These preliminary results and the results from this study performed on malformed calves support previous observation detected in lambs (14) but in our case also, most of the malformed calves had developed SBV antibodies, thus providing confirmation of the SBV infection in an additional number of calves. This data is consistent with recent publication suggesting that neutralizing antibodies could play a role in the clearance of the virus from the foetus resulting in a subsequent inability to detect SBV by RT-qPCR. Nevertheless, the implementation of the new RT-qPCR assay in the laboratory based on L-segment will be necessary to increase the sensitivity for detection of SBV in samples (3, 9).

Slovenia is the first SBV-infected country near the Balkan area. With the ongoing spread of the infection we may expect that this new virus will soon be detected in other countries of southeast Europe.

## Acknowledgments

Sampling and testing were carried out and financed within the framework of the official surveillance of SBV provided by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection. This research was also financially supported by the Slovenian Research Agency, programme group P4-0092 (animal health, environment and food safety). Special thanks to Bernard Hoffmann and Martin Beer from FLI, Germany, for providing the protocols and SBV-positive control.

## References

1. Beer M, Conraths FJ, van der Poel WH. "Schmallenberg virus": a novel Orthobunyavirus emerging in Europe. Epidemiol Infect 2013; 141: 1–8.

2. Elbers ARW, Loeffen WLA, Quak S, et al. Seroprevalence of Schmallenberg virus antibodies among dairy cattle, the Netherlands, Winter 2011-2012. Emerg Infect Dis 2012; 18: 1065-71.

3. Hoffmann B, Scheuch M, Höper D, et al., Novel Orthobunyavirus in cattle in Europe, 2011. Emerg Infect Dis 2012; 18: 469–72.

4. Garigliany MM, Bayrou C, Kleinen D, Cassart D, Desmecht D. Schmallenberg virus in domestic cattle, Belgium, 2012. Emerg Infect Dis 2012; 18: 1512–4.

5. Goller KV, Höper D, Schirrmeier H, Mettenleiter TC, Beer M. Schmallenberg virus as possible ancestor of Shamonda virus. Emerg Infect Dis 2012; 18: 1644–6.

6. European Food Safety Authority (EFSA). Schmallenberg virus: analysis of the epidemiological data and assessment of impact. EFSA J 2012; 10(6): e 2768 (89 str.) http://www.efsa.europa.eu/en/efsajournal/doc/2768.pdf (Dec. 2013)

7. Kaba J, Czopowicz M, Witkowski L. Schmallenberg Virus antibodies detected in Poland. Transbound Emerg Dis 2013; 60: 1–3.

8. Bouwstra RJ, Kooi EA, de Kluijver EP, et al. Schmallenberg virus outbreak in the Netherlands: routine diagnostics and test results. Vet Microbiol 2013; 165(1/2: 102–8.

9. Larska M, Polak MP, Grochowska M, Lechowski L, Zwiazek JS, Zmudzinski JF. First report of Schmallenberg virus infection in cattle

and midges in Poland. Transbound Emerg Dis 2013; 60: 97–101.

10. Mansfield KL, La Rocca SA, Khatri M, Johnson N, Steinbach F, Fooks AR. Detection of Schmallenberg virus serum neutralising antibodies. J Virol Methods 2013; 188: 139–44.

11. De Regge N, Deblauwe I, De Deken R, et al. Detection of Schmallenberg virus in different Culicoides spp. by real-time RT-PCR. Transbound Emerg Dis 2012; 59: 471–5.

12. Rasmussen LD, Kristensen B, Kirkeby C, et al. Culicoids as vectors of Schmallenberg virus. Emerg Infect Dis 2012; 18: 1204–6.

13. Bilk S, Schulze C, Fischer M, Beer M, Hlinak A, Hoffmann B. Organ distribution of Schmallenberg virus RNA in malformed newborns. Vet Microbiol 2012; 159: 236–8.

14. De Regge N, van den Berg T, Georges L, Cay B. Diagnosis of Schmallenberg virus in malformed lambs and calves and first indications for virus clearance in the fetus. Vet Microbiol 2013; 162: 595–600.

15. Schiefer P, Steinrigl A, Wodak E, Schmoll F. Rapid spread of Schmallenberg virus in Austrian domestic ruminants. In: International Meeting on Emerging Diseases and Surveillance. Vienna, 2013: 184.

16. Toplak I, Starič J, Cociancich V, Rihtarič D, Paller T. The first evidence of Schmallenberg virus infections in Slovenia: detection of antibodies in cattle since august 2012. In: 13th Middle European Buiatrics Congress. Belgrade, 2013: 46–49.

17. van Maanen C, van der Heijden H, Wellenberg GJ, et al. Schmallenberg virus antibodies in bovine and ovine fetuses. Vet Rec 2012; 171(112): 299.

