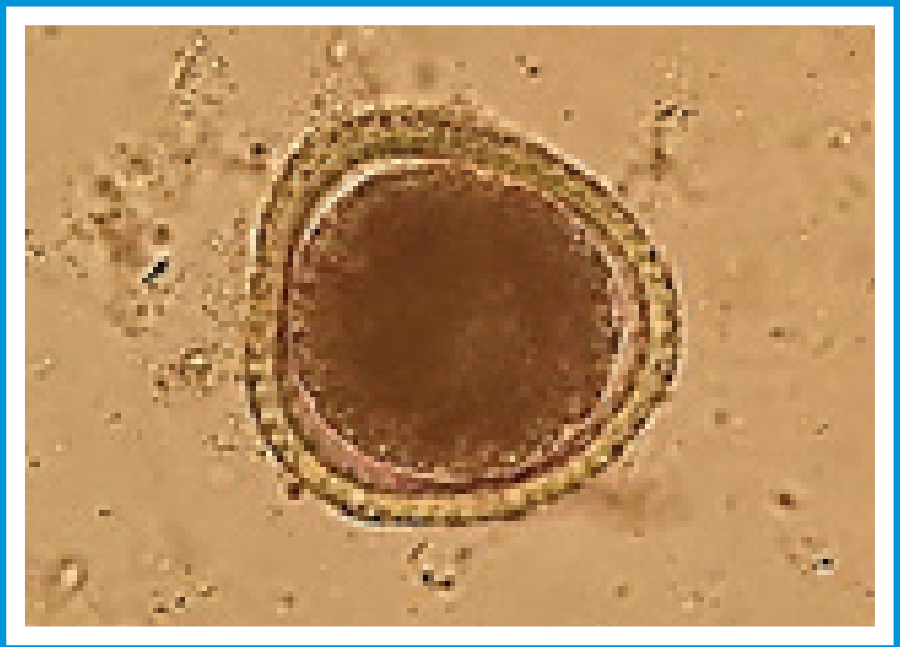
ISSN 1580-4003

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK



Slov Vet Res 2016; 53 (3): 181-240

Volume 53 4

Slov Vet Res • Ljubljana • 2016 • Volume 53 • Number 4 • 181-240

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA

SLOVENIAN VETERINARY RESEARCH

SLOVENSKI VETERINARSKI ZBORNIK

Volume 53 4

Slov Vet Res • Ljubljana • 2016 • Volume 53 • Number 4 • 181-240

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 Research Agency
Sofinancira: Javna agencija za raziskovalno dejavnost 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 2016; 53 (4)

Slov Vet Res 2016; 53 (4): 185-93
UDC 636.4.09:616.98-036.22:578

Review Article

EPIDEMIOLOGY AND STATUS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME IN THE WESTERN BALKAN REGION: CHALLENGES AND PROSPECTS

Dinko Novosel[1]*, Tamaš Petrović[2], Žaklin Acinger – Rogić[3], Marina Štukelj[4]

[1]Department of Pathology, Croatian Veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia; [2]Scientific Veterinary Institute "Novi Sad", Rumenacki put 20, 21000 Novi Sad, Serbia; [3]Veterinary and Food Safety Directorate, Ministry of Agriculture, Planinska 2a, 10000 Zagreb, Croatia; [4]Institute for Health Care of Pigs, University of Ljubljana, Veterinary Faculty, Grbiceva 60, 10000 Ljubljana, Slovenia

*Corresponding author, E-mail: dinko.novosel@gmail.com

Summary: Two decades after its emergence, porcine reproductive and respiratory syndrome (PRRS) remains a challenge to the sustainability of the porcine industry worldwide. In the Western Balkan region in particular, control of the disease is hampered by fragmentation of pig production; lack of farmer knowledge regarding health care; the fact that most farms are small, single-site pig operations with low biosecurity standards; and intensive trading and import of pigs from different countries without known health status and without quarantine. All these factors contribute to rapid disease transmission among pig operations. PRRS entered the Western Balkan region in 1995, when it appeared in Croatia and slightly later in Serbia, and again after 2004 when it entered Slovenia and potentially other countries. All PRRS cases originally described in the Western Balkans appear to have been caused by infection with type 1 subtype 1 PRRSV; more recently, infection with type 2 virus has also been reported. Veterinary services have an important role to play in monitoring and controlling spread of PRRS, but control programs in the region are either inconsistent or non-existent. Available epidemiological data suggest that new PRRSV introduction into Western Balkan countries is less likely to occur via animal transfers within the region and more likely to occur via arrivals from elsewhere in the EU. Strong efforts are needed to develop and implement guidelines for pig movement, implement biosecurity measures, establish consistent diagnostic testing for PRRS virus, and classified pig herds according to health status and farmer education.

Key words: PRRSV; Slovenia; Croatia; Serbia

Introduction

Two decades after its emergence, porcine reproductive and respiratory syndrome (PRRS) remains a challenge to the sustainability of the global porcine industry. As a result, many countries in North America and the European Union (EU) have implemented continuous monitoring programs. The picture is quite different among countries in the Western Balkans, where pig production and veterinary services are highly fragmented, and PRRS control measures are inconsistent or non-existent. This troubling state of affairs reflects a lack of harmonized diagnostic methods and sparse available data on PRRS prevalence, nature of virus and farm management practices. This poses a problem not only for control and reduction of PRRS virus (PRRSV) already present in the Western Balkans, but also for prevention of new virus introduction due to extensive trade with other EU countries.

The problem of PRRS monitoring and control in this region is even more challenging because

Received: 26 October 2015
Accepted for publication: 14 June 2016

Croatia, Slovenia and Serbia contain large numbers of sustainable farms where small numbers of animals are kept, mostly for household needs. For example, of approximately 168,000 breeding sows in Croatia, only 25,000 are in large pig production units; the remainder are on semi-intensive or -extensive farms, according to the Croatian Agriculture Agency (http://www.hpa.hr/, accessed 15 May 2016). Most pig farms in Slovenia are small, single-site production farms: according to a census of 4162 farms in 2013, 3909 farms had 1-20 breeding pigs; 206 farms, 21-50; 31 farms, 51-100; 12 farms, 101-200 breeding pigs; 2 farms, 501-1000 and 2 farms, 1001 or more (data from 1.2.2013 in the VOLOS database, Ministry for Agriculture, Forestry and Food, UVHVVR).

Though the first PRRS outbreak was reported in lower Saxony in 1992 (1), PRRSV appears to have emerged before that in Eastern European countries behind the "Iron Curtain" (2), when the present-day countries of the Western Balkans formed part of the single federation of Yugoslavia. This federation literally served as a bridge for PRRS: it was the only Communist country with soft borders, and it traded extensively with Eastern and Western Europe. The paths of live animal movements potentially carrying PRRSV changed often in the region as a result of complex political changes that molded and remolded trade routes. The Berlin Wall fell in 1989, Yugoslavia broke up into several republics in 1990, and war raged in Croatia, Bosnia and Herzegovina, Serbia and Montenegro in 1991-1995. Slovenia joined the Central European Free Trade Association (CEFTA) in 1996, followed by Croatia in 2003. The following year, Slovenia joined the EU and left CEFTA, while in 2007 most other former Yugoslav republics joined CEFTA. In 2013 Croatia joined the EU and left CEFTA (Figure 1). All these changes probably modified live animal movements in complex ways, making it difficult to track PRRSV epidemiology and predict prevalence.

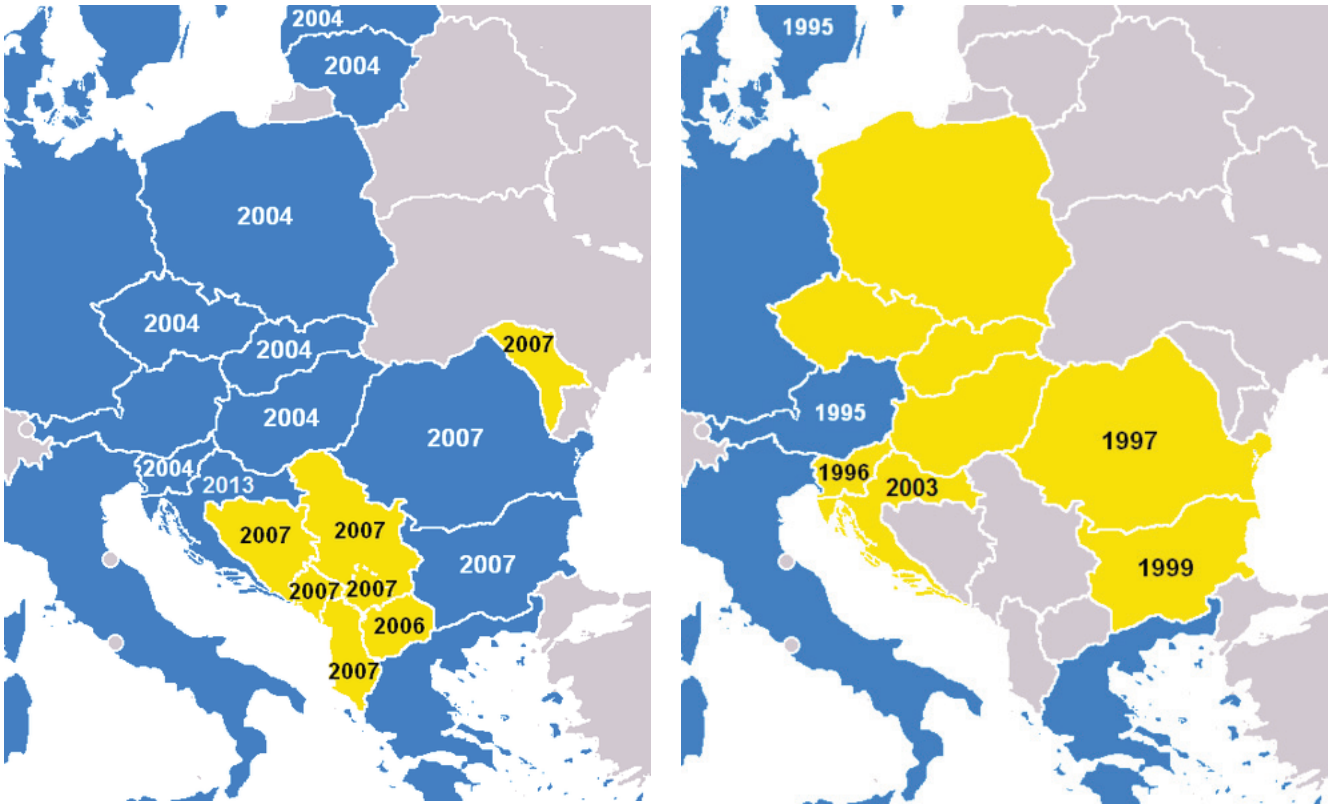


Figure 1. Evolution of political alliances affecting trade routes and therefore live animal movements in the Western Balkan region. EU member states are shown in blue; CEFTA member states, in yellow. Membership status is shown (A) before 2003 and (B) in 2015

Pig production and trade in the Western Balkans

Western Balkan countries import large amounts of pork and pigs from Western Europe. The biggest exporter to the Western Balkans in 2012 was Hungary, with the traded animals originating outside Hungary [Croatian Agriculture Agency (www.hpa.hr), accessed 15 May 2016]. This reflects the fact that large pig-exporting firms in Hungary had distribution centres for the Western Balkans but were not producing their own animals. In the same year, Croatia imported more pig and pork from the EU27 bloc than all other Western Balkan countries (Table 1). Croatia also exported large amounts to Bosnia and Herzegovina and Albania. In fact, membership in CEFTA facilitated pig and pork trading with Bosnia and Serbia (Table 2). Evidence also strongly suggests illegal trading among Western Balkan countries that bypassed veterinary inspections (3).

Before joining the EU in 2004, Slovenia was free of PRRS. The disease was introduced after entry as a result of pig import from other EU members; quarantine is not required for animals moving between EU members.

Epidemiology of PRRSV in selected Western Balkan countries and attempts at control

Epidemiological studies suggest that most pig pathogens in the Western Balkans came from outside the region, and that some local enzootic strains have emerged. Most strains of porcine circovirus type 2 (PCV2) in the Western Balkan region are highly homologous to Dutch strains (4), as are most strains of PRRSV (5, 6). One exception is porcine parvovirus genotype 3 (PPV3), which appears to have originated in Croatia (5). A different PCV2 genotype was recently discovered in wild boars (4). Some local enzootic strains appear to have spread from the Western Balkans to other regions, such as PPV3, while others have not, such as PCV2d (8).

Relatively good data exist about PRRSV seroprevalence in Slovenia and Serbia, but little is known about the situation in Croatia, and the few data available are sometimes contradictory. For example, only 11 ELISA and PCR tests were performed in 2009 (9) and only 5 in 2010 (10). Seroprevalence in Croatia is thought to be much higher than 25%, based on analyses of neighboring Slovenia and Serbia, suggesting that nearly all farms suffer economic losses due to PRRSV.

Table 1: Key data on Western Balkan countries. Source: FAO (www.fao.org, accessed 15 May 2016)

Country	Total area (10^3 Ha)	Agriculture area (10^3Ha)	Population (1000s)	GDP (mil €)	HDI	Export (%)	Import (MT)
Albania	2875	1201	3238	11781	0.19		8.6
Bosnia and Herzegovina	5121	2151	3736	16578	0.71		
Croatia	5659	1326	4379	60852	0.76		26.36
Montenegro	1381	512	633	411	0.769		7.3
Romania	23839	13982	21339	161624	0.767		65.8
Bulgaria	11100	5088	7349	47714	0.7343		37.9
Serbia	8836	5061	9835	38423	0.701		
FYROM	2571	1118	2069	9189	0.701		7.7
Slovenia	2027	458	2045	46908	0.828	18	
Western Balkans	63409	30897	54623	393480		18	153.66
EU	4381376		507890	17577000	0.876		

Abbreviation: [1] Area of country in 10^3 Ha; [2] Total agriculture area in 10^3 Ha; [3] Country population; [4] Gross domestic product; [5] Human development index; [6] Percentage of production that was exported to other countries; [7] Import in millions of tons

Table 2: Pig imports and exports to and from Western Balkan countries. Source: UN Comtrade database (http://comtrade.un.org/, accessed 15 May 2016)

Country	Pig type	Exports (MT)	Destination countries	Imports (MT)	Countries of origin
Albania	pure breed			7066	GR, H, HR, I
	< 50 kg			135	GR, NL, I
Austria	pure breed	1363	D, SLO, CZ, H	38402	D, DK, SLO, CZ
	< 50 kg	1028	SLO, H, HR, D	4814	D, SLO, CZ, H
Bosnia and Herzegovina	pure breed	16	MNE	4932	HR, SRB, H
	< 50 kg	1,6	MNE	711	H, D, SRB, HR
Croatia	pure breed	7983	BiH, SRB, AL	142	H, CZ, SLO
	< 50 kg	19	BiH	13887	NL, D, H, DK
Denmark	pure breed	46443	D, BY, I, E		
	< 50 kg	237313	D, PL, I, CZ		
Greece	pure breed				
	< 50 kg	34	AL	104	H, N, E
Montenegro	pure breed			833	H, SRB,BiH
	< 50 kg			651	SRB, BiH
Romania	pure breed			20686	H, BG, NL
	< 50 kg	11	MD	18709	NL, H ,D
Bulgaria	pure breed	89	GE, AL	18	DK, D, GR
	< 50 kg			574	NL, GR, RO
Serbia	pure breed				
	< 50 kg				
FYROM	pure breed	355	SRB		
	< 50 kg	43	SRB		
Slovenia	pure breed	2655	A, MNE, AL	306	A, H, I
	< 50 kg	19	A	1844	A, D, NL
Hungary	pure breed	55	AL	13	F, E
	< 50 kg	7948	RO, HR, NL	9042	NL, D, SK
EU27	pure breed	23443	RU, AL, SRB	1,8	CH, US
	< 50 kg	24622	HR, UA	20	CH
Western Balkans	pure breed	11098	HR, BiH	2691	
	< 50 kg	127,6		36615	

Abreviation: [1] Pure breed – reproductive gilts and boars, <50 kg – imported weaners for fattening; [2] Exports in million tons of body weight; [3] Imports in million tons of body weight

Croatia

The first outbreak of a severe reproductive disorder in breeding animals in Croatia occurred in 1995 (11), most probably resulting from sow insemination with imported Duroc semen contaminated with PRRSV. At that time, Croatia had not yet developed methods to diagnose PRRS, so serum samples were sent to the Veterinary Diagnostic Institute in Lelystad in the Netherlands; these samples tested positive for PRRSV by the enzyme-linked immunosorbent assay (ELISA). Subsequently the Croatian Veterinary Institute became a major testing centre for PRRSV, testing approximately 60,000 serum samples between 1996 and 2010 (12). During that time, the disease spread to nearly all major pig breeding herds, and seroprevalence in domestic swine was estimated to be over 90% (12).

The situation appeared to have improved in 2009, though only 709 serum samples were tested, followed by only 955 in 2010. Testing of sera from boars, sows, gilts and fatteners for the presence of anti-PRRSV antibodies using three commercial ELISA kits revealed no positives in 2009, compared to positive rates of 1.84% (5 of 272) in sows and 2.59% (5 of 193) in fatteners in 2010. These results likely underestimated the prevalence of PRRSV, since only 1.1% of sows were tested in 2009 and only 1.6% in 2010 (13). Indeed, testing of samples using nested reverse transcription (RT)-PCR, immunohistochemistry and pathology indicated the presence of PRRSV in nearly all large pig production units in Croatia.

This lack of progress in controlling or eradicating PRRS contrasts with the fact that PRRS is a notifiable disease in Croatia: every positive PRRS case must be notified to the Croatian veterinary directorate. The problem may be that the national authority has not mandated any specific control measures to combat PRRS. As a result, control measures are usually applied at the farm level by the local veterinary service assigned to that particular farm. Meanwhile, large pig production farms and units usually apply only nonspecific (mainly biosecurity) measures. Outbreaks of classical swine fever in 2006 and 2007-08 led the national authority to mandate biosecurity measures on all farms, with more stringent measures required on farms keeping more than 100 pigs.

Efforts to control and eradicate PRRS in Croatia continue to lack any coordination at the national level. As of 2015, the veterinary authority does not require PRRSV testing of national breeding stock, such as testing of boars before transfer to artificial insemination facilities or regular testing of sows and gilts. As a result, such testing is performed at farm level by the local veterinary service assigned to that particular farm based on individual health programs. No national-level data on PRRSV vaccination are available, nor are PRRSV-positive farms required to notify the veterinary authority of depopulation-repopulation programs or other control measures. In 2004, a PRRSV emergency was declared and imported inactivated vaccine was applied. The results were ambiguous and left many farmers disappointed and unconvinced of the efficacy of coordinated intervention at the national level. An attenuated vaccine against PRRSV entered the Croatian market in 2007, but it has been little used.

Epidemiological data on PRRSV-positive animals are less reliable because all animals that test positive locally are automatically recorded as PRRSV-positive, regardless of diagnostic criteria and testing method. Testing and confirmation methods have not been standardized at the national level. Available epidemiological data suggest persistence of the virus throughout Croatia: in 2009, 26 pigs on 6 farms tested positive for PRRSV; in 2010, 6 pigs on 6 farms; in 2011, 8 pigs on 3 farms; in 2012, 295 pigs on 48 farms; in 2013, 43 pigs on 7 farms; in 2014, 31 pigs on 10 farms. Positive farms were located mainly in Osijek-Baranja, Vukovar-Srijem and in Medjimurje County, consistent with the higher concentration of large pig production sites in these areas.

Slovenia

A survey of swine sera during 1999-2004 in Slovenia showed all herds to be free of PRRSV. In the beginning of 2005, soon after Slovenia joined the EU, animals positive for anti-PRRSV antibody using ELISA we first detected among breeding pigs in a few herds (14). In 2010, a survey of 267 herds revealed that 44.8% were seropositive for antibody (15). Positive herds manifested clinical signs of disease, including reproductive and/or respiratory disorders. To reduce economic losses, farms with positive animals relied on vaccination with the same

two live-attenuated vaccines currently available in the EU, as well as on herd closure, roll-over and serum inoculation. A volunteer project funded by the Slovenian Research Agency and the Ministry of Agriculture and Environment eliminated or eradicated PRRS from 7 of 19 farms (16).

The hypothesis that EU entry led to PRRSV introduction into Slovenia through import of infected animals seems more plausible given that something similar appears to have happened with Hungary. Shortly after Hungary acceded in 1996, the first seropositive pigs were recorded in 1996. In contrast to the situation in Slovenia, between 1995 and 2002, 27925 pig sera were tested and the seroprevalence remained below 3% until 2002, despite brisk pig trade between Hungary and Western Europe (17).

Although a national program to eradicate PRRS was proposed in 2011, it was never implemented. The main problem was that farmers did not want PRRS status to become publicly known.

Serbia

The first suspected cases of PRRS in Serbia occurred in 2001, when serious respiratory disorders associated with high mortality affected large numbers of pigs on two large industrial farms located in the northern region close to the borders with Croatia and Hungary. The suspected cause of the cases was boar semen illegally imported from neighbouring countries. Subsequently in 2001-02, respiratory syndrome with high morbidity and moderate mortality, which was diagnosed as PRRS, occurred on several large industrial farms in the northern Serbian province of Vojvodina, where pig production is intensive. This syndrome subsequently spread to parts of central Serbia. Severe health problems and high economic losses led the veterinary directorate to perform PRRS serology screening in 2002, 2004-2005 and 2006-2007 (Table 3). PRRSV monitoring using RT-PCR and immunofluorescence in 2002 detected the virus in 2 of 16 piglets who died on infected farms. Monitoring in 2006-2007 revealed PRRSV-positive herds in all Serbian regions at prevalences of 1.56-60.86%; the disease was most prevalent in northern, western and central parts of the country, where prevalence was 17.30-60.86%. In contrast, prevalence was only 1.56-8.98% among herds in the eastern and southern parts of the country (18).

These screening results suggest that two major PRRS introductions occurred in the Western Balkan region. The first one was in 1996 when the disease moved from Croatia into Hungary and shortly thereafter into Serbia. The second introduction occurred in Slovenia and Hungary after they joined the EU, when quarantine of imported animals from EU countries was no longer required. As a result of the first virus introduction and resulting outbreak, an emergency-vaccination campaign to control PRRSV was carried out on large industrial farms in northern Serbia in 2002-2003. Other than this limited intervention, no monitoring or control program against PRRS has ever been proposed at the national level.

Genetic diversity of PRRSV in the Western Balkan region

Extensive studies have identified only one PRRSV type (EU type 1 subtype 1) circulating in the Western Balkan region, with no evidence of other Eastern European subtypes (5, 6, 19, 20). ORF5 sequences from field samples in Croatia show 95.2-99.7% homology with the Lelystad vaccine strain used in the country (5). In Serbia, phylogenetics indicate that all 18 genetically typed isolates belong to EU subtype 1 or Lelystad-type viruses that are distributed across Europe as well as other parts of the world (19). In Slovenia, six genetically different PRRSV strains were found in circulation, all belonging to type 1 subtype 1.

Table 3: Results of PRRSV screening in Serbia

Period	Farms/animals, n	Positive animals, n / %	Positive farms, n / %
2002	32 / 880	511 / 58.07	20 / 62.50
2004-2005	43 / 1135	540 / 47.58	28 / 65.12
2006-2007	562 / 3069	No data	11 / 20.46

Median-joining (MJ) phylogenetic networks can illustrate microevolutionary process in viruses (21), particulary for viruses in which recombination frequently occurs (22). Therefore we generated an MJ phylogenetic network based on partial 281-bp ORF7 sequences from Slovenia (n = 22), Serbia (n = 17) and Croatia (n = 7) (6, 19, 20), as well as on seven additional ORF7 sequences from Croatia (5). Sequences were aligned using the ClustalW program in MEGA 5 software (23); sequence polymorphism and haplotype diversity were computed using DnaSP 5 (2). Finally, MJ networks of haplotypes were constructed using Network 4.6.0.0. (Fluxus Technology).

