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Emerging cases of chlamydial abortion in sheep and goats in Croatia and Bosnia and Herzegovina

Vorkommen von Chlamydienaborten bei Schafen und Ziegen in Kroatien und Bosnien und Herzegovina

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Summary

In a recent lambing season (2012/2013), the seroprevalence of ovine chlamydiosis was monitored in small ruminant abortion cases in Croatia. Blood samples of 93 sheep and 69 goats were examined. In addition, 50 sheep and 61 goat samples were tested using molecular methods. Furthermore, 14 sheep blood samples, one goat blood sample and one sheep placenta sample from Bosnia and Herzegovina (BIH) were also tested as a part of inter-laboratory cooperation. Overall high seroprevalence was detected in sheep, 19.6% with the ELISA IDEXX kit and 20.5% with the CIVTEST kit. Seroprevalence in goats was 11.4%. In BIH, four sheep and one goat blood sample were seropositive for chlamydiosis. The disease causing agent, *Chlamydia abortus* (*C. abortus*) was confirmed using molecular methods in two sheep flocks in continental Croatia and in one sheep flock in BIH. In this study, *C. abortus* infection in sheep was identified for the first time in Croatia using species specific molecular methods. Ovine chlamydiosis is present in national sheep and goat flocks in Croatia and BIH. Thus should be subject to ongoing controls in the case of abortion. A combination of serological and molecular methods should be used for optimal laboratory diagnostics of *C. abortus*.

Keywords: *Chlamydia abortus*, Croatia, Bosnia and Herzegovina, ovine enzootic abortion

Zusammenfassung

Während der Lammzeit 2012/2013 wurde die Seroprävalenz von oviner Chlamydiose in Fällen von Aborten bei Schafen und Ziegen in Kroatien dokumentiert. Hierfür wurden Blutproben von 93 Schafen und 69 Ziegen untersucht. Zusätzlich wurden 50 Proben von Schafen und 61 Proben von Ziegen mit molekularen Methoden getestet. Im Rahmen eines Ringversuches wurden außerdem noch 14 Blutproben von Schafen, eine Blutprobe einer Ziege und eine Probe einer Schaf-Plazenta aus Bosnien und Herzegowina untersucht. Eine hohe Seroprävalenz von 19,6 % mit dem IDEXX ELISA Kit und 20,5 % mit dem CIVTEST Kit wurde bei Schafen in Kroatien festgestellt. Die Seroprävalenz bei den untersuchten Ziegen lag bei 11,4 %. Von den Proben aus Bosnien und Herzegowina waren vier Blutproben von Schafen und die Blutprobe einer Ziege positiv. Das Vorkommen des krankheitsverursachenden Agens *Chlamydia* (*C.*) *abortus* konnte bei zwei Schafherden im kontinentalen Teil von Kroatien und bei einer Schafherde in Bosnien und Herzegowina mit molekularen Methoden bestätigt werden. Diese Untersuchung zeigt erstmalig eine Infektion mit *C. abortus* bei Schafen aus Kroatien mittels spezifischer molekularer Methoden. Da Infektionen mit *C. abortus* bei Schaf- und Ziegenherden in Kroatien und Bosnien-Herzegowina vorkommen, empfehlen wir beim Auftreten von Fehlgeburten regelmäßige Kontrollen. Zur optimalen Diagnosestellung von *C. abortus* sollten zukünftig sowohl serologische, als auch molekulare Untersuchungsmethoden angewendet werden.

Schlüsselwörter: *Chlamydia abortus*, Kroatien, Bosnien und Herzegowina, Chlamydienabort