## PRVI PRIMERI OKUŽB Z VIRUSOM SCHMALLENBERG V SLOVENIJI, 2012

I. Toplak, V. Cociancich, D. Rihtarič, P. Juntes, T. Paller

**Povzetek:** Konec poletja 2011 so iz Nemčije in Nizozemske poročali o prvih pojavih akutnih okužb goved povzročenih z virusom Schmallenberg (SBV) iz rodu *Orthobunyavirus*. Prve spačene zarodke zaradi SBV so opazili v decembru 2011 na Nizozemskem, v Belgiji in Nemčiji. V Sloveniji smo prvi primer okužbe z virusom SBV ugotovili v reji 23 ovac ob postavitvi suma na to bolezen po devetih zaporednih abortusih. Virusno nukleinsko kislino SBV smo v vzorcih dokazali iz možganov in vranice z metodo verižne reakcije s polimerazo v realnem času (RT-qPCR), ki jo je razvil Inštitut Friedrich-Loeffler iz Nemčije. Od januarja do aprila 2013 smo testirali 77 spačenih telet in pri 25 ugotovili prisotnost nukleinske kisline SBV z metodo RT-qPCR. Pri večini spačenih živalih smo ugotavljali eno ali več patoloških sprememb: artrogripozo, brahignatijo, tortikolis, skoliozo, hidranencefalijo ter nepravilen razvoj možganov in hrbtenjače. Dodatno smo tudi v dveh arhivskih vzorcih, odvzetih septembra 2012, ugotovili SBV, kar potrjuje prisotnost okužbe s SBV v Sloveniji že v septembru 2012. Z določanjem zaporedja nukleotidov v segmentu L smo potrdili, da je ugotovljeni sev v Sloveniji 100 % identičen z izolatom virusa Schmallenberg (JX853179), ki je bil ugotovljen v Nemčiji leta 2011. Pozitivne reje smo odkrili na celotnem območju Slovenije.

**Ključne besede:** prvo poročilo; virus Schmallenberg; diagnoza; Slovenija


Namen ustanovitve in delovanja podjetja MD svetovanje d.o.o. je pomagati podjetjem pri poslovanju z nudenjem produktov in storitev, ki ne spadajo v osnovno dejavnost podjetja. To dosegamo s celovito ponudbo predstavljenih produktov in storitev.

Zato smo naš moto Skupaj bomo uspešnejši! nadgradili še z motom in sloganom Vse za Vas na enem mestu!

## Vizija

Postati vodilna neodvisna družba s celotno ponudbo za podjetja in posameznike na enem mestu in na ta način prihraniti podjetjem in posameznikom čas in denar.

Vse to nam bo uspelo s trdim delom in kakovostno izvedbo storitev in zaupanih nam nalog, predvsem če bomo sledili naslednjim načelom:

- zagotavljanje celovite ponudbe,
- vedno delo v dobro stranke,
- strokoven razvoj,
- organizacijsko izpopolnjevanje,
- zagotavljanje visoke stopnje kakovosti storitev z upoštevanjem predlogov naših strank,
- ustvarjanje novih delovnih mest,
- povečanje produktivnosti in dobičkonosnosti,
- visoko motiviran in usposobljen kader s primernim vodenjem, kar zagotavlja
- kakovost izvajanja storitev,
- postati vodilno podjetje, ki ponuja rešitve, ki podjetju omogočajo da si na enem
- mestu zagotovi vse dejavnosti, ki ne spadajo v njegovo osnovno dejavnost.

## Prednosti poslovanja z nami:

- vse svoje potrebe in vizije uresničite s klicem na eno telefonsko številko,
- razbremenite se ukvarjanja z obrobnimi zadevami,
- posvetite se svojemu strokovnemu delu,
- informacijska tehnologija,
- prilagodljivost,
- zanesljivost,
- povečanje dobičkonosnosti,
- zmanjšanje stroškov dela,
- ...

MD svetovanje, poizvedbe in storitve d.o.o.
Dunajska cesta 421,
1231 Ljubljana – Črnuče

PE Ljubljana-Vič
Cesta dveh cesarjev 403,
1102 Ljubljana

01 / 620-47-01
01 / 620-47-04
041 / 614-090

www. mdsvetovanje. eu

## Zakaj MD Svetovanje d.o.o.

- visoka profesionalizacija,
- visoka strokovnost,
- visoka uspešnost,
- konkurenčne cene,
- vse na enem mestu.

mdinfo@mdsvetovanje.eu www.mdsvetovanje.eu

# SLOVENIAN VETERINARY RESEARCH
# SLOVENSKI VETERINARSKI ZBORNIK

**Slov Vet Res 2014; 51 (1)**

**Original Scientific Articles**