The MJ network clearly shows that all Serbian and Slovenian and most Croatian sequences belong to EU type 1, subtype 1. Eastern EU subtypes 2-4, previously identified in Belarus, Lithuania, Russia and Kazakhstan, were not present among the Western Balkan samples. The phylogenetic relationships suggest that the PRRSV genotype 1 was introduced six times into the Western Balkans, and these events involved the appearance of the following six strains: a strain with an ORF5 sequence similar to that of the Porcillis PRRSV strain used in vaccines in Serbia and Croatia (5), a strain that has been circulating for a long time in Slovenia and that has mutated several times, a strain recently introduced into Slovenia, a strain in Slovenia originating from an Amervac vaccine strain, a strain present in Croatia and Slovenia, and a strain circulating for a long time in Serbia that shows homology to Eastern European subtype 3. Two Croatian sequences clustered with American virus type - Genotype 2, one of which appears to be derived from a vaccine strain, while ResPRRS vaccine was never used and registered in Croatia. These sequences came from farms that imported breeding animals from the US.

These insights from MJ phylogenetic networks should be interpreted with caution when reconstructing the evolution of PRRSV strains in the Western Balkan region. The MJ analysis is based only on partial ORF7 sequences. Definitive determination of the origins of PRRSV strains in Croatia, Serbia and Slovenia requires more comprehensive analysis based on both ORF5 and ORF7 sequences. If the preliminary results in Figure 2 are verified, they may indicate that PRRSV movement among Western Balkan countries poses a much smaller threat than import of infected animals from Western Europe.

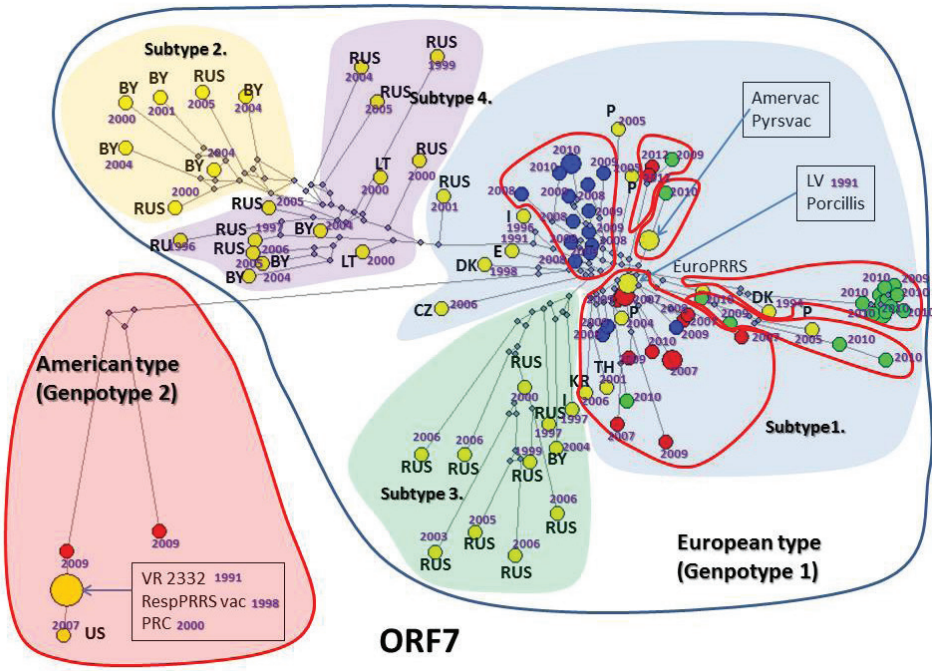


Figure 2: MJ network of PRRSV isolates from Croatia, Serbia and Slovenia based on partial ORF7 sequences, showing phylogenetic relationships among PRRSV sequences and related sequences in GenBank. Red dots indicate Croatian haplotypes; blue, Serbian; and green, Slovenian. Yellow dots indicate haplotypes isolated in other countries and deposited in GenBank. Haplotypes are labelled by geographic origin. Dot size is proportional to the frequencies of isolates belonging to the given haplotype

Conclusions

Complex trade relations and a poor understanding of the true economic costs of PRRSV have dampened political will to create control and eradication programs. Strong leadership from government agencies is needed throughout the Western Balkan region to develop guidelines for pig movements, develop diagnostic methods and screening approaches and implement requirements for clear division status at the herd and animal levels. Farmers and veterinarians need to be educated about diagnosis and biosecurity measures; uncertainties about these issues lead many to be unconcerned about PRRS as a threat. Systematic and rigorous epidemiological studies of PRRS are needed in Croatia in order to guide future monitoring, control and eradication efforts. Collaborative epidemiological studies involving the various Western Balkan countries may generate much-needed hard data on the economic impact of PRRSV, its prevalence, herd risk and effectiveness of diagnostic tools.

Acknowledgments

This work was conducted in the framework of COST Action FA0902 ("Understanding and combating porcine reproductive and respiratory syndrome in Europe – EuroPRRSnet"), the Ministry of Science, Education and Sport of the Republic of Croatia (project 0481153) as well as the Slovenian Research Agency (ARRS) and Ministry of Agriculture and Environment (CRP V4-1111). The work was also supported by grant TR31084 from the Serbian Ministry of Education, Science and Technological Development. The authors acknowledge Dr. Armando Chapin Rodriguez for English proofreading.

References

1. Busse FW, Alt M, Janthur I, Neuman W, Schoss P. Epidemiological studies on porcine epidemic abortion and respiratory syndrome (PEARS) in Lower Saxony of Germany. In: Proceedings of 12th Congress International Pig Veterinary Society. Hague, 1992: 115.

2. Stadejek T, Oleksiewicz MB, Scherbakov AV, et al. Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Arch Virol 2008; 153(8): 1479–88.

3. Kri I. Zbog šverca bikova sperme završili u jednomjesečnom pritvoru! Večernji List 2013; 6. 6. 2013.

4. Novosel D, Tuboly T, Csagola A, et al. Origin of porcine circovirus type 2 (PCV2) from swine affected by PCV2-associated diseases in Croatia. Vet Rec 2014; 174(17): 431.

5. Balka G, Cadar D, Novosel D, et al. Phylogenetic analysis of PRRSV strains from selected central and eastern European countries. In: 9th International congress of veterinary virology and joint meeting with the European Society of Clinical Virology. Madrid, Spain, 2012: 92.

6. Stadejek T, Balka G, Chrabos K, et al. Genetic diversity of European genotype PRRSV in Central and Eastern Europe: an update. In: 21st International Pig Veterinary Society Congress. Vancouver, 2010: 503.

7. Cadar D, Lorincz M, Kiss T, et al. Emerging novel porcine parvoviruses in Europe: origin, evolution, phylodynamics and phylogeography. J Gen Virol 2013; 94(10): 2330–7.

8. Franzo G, Cortey M, Olvera A, et al. Revisiting the taxonomical classification of Porcine circovirus type 2 (PCV2): still a real challenge. Virol J 2015; 12(1): e131 (8 pp.) http://virologyj.biomedcentral.com/articles/10.1186/s12985-015-0361-x (13. 9. 2016)

9. Hrvatski Veterinarski Institut. Pregled rada 2009. Zagreb : Hrvatski Veterinarski Institut, 2009: 64–8.

10. Hrvatski Veterinarski Institut. Pregled rada 2010. Zagreb : Hrvatski Veterinarski Institut, 2010: 67–71.

11. Lipej Z, Šoštarić B, Roić B, Lojkić M, Karačić V. Porcine reproductive and respiratory syndrome in Croatia. Vet Arh 1997; 67(3): 19–21.

12 Lipej Z, Novosel D. An overview about PRRS in Croatia. In: EuroPRRS.net. Balkan Meeting on PRRS Diagnostics. Split, Croatia, 2011: 31–2.

13. Jungić A, Roić B. Presence of antibodies against Porcine reproductive and respiratory syndrome virus in farm swines in eastern part of Croatia from 2009 to 2010. In: EuroPRRS2011: understanding and combating PRRS in Europe. Novi Sad, Serbia, 2011: 38.

14. Valenčak Z. Porcine reproductive and respiratory syndrome (PRRS) in Slovenia. Slov Vet

Res 2004; 41(2): 99–101.

15. Toplak I, Štukelj M, Zabavnik – Piano J, Hostnik P, Grom J, Valenčak Z. Študija o pojavnosti prašičjega reproduktivnega in respiratornega sindroma (PRRS) v Sloveniji v letu 2010. Ljubljana : Veterinarska fakulteta, Nacionalni veterinarski institut, 2010: 40 p.

16. Kovač M. Celovite rešitve sistemov rej prašičev z namenom izboljšanja konkurenčnosti slovenske prašičereje: zaključno poročilo ciljnega raziskovalnega projekta. Ljubljana : Biotehniška fakulteta, 2014: 62 str. (Poročilo: ARRS-CRP-ZP-2015/5)

17. Balka G. PRRS control in Hungary. In: Understanding and combating porcine reproductive and respiratory syndrome in Europe: final meeting. Heraklion, Greece, 2013: 40.

18. Petrović T, Milicević V, Prodanov-Radulović J, et al. PRRS situation in Serbia: what's next. In: EuroPRRS.net. Balkan Meeting on PRRS Diagnostics. Split, Croatia, 2011: 9–10.

19. Petrović T, Milicević V, Radulović-Prodanov J, et al. Molecular detection and genetic anylisis of Serbian PRRSV isolates. In: EuroPRRS2011: understanding and combating PRRS in Europe. Novi Sad, Serbia, 2011: 50–6.

20. 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.

21. Morrison DA. Networks in phylogenetic analysis: new tools for population biology. Int J Parasitol 2005; 35(5): 567–82.

22. Olvera A, Busquets N, Cortey M, et al. Applying phylogenetic analysis to viral livestock diseases: moving beyond molecular typing. Vet J 2010; 184(2): 130–7.

23. Tischer I, Rasch R, Tochtermann G. Characterization of papovavirus-and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl Bakteriol Orig A 1974; 226(2): 153–67.

NAČINI UKREPANJA IN STANJE V NEKATERIH DRŽAVAH ZAHODNEGA BALKANA GLEDE PRAŠIČJEGA REPRODUKCIJSKEGA IN RESPIRATORNEGA SINDROMA (PRRS)

D. Novosel, T. Petrović, Ž. Acinger – Rogić, M. Štukelj

Povzetek: Prašičji reprodukcijski in respiratorni sindrom (PRRS) so dokazali že pred 20 leti in še vedno povzroča velike izgube v prašičereji. V zahodnih balkanskih državah se srečujemo s specifično situacijo; v glavnem obstajajo majhne družinske reje, v katerih navadno ne izvajajo nikakršnih ukrepov zoper PRRS. Večina omenjenih držav ima nizek odstotek samooskrbe s prašičjim mesom, zato ga uvažajo iz drugih evropskih držav. Na podlagi rezultatov lahko sklepamo, da je do vnosa bolezni prišlo najprej na Hrvaškem leta 1995, malo kasneje pa v Srbiji. Drugi pomembni vnos bolezni se je zgodil pol leta po vstopu Slovenije v Evropsko unijo leta 2004. Najpogosteje v zahodnih balkanskih državah ugotavljamo genotip 1 virusa PRRS in podtip 1, medtem ko drugih podtipov, ki se pojavljajo v vzhodnoevropskih državah, nismo dokazali. Poglaviten vzrok neukrepanja zoper PRRS je v zahodnih balkanskih državah nepoznavanje dejanskih izgub, ki jih povzroča bolezen v posamezni reji, v nekaterih državah pa tudi slaba laboratorijska diagnostika. Verjetno je treba med vzroke prišteti tudi nezainteresiranost politike za izvajanje nacionalnih programov ukrepanja zoper PRRS. Izračuni so pokazali, da je PRRS na Hrvaškem leta 2011 povzročil za 17 milijonov EUR izgub v prašičereji. Poglavitni krivec za nastali položaj je veterinarska uprava, ki ni izpeljala potrebnih ukrepov. Širjene bolezni med omenjenimi zahodnimi balkanskimi državami ne predstavlja velikega tveganja, saj praktično ni trgovanja med njimi, nasprotno pa so uvozi iz drugih držav velika nevarnost za vnos novih sevov virusa PRRS. Na podlagi omenjenih dejstev bi bilo treba pripraviti načrt ukrepov zoper PRRS, ki bi med drugim vključevali tudi testiranje na prisotnost tako protiteles kot virusa PRRS in posledično vzpostavitev statusov čred, kar bi pripomoglo k zajezitvi širjenja bolezni.

Ključne besede: PRRSV; Slovenija; Hrvaška; Srbija

Slov Vet Res 2016; 54 (4): 195-204
UDC 614.31:637.56:578

Original Research Article

VIRAL CONTAMINATION IN MUSSEL PRODUCTION CHAIN ON THE SLOVENIAN COASTLINE

Mateja Ambrožič[1], Urška Henigman[2], Mojca Jevšnik[3], Andrej Kirbiš[2], Peter Raspor[1]

[1]Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, [2]Institute for Food Hygiene, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, [3]Department of Sanitary Engineering, Faculty of Health Sciences, University of Ljubljana, Zdravstvena pot 5, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: peter.raspor@guest.arnes.si

Summary: Noroviruses (NoVs) and Hepatitis A virus (HAV) are significant agents of foodborne human viral illness worldwide, both as agents of greatest concern from the consumption of shellfish. In the framework of official national monitoring shellfish samples have been collected since 2013 to determine the spread of NoVs and HAV. Contamination of shellfish samples with NoVs varied from 25% in year 2014 to 40% in year 2015. HAV was not found in any of the analyzed samples, which correlates with the low number of human infections in Slovenia. Alongside official surveillance, semi-structured interviews were carried out with local shellfish farmers regarding this subject. The qualitative analysis highlighted that Slovenian shellfish farmers are aware of food safety hazards, especially associated with hazards to primary production, but only in the context of *Escherichia coli* as an indicator microorganism, and not in the concept of viral food safety. Despite detected foodborne viruses in shellfish on the Slovenian market, local farmers are not aware of or do not recognize foodborne viruses as distinctive food safety hazards. It can be concluded that local farmers possess knowledge and information of critical points in the mussels food supply chain connected to food safety in general. However, in particular, they are not familiar with viruses that represent barrier and consequently critical point to food safety management practices. Training and education on all important aspects of viral food safety according to the current recommendations is strongly recommended for all actors within the shellfish supply chain.

Key words: mussel farmer; Slovenia; official control; food safety; foodborne viruses; semi-structured personal interviews

Introduction

Mariculture is a traditional activity in the Primorska region. Mediterranean mussels (*Mytilus galloprovincialis*) are the main harvested species and, of a smaller quantity, also warty venus (*Venus verrucosa*). Mussel farming takes place in a standard manner in lines of floating buoys linked together, with longline nets hung from them. In Slovenia, within 46.6 km of coastline there are three official harvesting areas of Mediterranean mussels: Seča, Strunjan, and Debeli rtič, with 56 registered shellfish farmers on a total area of 46 ha. In 2013, 231 persons were involved in aquaculture activities in Slovenia, and only 39 persons were involved in marine fish and shellfish farming. The aquaculture sector in Slovenia is mainly characterized by small self-employed family farms, most of which have one employee, and some are assisted by unpaid family members. Total shellfish production in 2013 was 311 tonnes, and current production covers mainly the needs of the domestic market. The major cultured shellfish species, Mediterranean mussel, accounts

Received: 20 November 2015
Accepted for publication: 10 May 2016

for 83 percent of total mariculture production in Slovenia (1, 2). Next to that there were about 153 tons of imports of mussels in Slovenia but only 23 tons of export, what is a negligible quantity in alimentation compared to consumption of fresh fruit and vegetables, which are also recognized as vulnerable to viral contamination. Yet, the level of shellfish consumption by country is very different. In some countries, the mussel consumption per capita is over 3 kg per year, while it is not even part of the local diet in others (3). Per capita consumption of fresh fruit in Slovenia was in 2013 75 kg and 73 kg of fresh vegetables. However, there were no contaminated samples with NoVs or HAV of fresh produce found within Slovenian national monitoring (27). Shellfish specialties are commonly part of the culinary and gastronomic specialties only along the Slovene coast and are generally prepared and served in restaurants.

The filter-feeding nature of bivalves and the traditional way of consuming them (often raw or slightly cooked) make shellfish one of the most common vehicles of viral foodborne illness. Shellfish are filter-feeding animals, which ingest and accumulate human pathogens (5). Lees (6) reported that shellfish grown in sewage-polluted waters tend to bio-accumulate environmentally stable enteric viruses. Processing interventions such as depuration do not eliminate viral particles (7), and food consumption practices increase the health risk related to shellfish consumption. The increasing amount of data on virus detection in shellfish (8, 9, 10) and shellfish-borne viral outbreaks (11) indicates the necessity of a constant surveillance system in European countries, including Slovenia (12). The management of the harvesting areas continues to rely uniquely on bacterial standards, such as *Escherichia coli*, despite the proven fact of being unreliable tools to indicate the viral presence in harvesting areas or to control the efficiency of the process, such as depuration (13, 14). From a virological point of view, shellfish safety continues to be a sanitation challenge and to protect consumers the EU strives towards establishing legislation on this matter.

With better diagnostic technology and investigative epidemiology, it is now accepted that enteric viruses are major contributors to foodborne disease as well. Enteric viruses are transmitted through contaminated food, but also in combination with person-to-person contact or through environmental contamination. They have been increasingly recognized a significant cause of foodborne disease, despite the measures already in place, mainly targeted at reducing bacterial contamination, because of the increasing consumption of ready-to-eat foods, raw and/or minimally processed shellfish, fruits, and vegetables. This is because products are often imported from areas lacking strict hygienic measures, they are often eaten uncooked, and they often come into contact with potentially contaminated animal manure, water, ice, human hands and surfaces from the "farm-to-table" continuum (10). Most foodborne viruses are more resistant (15) than bacteria to commonly used control measures, (e.g. refrigeration, freezing, pH, drying, UV radiation, heat, pressure, disinfection, etc.). There are currently no effective, realistic and validated risk management options to eliminate viral contamination prior to consumption without changing the normally desired characteristics of the food. Because of concerns about virus persistence during food processing, effective control strategies need to focus on the prevention of contamination. From the limited available information, foodborne viruses have a low infectious dose and are dispersed in stool or emesis in high numbers. Only a few viral/infectious particles are needed to cause an infection that may lead to illness (10, 15, 17).

Shellfish aquaculture is a marine-based industry that is affected by other land users such as tourism, recreation, forestry, agriculture, and urban development. In many cases, the public is unaware of the detrimental impact their activities have on the aquaculture sector and, consequently, also on shellfish food safety. Food safety embraces the absence or acceptable and safe levels of contaminants, adulterants, naturally occurring toxins or any other substances that can make food dangerous to human health. Microbial food safety is considered a significant public health issue but historically has focused mostly on the control of bacterial contamination; however, enteric viruses have been increasingly recognized as an important cause of foodborne disease, and control measures are being developed (16, 17). The food supply chain from stable to table includes activities such as production, processing, distribution, retail, packaging and labeling of foodstuffs, which are governed by a mass of laws, regulations, codes of practice and guidance. Nowadays, the distance that food travels from producer to consumer has increased as a result of globalization in the food

trade. Moving these food products safely and efficiently from farm to fork requires a highly coordinated series of links in a long chain of trading partners. Food miles, as a term that refers to the distance food is transported from where it is grown or raised to where it is purchased by a consumer, is part of the broader issue of sustainability that deals with a large range of environmental, social and economic issues. Therefore, keeping safety and quality along the food supply chain has become a significant challenge, whereas good traceability systems, defined as the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution (18), help to minimize the production and distribution of unsafe or poor quality products.

Epidemiology of foodborne viruses

Although shellfish consumption can contribute to a healthy diet they are often associated with outbreaks of foodborne disease. Viral foodborne outbreaks associated with shellfish consumption have occurred in many countries (11) despite existing strategies to prevent contamination. They are often attributed to water contamination by sewage and/or during processing and serving. According to epidemiological evidence, NoVs as the predominant agents of nonbacterial gastroenteritis in humans along with Hepatitis A virus (HAV), both as agents of greatest concern from the consumption of shellfish, are important agents of foodborne human viral illness worldwide (17, 19, 20, 21, 22, 23).

Of the approximately 600 million cases of illness caused by foodborne hazards in 2010 worldwide, infectious agents that cause diarrheal diseases accounted for the vast majority (550 million), in particular noroviruses (120 million cases) and Hepatitis A virus 14 million cases (24).

A total of 5251 foodborne outbreaks were reported in 2014 in the EU (20) within the framework of member states' national monitoring. In 2014, food-borne viruses were, for the first time, identified as the most commonly detected causative agent in the reported food-borne outbreaks. 1070 food-borne outbreaks caused by viruses were reported in 2014, implicated 11740 cases, 2486 hospitalizations and 2 deaths. In strong-evidence outbreaks caused by viruses, 'crustaceans, shellfish, mollusks and products thereof' was the most commonly implicated food vehicle (44.7% of outbreaks), followed by 'buffet meals' (15.8% of outbreaks), 'mixed food' (13.2%) and 'fruit' and 'berries and juices' (both 5.3%). The place of exposure most frequently reported was 'restaurant, café, pub, bar, hotel´, followed by the household. Norovirus was the most commonly reported virus implicated in the strong-evidence outbreaks and accounted for 97.6% of cases.

National statistics on foodborne viral disease are not easily available and, where present, likely to reflect significant under-reporting (17), because there is a lack of systematic surveillance for foodborne viral disease (25). Considering the scientific opinion from the EFSA, RASFF notifications and results of official controls, since 2013 the Slovenian National Zoonoses Monitoring Programme has included food sampling for the presence of NoVs and HAV in live shellfish at the retail level and distribution of local and foreign origin, which are recognized as potentially zoonotic viruses (26). In Slovenia, the Zoonoses Monitoring Programme (27) has been conducted at the national level since 1985. It is designed for the systematic collection, monitoring, analysis and communication of data on the emergence of zoonosis, zoonotic agents, and related antimicrobial resistance and comprises the recently emerging zoonotic agents, including foodborne viruses. The ultimate purpose is to capture high-quality information about infections in humans as well as in animals and the contamination of foods, providing important information that is integrated across sectors. It should provide a fundamental basis for making public health decisions with actions for reducing the risks to public health, document the impact of an intervention, track progress towards specified goals, and elucidate the epidemiology of health problems.

The results obtained within Slovenian official national monitoring have shown that live shellfish, purchased at retail stores in Slovenia during independent sampling times throughout the year were contaminated with NoVs, but HAV was not found in any of the samples analyzed (Table 1), which correlates with the low number of human infections in Slovenia (Table 2). In studies, contamination of mussels´ samples varies from 16.9% (9), to 34.4% NoVs in Italy (28), to 35.0% of contaminated mussels in France (29). Henigman et al. (8) reported

Table 1: Presence of NoVs and HAV in live shellfish samples within Slovenian official surveillance

Year	Virus	
	NoVs	HAV
2013	5/17 (29%)	0/15
2014*	3/12 (25%)	0/12
2015*	3/10 (40%)	0/10

*Preliminary results

Table 2: Reported infections caused by NoVs and HAV in humans in Slovenia from 2007–2014 (27)

N° of cases	Year							
	2007	2008	2009	2010	2011	2012	2013	2014*
NoVs	1094	1043	1393	2012	2231	1611	2146	1316
HAV	15	17	12	9	12	11	23	10

*Preliminary results

that mussels collected in Slovenian coastal waters were contaminated with NoVs, the highest at Debeli Rtič (25.9%), 21.2% in Strunjan, and only 8.1% in the Seča harvesting area. The difference in positive results is interlinked to the location of harvesting areas due to sea current, dense shipping, and the influx of streams and rivers.