Introduction

Ovine chlamydiosis (enzootic abortion of ewes [EAE]) is caused by *C. abortus* (OIE, 2012). The disease occurs in all countries with intensive or semi-intensive rearing of sheep and goats in the European Union and worldwide (OIE 2012, 2013). All currently known chlamydiae belong to the single genus *Chlamydia*, family *Chlamydiaceae*. The disease is characterised by abortion in late pregnancy, after 90 days of gestation, when the fetus starts to grow rapidly and *Chlamydiae* start to invade placentomes that produce a progressively diffuse inflammatory response, thrombotic vasculitis and tissue necrosis. Birth of avital lambs is also possible and generally these lambs do not survive longer than 48 h (OIE, 2012; Cvetnić, 2013). Infection can be established in a 'clean' flock by introducing infected replacements and results in a small number of abortions in the first year, which is followed by an 'abortion storm' in the second year. Abortions can affect up to around 30% of ewes (OIE, 2012). In the following years the number of infected ewes is reduced (Livingstone et al., 2009). Other manifestations of infection include pneumonitis, arthritis, pericarditis, enteritis, and conjunctivitis (Rodolakis et al., 1998). The infections were found in clinically healthy flocks (Lenzko et al., 2011). In nonpregnant ewes it can persist as sub-clinical infection (Rocchi et al., 2009). In abortion case, besides chlamydiosis as differential diagnose, brucellosis, *Brucella ovis* infection, coxiellosis, leptospirosis, salmonellosis, campylobacteriosis, toxoplasmosis and also *C. pecorum* have to be taken into account. *C. abortus* can also cause abortion in goats and, less frequently, in cattle, pigs, horses and deer. Small ruminants affected by *C. pecorum* could have symptoms of keratoconjunctivitis, polyarthritis and fertility disorders (Nietfeld, 2001). It can also be isolated accompanied by *C. abortus* from clinically healthy animals (Lenzko et al., 2011). Apart from a great economic importance, *C. abortus* also has a zoonotic importance. Farmers, veterinarians, pregnant women and laboratory personnel handling cultures and potentially infected tissues are at special risk. Different methods are used for serological diagnosis of chlamydiosis in sheep and goats. Complement fixation test (CFT) is the most widely used procedure, followed by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF).

Etiologic diagnosis is based on bacteriological testing on embryonated chicken eggs and on amplification of chlamydial DNA by polymerase chain reaction (PCR) and real-time PCR (Sachse et al., 2009; OIE, 2012). In Vlahović doctoral thesis (2000), ten cattle, five sheep and five goat herds with clinical signs of chlamydiosis were screened by ELISA. The *Chlamydia* antibodies were found in 33.2% of 184 blood samples. 111 swabs of 41 ruminants were tested by direct immunofluorescence and Clearview immunoassay and antigen was found in one bovine lung, one bovine liver and in one sheep conjunctiva. To our knowledge, up to now *C. abortus* infection has not been detected in Croatia. In BIH, the infection with *C. abortus* was reported in 2010 (OIE, 2013) and since 1992 small ruminant herds have been vaccinated with a vaccine produced by the Veterinary faculty in Sarajevo, BIH (personal communication). Krkalić et al. (2013) recently reported a high seroprevalence in sheep flocks in the western part of BIH. In our study, blood samples of goats and sheep, that had an abortion in the lambing season from December 2012 to May 2013, were serologically

TABLE 1: *Serological and molecular testing of sheep samples in Croatia*

County	Farm	Serological test			Molecular tests		
		Number of sheep tested	ELISA IDEXX	CIVTEST	Number of samples tested	PCR Berri et al., 2009)	Real Time RCR (Pantchev et al., 2010)
Zagreb	A1	2	neg	neg	1	neg	neg
Sisak-Moslavina	B1	2	2+	2+	2	2+	2+
Karlovac	C1–C3	7	neg	neg	6	neg	neg
	C4	4	2+	1+	NT	NT	NT
	C5	NT	NT	NT	1	neg	neg
Varaždin	D1–D3	4	4+	4+	NT	NT	NT
Bjelovar-Bilogora	E1	2	2+	1+	1	neg	neg
	E2	3	1+/-	1+	1	neg	neg
	E3	1	1+	1+	1	1+	1+
	E4–E7	6	neg	neg	NT	NT	NT
	E8	1	1+	neg	NT	NT	NT
	E9	NT	NT	NT	1	neg	neg
Primorje-Gorski Kotar	F1	2	neg	1+	NT	NT	NT
	F2	1	neg	neg	NT	NT	NT
Lika-Senj	G1	4	neg	neg	NT	NT	NT
	G2	5	neg	2+	NT	NT	NT
	G3	7	neg	1+	1	neg	neg
	G4	2	1+/-	2+	2	neg	neg
	G5–G7	5	neg	neg	6	neg	neg
Požega-Slavonija	H1	1	neg	neg	NT	NT	NT
	H2	1	neg	neg	1	neg	neg
	H3	NT	NT	NT	10	neg	neg
Virovitica-Podravina	I1	NT	NT	NT	2	neg	neg
Zadar	J1	6	neg	neg	4	neg	neg
Osijek-Baranja	K1	1	1+	1+	NT	NT	NT
	K2	1	neg	1+	NT	NT	NT
	K3	1	neg	neg	NT	NT	NT
Vukovar-Srijem	L1	1	neg	neg	NT	NT	NT
Split-Dalmatia	M1–M5	12	neg	neg	5	neg	neg
	M6	6	1+	1+	NT	NT	NT
Istria	N1–N4	4	neg	neg	4	neg	neg
	N5	1	1+	neg	1	neg	neg
Total	48	93	17	19	50	3	3

NT = not delivered for testing; 1+ = 1 positive; 2+ = 2 positive; 1+/- = 1 doubtful; neg = negative. The same animals were tested with both serological methods and molecular methods.

TABLE 2: *Serological and molecular testing of goat samples in Croatia*

County	Farm	Serological test		Molecular tests		
		Number of goats tested	ELISA IDEXX	Number of samples tested	PCR (Berri et al.,2009)	Real Time RCR (Pantchev et al., 2010)
Zagreb	A1–A2	2	neg	5	neg	neg
Krapina-Zagorje	B1	2	neg	NT	NT	NT
Karlovac	C1	10	1+/-; 2+	NT	NT	NT
	C2	7	1+	NT	NT	NT
	C3	1	neg	1	neg	neg
Koprivnica-Križevci	D1	1	1+	NT	NT	NT
	D2	1	neg	NT	NT	NT
Bjelovar-Bilogora	E1	1	1+	NT	NT	NT
	E2	1	neg	NT	NT	NT
Primorje-Gorski Kotar	F1	1	neg	NT	NT	NT
	F2	2	neg	2	neg	neg
Lika-Senj	G1	1	neg	1	neg	neg
	G2	5	neg	NT	NT	NT
Virovitica-Podravina	H1	1	neg	1	neg	neg
Zadar	I1	6	neg	NT	NT	NT
	I2-I5	14	neg	10	neg	neg
	I6	NT	NT	30	neg	neg
Šibenik-Knin	J1	1	neg	1	neg	neg
Požega-Slavonija	K1	NT	NT	2	neg	neg
Vukovar-Srijem	L1	6	neg	1	neg	neg
	L2	1	1+	NT	NT	NT
Split-Dalmatia	M1	1	neg	1	neg	neg
	M2	1	neg	NT	NT	NT
Istria	N1–N3	3	neg	6	neg	neg
Total	30	69	7	61	0	0

NT = not delivered for testing; 1+ = 1 positive; 2+ = 2 positive; 1+/- = 1 doubtful; neg = negative. The same animals were tested with serological and molecular methods.

examined. Molecular investigation on *C. abortus* was conducted on vaginal swabs, placental specimen and fetal organs of sheep and goats. Also, within inter-laboratory cooperation, the identical control of several suspicious samples from neighbouring BIH was performed.

Material and Methods

Sample collection

From December 2012 to May 2013, the presence of *C. abortus* antibodies was tested in cases of sheep and goat abortions. In Croatia, according to the annual order for control of causing agents of abortion, every case of abortion in domestic animals has to be serologically examined for infectious agents of diseases causing abortion, e. g. brucellosis, leptospirosis, coxiellosis and *Brucella ovis* infection). Bacteriological investigation for the same diseases was carried out in cases in which aborted fetus or placenta were available. The same material was used for the investigation of EAE. As a part of an inter-laboratory collaboration with the Public Institution "Veterinary Institute Herzegovina-Neretva Canton" in Mostar, BIH, samples found inconclusive concerning the causing agent of abortion were delivered for testing for EAE. Blood samples of 93 sheep from 44 flocks from 14 counties, 69 blood samples of goats from 28 flocks in 13 counties in Croatia were tested. Also, 14 sheep blood samples from three flocks from two BIH regions and one goat blood sample from the West Herzegovina Canton in BIH were tested. In four cases in sheep and two cases in goats serum samples from Croatia were not delivered for testing. Using molecular methods, 50 samples of vaginal swabs, placental specimen and fetal organs of sheep from 29 flocks from ten counties in Croatia and one sheep placental specimen from BIH were tested. In addition, 69 samples of vaginal swabs, placental specimen and fetal organs of goats from 18 flocks from eleven counties in Croatia were tested. Additionally, samples for molecular testing were delivered from the pathology department of the Croatian Veterinary Institute, Zagreb from four flocks with abortion history but without blood samples. In the case of 18 sheep flocks and twelve goat flocks no samples were delivered for molecular testing (Tab. 1, 2, 3).