Control and prevention of foodborne viruses

Virus contamination as a consequence of human handling can occur at any stage of food production, processing, and even preparation. At present, we are faced with insufficient knowledge and awareness of food safety issues among food handlers and accompanied by consumers being insufficiently informed about food safety principles in the home.

Today, we manage food safety through good practices at different levels within a food supply chain that can be described as a network of food-related businesses involved in the creation and consumption of food products that move from farm to table and are linked by information, material, and capital flows. Good practices are described in several different codes of practice designed by producers' organizations, importers and retailer's consortia and government bodies at different levels of production, processing and consumption within the food supply chain. All current active practices are segregated along the food supply chain and are not connected to a comprehensive system, resulting in the existence of exposure to potential of food hazards, especially emerging hazards, such as viruses (12, 30).

The development in different areas within the food production chain and in particularly in technological and technical means is moving very quickly. We encounter innovations in materials, and supporting measures almost daily. Consequently, the gap between knowledge and skills is widening. We are willing to accept the paradigm that drifting is the most dangerous challenge in analytical instruments. However, it is also extremely influential in technological practices. It occurs side by side with "industrial blindness", which develops as a personal characteristic of employees who do not see particular items although they are commonly present in routine operations.

Consumers play an important role in the transmission of hazards, including viruses. Implemented viral food safety guidelines (12) are not purposely designed for informing consumers, although studies in recent years have highlighted gaps in food safety knowledge and some critical safety violations regarding food handling at home (31, 32, 33, 34, 35, 36). Consumer behaviour and attitudes toward food safety have shown that the levels of understanding, motivation and trust need to be further cultivated, and their training and informing due to changes in lifestyle and food

consumption patterns encouraged (30, 37, 38, 39).

EFSA reported (20) that viral foodborne outbreaks most frequently occurred in 'restaurant, café, pub, bar, hotel", followed by the household. However, outbreaks of foodborne illness occurring in private homes are less likely to be reported than those in commercial and public premises, and it is believed that infections attributed to private homes are three times more frequent than those attributed to canteens (40).

Food handlers also play an important role in the transmission of enteric viruses in the shellfish supply chain (16, 20, 41, 42), especially because shellfish specialities are generally prepared and served in restaurants.

During production, harvest and packaging preparation, food can become contaminated with viruses by food handlers or after contact with virus-contaminated water and surfaces. A major contributor to the spread of disease in food production is poor hygiene practices or being in contact with faecal material or vomit (15). Food handlers are unaware of controls specific to enteric viruses (16). That is why training on all important aspects of NoVs and HAV according to the recently developed Codex Alimentarius guidelines to control viruses in food is strongly recommended. The primary purpose of the codex guidelines for the control of viruses in food is to give guidance on how to prevent or minimize the presence of human enteric viruses in food, especially NoVs and HAV, and to emphasize that management strategies regarding foodborne viruses and associated illnesses should be different from those for bacterial pathogens.

In 2011 Poklar Vatovec with co-workers (42) carried out the research to evaluate the offer of shellfish specialities in Slovene Istria restaurants and to assess food safety knowledge and behaviour of food handlers in preparing shellfish dishes. Results indicated poor food safety knowledge regardless the education of food handlers. The origin of shellfish is important in ensuring food safety; and restaurants should be convinced of good raw meat to exclude foodborne poisoning. Therefore, shellfish should be bought only at registered plants, since these are under official supervision. However they observed that shellfish were not always bought at registered plants, but supplied from the so called illegal »black market«. Next to that it was also observed that employed personnel were hardly acquainted with HACCP principles which represent major food safety hazard. Cooking (at least 90 °C for at least 90 seconds) is a critical point for ensuring food safety. The survey showed that the mid temperature was measured by only 26.8% of the interviewees with formal education and 7.3% with informal education. The remaining did not perform this procedure or it was not known whether it was performed. The results of the survey demonstrated that only 4.9% of the interviewees, regardless their education, are familiar with the correct temperature for heat treatment of shellfish. Research also pointed out that food handlers employed in Slovene Istria restaurants have insufficient knowledge on storing temperatures, storing time and the adequate methods of storing shellfish.

Pilot study: Semi-structured interview with Slovenian mussel farmers

Pilot study illustration

In order to determine eventual connection between comprehension of viral food safety and the shellfish growing practices, the four semi-structured interviews were carried out with Slovenian mussel farmers. The interview guide covered the following topics:

- Factors responsible for food safety within the shellfish food supply chain,
- Conditions related to food safety hazards with an emphasis on foodborne viruses due to virus-commodity combination, which has been identified as one of the greatest public health concerns.

In this pilot study, an empirical grounding was important because an exploration of local farmers' viral food safety perceptions and their good hygiene practices, together with results from the National Zoonoses Monitoring Programme, outlined and gave insight into the current situation in Slovenia. The semi-structured interviews were chosen due to the sensitivity and complexity of the subject discussed.

The semi-structured interview started with questions concerning food safety in general. The first open thematic question was: "Tell me as much as you can about the importance of the mussels production process, and about the factors that could affect its food safety." Discussions continued with the questions: "Could you please

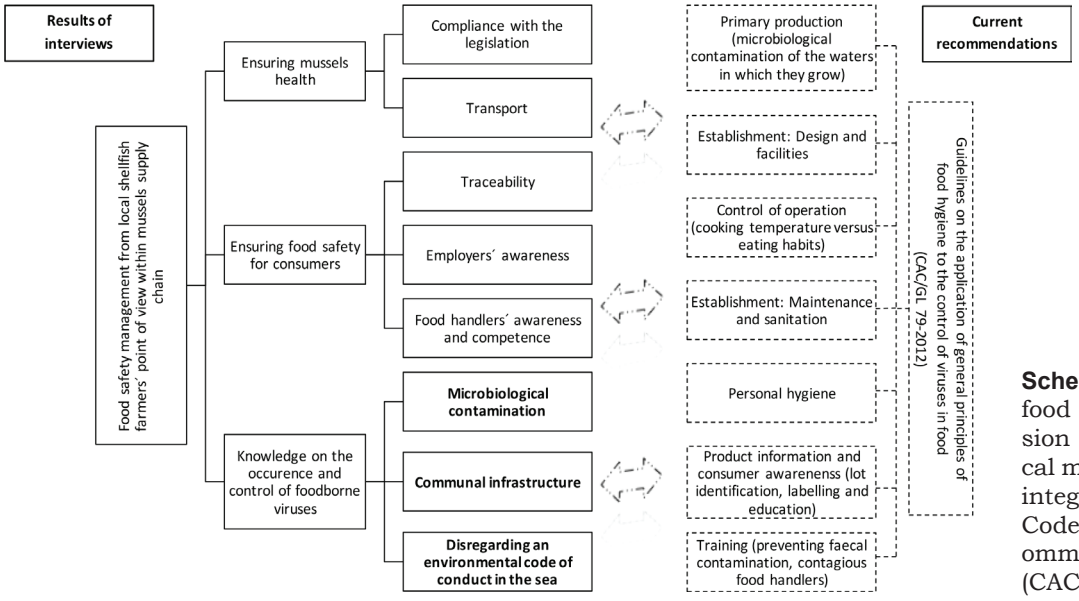
explain what food safety means from your point of view and when mussels are considered safe for a consumer?" and: "Have you ever heard about viruses that are transmitted by shellfish?" Follow-up questions were posed to complement and facilitate the dialogue. The discussions were concluded with the question: "Do you use working documents that have resulted from food safety legislation, like the HACCP plan, good practices that includes viruses?" The semi-structured interview ended with a question on whether the interviewee had something to add. Efforts were made to create trust, since issues of guilt and failure may easily arise. The results of national monitoring are supported by the responses recorded by the interviewers, which were clearly marked due to the assurance of anonymity. The letter "I" (I1) signifies "interview", while the number represents a running number of interviews.

The semi-structured interviews with the local mussel farmers were qualitatively analyzed, using the grounded theory method. This theory produces theoretical models of individuals' perspectives of a given phenomenon and the strategies they use to resolve or cope with the problem in a distinct and bounded context (43, 44, 45). Interviews were recorded using a Dictaphone and later transcribed. Transcripts of the interviews were analyzed by coding the statements of the respondents using identified notions. These notions were gathered into topic categories (44, 45, 46). The validity is justified by triangulation. Three researchers with different disciplinary basic knowledge and different experiences in research were included in the analysis and interpretation process.

Findings from semi-structured interviews

Eight topics (Scheme 1) were identified following text analysis of the respondents' answers during semi-structured interviews: compliance with the legislation, transport, traceability, employer's awareness, food handlers' awareness and competencies, microbiological contamination, communal infrastructure, and disregarding an environmental code of conduct in the sea. These topics were obtained after identifying the topics in the statements. The interviewee's answers were very guarded; consequently, more comprehensive data processing cannot be achieved.

With the intention to show comprehension within interviewee reflections, these identified topics (Scheme 1) were integrated with a specified section in Guidelines for the control of viruses in food (15) as follows: Primary production/Harvesting area; Establishment: Design and facilities; Control of operation; Establishment: Maintenance and sanitation; Personal hygiene; Product information and consumer awareness; and Training. Specific topics (microbiological contamination, communal infrastructure, and disregarding an environmental code of conduct in the sea) represent an unrecognized threat to



Scheme 1: Topics of viral food safety comprehension among Slovenian local mussel farmers and its integration within current Codex Alimentarius recommendations key points (CAC/GL 79-2012)

food safety in the mussels supply chain from viral point of view and are marked bold in the Scheme 1 due to their significance.

The identified topics are in accordance with the Codex Guidelines (15) sections, but not from the viral point of view, but in the context of bacterial contamination associated with hygiene practices. The results indicated that the respondents rarely comprehend viral food safety separately, but view it in different combinations with already obtained knowledge and skills. This can be demonstrated by the question "Are you familiar with viruses, which are transmitted by shellfish?", which yielded no answers dealing with foodborne viruses. With other questions, dialogue was maintained, but replies always approached local farmers' familiar topics in the field of food safety as ensuring cold chain management practices; microbiological contamination connected to biotoxines and *E. coli*, but *E. coli* in conjunction with gulls' and cormorants' excrement and as obligatory indicator microorganisms; and food handlers' awareness. This aspect is most obvious in the following statement:

Citation III (I3) […] A couple of years ago, biotoxins, now viruses […] we depend on water, because mussels grow themselves […] the biggest problem is the buoys […] in summer every buoy is covered with gulls' and cormorants' faeces, and you are not able to see the color of the buoy […] I was asking if this may be an *E.coli* reservoir, but they said no […]

Local farmers link food safety to employers' awareness and food handlers' awareness and competencies, which often intersect and obstruct food safety system implementation. They associated food safety with compliance with legislation and regulations and transport practices, which can be illustrated by answers to the question "What is important for food safety within the mussels food supply chain?". This aspect is the most obvious in the following statement:

Citation I (I1) […] if anything goes wrong, it can be seen immediately due to inspection control and traceability issues, because we export all harvested mussels to wholesalers […] Anyway, I would not sell mussels, which I would not give to my children to eat […]

Citation II (I2) […] I think that the problem is not only in growing conditions but also in the awareness of employees, especially in restaurants.

Respondents also pointed out the quality of growing waters and its linkage to the communal infrastructure and sewage discharge, and failures to comply with hygiene practices on the sea was also observed. This aspect is seen in the statement:

Citation IV (I3) […] heavy rainfall and storms may flush sewage overflow or farm run-off into the growing waters […]

Recreational and economic activities in the sea were also pointed out as hazards:

Citation V (I4) […] tourists are rascals and throw garbage and discharge sewage into the sea, even though they know that it is prohibited […] next to that problems are large transport ships, which are regularly present in the area with and river estuary […] we only exploit what nature offers to us, and the quality of growing waters is not solely under our responsibility.

This overview has clearly indicated that the development of new concepts is far from sufficient to enhance implementation in real practice. It is a fact that approximately one third of the live shellfish bought on the Slovenian market are contaminated with NoVs. The qualitative analysis alongside official surveillance highlighted that Slovenian mussel farmers are aware of food safety hazards in the mussel food supply chain connected to compliance with the legislation requirements. Despite detected foodborne viruses in samples bought on the Slovenian market, local farmers are not aware of or do not recognize foodborne viruses as distinctive food safety hazards, which represent barrier and consequently critical point to food safety management practices. Despite the fact that guidelines on viruses in food (15) are enforced are mostly unknown to professionals. There is a need to disseminate current guidelines as good viral food safety practice via food safety authorities and professional associations, chambers and societies, because we have demonstrated that even professionals in the field are generally unaware of its recommendations or even existence. Generally, despite a quite long tradition of aquaculture in Slovenia, there is no leading research institution dealing with fisheries and aquaculture. The research programmes are dispersed to different government and public institutions. Non-government institutions and farmers are only exceptionally included in research activities. Advanced level training in aquaculture is not well developed; consequently, shellfish farmers are thus more or less self-educated in accordance with the requirements of existing legislation.

Conclusions

The filter-feeding nature of shellfish and their tendency to concentrate any environmental or man-made contaminant present in their growing waters requires attention to these food safety issues and compliance with applicable requirements. As viruses do not grow in food, do not cause deterioration of the product, and the organoleptic properties of the food are not affected, it is questionable if control measures aiming at microbial growth inhibition are effective to reduce viral contamination. There is a need to assess whether the control measures in place for bacterial hazards require adjustments to be effective against viruses. For the time being, HACCP studies need to address prerequisite programs, such as good hygiene, agricultural and aquacultural practices, especially the origin and quality of water used in food supply chains, and adequate hand hygiene as the most effective prevention measure. Compliance with prerequisite programs, such as codex guidelines, is essential to reduce the risk of contamination. It is also beneficial to have the harmonized integration of monitoring and control to be able to routinely monitor that compliance measures are being undertaken effectively.

Food safety education is most effective when messages are targeted at changing the behaviors most likely to result in foodborne illness, such as personal hygiene, adequate cooking, avoiding cross-contamination, keeping food at safe temperatures, and avoiding foods from unsafe sources. Food safety education is most likely to be effective if the messages are targeted toward specific audiences. The results emphasize the need for tailored educational programs to improve awareness with respect to viruses and to implement innovations into good practices. Not just connected to hygiene, but even more to integrate it into comprehensive good aquaculture practice.

Acknowledgments

The authors thank the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection for the results of national monitoring used in the study.

References

1. Food and Agriculture Organization of the United Nations (FAO). National aquaculture sector overview: Slovenia. http://www.fao.org/fishery/countrysector/naso_slovenia/en (31. Mar. 2016)

2. Statistical Office Republic of Slovenia (SURS). Fishery and aquaculture. Ljubljana : SURS, 2016. http://www.stat.si/StatWeb/en/field-overview?idp=94&headerbar=0# (31. Mar. 2016)

3. Monfort MC. The European market for mussels. GLOBEFISH Research Programme: Vol. 115. Rome: Food and Agriculture Organization of the United Nations (FAO), 2014: 65 pp.

4. Kmetijski inštitut Slovenije. Poročilo o stanju kmetijstva, živilstva, gozdarstva in ribištva v letu 2013. Pregled po kmetijskih trgih. Ljubljana : KIS, 2016. http://www.kis.si/Porocila_o_stanju_v_kmetijstvu_OEK. (15. Mar. 2016)

5. Rippey SR. Infectious diseases associated with molluscan shellfish consumption. Clin Microbiol Rev 1994; 7(4); 419–25.

6. Lees D. Viruses and bivalve shellfish. Int J Food Microbiol 2000; 59(1/2): 81–116.

7. Loisy F, Atmar RL., Guillon P, et al. Real-time RT-PCR for norovirus screening in shellfish. J Virol methods 2005; 123(1): 1–7.

8. Henigman U, Biasizzo M, Vadnjal S, et al. Molecular characterisation of noroviruses detected in mussels (*Mytilus galloprovincialis*) from harvesting areas in Slovenia. New Microbial 2015; 38: 225–33.

9. Diez-Valcarce M, Kokkinos P, Söderberg K, et al. Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. Food Environ Virol 2012; 4(2): 73–80.

10. EFSA. Scientific Opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. EFSA J 2011; 9(7): e2190 (96 pp.) http://www.efsa.europa.eu/en/efsajournal/pub/2190

11. Bellou M, Kokkinos P, Vantarakis A. Shellfish-borne viral outbreaks: a systematic review. Food Environ Virol 2013; 5(1): 13–23.

12. Ambrožič M, Božič T, Jevšnik M, et al. Compliance of proposed Codex Alimentarius Guidelines for virus management with principles of good practice. Acta Aliment Hung 2011; 40 (3): 364–75.

13. Richards GP, McLeod C, Le Guyader FS. Processing strategies to inactivate viruses in shell-

fish. Food Environ Virol 2010; 2: 183–93.

14. Chalmers JWT, McMillan JH. An outbreak of viral gastroenteritis associated with adequately prepared oysters. Epidemiol Infect 1995; 115: 163–7.

15. CAC/GL 79-2012. Guidelines on the application of general principles of food hygiene to the control of viruses in food. Codex Alimentarius. International Food Standards. Rome : FAO ; WHO, 2012: 13 pp.

16. Verhoef L, Gutierrez GJ, Koopmans M, et al. Reported behavior, knowledge and awareness toward the potential for norovirus transmission by food handlers in Dutch catering companies and institutional settings in relation to the prevalence of norovirus. Food Control 2013; 34(2): 420–7.

17. Koopmans M. Foodborne viruses from a global perspective. In: Improving food safety through a one health approach: workshop summary. Washington : National Academies Press, 2012: 225–57.

18. Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Off J Eur Commun 2002; L 31: 1–24.

19. Centers for Disease Control and Prevention (CDC). Surveillance for foodborne disease outbreaks, United States, 2013, Annual Report. Atlanta, Georgia: US Department of Health and Human Services, 2015: 20 pp.

20. EFSA. The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2014. EFSA J 2015; 13(12): e4329 (191 pp) https://www.efsa.europa.eu/en/efsajournal/pub/4329

21. Kirk M, Ford L, Glass K, et al. Foodborne illness, Australia, circa 2000 and circa 2010. Emerg Infect Dis 2014; 20(11): 1857–64.

22. Scallan E, Hoekstra RM, Angulo FJ, et al. Foodborne illness acquired in the United States: major pathogens. Emerg Infect Dis 2011; 17(1):7–15.

23. Mead PS, Slutsker L, Dietz V, et al. Food-related illness and death in the United States. Emerg Infect Dis 1999; 5(5): 607–25.

24. Havelaar AH, Kirk MD, Torgerson PR, et al. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLoS Med 2015; 12(12): e1001923 (23 pp.). http://journals.plos.org/plosmedicine/article?id=10.1371/journal.pmed.1001923

25. Newell DG, Koopmans M, Verhoef L, et al. Foodborne diseases: the challenges of 20 years ago still persist while new ones continue to emerge. Int J Food Microbiol 2010; 139(1): S3–15.

26. Wilhelm B, Waddell L, Greig J, et al. A scoping review of the evidence for public health risks of three emerging potentially zoonotic viruses: hepatitis E virus, norovirus, and rotavirus. Prev Vet Med 2015; 119(1/2):61–79.

27. Program monitoringa zoonoz in povzročiteljev zoonoz 2015. Ljubljana : Ministrstvo za kmetijstvo, gozdarstvo in prehrano Republike Slovenije ; Ministrstvo za zdravje Republike Slovenije http://www.uvhvvr.gov.si/si/delovna_podrocja/zivila/zoonoze (25. Mar. 2016)

28. Suffredini E, Lanni L, Arcangeli G, et al. Qualitative and quantitative assessment of viral contamination in bivalve molluscs harvested in Italy. Int J Food Microbiol 2014; 184: 21–6.

29. Le Guyader F, Haugarreau L, Miossec L, et al. (2000). Three-year study to assess human enteric viruses in shellfish. Appl Environ Microbiol 2000; 66(8): 3241–8.

30. Raspor P, Ambrožič M, Jevšnik M. Food chain safety management systems: the impact of good practices. In: Yanniotis S, ed. Advances in food process engineering research and applications. New York : Springer, 2013: 607-25. (Food engineering series)

31. Wills WJ, Meah A, Dickinson AM, et al. I don't think I ever had food poisoning'. A practice-based approach to understanding foodborne disease that originates in the home. Appetite 2015; 85: 118–25.

32. Taché J, Carpentier B. Hygiene in the home kitchen: changes in behaviour and impact of key microbiological hazard control measures Food Control 2014; 35(1): 392–400.

33. Byrd-Bredbenner C, Schaffner DW, Abbot JM. How food safe is your home kitchen? A self-directed home kitchen audit. J Nutr Educ Behav 2010; 42: 286–9.

34. Jevšnik M, Hlebec V, Raspor P. Consumers' awareness of food safety from shopping to eating. Food control 2008; 19(8): 737–45.

35. 41. Jevšnik M, Hlebec V, Raspor P. Food safety knowledge and practices among food handlers in Slovenia. Food Control 2008; 19(12): 1107–18.

36. Jevšnik M, Hlebec V, Raspor P. Meta-anal-

ysis as a tool for barriers identification during HACCP implementation to improve food safety. Acta Aliment Hung 2006; 35(3): 319–53.

37. Ergönül B. Consumer awareness and perception to food safety: a consumer analysis. Food Control 2013; 32: 461–71.

38. Raspor P. Total food chain safety: how good practices can contribute. Trends Food Sci Technol 2008; 19(8): 405–12.

39. Raspor P, Jevšnik M. Good nutritional practice from producer to consumer. Crit Rev Food Sci Nutr 2008; 48(3): 276–92.

40. Scott E. Food safety and foodborne disease in 21st century homes. Can J Infect Dis Med Microbiol 2003; 14: 277–80.

41. Boxman ILA, Verhoef L, Hägele G, et al. Environmental testing for norovirus in various institutional settings using catering companies as sentinels for norovirus prevalence among the general population. Food Control 2015; 47: 98–102.

42. Poklar Vatovec T, Jakus T, Bizjak M. Seashell specialties and food handling in Slovene Istria restaurants. Acta Agric Slov 2014; 104(2): 81–90.

43. Stave C, Törner M. Exploring the organisational preconditions for occupational accidents in food industry: a qualitative approach. Safety Sci 2007; 45, 355–71.

44. Strauss LA. Qualitative analysis for social scientists. Cambridge : University Press, 1987: 319 pp.

45. Glaser B, Strauss A. The discovery of grounded theory: strategies for qualitative research. Chicago : Aldline, 1967.

46. Hollway W, Jefferson T. Doing qualitative research differently: free association, narrative and the interview method. London, Thousand Oaks, New Delhi : Sage, 2003: 166 pp.

OKUŽBA Z VIRUSI V PROIZVODNO-OSKRBOVALNI VERIGI ŠKOLJK NA SLOVENSKI OBALI

M. Ambrožič, U. Henigman, M. Jevšnik, A. Kirbiš, P. Raspor

Povzetek: Med živili, ki so pogosto povezana z norovirusnimi izbruhi in izbruhi hepatitisa A svetovnih razsežnosti, so tudi školjke. V okviru uradnega nadzora v Sloveniji od leta 2013 vzorčimo školjke, pri katerih se ugotavlja prisotnost norovirusov in virusov hepatitisa A. Prisotnost norovirusne RNK je bila ugotovljena v 25 % testiranih vzorcih v letu 2014 do 40 % v letu 2015. Prisotnosti virusov hepatitisa A ni bilo možno potrditi v nobenem od analiziranih vzorcev, kar povezujemo z nizkim številom okužb pri ljudeh v Sloveniji. Poleg ugotavljanja prisotnosti norovirusov in virusov hepatitisa A v školjkah smo opravili tudi polstrukturirane intervjuje s slovenskimi školjkarji. Kvalitativna analiza je razkrila, da se slovenski školjkarji zavedajo možnih tveganj na področju gojenja školjk, ampak samo v povezavi s prisotnostjo bakterije *Escherichia coli* kot indikatorskega mikroorganizma in biotoksinov. Kljub ugotovljeni prisotnosti norovirusne RNK v školjkah, prisotnih na slovenskem tržišču, lokalni školjkarji ne prepoznajo virusov kot dejavnikov tveganja, pomembnih za zagotavljanje varnosti živil. Ugotovitve kažejo, da se lokalni školjkarji zavedajo možnih mikrobioloških tveganj na področju gojenja školjk. Vendar pa kljub temu ne prepoznajo virusov kot možnih dejavnikov tveganja, kar izpostavi pomembnost kontinuiranega, rednega usposabljanja in izobraževanja pri obvladovanju virusnih okužb v proizvodni in oskrbovalni verigi školjk.