Serological tests

Serological tests of sheep and goat blood samples were performed using the CHEKIT *Chlamydia* Antibody Test Kit (IDEXX ELISA; IDEXX, Switzerland). The indirect ELISA CIVTEST OVIS *Chlamydia* HP (CIVTEST; HiproLaboratorios, Spain) was used for further testing of sheep blood samples (Tab. 1). The tests were performed according to the manufacturer's instructions. In short, in the case of IDEXX ELISA test results were expressed as a percentage of the optical density (OD) in relation to the control sera after measuring on the spectrophotometer with filter at 450 nm. Samples with less than 30% were negative, suspicious samples were those with values ≥ 30% and < 40%, while samples with values ≥ 40% were considered positive. CIVTEST was intended only for sheep blood tests. The value of ≥ 40% was considered as a positive reaction. Flocks were considered seropositive if at least one test showed a positive or suspicious reaction.

Molecular investigation

DNA from vaginal swabs, placenta and tissues of aborted fetuses was isolated using QIAcube system (Qiagen, Hilden, Germany). Detection of *C. abortus* DNA was carried out by conventional PCR using primers pmp-F and pmp-R821 designed to target gene pmp 90/91 (Berri et al., 2009). The expected size of the PCR product is 821 bp for *C. abortus* and 650 bp for *C. pecorum*. Species-specific real-time PCR for *C. abortus* was performed with primers CpaOMP1-F, CpaOMP2-R and probe CpaOMP1-S created to the target gene ompA (Pantchev et al., 2010). Threshold value (Ct) was calculated automatically by the 7500 Real Time PCR System (Applied Biosystems, Singapore). Content of reaction mixtures and cycling parameters for both tests were performed according to the references (Berri et al., 2009; Pantchev et al., 2010). The positive control (*C. abortus* DNA) was provided by the Institute for Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, Slovenia.

Results

Positive or suspicious serological reactions with IDEXX ELISA were identified in 17 out of 93 sheep tested in eight counties in the Republic of Croatia and in four out of 14 sheep serums in two regions in BIH. With CIVTEST, positive reactions were identified in 19 out of 93 sheep tested in eight counties in Croatia and three out of 14 sheep serums in two regions in BIH.

TABLE 3: *Serological and molecular testing of sheep and goat samples in BIH*

Region of BIH	Origin	Farm	Serological test			Molecular tests		
			Number of sheep/ goats tested	ELISA IDEXX	CIVTEST	Number of samples tested	PCR (Berri et al.,2009)	Real Time RCR (Pantchev et al., 2010)
Republic of Srpska	sheep	BIH1	1	1+	1+	1	1+	1+
Herzegovina-Neretva Canton	sheep	BIH2	12	1+; 1+/-	2+	NT	NT	NT
Herzegovina-Neretva Canton	sheep	BIH3	1	1+	neg	NT	NT	NT
West Herzegovina Canton	goat	BIH4	1	1+	NT	NT	NT	NT

NT = not delivered for testing; 1+ = 1 positive; 2+ = 2 positive; 1+/- = 1 doubtful; neg = negative.
The same animals were tested with both serological methods and molecular methods.

29 positive or suspicious reactions were identified using IDEXX ELISA in 13 sheep (B1, C4, D1, D2, D3, E1, E2, E3, E8, G4, K1, M6, N5) and five goat (C1, C2, D1, E1, L2) flocks from Croatia, as well as five serums from three sheep (BIH1, BIH2, BIH3) and one goat (BIH4) flocks from BIH. Both tests showed a positive reaction in 13 sheep flocks (B1, C4, D1, D2, D3, E1, E2, E3, G4, K1, M6, BIH1, BIH2) in Croatia and BIH. Positive reactions with only IDEXX ELISA were assessed in three (E8, N5, BIH3) flocks and positive reactions with only CIVTEST were found in four (F1, G2, G3, K2) sheep flocks (Tab. 1, 3).

Using conventional and real-time PCR *C. abortus* was confirmed in one sheep flock (B1) from Sisak-Moslavina county in Croatia and one sheep flock (BIH1) in the neighbouring region of BIH (Republic of Srpska). No simultaneous infections with *C. pecorum* were observed by conventional PCR.

Discussion

The IDEXX ELISA can be used for monitoring sheep and goat flocks for the presence of *Chlamydia* (Lenzko et al., 2011) but cannot distinguish whether the infection is caused by *C. abortus*, *C. pecorum* or *C. psittaci* (Vretou et al., 2007; Wilson et al. 2009). CIVTEST also uses *C. abortus* antigen and the same drawbacks are expected. The most specific serological test (Institut Pourquier) based on highly immune-reactive complex antigens (POMPs) with high specificity for *C. abortus* and no cross-reactions with sera from *C. pecorum* infected animals (Sachse et al., 2009) unfortunately is not commercially available any more. Our investigation was based on samples collected at abortion. We carried out serological and molecular testing in order to get information about presence of EAE in national flocks of sheep and goats in Croatia. Same was done in limited material delivered from neighbouring BIH.