Ključne besede: školjkar; Slovenija; uradni nadzor; varnost živil; virusi v hrani; polstrukturirani pogovor

Slov Vet Res 2016; 53 (4): 205-10
UDC 579.62:617.711-002: 639.112

Original Research Article

EVALUATION OF THE CONJUNCTIVAL BACTERIAL FLORA IN 140 RABBITS (*Oryctolagus cuniculus*) FARMED IN SICILY ISLAND

Michela Pugliese[1], Filippo Spadola[1], Manuel Morici[1], Antonino Piazza[1,2], Giulia Caracappa[2], Maria Flaminia Persichetti[2], Alessandra Lommi[1]

[1]Department Of Veterinary Sciences, University Of Messina, Polo Didattico dell'Annunziata, 98168, Messina, [2]Istituto Zooprofilattico Sperimentale Della Sicilia A. Mirri, Via G. Marinuzzi 4, Palermo, Italy

*Corresponding author, E-mail: mpugliese@unime.it

Summary: Normal conjunctival flora of animal eyes comprises of both bacterial and fungal organisms. Bacterial and fungal species isolated from healthy conjunctiva appear to vary with geographic location, as well as is influenced by age, sex, housing, and climate. The aim of this work was to evaluate the bacterial flora of the conjunctiva of rabbits from intensive livestock farming in Sicily Island, comparing differences in the isolation of bacteria, and evaluating the potential zoonosis risk for humans related to the bacterial species isolated. 140 rabbits of California and New Zealand breeds were examined, while 280 eyes swab were obtained. Isolation techniques, biochemical and enzymatic tests were performed. Statistical analysis performed shown that the zoonotic risk is statistically not significant. A 4 % of *Moraxella spp.*, 9 % of *Staphylococcus spp.*, 5 % of *Pasteurella multocida* and 9 % of *Staphylococcus aureus* were isolated on eye swabs. In the examined area may be excluded a human-health risk related to isolated pathogens, both because of low percentage of isolations and for small number of involved farms. Further investigations are necessary to continuous monitor the related epidemiological risk.

Key words: rabbit; microbiology; eye; Sicily Island

Introduction

A poorly defined microbial and mycological flora is present on domestic animals conjunctival mucous membranes. These microorganisms have an important role in defending ocular mucous membranes, by competing with pathogenic species and by limiting their capability to colonize ocular surface. Changes of the microbial equilibrium creates favourable conditions for the development of pathologic process (1). Under normal conditions, microbial flora of the conjunctival sac is wide-ranging in relation to several factors, including geographical area and climate conditions, season, environmental hygiene, species, and specimen's immune system or performed medical treatments. Knowing the composition of the normal conjunctival flora during physiological conditions helps to recognize possible abnormalities. Gram-positive and Gram-negative bacteria are the most common microorganism isolated in most studies focused in ocular microbiology. Pathogenic bacteria frequently isolated by ocular surface of various domestic species are *Staphylococcus sp.*, *Streptococcus sp.*, *Corynebacterium sp.* and *Rhodococcus sp.*, *Listeria*

Received: 15 February 2016
Accepted for publication: 9 July 2016

monocytogenes, and other Gram-negative bacteria such as *Pasteurella multocida, Pseudomonas sp.*, and *Moraxella sp* (2-7).

Bacterial flora show a discrepancy within geographic variables; therefore conclusions from others research performed in rabbits from different area, cannot be related to rabbits farmed in southern Europe, and particularly in an island. To authors' knowledge, no studies have been performed to evaluate physiological conjunctival flora of farmed rabbits in Sicily. The aim of this research is to evaluate the bacterial flora of the conjunctiva of rabbits from intensive livestock farming in Sicily Island, by comparing differences, the current literature, and therefore possible zoonotic risk.

Materials and methods

Animals

One hundred-forty (140) rabbits for meat production, of Californian and New Zealand breeds, have been included in this study (Table 1). Rabbits were sampled in eight (n=8) farms in Sicily Island. All of them were farmed in enclosed and climate-controlled barns. Rabbits were categorized into four groups according to production phases: fattened up rabbits (FR, ranging in age between 37 and 51 day, both male and female); bucks (ranging in age between 1 and 2 years); does (ranging in age from 7 months and 1.5 years); young does (ranging in age from 5 months to 1.5 years). Of the 140 examined rabbits, 59 were males (40 fattened; 19 bucks) and 81 females (24 does, 20 in fattening period; 37 young does). All examined rabbits were individually housed without litter except for the does; for does a litter with swarf was set a week before the expected date of parturition. Two days after parturition, litter was replaced with a new swarf, leaving just the fur used by the does to prepare the nest. Rabbits were exclusively feed with cubed feedings tuff. The temperature inside the shed ranged from 20/25 °C overnight, 25/30 °C during the day, constantly for the whole year. Humidity ranged from 33 % to 75 %, often depending on the external weather.

Before performing this the conjunctival swabs for each rabbit a medical data (race, gender, age, productive capacity, therapeutic treatments, type of litter used, kind of feeding, climate in the enclosures) were collected and a complete physical examination was performed. Upon complete physical examination, all sampled rabbits have good health conditions. Physical examination and sampling swabs were executed by an operator and an assistant, working in sterility. For each rabbits two eye-conjunctival swabs (1 right eye, 1 left eye, a total of 280 samples) were obtained. The swabs (Copan Transystem, Copan, Italy) were immediately placed in Amies transport medium (Dominique Dutscher , France) and then transported on ice to the Instituto Zooprofilattico Sperimentale "A. Mirri ", Infectious Disease Section, Palermo (Italy).

Bacterial isolation and identification

The swabs were smeared directly on plates containing solid media, AS (Blood Agar) and MSA (Agar salt and mannitol), and incubated at the temperature of 37 °C in aerobic conditions and with enriched atmosphere of 5 % CO_2. After 24 h of incubation, bacterial colonies growth. Most noteworthy colonies were placed in pure culture. Further the identification of genus and species by using several biochemical and enzymatic assays were performed. Gram stain, catalase test, oxidase test, test of mobility, test of KIA (Kligler Iron Agar), growth in AGAR MacConkey, test of coagulase :, API 20 NE, API Staph were performed in order to identify the microorganism. On isolated strains antibiogram was performed (using Kirby Bauer method).

Table 1: Number of sampled rabbit

	Male		Female			
	Bucks	FR	Does	Young Does	FR	Total in Farm
Total	19	40	24	37	20	140

Statistical analysis

Statistical analysis was performed. Normal distribution of the studied parameters was verified (Kolmogorov-Sminrov). Statistical analysis of the data was performed by applying one-way analysis of variance for repeated measures (ANOVA) and by performing a Duncan's multiple comparison tests in order to evaluate the impact of causal agents on groups "farm", "phases", and "microorganism". For statistical analysis software Prism v. 5.00 (Graphpad Software Ldt., USA, 2003) was used. All data were expressed as mean ± standard deviation (SD).

Results

A blood agar showed colonies of *Pasteurella spp.*. A Pure culture and identification of the genus by using biochemical and enzymatic tests was performed. Tests showed: Gram stain: negative coccobacillus; Catalase test: positive; Oxidase test: negative; mobility test: negative; Test KIA (Kligler Iron Agar): fermentation of glucose without gas production; Growth in Agar-MacConckey: no colonies growth; API 20 NE: identified *Pasteurella multocida*. In agar-blood plates, several colonies with smooth margins and regular cream white had grown, attributable to the genus *Staphylococcus* spp.; a seeding phase on MSA was performed. Preparation of pure culture and identification of genus and species by using biochemical and enzymatic tests were performed; test results showed: Gram stain: positive cocci; Catalase test: positive; Oxidase test: negative; mobility test: negative; coagulase test: positive. Further identification was performed in micro method by using API Staph that confirmed presence of *Staphylococcus aureus*. Again on Blood Agar, other colonies were smooth, with regular margins and creamy white, due to the genus *Staphylococcus spp*. A pure culture and identification of the genus by using biochemical and enzymatic tests was performed. Results showed: Gram stain: positive cocci; Catalase test: positive; oxidase test: negative; mobility Test: negative; coagulase Test: negative. Considering the negativity in this last test, it was excluded presence of *Staphylococcus aureus* and *Staphylococcus pseudintermedius*, so the subspecies remained undefined (ie *Staphylococcus spp.*). The second type of colonies present on Blood Agar, were several minute colonies of 1-2 mm in diameter, smooth, flat, brown, attributable to the genus *Moraxella spp.*. A pure culture and identification of the genus by using biochemical and enzymatic tests was performed. Results showed: Gram stain: negative coccobacillus; catalase test: positive oxidase test: positive; Test mobility: negative; API 20 NE: *Moraxella spp*. was identified.

Results of 280 samples were the following (Table 2): 9 positive for *Pasteurella multocida*, 6 tampons were from females (3 does and 3 young does) and 3 males (2 in fattening period and 1 buck); 16 positive for *Staphylococcus aureus*; 14 tampons were from females (6 in fattening period, 5 does and 3 young does) and two males in fattening period; 4 positive for *Moraxella spp.*, 3 from to females (does only) and 1 to males (only during fattening period); 9 positive for *Staphylococcus spp.*, 6 from females (4 young does and 2 does) and 3 from males (fattening period). Totally, in farmed rabbit 38/180 samples were positive.

Table 2: Positivity to bacteria in sampled rabbits distinguished by sex and breeding/fattening period

	Male		Female			
Bacteria	Bucks	FR	Does	Young Does	FR	Total
Pasteurella multocida	1	2	3	3	0	9
Saphylococcus aureus	0	2	5	3	6	16
Moraxella spp.	0	1	3	0	0	4
Spahylococcus spp.	0	3	2	4	0	9

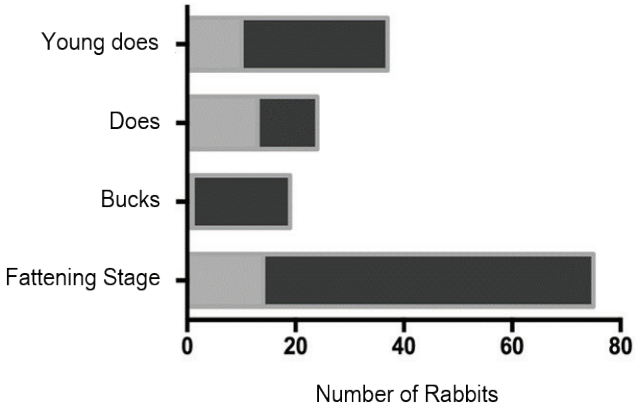


Figure 1: Relationship between positive and negative samples according to the type of farming. This chart shows that the number of samples obtained in rabbits fattening, was statistically lower compared to the negative

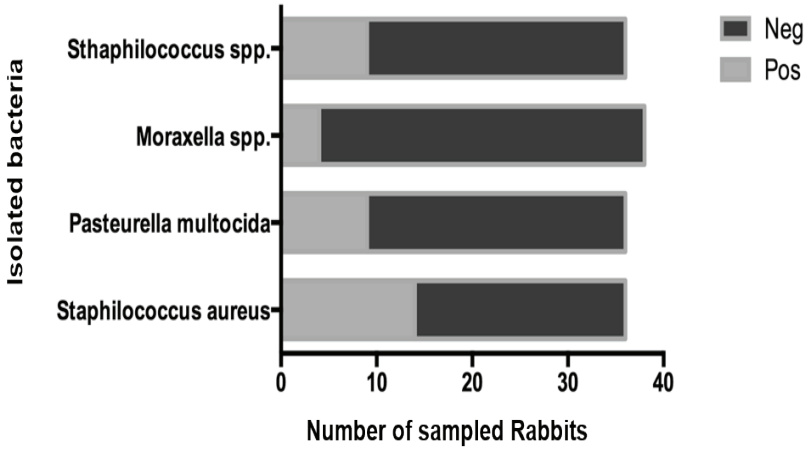


Figure 2: Number of positive and negative samples sorted by isolated bacteria. In the groups Moraxella and Staphylococcus spp. the number of positive samples was statistically lower than the negative

After grouping the obtained results, statistical analyses were performed and they showed a significant impact of the etiological agent (i.e. any of pathogens obtained in our investigation) on: Farm = $P < 0.05$; phases = $P < 0.05$; Single microorganism = $P < 0.05$. Duncan's multiple comparison test, revealed that, positive samples was statistically lower than negative. In addition number of positive samples was statistically lower than negative in the group of fattening rabbit (Fig.1). Finally, we could admit that the *Moraxella spp.* and *Staphylococcus spp.* positive samples were statistically lower than the negative (Fig.2).

Discussion

Gram positive organisms predominated in current study. The most frequently isolated Gram-positive bacteria were *Staphylococcus spp., Pasteurella spp.* and *Moraxella spp.*. *Staphylococcus spp.* has the highest isolation rate in our study, and as reported in literature is commonly found on health conjunctiva of most domestic animals (8-10). Our findings are not similar to the study carried out by Okuda and Campbell (11) on 54 New Zealand white rabbits where *Bacillus* species have been found as the most common isolated organism. However, they are just opportunistic pathogens and the most frequently isolated organisms in cases of canine bacterial conjunctivitis (12). The evolution of farming techniques and the emergence of antibiotic resistance over time have increased the spread of staphylococcal infections, especially in backyard flocks intensive cattle, sheep and goats, rabbits, equids and pigs (13-14). In recent years Foti et al. (15) have isolated methicillin-resistant *Staphylococcus aureus* from the healthy conjunctival sac of donkeys. *Staphylococcus aureus* MRSA, is an important cause of nosocomial and emerging infections. The widespread use of cephalosporin and fluoroquinolones on medicated feed in farmed rabbits may be promote in the coming years further spread of MRSA, with dangerous consequences for rabbits farming. Luckily in our study, this strain was

not encountered. In addition, our findings were compatible to a study performed on rabbits by Cooper et al. (16) in terms of species of bacteria. In Cooper et al. study *Staphylococcus* species were the most commonly recovered organisms, and other organisms isolated with less frequency were *Pasteurella* species, and *Moraxella* species. In contrast with this report, in present study *Micrococcus spp.*, *Bacillus spp.*, and other bacteria were not founded. Frequency of isolation was similar for *Pastorella spp.* (6 %) and *Moraxella spp.* (4 %). If compared with the finding in other farmed animals like pigs, results from our study is similar to the existent literature (17), which shown a high prevalence (78 %) of *Staphylococcus spp.* Unlike, a recent report in horses, shown a high presence of *Acinetobacter spp.* (18), but not isolated in our report. Regarding *Moraxella spp.*, they have been isolated from the healthy conjunctival sac of dogs, horses, cattle, goats, sheep (19-24). In Human *Moraxellaeceae* are normally was isolated at the level of the oropharynx, mucous membranes, skin and genital tract, and although the pathogenic and zoonotic potential of this bacterium has not been clearly documented, this possibility may occur (25). *Pasteurella spp.* are frequently encountered in rabbit farms, causing enormous economic damage direct and indirect (26); pasteurellosis is one of the most important diseases of the rabbit and this species may act as a carrier of the pathogen. Although *Pasteurella* infections in humans are still very rare, especially for children or for farms technician could be an significant issue (27).

From obtained data on our study, we can admit that since the limited number of isolations, the zoonotic risk is negligible despite isolated bacteria are potentially pathogenic to humans. Particularly in studied farms *Moraxella spp.* was found in 4 % and *Staphylococcus spp.* was isolated in 9 % of eye swabs, and *Pasteurella multocida* was revealed in 5% and *Staphylococcus aureus* was isolated in 9 % of eye swabs. However, must be admitted that in the examined area, sanitary risk related to the presence of these pathogens may be excluded. Further investigations are needed to increase a constant monitoring of the Island. Moreover, because of zoonosis are transmitted also through ingestion of infected meat, obtained data are encouraging, as we have recorded a prevalence of negativity in rabbits during fattening period in both farms.

References

1. Gould D, Papasouliotis K. Clinical microbiology and parasitology. In: Gelatt KN, Gilger BC, Kern TJ, eds. Veterinary ophthalmology. 5th ed. Ames : Wiley-Blackwell, 2007: Vol. 2: 300–50.

2. Urban M, Wyman M, Rheins M, et al. Conjunctival flora of clinically normal dogs. J Am Vet Med Assoc 1972; 161: 201–6.

3. McDonald PJ, Watson DJ. Microbial flora of normal canine conjunctivae. J Small Anim Pract 1976; 17: 809–12.

4. Barber DM, Jones GE, Wood A. Microbial flora of the eyes of cattle. Vet Rec 1986; 118: 204–6.

5. Moore CP, Heller N, Majors LJ, et al. Prevalence of ocular microorganisms in hospitalized and stabled horses. Am J Vet Res 1988; 49: 773–7.

6. Gionfriddo JR, Rosenbusch R, Kinyon JM, et al. Bacterial and mycoplasmal flora of the healthy camelid conjunctival sac. Am J Vet Res 1991; 52: 1061–4.

7. Espinola MB, Lilenbaum W. Prevalence of bacteria in the conjunctival sac and on the eyelid margin of clinically normal cats. J Small Anim Pract 1996; 37: 364–6.

8. Kakoma I, Gerding PA, Weisiger R, et al. Identification of Staphylococci and Micrococci from eyes of clinically normal dogs: relative frequency of isolation, b-lactamase production, and selected antimicrobial sensitivity profiles. Canine Pract 1993; 18: 11–7.

9. Prado MR, Rocha MF, Brito EH, et al. Survey of bacterial microorganisms in the conjunctival sac of clinically normal dogs and dogs with ulcerative keratitis in Fortaleza, Ceara, Brazil. Vet Ophthalmol 2005; 8: 33–7.

10. Wang L, Pan Q, Zhang L, et al. Investigation of bacterial microorganisms in the conjunctival sac of clinically normal dogs and dogs with ulcerative keratitis in Beijing. China. Vet Ophthalmol 2008; 11: 145–9.

11. Okuda H, Campbell LH. Conjunctival bacterial flora of the clinically normal New Zealand white rabbit. Lab Anim Sci 1974; 24(5): 831–3.

12. Gerding PA Jr, McLaughlin SA, Troop MW. Pathogenic bacteria and fungi associated with external ocular diseases in dogs: 131 cases (1981–1986). J Am Vet Med Assoc 1988; 193: 242–4.

13. Anderson ME, Lefebvre SL, Weese JS. Evaluation of prevalence and risk factors for

methicillin-resistant *Staphylococcus aureus* colonization in veterinary personnel attending an international equine veterinary conference. Vet Microbiol 2008; 129: 410–7.

14. Quinn PJ, Markey BK, Leonard FC, et al. Antibacterial resistance. In: Quinn PJ, et al., eds. Veterinary microbiology and microbial disease. Chichester, UK : Wiley-Blackwell, 2005: 157–64.

15. Foti M, Fisichella V, Giacopello C. Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in the microbial flora from the conjunctiva of healthy donkeys from Sicily (Italy). Vet Ophthalmol 2013; 16: 89–92.

16. Cooper SC, McLellan GJ, Rycroft AN. Conjunctival flora observed in 70 healthy domestic rabbits (*Oryctolagus cuniculus*). Vet Rec 2001; 149(8): 232–5.

17. Davidson HJ, Rogers DP, Yeary TJ, et al. Conjunctival microbial flora of clinically normal pigs. Am J Vet Res 1994; 55(7): 949–51.

18. Johns IC, Baxter K, Booler H, et al. Conjunctival bacterial and fungal flora in healthy horses in the UK. Vet Ophthalmol 2011; 14(3): 195–9.

19. Wilcox GE. Bacterial flora of the bovine eye with special reference to the Moraxella and Neisseria. Aust Vet J 1970; 46: 253–6.

20. Whitley RD, Moore CP. Microbiology of the equine eye in health and disease. Vet Clin North Am Large Anim Pract 1984; 6: 451–66.

21. Dagnall GJ. An investigation of colonization of the conjunctival sac of sheep by bacteria and mycoplasmas. Epidemiol Infect 1994; 112: 561–7.

22. Andrew SE, Nguyen A, Jones GL, et al. Seasonal effects on the aerobic bacterial and fungal conjunctival flora of normal thoroughbred brood mares in Florida. Vet Ophthalmol 2003; 6: 45–50.

23. Angelos JA. *Moraxella bovoculi* and infectious bovine keratoconjunctivitis: cause or coincidence? Vet Clin North Am Food Anim Pract 2010; 26: 73–8.

24. Galvão KN, Angelos JA. Ulcerative blepharitis and conjunctivitis in adult dairy cows and association with *Moraxella bovoculi*. Can Vet J 2010; 51: 400–2.

25. Epling J. Bacterial conjunctivitis. BMJ Clin Evid 2012; 2012: e 0704 (22 pp.)
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3635545/ (Jan. 2016)

26. Deeb BJ, DiGiacomo RF. Respiratory diseases of rabbits. Vet Clin North Am Exot Anim Pract 2000; 3(2): 465–80.

27. Nelson JD. Diseases acquired from pets. Pediatr Infect Dis 1983; 2(3): S56–60.

OCENA BAKTERIJSKE FLORE OČESNIH VEZNIC 140 KUNCEV (*Oryctolagus cuniculus*) GOJENIH NA SICILIJI

M. Pugliese, F. Spadola, M. Morici, A. Piazza, G. Caracappa, M.F. Persichetti, A. Lommi

Povzetek: V normalni flori veznice živalskih oči so bakterijski in glivični mikroorganizmi. Bakterijske in glivične vrste, izolirane iz zdravih veznic, se spreminjajo z geografsko lokacijo, nanje pa vplivajo tudi starost, spol, način reje živali in podnebje. Namen dela je bil oceniti bakterijsko floro veznic kuncev intenzivne reje na Siciliji ter primerjati razlike v izolaciji bakterij in ocenjevanju potencialnih tveganj za obolevanje ljudi, povezanih z najdenimi bakterijskimi vrstami. Pregledanih je bilo 140 kuncev kalifornijske in novozelandske pasme in tako je bilo pridobljenih 280 očesnih brisov. Opravljeni so bili izolacija mikroorganizmov ter biokemijski in encimski testi. Statistična analiza je pokazala, da je tveganje za pojav zoonoz zelo majhno. Iz brisov očes so bili izolirani v 4 % *Moraxella spp.*, v 9 % *Staphylococcus spp.*, v 5 % *Pasteurella multocida* in v 9 % *Staphylococcus aureus*. V proučevanem območju se lahko izključi nevarnost za zdravje ljudi, povezano z izoliranimi patogeni, tako zaradi nizkega odstotka izolacij kot majhnega števila vključenih rej. Nadaljnje raziskave so potrebne za nadaljevanje spremljanja, povezanega z epidemiološkim tveganjem.

Ključne besede: kunec; mikrobiologija; oko; Sicilija

Slov Vet Res 2016; 53 (4): 211-7
UDC 611.013:577.218

Original Research Article

DISTRIBUTION OF PRIMITIVE ENDODERM AND EPIBLAST LINEAGE SPECIFIC FACTORS IN LATE STAGE BLASTOCYSTS

Duygu Mutluay

Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Burdur, Turkey

*Corresponding author, E-mail: duygumutluay@gmail.com

Summary: Mouse preimplantation development leads to the formation of three distinct cell types by the blastocyst, namely, the trophectoderm (TE) which is an apolarized epihthelial layer, the inner cell mass which forms primitive endoderm, and pluripotent lineage epiblast. Segregation of the lineages is related to the expression of some key factors such as Cdx2, Oct4, Nanog, Gata4 and Sox17. We aimed to understand the role and distribution of these factors in mouse late blastocyst stage embryos. Two cell stage embryos were flushed from mated female B6D2F1 mice 44 hours after human chorionic gonadotropin injection. They were cultured in KSOM-AA medium at 37 °C with 5 % CO_2 in humidified air up to the late blastocyst stage. We performed immunofluorescent staining with antibodies specific to these factors. After that blastocysts were imaged and assessed using an fluorescence and confocal laser scanning microscope. We show that lineage specific factors, Gata4 and Sox17, were restricted to primitive endoderm while Nanog was restricted to epiderm lineage at late blastocyst stage. Moreover, the Oct4 protein is present in the nuclei in all cells but is strongly expressed in inner cell mass cells, whereas Cdx2 is localized only in TE cells. We conclude that lineage specific factors, Gata4, Sox17 and Nanog are essential for segregation of distinct lineages and blastocyst development.

Key words: blastocyst; epiblast; preimplantation; primitive endoderm; transcription factors

Introduction

Embriyonic development in mammals is initiated with a series of mitotic cell divisions and cleavages to generate blastomeres. After the end of the many rounds of cleavages with compaction, an embryo with single fluid-filled large cavity is formed, and this embryo is specifically called blastocyst (1). In eutherian preimplantation development, two extraembryonic lineages, the trophectoderm (TE) and the primitive endoderm (PrE/hypoblast) are essential to support fetal development and survival (2). The TE, outer layer of cells that forms an epithelium to surround the blastocyst cavity, is segregated from the small cluster of cells called inner cell mass (ICM). While TE establishes the connection to the mother's uterus to initiate blastocyst implantation and differentiates into trophoblast to constract the placenta, the ICM is the source of cells that give rise to the fetus and additional extra-embryonic tissues (3, 4). Several transcription factors have been identified to be involved in TE and ICM development. The essential transcription factors are Oct4 (Pou5f1) and Cdx2, which are

Received: 3 March 2016
Accepted for publication: 21 June 2016

POU-domain and caudal-type homeodomain transcription factors, respectively. Oct4, a key regulator of pluripotency, is strongly expressed in ICM and Cdx2 is specifically expressed in TE cells (1). Oct4 protein is present in nuclei of the all cells declining gradually in TE by early blastocyst stage, while only ICM cells show intense Oct4 staining by the late blastocyst stage (3, 5). Cdx2 is essential for maintenance of the TE lineage in mouse blstocysts, and suppresses the ICM lineage formation (6, 7).

As the blastocyst cavity expands between E3.5 and E4.5, ICM needs to segregate into two distinct layers of PrE and epiblast (Epi). Cells of the PrE, as a morphologically-distinct layer, congregate on the surface of the ICM. PrE forms an epithelial layer positioned at the interface between the ICM and blastocyst cavity, and the pluripotent epiblast appears in the ICM (2, 8, 9). After the implantation of the embryo to the uterus the PrE is eventually forms the visceral and parietal endoderm layers which contributes the yolk sac. Visceral endoderm initially covers the outside of the epiblast prior to gastrulation, later it is displaced by the definitive endoderm. And ends up as the outer layer of the extraembryonic visceral yolk sac. The Epi gives rise to most of the cells of the embryo proper as well as amnion, allantois and the extraembryonic mesoderm cells that line the visceral yolk sac. (2, 10, 11). It is also known that PrE extraembryonic lineages are not only essential to perform nutritive functions but they also are sources of signals to the Epi to initiate axial patterning (2, 8, 10). During the post-implantation period, an embryo convert with two germ layers (ectoderm and endoderm) into three germ layers. This process that ectoderm, mesoderm and endoderm germ layers are established named gastrulation (12).

The homeodomain transcription factors Oct4 and Nanog are expressed in the ICM and are required for proper formation of the epiblast (13-16). Nanog is expressed in ICM at E3.5 and becomes epiblast specific at E4.5 blastocyst stage (6). These transcription factors are also essential to maintain the embryonic stem cell pluripotency and self-renewal (17). The specification and differentiation of the PrE rely on the expression of key transcriptional regulators, primarily those belonging to GATA, SOX, and HNF protein families. Nanog and Gata are SOX family transcription factors that are used to mark the Epi and PrE, respectively. Epi cells are labelled by Nanog, a homeodomain protein that is essential for the maintenance of pluripotency in mouse epiblast and embryonic stem cells (16). Sox17 is a member of SOX (SRY-releated high mobility group box) transcription factor family and is first detected within the ICM, and is subsequently restriced to the PrE epithelium. Artus et al. (2011) indicate that distribution of Sox17 look like the localization of zinc finger transcription factors Gata binding protein 4 and 6 (Gata6, Gata4) in E4.5 blastocysts (11). Gata6 and Gata4 are expressed in the extra-embryonic endoderm lineages, primitive endoderm and the PrE derivates; visceral endoderm and parietal endoderm (18, 19). Gata4 and Gata6 proteins have essential roles on differentation of embryonic stem cells into extra-embryonic endoderm (19). It has also been suggested that Sox17 is a transcriptional regulator, and functions in the differentiation of pluripotent cells toward the extra-embryonic endoderm (17).

With this study, we demonstrate and review the current knowledge about essential transcription factors for mouse embryo development and their roles on maintanence of pluripotency in mouse late stage blastocysts (E4.5).

Material and methods

Animals

B6D2F1 (C57BL/6 x DBA/2) mice were purchased from National Cancer Institute. The protocol for animal handling and use was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Hawaii. The animals were maintained and treated according to the regulations and guidelines of the Animal and Veterinary Service at the University of Hawaii and the Committee for the Update of the Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research of the National Research Council of the National Academies (8th ed., 2011).

Embryo Collection

B6D2F1 female mice 6-8 weeks old, were induced to superovulate by intraperitoneally injections of 5 IU of equine chorionic gonadotropin (PMSG) and human chorionic gonadotropin (hCG)

at 48 hours (h) apart. Female mice were mated overnight with fertile males of the same strain. Following morning, inseminated females were selected by the presence of vaginal plug indicated the 1st day (day 1) of pregnancy. At day 2, 44 h after hCG injection female mice were sacrificied by cervical dislocation and two-cell stage embryos were flushed from the dissected oviducts with FHM HEPES-buffered medium (MR-024-D;EMD Milipore) under the stereo-microscope. After that, embryos were cultured in 20µl drops of KSOM-AA medium (MR-121-D;EMD Milipore) under mineral oil at 37 °C in a 5 % CO_2 humidified air incubator for the experiments.

Immunofluorescent Staining

Embryos were fixed in 4 % paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS) for 30 minutes (min) at room temperature. Embryos were subsequently permeabilized in PBS containing 0.5 % Triton X-100 for 15 min at room temperature. After blocking with 5 % bovine serum albumin in PBS containing 0.1 % Tween-20 (PBSw), samples were incubated in the primary antibody overnight at 4 ºC and embryos were incubated in secondary antibody for 2-3 h at 25 ºC. Primary antibodies used were, mouse anti-Cdx2 (1:200; Cdx2-88; BioGenex), goat anti-Gata4 (1:400; C-20, #sc-1237; Santa Cruz Biotechnology), goat anti Sox17 (1:100; S-20,# SC-17355), rabbit anti-Nanog (1:800; #RCAB0002P-F; Cosmo Bio) and goat anti-POU5F1 (1:200; N-19, #sc-8628; Santa Cruz Biotechnology), and Secondary antibodies (1:1000; Life Technologies) used were conjugated with Alexa Fluor 546 namely rabbit anti-mouse, donkey anti-goat and conjugated with Alexa Fluor 488, namely donkey anti-rabbit, rabbit anti-goat. Stained samples were mounted in ProLong Gold antifade reagent containing 4',6'-diamidino-2-phenylindole (DAPI; Life Technologies) (2).

Microscopy and Image Analysis

Embryos were imaged using an Axiovert 200 fluorescence microscope (Carl Zeiss) and FV1000 confocal laser scanning microscope (Olympus). For confocal microscopy, serial optical sections were imaged at 2 µm intervals under a 40x objective lens with oil.

Results

Expression of Cdx2 and Oct4 proteins in blastocyst stages of mouse embryos

Cdx2 expression is known as a marker of TE and TE precursors is absent from ICM (6, 20). Moreover, Oct4 is regarded as a marker of pluripotent cells, and is also expressed in TE cells of mouse blastocysts (3). We firstly examined Cdx2 and Oct4 protein expression of blastocysts in different embryos by immunofluorescence staining. Strong Cdx2 protein localization persisted in the nuclei of the TE cells (Figure 1A) while Oct4 protein localization was seen in both TE and ICM cells (Figure 1B). We next analized the Oct4 and Cdx2 expression in the same embryo. TE cells showed clear nuclear Cdx2 staining whereas Oct4 expression was revealed in nuclei of all the cells in blastocyst. Moreover, comparison of mean fluorescence intensities of Oct4 within the nuclei of ICM and TE cells revealed that ICM cells showed intense Oct4 staining (Figure 1C). We did not observe co-existence of Cdx2 within the ICM cells, suggesting that Cdx2 is localized exclusively in the nuclei of TE and is a TE specific transcription factor (Figure 1C). We also noted the same results in the literature that Cdx-2 staining cells in blastocysts were trophectoderm while the ICM was positive only for Oct4 (21).

Expression of lineage-specific transcription factors for PrE and Epi Tissue Segregation

To elucidate the PrE and Epi tissue segregation, we assessed the localization of the EPI-specific homeobox transcription factor Nanog and the PrE-specific factors Gata4 and Sox17 during this period (9, 22). To determine the expression domain of these transcription factors, E4.5 stage blastocysts were immunostained with specific antibodies and imaged using confocal and immunflorescence microscopy. We confirmed that E4.5 blastocysts had an ICM whose cells were clumped and consisted of two distinct PrE and Epi cell lineages. PrE cells that are in contact with the blastocyst cavity, form the superficial layer of the ICM and while Epi cells form the deeper layer of ICM cells (Figure 2 and 3).

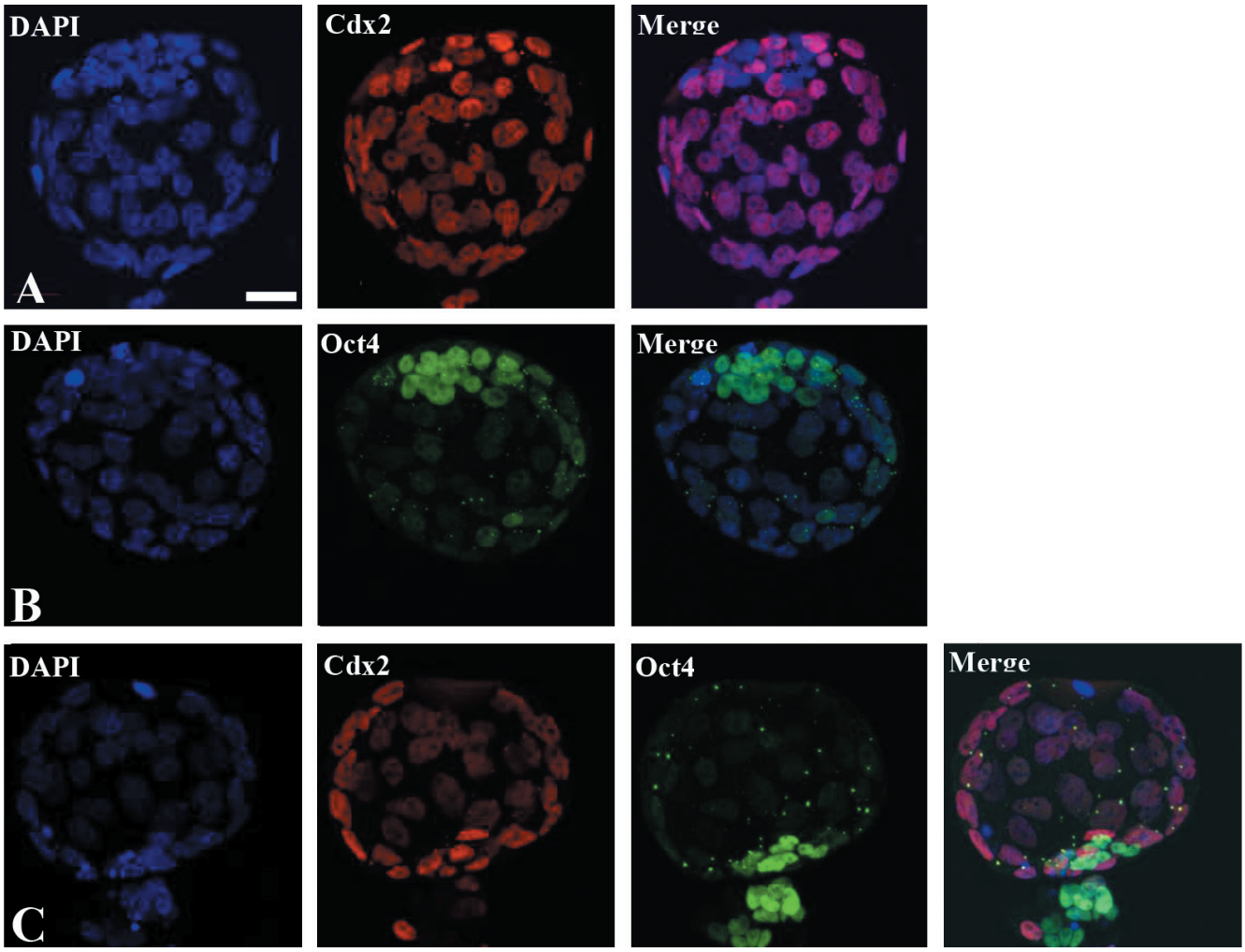


Figure 1: Localisation of transcription factors, Cdx2 and Oct4 in blastocyst

Embryos were imaged by confocal microscopy. (A)-TE cells were immunostained with Cdx2 (red) protein. (B)- Blastocyst was immunostained for pluripotency marker Oct4 (green) protein. (C)- Blastocyst was double labeled for Cdx2 (red) and Oct4 (green). Oct4 was predominantly located in the ICM, but some weak Oct4 staining was detected in the TE and Cdx2 is exclusively expressed in TE with a clear lineage segregtion (A-C) Nuclei were stained with DAPI (blue). Confocal images are z-series projections. Scale bar represents 20μm.

By the E4.5 blastocyst stage, nuclei of the Epi cells were positively stained with Nanog protein. We demonstrate that Gata4, a zinc-finger-containing transcriptional regulator, and Sox17 were localised in the nuclei of the PrE, the ICM cells immediately adjacent to the blastocyst cavity (Figure 2 and 3). These data revealed that Sox17 and Gata4 expression is specific to PrE cells while Nanog is specific to Epi cells. We also did not observe any coexpression of Nanog with Sox17 nor Gata4, suggesting that these factors are specific for PrE and Epi cell lineages and demonstrate salt and pepper distribution during late blastocyst stage.

Discussion

We detailed the formation of three distinct cell lineages by the late blastocyst: the pluripotent epiblast, trophoblast and primitive endoderm. We demonstrated that cells from late blastocyst stage express several key transcription factors to achive segregation of the lineages. We observed that the distribution of the Epi-specific transcription factor Nanog and the PrE-specific factors Gata4 and SOX17 during the late blastocyst stage. Expression of Nanog, Gata4 and Sox17 have previously been reported in late blastocysts (2, 5, 11, 17) and our

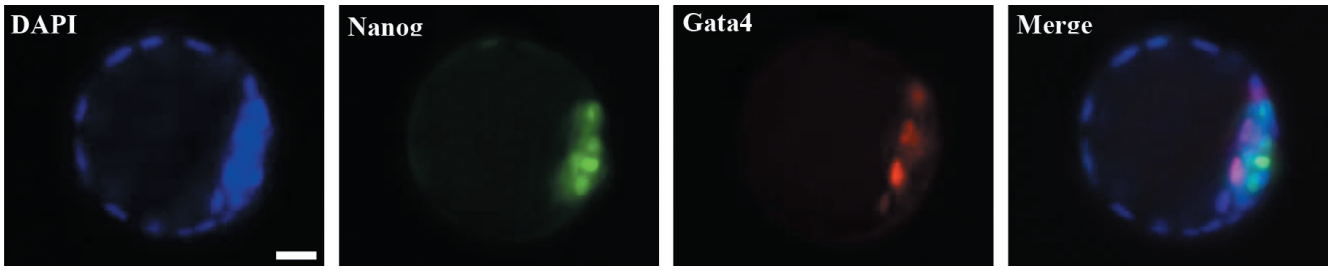


Figure 2: Salt and pepper distribution of Nanog and Gata4 in E4.5 blastocysts. Gata4 is expressed in PrE cells (green) and Nanog is expressed in Epi cells (red) . Nuclei were stained with DAPI (blue). Embryos were imaged by fluorescence microscopy. Scale bar represents 20µm.

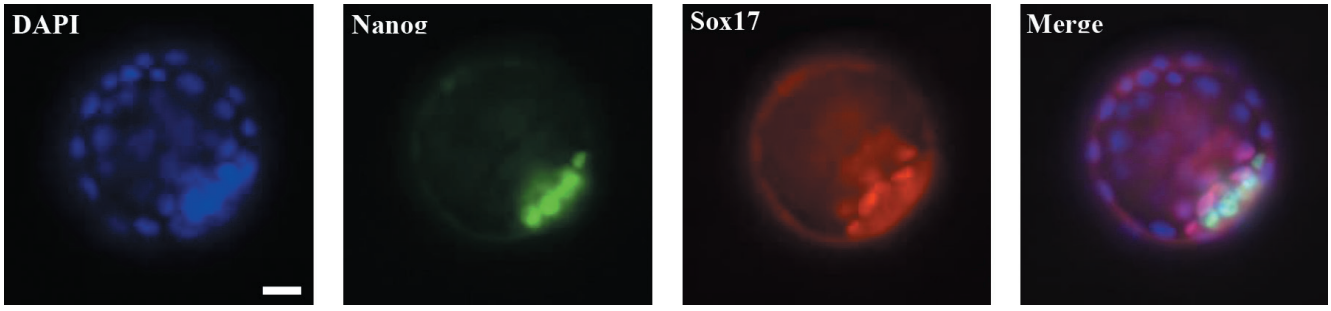


Figure 3: Sox17 and Nanog distribution pattern in E4.5 blastocysts. SOX17 is restricted to PrE epithelium and Nanog is restricted to Epi lineage. PrE marker (Sox17) is exclusive from the Epi marker (Nanog). Sox17 positive cells (red), Nanog-positive cells (green) and nuclei were labeled with DAPI (blue). Embryos were imaged by fluorescence microscopy. Scale bar represents 20µm

data correlate well with these (22). Our results revealed that Gata4 and Sox17 were restricted to PrE epithelium reported results while Epi marker Nanog was restricted to Epi lineage. We observed that cells expressing PrE and Epi markers, Gata4 and Sox17, Nanog, respectively are distributed in a salt and pepper pattern, showed that these lineages have a mutually exclusive distribution and these data are consistent with the cell sorting hypothesis (23, 24).

Several transcription factors are known to play important roles in TE and ICM fate. Pou5f1 deficient embryos, which encode Oct4, generate an ICM that expresses TE markers (13, 25, 26). Loss of Cdx2 embryo forms an expanded blastocyst including TE, but inability to sustain TE function and developmet. In addition, Oct4 is strongly expressed in the external cells of these embryos suggesting that Cdx2 is essential to repress the expression of Oct4 in TE (1, 6, 20).

By the E3.5, Cdx2 expression becomes restricted exclusively to the cells located on the outside TE cells, whereas Oct4 protein is present in all cells until late blastocyst stage, declining gradually in the TE thereafter (3, 5). The early restriction of Cdx2 expression indicates that Cdx2 is an essential factor for divergence of TE and ICM lineages (26). By the E3.5 stage, Cdx2 expression becomes restricted exclusively to trophectoderm (3, 20, 25). As expected, we demonstrated that Oct4 was present in nuclei of all cells, but stronger staining was associated with ICM cells and Cdx2 was localized exclusively to the nuclei of TE cells at E4.5 blastocyst stage.

The establishment of ICM and Epi relies on the interactions of transcription factors like Oct4, Nanog and Sox2 (13, 14, 16, 27). It has been demonstrated that Oct4, Nanog and Sox2 are also key players and promote embryonic stem cell pluripotency and self-renewal (17). Oct4 deficient embryos fail to form pluripotent ICM cells, which will differentiate into the extraembryonic trophoblast lineage (13). On the other hand, ablation of Nanog in ICM cells of embryonic

stem cells cause the loss of self-renewal and differentiation of endoderm-like cells (16, 28).

At the late blastocyst stage, extraembryonic endoderm is differentiated from the ICM to generate visceral and parietal endoderm. Gata4 and Gata6 have essential roles on differentiation of visceral endoderm. Moreover, Gata4 and Gata6 in embryonic stem cells are sufficient for controlling differentation program towards extra-embryonic endoderm (17, 19). It is known that Sox17 acts in the differentation of mouse embryonic stem cells toward the extra-embryonic endoderm (17, 29) and embryonic stem cells deficient in Sox17 fail to differentiate into extraembryonic cell types (17). Niakan et al. (2010) also demonstrate that Sox17 is a transcriptional regulator of differentation in embryonic stem and ICM cells (17).

In this study, we showed the distribution of Cdx2, Oct4, Nanog, Gata4 and Sox17 in blastocyst stage murine embryos. Our data showed that lineage specific factors Gata4, Sox17 and Nanog are expressed by PrE and Epi cells respectively, and these lineage specific factors, Gata4, Sox17 and Nanog play essential roles in regulating and maintaining the characteristics of each lineage in blastocyst stage embryos.

Acknowledgements

I would like to thank Dr. Vernadeth B. Alarcon for opening up her laboratory and providing all the equipments and antibodies to do this research.

References

1. Marikawa Y, Alarcon VB. Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. Mol Reprod Dev 2009; 76(11): 1019–32.

2. Laeno AMA, Tamashiro DAA, Alarcon VB. Rho-associated kinase activity is required for proper morphogenesis of the inner cell mass in the mouse blastocyst. Biol Reprod 2013; 89(5): 122.

3. Dietrich JE, Hiiragi T. Stochastic patterning in the mouse preimplantation embryo. Development 2007; 134(23): 4219–31.

4. Marikawa Y, Alarcon VB. Creation of trophectoderm, the first epithelium, in mouse preimplantation development. Results Probl Cell Differ 2012; 55: 165–84.

5. Palmeri SL, Peter W, Hess H, Schöler HR. Oct4 Transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. Dev Biol 1994; 166(1): 259–67.

6. Strumpf D, Mao CA, Yamanaka Y, et al. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 2005; 132(9): 2093–102.

7. Ralston A, Rossant J. Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo. Dev Biol 2008; 313(2): 614–29.

8. Chazaud C, Yamanaka Y, Pawson T, Rossant J. Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. Dev Cell 2006; 10(5): 615–24.

9. Plusa B, Piliszek A, Frankenberg S, Artus J, Hadjantonakis AK. Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. Development 2008; 135(18): 3081–91.

10. Rossant J. Lineage development and polar asymmetries in the peri-implantation mouse blastocyst. Semin Cell Dev Biol 2004; 15(5): 573–81.

11. Artus J, Piliszek A, Hadjantonakis AK. The primitive endoderm lineage of the mouse blastocyst: sequential transcription factor activation and regulation of differentiation by Sox17. Dev Biol 2011; 350(2): 393–404.