Positive or suspicious serological reactions in sheep with both tests were found in 13 (B1, C4, D1-D3, E1-E3, G4, K1, M6, BIH1, BIH2) out of 47 tested flocks. Additionally, three flocks (E8, N5, BIH3) were positive only with IDEXX ELISA and four flocks (F1, G2, G3, K2) only with CIVTEST. Seropositive flocks belonged to nine of out 13 counties represented by samples in the Republic of Croatia and all three sheep flocks from Bosnia and Herzegovina. In our investigation, seroprevalence in sheep tested by IDEXX ELISA and CIVTEST was 19.6% and 20.5% respectively, and in goats 11.4%. Values for positive samples ranged from 30.5 to 172.1% for IDEXX ELISA (suspicious included) and 40.0 to 87.9 for CIVTEST. Infection with *C. abortus* was confirmed using molecular tests in sheep positive with both serological tests. Positive serological reactions with IDEXX ELISA were found in five flocks (16.6%) in four counties (28.6%) in Croatia and in one flock in BIH (Tab. 2, 3). In Karlovac and Bjelovar-Bilogora County positive serological reactions were shown in both, sheep and goat blood samples. Seroprevalence in small ruminants in other studies, ranged from 4.8% in Sardinia (Masala et al., 2005) to 94% in Germany (Lenzko et al., 2011). These findings depend on numerous parameters (e. g. applied methods, abortion rates, flock size, management, etc.). In our investigation we had no data about flock size, management or other relevant epidemiological data except confirmation of abortion.

Conventional PCR allows rapid and specific detection of *Chlamydia* from clinical specimens (Berri et al., 2009; Sachse et al., 2009). The chosen method in our investigation allows us to distinguish between two species *C. abortus* and *C. pecorum*, often tested simultaneously at abortions (Berri et al., 2009; Lenzko et al. 2011). In the last few years real-time PCR has become the preferred method in laboratories due to high sensitivity and specificity (Ehricht et al. 2006; Pantchev et al., 2010; OIE, 2012). In this study, comprising data from Croatia and BIH, three sheep samples were positive with both molecular methods. There were no positive results for goat samples. *C. abortus* was confirmed in 2 (4.4%) out of 45 overall tested sheep and goat flocks. In a similar investigation in Mexico, in sheep with clinical history of abortion, Jiménez-Estrada et al. (2008) found that seroprevalence was 21.3% and *C. abortus* infection was confirmed by PCR in 0.65% of vaginal swabs. Lenzko et al. (2011) also found seropositive sheep in 30 out of 32 (94%) flocks, *Chlamydiaceae*-specific real-time or conventional PCR revealed the presence of *Chlamydia* in 25 out of 32 (78%), and species-specific real-time PCR detected *C. abortus* in 15 out of 32 (47%), *C. pecorum* in 13 out of 32 (41%) and *C. psittaci* in eight out of 32 (25%) flocks. In 31% (10/32) of flocks more than one chlamydial species was found. Sensitivity of serological testing is higher than molecular so it is necessary to repeat molecular testing for a few times and from different materials in order to confirm or exclude disease presence in a flock.

EAE is a notifiable disease in the Republic of Croatia but it still has not been controlled by routine laboratory testing regime in small ruminants. Based on passive surveillance (abortion cases) in Croatia and randomly picked etiologically inconclusive cases of abortion in BIH, our research was carried out in a limited form.

The results of this work confirmed the recent existence of a disease in national flocks in the Republic of Croatia and BIH. High seroprevalence in sheep and goats suggests that commercially available kits should be used as a screening method. Whenever possible, molecular identification of the causing agent should be performed. *C. abortus* confirmed in sheep samples represents the first published evidence of this agent in Croatia. Furthermore, a disease confirmation in neighbouring BIH stresses the need to permanently control EAE too. Besides zoonotic potential, EAE should be considered as a serious problem of sheep and goat production in many countries, particularly in the European Union. Our proposal for disease control in Croatia, at minimum, is serological testing of abortion cases in sheep and goats and at the same time molecular testing of abortion material using PCR (e. g. fetus organs, placentas and vaginal swabs). Evidence of infected flocks and introduction of a disease control program with objective to define flocks with officially disease free status should be a long-term goal. As a measure of disease control program, introduction of genotyping by multiple loci variable number of tandem repeats analysis (MLVA) for exploring the diversity of *C. abortus* could be helpful.

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