12. Tam PP, Behringer RR. Mouse gastrulation: the formation of a mammalian body plan. Mech Dev 1997; 68(1/2): 3–25.

13. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998; 95(3): 379–91.

14. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 2003; 17(1): 126–40.

15. Chambers I, Colby D, Robertson M, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 2003; 113(5): 643–55.

16. Mitsui K, Tokuzawa Y, Itoh H, et.al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003; 113(5): 631-42.

17. Niakan KK, Ji H, Maehr R, Vokes SA, Rodolfa KT, Sherwood RI, et al. Sox17 promotes differentiation in mouse embryonic stem cells by

directly regulating extraembryonic gene expression and indirectly antagonizing self-renewal. Genes Dev 2010; 24(3): 312–26.

18. Morrisey EE, Ip HS, Lu MM, Parmacek MS. GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. Dev Biol 1996; 177(1): 309–22.

19. Fujikura J, Yamato E, Yonemura S, et al. Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev 2002; 16(7): 784–9.

20. Niwa H, Toyooka Y, Shimosato D, et al. Interaction between Oct3/4 an and Cdx2 determines trophectoderm differentiation. Cell 2005; 123(5): 917–29.

21. Katayama M, Roberts RM. The effect of superovulation on the contributions of individual blastomeres from 2-cell stage CF1 mouse embryos to the blastocyst. J Dev Biol 2010; 54(4): 675–81.

22. Yamanaka Y, Ralston A, Stephenson RO, Rossant J. Cell and molecular regulation of the mouse blastocyst. Dev Dyn 2006; 235(9): 2301–14.

23. Rossant J, Chazaud C, Yamanaka Y (2003) Lineage allocation and asymmetries in the early mouse embryo. Philos Trans R Soc Lond B Biol Sci 2003; 358(1436): 1341-8.

24. Morris SA, Teo RT, Li H, Robson P, Glover DM, Zernicka-Goetz M. Origin and formation of the first two distinct cell types of the inner cell mass in the mouse embryo. Proc Natl Acad Sci U S A 2010; 107(14): 6364–9.

25. Ralston A, Cox BJ, Nishioka N, et al. Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. Development 2010; 137(3): 395–403.

26. Stephenson RO, Yamanaka Y, Rossant J. Disorganized epithelial polarity and excess trophectoderm cell fate in preimplantation embryos lacking E-cadherin. Development 2010; 137(20): 3383–91.

27. Madeja ZE, Sosnowski J, Hryniewicz K, et al. Changes in sub-cellular localisation of trophoblast and inner cell mass specific transcription factors during bovine preimplantation development. BMC Dev Biol 2013; 13: e32 (17 pp.) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3751447/ (Feb. 2015)

28. Pan G, Li J, Zhou Y, Zheng H, Pei D. A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. FASEB J 2006; 20(10): 1730–2.

29. Shimoda M, Kanai-Azuma M, Hara K, et al. Sox17 plays a substantial role in late-stage differentiation of the extraembryonic endoderm in vitro. J Cell Sci 2007; 120: 3859–69.

RAZPOREDITEV PRIMITIVNEGA ENDODERMA IN EPIBLASTNIH RODOVNO SPECIFIČNIH DEJAVNIKOV V POZNI FAZI BLASTOCIST

D. Mutluay

Povzetek: Mišji predimplantacijski razvoj vodi do tvorbe treh različnih tipov celic blastociste - trofektoderma (TE), ki je nepolarizirana epitelijska plast, notranje celične mase, ki tvori primitivni endoderm in pluripotentni epiblast. Ločevanje teh celičnih linij je povezano z izražanjem nekaterih ključnih genov oziroma prepisovalnih dejavnikov, kot so Cdx2, Oct4, Nanog, Gata4 in Sox17. Naš namen je bil raziskati vlogo in porazdelitev izraženosti teh genov v mišjih zarodkih v pozni fazi blastociste. Zarodki so bili izprani iz brejih samic miši B6D2F1 44 ur po injiciranju humanega horionskega gonadotropina. Do faze pozne blastociste so bili zarodki gojeni v mediju KSOM-AA pri 37 °C s 5 % CO_2 v navlaženi atmosferi. Izvedli smo imunofluorescenčno barvanje s protitelesi, specifičnimi za prej naštete gene oziroma beljakovine. Blastociste so bile opazovane in ocenjene s pomočjo fluorescenčnega in konfokalnega mikroskopa. Pokazali smo, da je bila izraženost rodovno specifičnih genov Gata4 in Sox17 omejena na primitiven endoderm, medtem ko je bil gen Nanog omejeno izražen v epidermu v poznem stadiju blastociste. Poleg tega je bila beljakovina Oct4 prisotna v jedrih v vseh celicah, vendar najmočneje v notranji masi, medtem ko je bil Cdx2 lokaliziran samo v celicah TE. Iz rezultatov sklepamo, da je rodovno specifična izraženost genov Gata4, Sox17 in Nanog bistvenega pomena za ločevanje različnih linij in razvoja blastociste.

Ključne besede: blastocista; epiblast; predimplantacija; primitivni endoderm; prepisovalni dejavniki

Slov Vet Res 2016; 53 (4): 219-27
UDC 636.7.09:615.849:616-007.271:617.559

Original Research Article

RADIOLOGICAL COMPARISON OF LUMBOSACRAL ANATOMY BETWEEN GERMAN AND BELGIAN SHEPHERD (MALINOIS) WORKING DOGS

Estera Pogorevc[1], Barbara Lukanc[1], Alenka Seliškar[1], Rok Pelc[2], Bojan Zorko[1]*

[1]Small Animal Clinic, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, [2]Department for Working Dogs, Police, Ministry of the Interior, Gmajnice 34, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: bojan.zorko@vf.uni-lj.si

Summary: The objective of this study was to assess the radiologic differences and the incidence of clinical and radiological signs of degenerative lumbosacral stenosis (DLSS) in 36 active working dogs; 24 German Shepherd (GSDs) and 12 Belgian Shepherd (Malinois; MNs). The medical record was evaluated and pertinent historical data recorded. Thorough clinical and neurological examinations were performed, as well as plain and contrast radiography (myelography) of caudal lumbal and sacral vertebrae. Thirty-three (92%) dogs were able to perform their duties without restrictions. Three (8%) dogs were excluded from active duty due to DLSS (2 dogs) or thoracolumbar disc disease (1 dog). Sixteen GSDs showed clinical signs of DLSS, and the most consistent finding was lower back pain (15 of 16; 94%). Radiological signs of DLSS were confirmed in 10 of them. The differences between GSDs and MNs were found in the bodyweight (GSDs>MNs; $p<0.001$) and anatomical conformation of the lumbosacral area, which was correlated with the incidence of DLSS. GSDs had significantly higher bodies of L7 ($p<0.001$) and S1 ($p<0.01$), higher L7/S1 step ($p<0.01$) and shorter CrS1/SL distance ($p<0.05$) than MNs. There was a significant association between DLSS and spondylosis deformans ($p<0.05$) and sclerosis of S1 cranial endplate ($p<0.05$). In MNs, no dog was radiographically confirmed for DLSS, although the age of dogs of both breeds was comparable. Fewer eventual radiological changes of the lumbosacral spine were also found in MNs. Regarding our findings, MNs seem to be more suitable for working dogs. The limitations of our study are the small number of MNs and the lack of MNs with DLSS. Our study confirmed radiographic differences of the lumbosacral junction between GSDs and MNs. Nevertheless, we could not confirm any radiographic parameter as a predisposing sign.

Key words: lumbosacral junction; radiography; myelography; working dogs; German Shepherd dogs; Belgian Shepherd dogs (Malinois); degenerative lumbosacral stenosis

Introduction

Cauda equina syndrome (CES) refers to a complex of clinical signs resulting from compression of cauda equina nerve roots. Degenerative lumbosacral stenosis (DLSS) is the most common cause of cauda equine syndrome reported in working dogs. German Shepherd dogs (GSDs) are the most commonly affected by clinical signs related to DLSS (1, 2, 3, 4, 5, 6, 7, 8, 9). To our knowledge there is no data published about incidence of DLSS in MNs. DLSS is the most common abnormality of the LS junction in dogs, particularly working GSDs (4, 6, 8, 9, 10). It is a multifactorial degenerative disorder resulting in stenosis of the spinal canal and compression of the cauda equina or its blood supply (4, 6, 8). Spinal cord diseases and CES in particular are common causes for early exclusion of working dogs (11, 12, 13).

The most common and typically earliest finding in dogs with DLSS is pain during palpation and hyperextension of the LS junction (10). The clinical

Received: 14 March 2016
Accepted for publication: 29 July 2016

signs of dogs that are affected by DLSS may show considerable variation related to the severity of compression of the cauda equina. The most common clinical signs are pelvic limbs lameness, abnormal gait, and caudal lumbar pain (3, 4, 5). Each dog can have one or more clinical signs.

Diagnosis is based on history, clinical and neurological assessment and the correlation of clinical findings and ancillary diagnostic imaging findings. Radiography, stress radiography and contrast studies (myelography, epidurography) may aid in ruling out pathological osseous changes of the LS region as cause of CES (14, 15, 16). Myelography represents a useful technique for assessing stenotic lesions in the lumbar spinal canal as well as detection of DLSS when a dural sac extends into the sacrum. The diagnostic sensitivity of myelography may be enhanced by using flexion-extension myelography of the lumbosacral junction (17)»=B.

Slovenian police GSDs and MNs are examined radiographically for hip and elbow dysplasia before accepted to working unit (18). To our knowledge, there are no published data elsewhere in the world regarding working dogs being regularly evaluated for spinal changes in lateral projection of the spine (ie. lumbal transitional vertebra (LTV), evaluation of LS conformation) before acceptance to the working unit.

The aim of this study was to radiographically assess LS area in working police GSDs and MNs and compare the radiographic (myelography) and clinical findings of DLSS. This study also attempts to identify whether radiography of whole lumbar and sacral area in lateral projection before acceptance to the working unit is of clinical relevance.

Materials and methods

Animals

Thirty-six working police dogs, 35 intact males and 1 intact female, were included in the study. Twenty-four of them were GSDs and 12 MNs. Mean age of the examined dogs was 68.3±33.5 months (76.6±31.9 months in GSDs and 51.8±31.3 months in MNs). All dogs were regularly used as patrol and attack dogs at a Slovenian police unit during the time of examination. The consent of the owner was obtained, and protocols were approved by the Veterinary Administration of the Republic of Slovenia (No. 34401-25/2010/3).

Evaluation of the dogs consisted of the observation of a handler, clinical and neurological examination and native and contrast (mielography) radiography. Final diagnosis of DLSS was based on clinical signs of pain and mielographically confirmed compression of cauda equina. Compression was pronounced with extension and relieved with flexion. In this study, dogs were assigned into three groups based on a dog breed and confirmed lumbosacral stenosis, namely 1) GSDs without DLSS(GSD/NOrDLSS; n=14), 2) GSDs with DLSS(GSD/rDLSS; n=10) and 3) MNs without DLSS (MN/NOrDLSS; n=12).

Study protocol

Handlers were asked about the general health status of their dog and any concurrent disorders diagnosed by their veterinarian. They were also asked about their dog's performance of expected duties and if they noticed any signs of pain, lameness or weakness (especially of the pelvic limb and tail). Each dog underwent a neurological examination that included assessments of attitude, posture and gait outside the clinic. Later on, the quality of conscious proprioception, spinal reflexes, the anal and tail tone were assessed. Deep palpation, lumbosacral hyperextension and hyperextension of the tail were performed to evaluate lumbosacral hyperesthesia. A physical examination, including auscultation of the heart and lungs, palpation of the peripheral pulse and blood sampling for evaluation of general health status before anaesthesia was performed.

A pre-sedation complete blood count, white cell differential count, and serum biochemistry profile including blood urea nitrogen, creatinine, total protein, albumin, glucose, sodium, potassium, chloride, alkaline phosphatase and alanine aminotransferase (data not shown) were determined to exclude underlying diseases.

Radiography

Following the neurological examination all the dogs underwent survey and contrast radiography (myelography) under general anaesthesia. Dogs were premedicated with methadone (Heptanon; Pliva, Zagreb, Croatia) 0.28-0.3 mg/kg

subcutaneously. General anaesthesia was induced with midazolam (Dormicum; F. Hoffmann-La Roche, Basel, Switzerland) 0.07-0.2 mg/kg and thiopental (Nesdonal, Merial, Lyon, France) 7.8-17.4 mg/kg given intravenously. After intubation with a cuffed endotracheal tube, anaesthesia was maintained with isoflurane (Forane; Abbott Laboratories, Baar, Switzerland) in 100 % oxygen, using a circle circuit.Radiographic images were taken by AXIOM Iconos R100, Siemens AG, Munich, Germany and films developed by CLASSIC E.O.S., Agfa, Munich, Germany.

First, a right lateral (RL) radiograph of the lumbosacral area with hind legs in a neutral position was obtained. After collecting cerebrospinal fluid (data not shown), a non-ionic contrast medium iohexol (Omnipaque 240 mgI/ml, Nycomed Inc, Princeton, NJ) 0.3–0.5ml/kg was injected into the subarachnoid space at the cisterna magna. Dogs were tilted at 15-20° with their heads up and the fluoroscopy/radiograph was taken immediately. When contrast reached the lumbosacral region, three more radiographs of the lumbosacral junction were obtained: 1) a RL with hind legs in neutral position, 2) a RL with hind legs in flexion, 3) a RL with hind legs in extension. In total, the radiographic examination included four lateral radiographs in three different positions. All radiographs were assessed by the same radiologist (BZ) for evidence of spondylosis, L7 and S1 endplate sclerosis, LTV and sacral osteochondrosis (SOC). The number of lumbar vertebra was counted in each dog in order to identify LTV (8 lumbar vertebra). Next, the ventral displacement of the sacrum in respect to L7, also named misalignment of L7 or L7/S1 step formation, was evaluated. It was defined as a distance between two lines: a first line was drawn along the dorsal aspect of the body of L7, and a second line, parallel to the first, was drawn at the height of the craniodorsal edge of the sacrum. The height of the caudal endplate of L7 and the cranial endplate of S1 were measured axially. At the same place, the height of the spinal canal at the level of L7 and S1 was measured, defined as L7/S1 spinal canal ratio. The extension of the dural sac over the lumbosacral junction was evaluated on contrast radiographs with hind legs in neutral position. All measured parameters are shown in Figure 1.

Cranially prolonged sacral lamina with or without sacral overhang could contribute to the compression of cauda equina, so the measurement of CrS1/SL distance was also made between the cranial endplate of S1 and cranial extent of the sacral lamina(19), as shown in Figure 2.

Statistical analysis

Data were analysed with the commercial software SPSS 22.0. (Chicago, Illinois, USA). Descriptive statistics was used to describe the basic features of the data. The association of the all three dog groups with anatomical conformation of the LS region was examined using a x^2-square test. The difference between MN/NOrDLSS, GSD/NOrDLSS and GSD/rDLSS in body weight, vertebral body height of L7, vertebral body height of S1 and CrS1/SLdistance was examined using a one-way ANOVA. Spinal canal height at L7, spinal canal height at S1 and L7/S1 misalignment were examined using a non-parametric Mann-Whitney U test. A Shapiro-Wilks test was used to test the normality. Statistical differences were considered significant with $p< 0.05$.

Results

History

Handlers of 12 of 36 (33%) dogs (11 GSDs and 1 MN) reported problems potentially associated with a lumbosacral disorder. These included mild lameness or occasional weakness of the pelvic limbs (10 dogs) difficulty jumping (6 dogs), and signs of lower back pain (4 dogs). Handlers of 33 of 36 (92%) dogs considered their dogs to be able to perform their duties without restrictions and 3 (all GSDs) dogs were reported to be on restricted duties (dogs were used as patrol dogs, but excluded from heavy attack training). Reason for restricted duty was DLSS in 2 dogs and thoracolumbar disc disease in 1 dog, all diagnosed previously.

Gait assessment

Of 36 dogs, posture and gait were normal in 20 (55%). Changes were seen intermittently in 8 (22%) dogs, persistent but mild changes (intermittent foot misplacement and toe knuckling, mild ataxia) were seen in 6 (17%) dogs, persistent and obvious (ataxia, uneven distribution of weight on the limbs, improper limb positioning, toe knuckling, occasional circling) changes in 2 (6%).

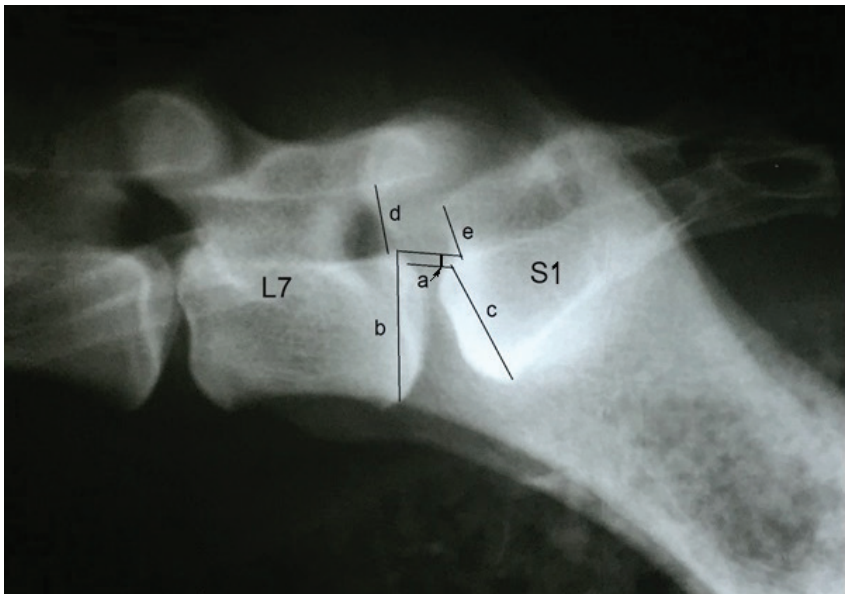


Figure 1: Schematic presentation of parameters of the lumbosacral junction measured on RL radiographs. L7- seventh lumbar vertebra; S1- first segment of sacrum; a- L7/S1 misalignment; b- L7 height; c- S1 height; d- spinal canal height L7; e- spinal canal height S1

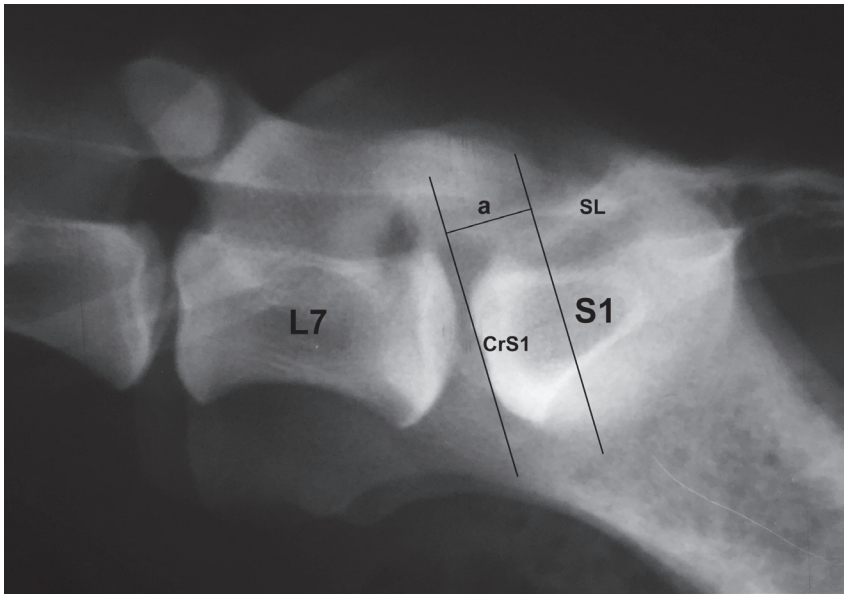


Figure 2: Schematic presentation of CrS1/SL distance on a RL radiograph. CrS1/SL distance (a) was defined as distance between the cranial endplate of sacrum (CrS1) and cranial point of sacral lamina (SL); L7- seventh lumbar vertebra; S1- first segment of sacrum

Clinical signs

Sixteen GSDs showed clinical signs of DLSS. On clinical exam, the most consistent finding was lower back pain (15 of 16), elicited by hyperextension of the lumbosacral junction (11 of 15; 2 GSD/NOrDLSS and 9 GSD/rDLSS), by digital palpation of the paraspinal muscles at the level of the lumbosacral joint (2 of 15) or by hyperextension of the tail (2 of 15). Digital palpation of paraspinal muscles was painful in 2 GSD/NOrDLSS and hyperextension of the tail was painful in two other GSD/NOrDLSS.

Neurological examination

The neurological exam was abnormal in 17 of 36 (47%) dogs, including 11 dogs with a history of pain or pelvic gait abnormalities. Abnormal findings included proprioception deficits (5 of 17; 2 GSD/NOrDLSS and 3 GSD/rDLSS), reduced withdrawal reflex (1 of 17; GSD/rDLSS) and gait abnormalities seen in 5 GSD/NOrDLSS and 5 GSD/rDLSS. Each dog could have one or more clinical signs. None of the MN/NOrDLSS showed any clinical abnormalities.

Table 1: Body weight and vertebral parameters at the level of L7-S1 in GSD/NOrDLSS, GSD/rDLSS and MN/NOrDLSS dogs. Data are presented as mean ± S.E.M.

		GSD/NOrDLSS (n= 14)	GSD/rDLSS (n= 10)	MN/NOrDLSS (n= 12)
BW [kg]		37.5 ± 1.2	36.0 ± 1.2	31.2 ± 0.8[a]
L7/S1 misalignment [mm] (rate)		0.5 ± 0.2	0.9 ± 0.3	0.1 ± 0.1[c]
incidence of L7/S1 misalignment [%]		43	70	8
CrS1/SL distance [mm]		8.1 ± 0.8	8.6 ± 1.1	10.5 ±0.4[c]
vertebral body height [mm]	L7	21.1 ± 0.4	21.3 ± 0.6	18.5 ± 0.3[a]
	S1	19.0 ± 0.5	19.6 ± 0.7	16.6 ± 0.3[b]
spinal canal height [mm]	L7	9.6 ± 0.2	10.2 ± 0.4	10.1 ± 0.3
	S1	7.1 ± 0.2	7.1 ± 0.4	7.3 ± 0.2

[a] p< 0.001; different than GSD/NOrDLSS and GSD/rDLSS
b p< 0.01; different than GSD/NOrDLSS and GSD/rDLSS
c p< 0.05; different than GSD/NOrDLSS and GSD/rDLSS

Survey and contrast radiography

The group of GSD/NOrDLSS consisted of 14 dogs without myelographically detected DLSS. Two dogs showed clinical signs of CES (both had painful hyperextension of the tail and one had mild gait abnormalities), but were radiographically normal. In this group, there was also one dog with LTV. Radiographic changes were seen in 5 dogs, none of them showing clinical signs. Spondylosis of lumbar area was found in 4 dogs. Mild sclerosis of the adjacent endplates was found in 4 dogs. The dural sac extended over lumbosacral junction in all GSD/NOrDLSS.

The group of GSD/rDLSS consisted of 10 dogs with radiographic/myelographic signs of lumbosacral stenosis. All of them showed clinical signs of CES; painful lumbar extension was observed in 8 dogs and painful extension of the tail in 5 dogs. Spondylosis of the lumbar area was found in 7 dogs. Sclerosis of the adjacent endplates was found in 8 dogs. The dural sac extended over the lumbosacral junction in 9 dogs. None of GSD/rDLSS dogs had a LTV. Cauda equina compression was greater at lumbosacral spine in extension in comparison to lumbosacral spine in flexion.

The group of MN/NOrDLSS consisted of 12 dogs without myelographically detected lumbosacral stenosis. None of them showed any clinical signs of CES. On the radiographs, none of them had spondylosis, and one of them had a LTV. There was mild sclerosis of S1 endplate, but not seen on the caudal endplate of L7. The dural sac extended over the lumbosacral junction in 10 dogs. Statistical analysis showed significant association between radiographically confirmed DLSS and the presence of 1) spondylosis ($p< 0.05$) and 2) sclerosis of S1 endplate ($p< 0.05$) in GSDs only. The presence of a dural sac extending over the lumbosacral junction, osteophytes of the articular processes, sclerosis of the L7 endplate or LTV were not associated with DLSS in GSDs. No dog in our study had radiographic evidence for SOC.

The incidence of L7/S1 misalignment was the highest in GSD/rDLSS dogs (70%), which also exhibited the largest maximal S1 displacement (GSD/rDLSS = 3 mm, GSD/NOrDLSS = 2 mm, MN/NOrDLSS = 1 mm). In line with this, GSDs had significantly higher L7/S1 misalignment ($p< 0.05$) and shorter CrS1/SL distance ($p< 0.05$) than MNs (Table 1). GSDs had significantly higher vertebral bodies (L7; $p< 0.001$, S1; $p< 0.01$) but not corresponding spinal canals at L7 and S1 than MNs (Table 1). GSDs were also heavier ($p< 0.001$) than MNs (Table 1).

Discussion

German Shepherd dogs and Belgian Shepherd dogs (Malinois) are the most common breeds used as working police dogs worldwide. A common cause for the exclusion of police dogs from working

units is CES (11, 12, 13). In our study, some dogs showed difficulty and reluctance to get up and to jump. The dogs seemed to have more problems with extension of the caudal lumbar spine than with flexion. This is probably due to the increase of cauda equina compression that occurs when the caudal lumbar spine is extended.

Dogs that are affected with DLSS may not simultaneously show all clinical signs, but caudal lumbar pain is usually predominant (3, 4, 5). Most often, pain arises as a result of compression of the nerve roots of the cauda equina, although other potential sources of pain include the lumbosacral disc and the articular facets (10). Pain is usually evoked during palpation and hyperextension of the lumbosacral junction, which is highly sensitive, with responses to painful stimuli in 91% to 100% dogs with DLSS (6). In our study 13 GSDs showed pain and 9 (69%) of them were later mielographically confirmed for DLSS.

During clinical examination, exerting pressure over the lumbosacral region, and hyperextension of the caudal lumbar spine and hip joint extension also evoked signs suggesting pain and discomfort in dogs with DLSS. The dogs were radiographed for absence of hip displasia before they were recruited as working dogs, so there is strong evidence that hip dysplasia was not the cause of pain.

DLSS commonly affects medium sized to large breed dogs, at a mean age of six to seven years (20) and occurs more often in male dogs (3, 4, 5). All dogs in our study were large breed dogs. The mean age of affected GSDs was 95.2 months (61 to 117months). Male-to-female predisposition from 1.7:1 up to 5:1(3, 5, 7), with a higher mean body weight for male dogs than for female dogs with DLSS, suggests that biomechanical loading plays a role in the pathogenesis (7). In our study, male dogs were overrespresented, so we cannot define male-to-female comparison. Nevertheless, our GSDs had significantly ($p<0.001$) higher body weight than MNs, which would support the suggestion about biomechanical loading as another predisposing factor for greater incidence of DLSS in GSDs than in MNs. Mainly large breed dogs with a high level of physical activity are predominately affected. The influence of the increased load is supported by the fact that DLSS is extremely rare in small dogs and cats and also in large dogs with less physical activity.

The incidence of the radiologically confirmed DLSS in GSDs in our study was 42 % which is higher than in previous reports (5, 7, 21). This supports the suggestion of genetic predisposition of GSDs to DLSS (5, 7). The overall incidence of DLSS in our study was 28% which is probably due to presence of only normal MNs.

Diagnostic investigation of CES begins with survey radiographs of the lumbosacral joint, to rule out bone-associated neoplasia, discospondylitis, trauma, and vertebral abnormalities (14). The next indication to take survey radiographs is to identify conditions which may predispose a dog for development of DLSS such as LTV segments and osteochondritis dissecans of the endplate of S1 or L7 (17, 22). Survey radiography (+/- mielography) as a widely available modality still has potential as a screening technique (6).

In our study, survey radiography revealed L7/S1 misalignment in 14 of 36 dogs (13 GSDs and 1 MN), 7 GSDs also had clinical signs. This is considered to be a sign of lumbosacral instability, although the size of the lumbosacral step formation does not always correlate with the clinical signs (3, 6, 7). In our study, lumbosacral step formation was no higher than 3 mm. It was previously reported that lumbosacral step formation is of clinical relevance only when higher than 4 mm (21). In contrast, Suwangkong et al. (2006) suggested that lumbosacral misalignment as low as 2 mm may be clinically relevant.

The height of the body of L7 ($p<0.001$) and the height of the body of S1 ($p<0.01$) were significantly greater in GSDs in comparison to MNs. In contrast, there was no significant difference between breeds when comparing the height of the spinal canal at the level of caudal L7 and cranial S1 endplate. These findings support the hypothesis of primary stenosis of the spinal canal in GSDs (1). The L7/S1 spinal canal ratio was less than 2 in all dogs in our study, the highest being 1.7. There were no significant differences between groups nor between breeds. Primary canal stenosis is assumed to be a hereditary disease in large breed dogs and may be a cause of CES (14, 20).

The only anatomical conformation that is correlated with DLSS is a LTV (22, 23). In our study, we diagnosed 2 of 36 dogs (5%) with LTV (1 GSD/NOrDLSS and 1 MN/NOrDLSS). A LTV is an abnormally formed vertebra between the last normal lumbar vertebra and the first normal sacral vertebra (22). While both symmetrical and asymmetrical LTV may be associated with DLSS, asymmetrical LTV results in a specific pattern of

DLSS consisting of unilateral protrusion of a disk and degeneration of the adjacent bone marrow (24). Lacking ventrodorsal or dorsoventral projection, we could not define whether it was symmetrical or asymmetrical LTV. None of the dogs in our study had SOC, which is in accordance with study of Scharf (25), although SOC was reported in over 30% of predominately male GSDs with CES and 6.4% in clinically normal GSDs (26).

Myelography is a useful diagnostic procedure in the assesment of the stenotic lesions of the spinal canal, but it depends on the extension of the dural sac over the lumbosacral junction. In large dogs the spinal cord ends at L6 and the dural sac extends further caudally (8). Myelography is not diagnostic when the dural sac ends cranial to the lumbosacral junction, when the sac is elevated from the vertebral floor or the compressive lesion is located in the lateral recess or the intervertebral foramen (6). DLSS can be detected when the dural sac extends into the sacrum, which is observed in 80% of dogs with DLSS (17). In our study dural sac extended over LS junction in 33 of 36 (91%) dogs. The diagnostic sensitivity of myelography may be enhanced by using flexion-extension position of the lumbosacral junction (17).

The CrS1/SL distance could play a role in development of DLSS. The greater the distance, there is less possibility of compression during extension. In our study GSDs had significantly ($p<0.05$) smaller distance and additionally higher lumbosacral step. This could lead to excessive stenosis during the extension of the spine and cause pain even in younger dogs or dogs without radiographically visible degenerative changes.

Advanced imaging procedures, as CT and MRI, are used for more exact evaluation of soft tissues of lumbosacral and foraminal area as well as for the evaluation of the facet joints geometry (6, 16, 27, 28). Due to their accuracy, they are very imporant in diagnostics as in surgical treatment planning, but so far they remain expensive and frequently not available everywhere. MRI provides excellent soft tissue contrast, so it appears to be superior in detection of spinal stenosis caused by soft tissue proliferation or recognition of early disc degeneration (16, 29, 30, 31). However, there is also lack of correlation between the severity of clinical signs and the severity of compression as determined even by MRI (30).

Regarding to our findings, MNs seem to be more suitable for working dogs. There were only 12 MNs included in this study, but they were approximately of the same age as GSDs (range from 15 to 119 months). Nevertheless, we did not confirm any clinical or radiographical signs of DLSS in this breed.

Slovenian police GSDs and MNs are examined radiographically for hyp displasia and elbow dysplasia before accepted into working unit. In addition, radiographic examination of whole lumbosacral spine would be recommended in order to determine LTV, although there was no correlation found in our study.

Acknowledgement

Author would like to thank Rok Korljan for his assistance and technical support during this study. This research was financially supported by the Slovenian Research Agency, program group P4-0053.

References

1. Ondreka N, Amort KH, Stock KF, et al. Skeletal morphology and morphometry of the lumbosacral junction in German shepherd dogs and an evaluation of the possible genetic basis for radiographic findings. Vet J 2013; 196(1): 64–70.

2. Indrieri RJ. Lumbosacral stenosis and injury of the cauda equina. Vet Clin North Am Small Anim Pract 1988; 18(3): 697–710.

3. Watt PR. Degenerative lumbosacral stenosis in 18 dogs. J Small Anim Pract 1991; 32(3): 125–34.

4. Ness MG. Degenerative lumbosacral stenosis in the dog: a review of 30 cases. J Small Anim Pract 1994; 35(4): 185–90.

5. Danielsson F, Sjostrom L. Surgical treatment of degenerative lumbosacral stenosis in dogs. Vet Surg 1999; 28(2): 91–8.

6. De Risio L, Thomas WB, Sharp NJ. Degenerative lumbosacral stenosis. Vet Clin North Am Small Anim Pract 2000; 30(1): 111–32, vi.

7. Suwankong N, Meij BP, Voorhout G, et al. Review and retrospective analysis of degenerative lumbosacral stenosis in 156 dogs treated by dorsal laminectomy. Vet Comp Orthop Traumatol 2008; 21(3): 285–93.

8. Meij BP, Bergknut N. Degenerative lumbosacral stenosis in dogs. Vet Clin North Am Small Anim Pract 2010; 40(5): 983–1009.

9. Bergknut N, Egenvall A, Hagman R, et al. Incidence of intervertebral disk degeneration-related diseases and associated mortality rates in dogs. J Am Vet Med Assoc 2012; 240(11): 1300–9.

10. Steffen F, Hunold K, Scharf G, et al. A follow-up study of neurologic and radiographic findings in working German Shepherd dogs with and without degenerative lumbosacral stenosis. J Am Vet Med Assoc 2007; 231(10): 1529–33.

11. Moore GE, Burkman KD, Carter MN, et al. Causes of death or reasons for euthanasia in military working dogs: 927 cases (1993–1996). J Am Vet Med Assoc 2001; 219(2): 209–14.

12. Evans RI, Herbold JR, Bradshaw BS, et al. Causes for discharge of military working dogs from service: 268 cases (2000–2004). J Am Vet Med Assoc 2007; 231(8): 1215–20.

13. Worth AJ, Sandford M, Gibson B, et al. Causes of loss or retirement from active duty for New Zealand police German Shepherd dogs. Anim Welfare 2013; 22(2): 166–73.

14. Morgan JP, Bailey CS. Cauda equina syndrome in the dog: radiographic evaluation. J Small Anim Pract 1990; 31(2): 69–77.

15. Mattoon JS, Koblik PD. Quantitative survey radiographic evaluation of the lumbosacral spine of normal dogs and dogs with degenerative lumbosacral stenosis. Vet Radiol Ultrasound 1993; 34(3): 194–206.

16. Ramirez O, Thrall DE. A review of imaging techniques for canine cauda equina syndrome. Vet Radiol Ultrasound 1998; 39(4): 283–96.

17. Lang J. Flexion-extension myelography of the canine cauda equina. Vet Radiol 1988; 29(6): 242–57.

18. Zorko B, Ivanuša T, Pelc R. Progression of hip dysplasia in 40 police working dogs. Eur J Compan Anim Pract 2007; 17(2): 145–8.

19. Blume LM, Worth AJ, Cohen EB, et al. Accuracy of radiographic detection of the cranial margin of the dorsal lamina of the canine sacrum. Vet Radiol Ultrasound 2015; 56(6): 579–88.

20. Morgan JP, Wind A, Davidson AP. Hereditary bone and joint diseases in the dog. Hannover : Schluetersche, 2000: 209–29.

21. Schmid V, Lang J. Measurements on the lumbosacral junction in normal dogs and those with cauda equine compression. J Small Anim Pract 1993; 34(9): 437–42.

22. Fluckiger MA, Damur-Djuric N, Hassig M, et al. A lumbosacral transitional vertebra in the dog predisposes to cauda equina syndrome. Vet Radiol Ultrasound 2006; 47(1): 39–44.

23. Morgan JP, Bahr A, Franti CE, et al. Lumbosacral transitional vertebrae as a predisposing cause of cauda equina syndrome in German shepherd dogs: 161 cases (1987–1990). J Am Vet Med Assoc 1993; 202(11): 1877–82.

24. Steffen F, Berger M, Morgan JP. Asymmetrical, transitional, lumbosacral vertebral segments in six dogs: a characteristic spinal syndrome. J Am Anim Hosp Assoc 2004; 40(4): 338–44.

25. Scharf G, Steffen F, Grunenfelder F, et al. The lumbosacral junction in working german shepherd dogs: neurological and radiological evaluation. J Vet Med A Physiol Pathol Clin Med 2004; 51(1): 27–32.

26. Lang J, Häni H, Schawalder P. A sacral lesion resembling osteochondrosis in the german shepherd dog. Vet Radiol Ultrasound 1992; 33(2): 69–76.

27. Sisson AF, LeCouteur RA, Ingram JT, et al. Diagnosis of cauda equina abnormalities by using electromyography, discography, and epidurography in dogs. J Vet Intern Med 1992; 6(5): 253–63.

28. Seiler GS, Hani H, Busato AR, et al. Facet joint geometry and intervertebral disk degeneration in the L5-S1 region of the vertebral column in German Shepherd dogs. Am J Vet Res 2002; 63(1): 86-90.

29. Adams W H, Daniel G B, Pardo A D, et al. Magnetic resonance imaging of the caudal lumbar and lumbosacral spine in 13 dogs (1990–1993). Vet Radiol Ultrasound 1995; 36(1): 3–13.

30. Mayhew PD, Kapatkin AS, Wortman JA, et al. Association of cauda equina compression on magnetic resonance images and clinical signs in dogs with degenerative lumbosacral stenosis. J Am Anim Hosp Assoc 2002; 38(6): 555–62.

31. Suwankong N, Voorhout G, Hazewinkel HA, et al. Agreement between computed tomography, magnetic resonance imaging, and surgical findings in dogs with degenerative lumbosacral stenosis. J Am Vet Med Assoc 2006; 229(12): 1924–9.

RADIOLOŠKA PRIMERJAVA ANATOMSKIH ZNAČILNOSTI LUMBOSAKRALNEGA OBMOČJA MED NEMŠKIMI IN BELGIJSKIMI (MALINOIS) OVČARJI – DELOVNIMI PSI

E. Pogorevc, B. Lukanc, A. Seliškar, R. Pelc, B. Zorko

Povzetek: Namen raziskave je bili oceniti medpasemske rentgenske razlike in pojavnost kliničnih in rentgenskih znakov degenerativne lumbosakralne stenoze (DLSS) pri 36 aktivnih policijskih delovnih psih; 24 pasme nemški (GSD) in 12 pasme belgijski ovčar (Malinois; MN). Po razgovoru z vodniki psov smo opravili natančen klinični in nevrološki pregled ter rentgensko slikanje lumbalnega in sakralnega dela hrbtenice brez in s kontrastnim sredstvom (mielografija). Svoje delo je brez omejitev opravljalo 33 psov, trije pa omejeno in sicer dva zaradi DLSS in eden zaradi bolezni diska v prsno-ledvenem delu hrbtenice. Šestnajst GSD je imelo znake DLSS, najpogostejši klinični znak je bila bolečina v zadnjem delu hrbta (15 od 16; 94%). Rentgensko smo potrdili DLSS pri 10 GSD (GSD/rDLSS). Potrdili smo razlike v anatomski konformaciji lumbosakralnega dela med pasmama, ki so korelirale s pojavnostjo DLSS pri GSD. Nemški ovčarji so imeli statistično značilno višja telesa sedmega ledvenega vretenca (L7; $p<0.001$) in prvega segmenta križnice (S1; $p<0.01$) kot pa MN. Pri GSD je bila stopnica L7/S1 ($p<0,01$) višja in razdalja CrS1/SL nižja ($p<0,05$) kot pri MNs. Nemški ovčarji so imeli tudi večjo telesno maso kot MN ($p<0.001$). Ugotovili smo tudi statistično značilno povezavo med DLSS in spondilozo deformans ($p<0.05$) ter brstenjem kranialne površine S1 ($p<0.05$). Pri MN bolezni nismo potrdili, čeprav je bila starost psov obeh pasem med seboj primerljiva. Tudi sicer smo pri MN rentgensko našli manj sprememb na hrbtenici kot pri nemških ovčarjih. Glede na naše ugotovitve, so MN bolj primerni za delovne pse. Omejitev naše študije je majhno število MN, in to, da v študijo ni bil zajet noben MN z DLSS. Med pasmama smo potrdili rentgenske razlike v konformaciji lumbosakralnega predela. Rentgenskega parametra, ki bi lahko bil predispozicijski faktor za razvoj bolezni, nismo odkrili. Za oceno pojavnosti bolezni pri MN bodo potrebne nadaljnje študije.

Ključne besede: lumbosakralni predel; rentgenologija; mielografija; delovni psi; nemški ovčar; belgijski ovčar (Malinois); degenerativna lumbosakralna stenoza

Slov Vet Res 2016; 53 (4): 229-35
UDC 636.7.09:616.993.1-078:57.088.7

Case Report

Pentatrichomonas hominis COINFECTION IN A PUPPY FROM A SLOVENIAN ANIMAL SHELTER

Maja Brložnik[1], Siniša Faraguna[2], Brigita Slavec[3], Rok Kostanjšek[5], Aleksandra Vergles Rataj[4], Igor Gruntar[4]

[1]PRVA-K, Small Animal Clinic, Gorkičeva 6, Ljubljana, [2]Veterinary faculty, [3]Institue for Health Care of Poultry, [4]Institute of Microbiology and Parasitology, Veterinary Faculty, Gerbičeva 60, Ljubljana, [5]Department of Biology, Biotechnical Faculty, Jamnikarjeva 101, Ljubljana, Slovenia

*Corresponding author, E-mail: igor.gruntar@vf.uni-lj.si

Summary: A 3-month-old dog originating from a Slovenian animal shelter presented with acute bloody, soft, foamy and malodorous diarrhoea. The clinical examination, haematology and serum biochemistry were unremarkable. Ultrasonography of the abdomen showed prominent mesenteric lymph nodes and the presence of echogenic content within the small intestine. Light microscopy of a native smear and a wet mount darkfield microscopy examination of the faecal material showed motile trichomonad-like organisms with a particular circular motion. The flotation and SAF (Sodium acetate - acetic acid - formalin solution) method using light microscopy revealed eggs of nematode *Toxocara* canis and protozoan oocysts of *Isospora* spp. Trichomonad-like organisms were successfully isolated and cultivated in axenic culture. Light microscopy of Giemsa-stained trichomonads showed the presence of five flagella, and *Pentatrichomonas hominis* (*P. hominis*) was presumptively diagnosed. The diagnosis was confirmed by the Polymerase Chain Reaction (PCR) followed by DNA sequencing and the Scanning Electron Microscopy (SEM) of cultured trichomonad isolates. The PCR and sequencing results confirmed a 99% homology of the *P. hominis* isolates with isolates from other studies, originating both from humans and animals, which suggests that *P. hominis* could have zoonotic potential and have been transmitted from animals to people via the per-oral route. This is also the first report on *P. hominis* involvement in clinical diseases in dogs in Slovenia.

Key words: *Pentatrichomonas hominis*; dog; isolation; cultivation; PCR; DNA sequencing; scanning electron microscopy

Introduction

Young dogs are commonly infested with different zoonotic intestinal parasites. Conventional canine intestinal parasites of zoonotic importance, such as *Toxocara*, *Taenia, Ancylostoma*, *Giardia, Cryptosporidium*, etc., have been studied thoroughly, but less is known about the zoonotic trichomonad species *Pentatrichomonas hominis (P. hominis)*, formerly *Trichomonas intestinalis* or *Trichomonas hominis* (1). This flagellated protozoan has recently been identified in faeces of dogs with diarrhoea (2, 3, 4, 5). The pathogenicity of the parasite remains unclear. Due to the lack of evidence of cases where *P. hominis* was the only infecting agent, this trichomonad is presumed to be a commensal organism that may overgrow in patients with other causes of diarrhoea. As enteropathogens have always been found in dogs infected by *P. hominis* (6), the pathogenic potential of this trichomonad species has to be further evaluated by experimental infection studies (4). For such trials an axenic P. *hominis* culture of

Received: 19 November 2015
Accepted for publication: 21 April 2016

dog origin is needed, which, to the best of our knowledge, has not yet been available. In humans, *P. hominis* has been reported as the causative agent of gastrointestinal disturbances in children (7, 8, 9). Therefore the assessment of the zoonotic potential of canine *P. hominis* is important. It is also necessary to establish whether host-specific genotypes exist as demonstrated among *Tritrichomonas foetus* isolates from cats and cattle (10, 11). To prove this, the characterisation of as many isolates as possible from diverse hosts should be performed. A study carried out last year (12), however, demonstrates that even when using the high-resolution gene locus of the ITS (internal transcribed spacer) regions, all *P. hominis* strains from diverse hosts are genetically identical. This suggests that zoonotic transmission between humans and animals may occur in the area investigated. Consequently, further research is required to clarify the role *of P. hominis* in human and animal diseases. This article provides the first description of *P. hominis* involved in a clinical disease in dogs in Slovenia. The trichomonad was successfully isolated and cultivated in axenic culture. The presumptive diagnosis based on light microscopy was confirmed by SEM and PCR.

Material and methods

Case Description and Sampling

A 3-month old, 9 kg mixed breed and regularly vaccinated dog that had recently been adopted from a Slovenian shelter, presented at a small animal clinic with acute bloody, soft, foamy and malodorous diarrhoea. Clinical examination, haematology, blood serum biochemistry and abdominal ultrasonography were performed. According to the results viral etiology was ruled out. Consequently, coprological and bacteriological analyses of a faecal sample were carried out. The faecal sample was analysed using light microscopy, bacteriological examination, flotation, sedimentation and the SAF (Sodium acetate-acetic acid-formalin solution) method. Bacteriological analyses of faecal samples were conducted on blood agar and Drigalski agar plates, incubated aerobically and anaerobically at 37 °C overnight. The isolation of trichomonads followed. SEM and PCR were used for definitive diagnosis.

Isolation of Trichomonads

Trichomonads were isolated using the following procedure: a set of 3 tubes containing Modified Diamond's growth medium (MDM) was inoculated with a loopful (approx. 0.1 g) of faecal sample and incubated at 37°C for seven days (13). In parallel, another set of tubes was inoculated in the same way, this time with MDM supplemented with meropenem (6 µg/ml; MeMDM), to provide additional prevention against bacterial contamination (14). The inoculated tubes were checked for trichomonad growth at intervals during this period. An aliquot was taken from the bottom of the tube and wet mount-examined by darkfield microscopy. When motile flagellates were observed, an aliquot (0.1 ml) of fresh culture was transferred to fresh MDMs/MeMDMs.

Staining of Isolated Trichomonads

Thin smears of cultivated trichomonad suspension were air-dried, fixed and stained with Giemsa, trichrome and methylene blue stain.

Scanning Electron Microscopy

For scanning electron microscopy, the trichomonads in the cultivation suspension were washed with phosphate buffered saline (PBS) by centrifugation at 100 xg for 5 min before overnight fixation in a combination of 1 % glutaraldehyde and 0.5 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C. Fixed cells were transferred to pre-cleaned cover slides, washed by PBS and postfixed in 2 % OsO_4 for one hour at 4 °C. After being washed in deionized water, the cells on the slides were dehydrated in a graded series of ethanols, then dried by hexamethyldisilazane (HMDS), mounted on aluminum stubs and coated with platinum, as described above (15). The samples were examined with the JEOL JSM-7500F field emission scanning electron microscope.

Molecular Diagnosis

For the molecular detection of trichomonads, two hundred microlitres of protozoal culture suspension were used. Total DNA was extracted using the QIAamp® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions for

blood and body fluid spin protocol. Finally, the DNA was eluted with 100 µl of AE buffer and stored at -20 °C until examination. Specific pairs of primers TFR1 and TFR2 amplifying the 350 bp long ITS1-5.8S-ITS2 region were used (16). PCR was performed in a total volume of 20 µl containing 10 µl of 2X Thermo Scientific DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, CA, USA), 0.8 µl of each primer (0.4 µM), 6.4 µl of nuclease-free water and 2 µl of extracted DNA. The reaction was performed on an ABI 2720 Thermo Cycler (Applied Biosystems, Foster City, CA, USA). The cycling profile included initial denaturation at 95 °C for 5 min, which was followed by 40 cycles of heat denaturation at 94 °C for 30 sec, oligonucleotide annealing at 55 °C for 1 min, oligonucleotide extension at 72 °C for 1 min, and final oligonucleotide extension step at 72 °C for 10 min. The extracted DNA of *Trichomonas gallinae* was used as positive control in the PCR assay.

The PCR products were analysed by electrophoresis on a 1.8 % ethidium bromide-stained agarose gel. DNA fragments were excised from the gel and, after being purified with the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA), sent for sequencing to the Macrogen laboratory (Macrogen Inc, Amsterdam, the Netherlands).

The nucleotide sequences were downloaded using Chromas software (Technelysium Pty Ltd., Queensland, Australia), and the nucleotide sequence data were analysed by BLAST (17) to find similar sequences in the Genbank NCBI sequence database.

Results

The clinical examination of the diarrhoeic 3-month old mixed breed dog was unremarkable. Haematology and serum biochemistry did not reveal any abnormalities. Ultrasonography of the abdomen showed prominent mesenteric lymph nodes and the presence of echogenic content within the small intestine.

Light microscopy of the native faecal smear and darkfield microscopy (wet mount examination) of the diarrhoeic material showed numerous motile trichomonad-like organisms with a particular circular motion. Flotation and the SAF method using light microscopy revealed *Toxocara canis* nematode eggs (Figure 1), *Isospora* sp. oocysts (Figure 2) and the above-mentioned trichomonad species. Bacteriological analyses of faecal samples were negative for aerobic and anaerobic pathogens. Trichomonad isolation and cultivation attempts were successful. Numerous motile trichomonads were observed after seven days incubation in MDM (xenic culture; with other organisms - bacteria - present). In MeMDM, the trichomonads were less abundant but other organisms were absent (axenic culture). The cultures were stored

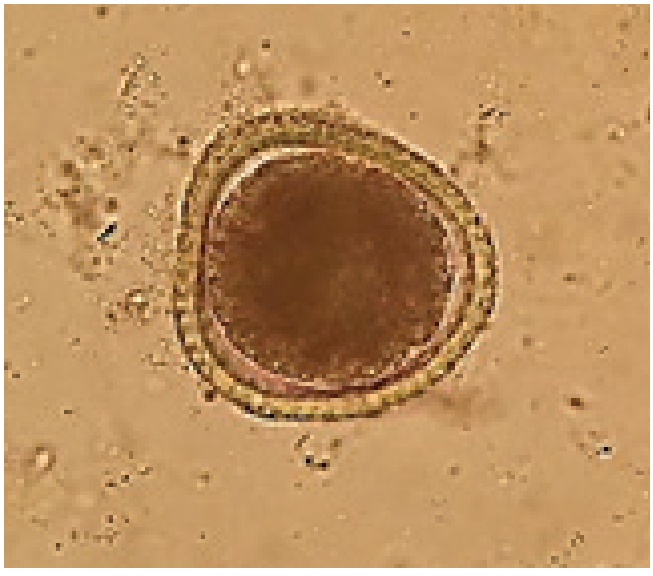


Figure 1: *Toxocara canis* egg, size 90 x 80 µm, flotation method, x400

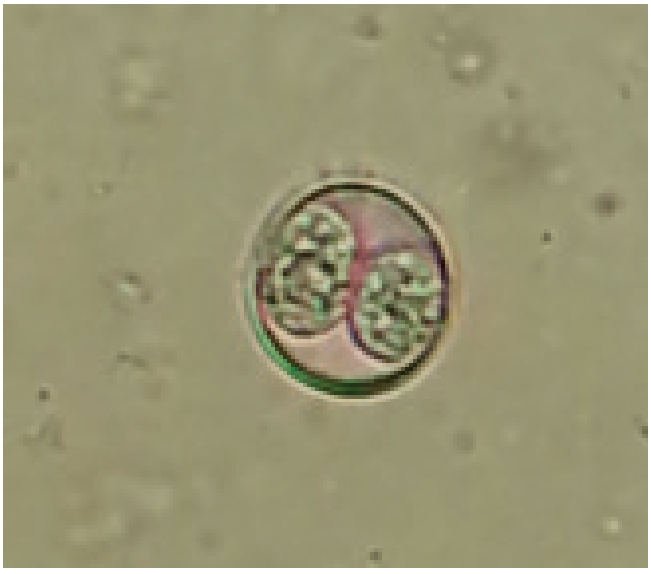


Figure 2: *Isospora* sp. oocyst, size 23 x 18 µm, flotation method, x400

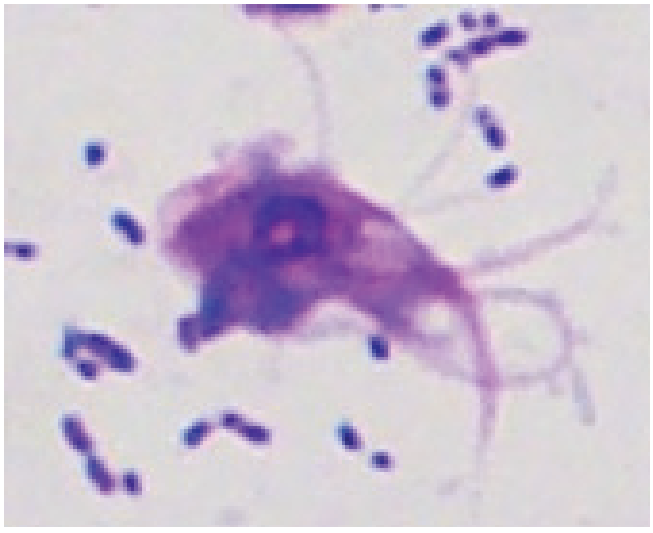


Figure 3: *Pentatrichomonas hominis* trophozoite, Giemsa, x1000

using 10 % dimethylsulphoxide (18). The isolated trichomonads were stained with Giemsa, trichrome and methylene blue stain. When examined by light microscopy, the Giemsa stain improved the visibility and enabled the enumeration of flagella (Figure 3).

The SEM and PCR assay confirmed the diagnosis of *P. hominis*. SEM observations revealed the presence of five flagella in the anterior part of the trichomonad (four flagella in a group and a single independent flagellum) and one in the posterior part (Figure 4). The latter run from the anterior part, alongside the cell in a posterior direction, forming a distinct undulating membrane displaying three undulations. It ended freely at its distal end. The axostyle was observed as a discrete tip in the posterior region of the cell.

With PCR and sequencing, a high similarity of obtained sequence (Accession No. KU670675) with *P. hominis* was confirmed. The comparison of the 300 bp long sequence of the complete ITS1-5.8S-ITS2 gene had 100 % homology with *P. hominis* isolate from empyema thoracis (Accession No. AF156964), and 99 % homologies with the *P. hominis* isolates from a human (Acc. No. JN007007) and a dog (Acc. No. KJ404270).

The dog was treated with metronidazole 200 mg/12 h p/o for 5 days and Dehinel plus® (febantel 150 mg, pyrantel embonate 144 mg, praziquantel 50 mg) 1 tbl/day for 5 days. The dog's condition improved immediately, and the faecal exam was negative two weeks after the treatment. Since then, the dog has been asymptomatic for one year.

Discussion

P. hominis, a flagellated protozoan of the order *Trichomonadida*, inhabits the large intestine of many mammalian hosts, including humans (6, 7, 8, 9, 19). Its prevalence in humans is low in developed countries (20), but is much higher in subtropical and tropical zones (21). In dogs, the prevalence of the trichomonad infection in a study analysing 215 puppies from French kennels was 15.8% (4). This study also reports that *P. hominis* was the only trichomonad infecting the studied canine population, whereas some older papers suggest that *P. hominis* was far more frequent than *Tritrichomonas foetus* in diarrhoeic dogs suffering from trichomonosis (2, 3, 6).

In the majority of studies (2, 3, 4), the reported age of dogs with trichomonosis ranged from 7 weeks to 6 months which is consistent with the age of the 3-month-old dog in this case. A more recent study reported that trichomonosis was also diagnosed in 10-year-old dogs (6).

Differential diagnoses for bloody, soft, foamy and malodorous diarrhoea are dietary intolerance, infection, partial obstruction, motility disorders, inflammatory/immune-mediated disease, drugs/toxins, idiopathic disease, neoplasia and extra-gastrointestinal disease (22). Given the age of the dog in this study, the clinical signs and results of the clinical investigation, infection was considered the most likely cause. Considering that the dog has been routinely vaccinated against viral diseases, the absence of leukopenia in haematology results, as well as lack of other usual clinical signs, parvoviral enteritis was not suspected and additional diagnostics regarding shedding the parvoviruses was not performed. The excretion of other viruses is generally not evaluated in practice, since viral diarrhoea is usually self-limiting and does not require a positive diagnosis. The negative results of the bacteriological analyses led to the conclusion that the mixed trichomonad-parasitic-coccidial infection was responsible for the symptoms. However, it is difficult to presume about the origin of the diarrhea from amongst the three organisms. It is most likely that the concurrency of the pathogens was significant.

Microscopical observations of unusual and copious trichomonad-like organisms in the native sample was a surprising finding, which triggered additional efforts to characterise these organisms. Microscopy cannot be used to identify the

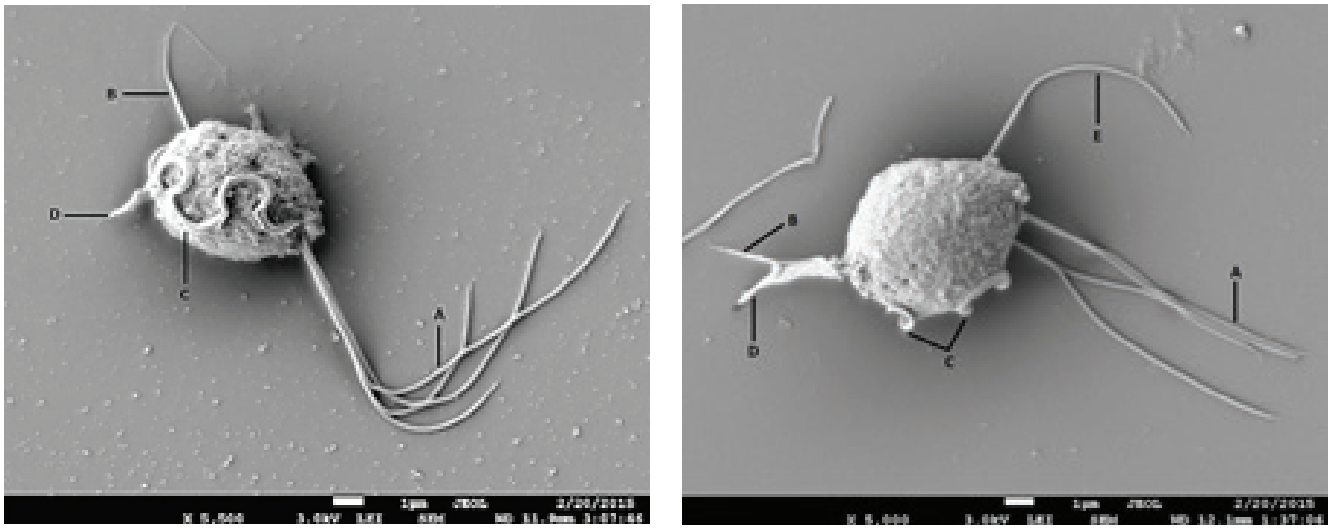


Figure 4: *Pentatrichomonas hominis* trophozoite, A – anterior flagella, B – posterior flagellum, *C* – undulating membrane, *D* – axostyle, E – independent flagellum, SEM x 550

trichomonad species in native samples (23, 24, 25). Flagella are not well discernible in such preparations, but their visibility improves with the use of stains, thus supporting the species differentiation.

Trichomonad species can accurately be determined by SEM: for the purposes of this study the isolate was characterised as a pear-shaped organism with distinctive undulating membrane, an axostyle, a single posterior (recurrent) flagellum and five anterior flagella, comprising a group of four flagella of unequal length and a single independent flagellum. The undulating membrane displayed three undulations. An axostyle was an elongated rod-like structure projecting out of the posterior end to form a pointed spine. The axostyle is an important criterion for distinguishing tritrichomonads from pentatrichomonads (5, 26). In this study, the axostyle of the trichomonad had a relatively heavy terminal segment and was observed as a discrete tip in the posterior region of the cell. By contrast, the axostyle of the *T. foetus* has a short conical projection with a small spherical structure at the end (27). Furthermore, it has only 3 anterior flagella. The long undulating membrane is also suggestive of *P. hominis* because the other trichomonads have a shorter undulating membrane (27).

For the detection of *P. hominis* in biological specimens, a highly specific and sensitive PCR assay can be used (28, 29, 30). In this case, the PCR assay for amplification of the 5.8S rRNA gene and two flanking internal transcribed spacer regions ITS1 and ITS2 of trichomonads was used. This region is one of the most used for taxonomic classification of trichomonads (16). The results confirmed high homology of this isolate with the previously described genetic loci of *P. hominis* from various mammalian hosts (humans, dogs, cattle, pigs, cats, goats, water buffaloes), suggesting a low genetic diversity in *P. hominis* isolates and a very broad host range for this species (5, 12, 28, 29, 30).

Therefore, it is possible that *P. hominis* strains may circulate between different hosts, including humans, where they, under certain circumstances, may cause clinical disease (7, 8, 9) or exacerbate symptoms of an existing illness (31). To elucidate the actual role and pathogenicity of *P. hominis* in human and animal disease, further research is needed.

Conclusion

Routine coprological examinations, at least microscopic evaluation of the stool for protozoans, parasites and the ova (eggs, cysts) of parasites, are essential for a correct diagnosis in a dog with diarrhoea. In the case of trichomonads, SEM or PCR assays are required for the definite diagnosis of trichomonad species. Molecular diagnostic data of trichomonads suggest that *P. hominis* is a zoonotic species with the potential for transmission via the per-oral route from animals to people and vice-versa.

References

1. Pentatrichomonas. In: Studdert VP, Blood DC, Gay CC, eds. Saunders comprehensive veterinary dictionary. 4th ed. Edinburgh ; New York : Elsevier Saunders, 2012: 831.

2. Gookin JL, Stauffer SH, Coccaro MR, Marcotte MJ, Levy MG. Optimization of a species-specific polymerase chain reaction assay for identification of *Pentatrichomonas hominis* in canine fecal specimens. Am J Vet Res 2007; 68: 783–7.

3. Kim YA, Kim HY, Cho SH, Cheun HI, Yu JR, Lee SE. PCR detection and molecular characterization of *Pentatrichomonas hominis* from feces of dogs with diarrhea in the Republic of Korea. Korean J Parasitol 2010; 48: 9–13.

4. Grellet A, Polack B, Feugier A, et al. Prevalence, risk factors of infection and molecular characterization of trichomonads in puppies from French breeding kennels. Vet Parasitol 2013; 197: 418–26.

5. Li WC, Gong PT, Ying M, Li JH, et al. *Pentatrichomonas hominis:* first isolation from the feces of a dog with diarrhea in China. Parasitol Res 2014; 113: 1795–1801.

6. Tolbert MK, Leutenegger CM, Lobetti R, Birrell J, Gookin JL. Species identification of trichomonads and associated coinfections in dogs with diarrhea and suspected trichomonosis. Vet Parasitol 2012; 187: 319–22.

7. Yang CR, Meng ZD, Wang X, Li YL, Zhang YX, Zhao QP. Diarrhoea surveillance in children aged under 5 years in a rural area of Hebei Province, China. J Diarrhoeal Dis Res 1990; 8: 155–9.

8. Chunge RN, Nagelkerke N, Karumba PN, et al. Longitudinal study of young children in Kenya: intestinal parasitic infection with special reference to *Giardia lamblia*, its prevalence, incidence and duration, and its association with diarrhoea and with other parasites. Acta Trop 1991; 50: 39–49.

9. Meloni D, Mantini C, Goustille J, et al. Molecular indentification of *Pentatrichomonas hominis* in two patients with gastrointestinal symptoms. J Clin Pathol 2011; 64: 933–5.

10. Šlapeta J, Craig S, McDonell D, Emery D. *Tritrichomonas foetus* from domestic cats and cattle are genetically distinct. Exp Parasitol 2010; 126: 209–13.

11. Reinmann K, Müller N, Kuhnert P, et al. *Tritrichomonas foetus* isolates from cats and cattle show minor genetic differences in unrelated loci ITS-2 and EF-1α. Vet Parasitol 2012; 185: 138–44.

12. Kamaruddin M, Tokoro M, Rahman M, et al. Molecular characterization of various trichomonad species isolated from humans and related mammals in Indonesia. Korean J Parasitol 2014; 52: 471–8.

13. Trichomonosis. In: OIE Manual of diagnostic tests and vaccines for terrestrial animals. 7th ed. [Online]. Paris: The World Organization of Animal Health, 2012: chapter 2.4.17 (8 pp.) (adopted ver. May 2012) http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2008/pdf/2.04.17_TRICHOMONOSIS.pdf (14. 10. 2015)

14. Amin A, Neubauer C, Liebhart D, Grabensteiner E, Hess M. Axenization and optimization of in vitro growth of clonal cultures of *Tetratrichomonas gallinarum* and *Trichomonas gallinae*. Exp Parasitol 2010; 124: 202–8.

15. Kostanjšek R, Pirc-Marolt T. Pathogenesis, tissue distribution and host response to *Rhabdochlamydia porcellionis* infection in rough woodlouse *Porcellio scaber*. J Invertebr Pathol 2015; 125: 56-67.

16. Felleisen RS. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitology 1997; 115: 111–9.

17. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 215: 403–10.

18. Miyake Y, Karanis P, Uga S. Cryopreservation of protozoan parasites. Cryobiology 2004; 48: 1–7.

19. Gookin JL, Stauffer SH, Levy MG. Identification of *Pentatrichomonas hominis* in feline fecal samples by polymerase chain reaction assay. Vet Parasitol 2007; 145: 11–5.

20. Ackers JP. Trichomonads. In: Gillespie SH, Pearson RD, eds. Principles and practice of clinical parasitology. Chichester: John Wiley & Sons, 2001: 258–60.

21. Saksirisampant W, Nuchprayoon S, Wiwanitkit V, Yenthakam S, Ampavasiri A. Intestinal parasitic infestations among children in an orphanage in Pathum Thani province. J Med Assoc Thai 2003; 86: 263–70.

22. Gough A. Differential diagnosis in small animal medicine. Oxford: Blackwell, 2007: 26–31.

23. Romatowski J. *Pentatrichomonas hominis* infection in four kittens. J Am Vet Med Assoc 2000; 216: 1270–2.

24. Gookin JL. Trichomoniasis. In: Greene

CE, ed. Infectious diseases of the dog and cat. 4th ed. St. Louis: Saunders, 2012: 797–801.

25. Rivera WL, Lupisan AJ, Baking JM. Ultrastructural study of a tetratrichomonad isolated from pig fecal samples. Parasitol Res 2008; 103: 1311–6.

26. Levy MG, Gookin JL, Poore M, Birkenheuer AJ, Dykstra MJ, Litaker RW. *Tritrichomonas foetus* and not *Pentatrichomonas hominis* is the etiologic agent of feline trichomonal diarrhea. J Parasitol 2003; 89: 99–104.

27. Warton A, Honigberg BM. Structure of trichomonads as revealed by scanning electron microscopy. J Protozool 1979; 26: 56–62.

28. Crucitti T, Abdellati S, Ross DA, Changalucha J, van Dyck E, Buve A. Detection of *Pentatrichomonas hominis* DNA in biological specimens by PCR. Lett Appl Microbiol 2004; 38: 510–6.

29. Gookin JL, Birkenheuer AJ, St John V, Spector M, Levy MG. Molecular characterization of trichomonads from feces of dogs with diarrhea. J Parasitol 2005; 91: 939–43.

30. Mostegl MM, Wetscher A, Richter B, Nedorost N, Dinhopl N, Weissenböck H. Detection of *Tritrichomonas foetus* and *Pentatrichomonas hominis* in intestinal tissue specimens of cats by chromogenic in situ hybridization. Vet Parasitol 2012; 183: 209–14.

31. Jongwutiwes S, Silachamroon U, Putaporntip C. *Pentatrichomonas hominis* in empyema thoracis. Trans R Soc Trop Med Hyg 2000; 94: 185–6.

SOOKUŽBA S PROTOZOJEM *Pentatrichomonas hominis* PRI PSU IZ SLOVENSKEGA ZAVETIŠČA

M. Brložnik, S. Faraguna, B. Slavec, R. Kostanjšek, A. Vergles Rataj, I. Gruntar

Povzetek: Na kliniko smo zaradi akutne krvave, penaste in smrdljive driske sprejeli tri mesece starega psa, ki so ga lastniki nedavno posvojili iz zavetišča. Klinični pregled ter hematološke in biokemične preiskave krvi so bili brez posebnosti. Pri pregledu trebuha z ultrazvokom smo ugotovili velike mezenterialne bezgavke, tanko črevo pa je bilo polno ehogene vsebine. Z mikroskopskim pregledom nativnih preparatov smo v vzorcu blata opazili številne gibljive organizme, podobne trihomonasom, s specifičnim krožnim gibanjem. Z mikroskopiranjem ter s flotacijo in metodo SAF smo diagnosticirali še jajčeca nematodov *Toxocara canis* in oociste praživali *Isospora sp*. Trihomonasom podobne organizme smo uspešno izolirali in jih vzgojili v čisti kulturi. Mikroskopski pregled organizmov, obarvanih po Giemsi, je omogočil določitev števila bičkov in vzpostavitev suma na okužbo s *Pentatrichomonas hominis* (*P. hominis*). Diagnozo smo dokončno potrdili s polimerazno verižno reakcijo (PCR) in določitvijo baznega zaporedja, ter z vrstičnim elektronskim mikroskopom. PCR in določitev baznega zaporedja sta pokazala 99 % skladnost našega izolata z izolati *P. hominis* iz drugih gostiteljev/študij. Ta podatek kaže na zoonotski potencial *P. hominis* in na možnost peroralnega prenosa med živalmi in ljudmi. Članek predstavlja tudi prvo poročilo o trihomonadni okužbi s *P. hominis* pri psih v Sloveniji.

Ključne besede: *Pentatrichomonas hominis*; pes; izolacija; gojenje; PCR; določanje zaporedja DNK; vrstična elektronska mikroskopija

AUTHOR INDEX VOLUME 53, 2016

SLOVENIAN VETERINARY RESEARCH
SLOVENSKI VETERINARSKI ZBORNIK

Slov Vet Res 2016; 53 (4)

Review Article

Original Scientific Articles

Case Report