Identification and MLVA genotyping of *Chlamydia abortus* from abortion cases in small ruminants in Croatia

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Abstract

In addition to zoonotic potential, *Chlamydia (C.) abortus* is a very important bacterium causing serious disease in small ruminants. The main outcome of the disease is abortion in the late stages of pregnancy and the economic impact for farms is significant. During a three-year period (2015–2017), 191 vaginal swabs, 24 placentas, 210 foetal organs and 2 milk samples from small ruminant abortion cases were tested for *C. abortus* by real-time PCR. Positive samples were detected on eight sheep farms and two goat farms, with 8.4% of total samples testing positive samples. These samples were characterised using the MLVA method, and a single MLVA genotype (genotype [2]) was identified from sheep and goat samples, suggesting highly conserved *C. abortus* strains among the national flock. This study is the first description of *C. abortus* as a causative agent of abortion in goats in Croatia. More detailed study is required to recognize the epidemiological relevance of the abortion chlamydiosis. An open register of farms with defined health status should be established for each farm at the national level for better disease(s) control.

Key words: *small ruminants; Chlamydia abortus; chlamydial infection; abortion; Croatia*

Introduction

The genus *Chlamydia* (C.) is a group of obligate intracellular bacteria capable of causing severe disease in a range of animal species, and in humans. Recent classification recognizes a single genus (*Chlamydia*) with 11 species

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(Sachse et al., 2015). *C. abortus* is among several microbial species that play an important role in ruminant abortions. It is considered the most important cause of infectious abortions in sheep and goat flocks worldwide (http://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Countrytimelines; Rodolakis and Mohamad, 2010; Sachse et al., 2015; Selim, 2016; OIE, 2018).

C. abortus also causes sporadic abortions in cattle, though less frequently than in sheep and goats (Livingstone and Longbottom, 2006). Apart from *C. abortus* infection, other chlamydial species play a significant role associated with infertility: *C. pecorum* and several newly recognized species of *Chlamydia*-related organisms (CROs), order *Chlamydiales*, members of families *Parachlamydiaceae* and *Waddliaceae* (Borel et al., 2007; Godin et al., 2008; Wheelhouse et al., 2015). The disease is not easily diagnosed. Characteristics such as a unique replication cycle inside and outside the host, nonspecific symptoms at abortion or other clinical manifestations, short and late antibody response after abortion, cross reactivity with other chlamydial species and *Chlamydia*-like organisms, as well as inadequate selection and collection of samples (Borel et al., 2014) make diagnosis very challenging. The most effective means of etiological diagnosis are molecular methods. Real-time PCR (RT-PCR) (Pantchev et al., 2010) and restriction fragment length polymorphism (RFLP-PCR) (Ababneh et al., 2014) targeting the *omp*A gene are often used for *C. abortus* identification. Agent identification is predominantly conducted on vaginal discharge and swabs, placenta and foetuses as the prime targets (Wheelhouse et al., 2015; Selim, 2016; OIE, 2018).

A contaminated environment and infected animals shedding after abortion are the main sources of infection for contact animals. Recent molecular investigations of European *C. abortus* strains found a limited genomic diversity pointing to high stability of the genome (Seth-Smith et al., 2017). A typing method called multiple loci variable number of tandem repeats (VNTR) analysis, also known as multiple locus variable number of tandem repeats analysis (MLVA), has limited discriminatory power and to date has identified 7 different genotypes (Laroucau et al., 2009; Siarkou et al., 2015). The first study of abortion cases in sheep and goats in Croatia was conducted in 2012/2013. *C. abortus* was confirmed in sheep for the first time in Croatia based on molecular investigation of abortion cases (Špičić et al., 2015). As a result, identification of *C. abortus* became a routine test in abortion cases in sheep and goats in 2015, and in cattle in 2016. In 2016, the Croatian national flock included 632,087 sheep in 19,249 herds and 75,527 goats in 5,425 herds. To increase overall disease diagnosis, it is necessary to apply molecular methods to confirm cases of infection with *C. abortus* (Špičić et al., 2015; OIE 2018).

Materials and methods

Specimens

Routine monitoring of *C. abortus* was conducted on sheep and goat samples delivered to our laboratory after abortion. During a three-year period, 427 samples, predominately vaginal swabs and foetal organs (liver or stomach contents) were tested for *C. abortus* (Table 1).

DNA extraction

DNA was extracted from clinical samples (vaginal swabs, placenta and tissue of aborted foetuses) using the QIAamp DNA Mini QIAcube Kit (QIAGEN, Hilden, Germany) on the QIAcube system (Qiagen, Hilden, Germany). A total of 400 µL of tissue homogenized in saline solution was placed in a 2 mL screw cap tube containing 0.5 g of 0.10 – 0.25 mm glass

beads. Then 360 µL Buffer ATL and 40 µL Proteinase K were added. Tubes were placed in the Tissue Lyser for 2 min at 30 Hz, incubated in the thermomixer at 56 °C for 15 min with shaking at 450 rpm. Tissue Lyser homogenisation was repeated twice more, with the exception that the final incubation step lasted overnight at 56 °C. The samples were centrifuged at 10 000 rpm for 5 min to spin-down the beads. Then, 400 µL of supernatant were transferred to a new 2 mL microcentrifuge tube and samples were further processed on the QIAcube system, using the recommended tissue protocol. The DNA was stored at 4 °C while testing and then at -20 °C until further use. During the extraction process, a negative control containing PBS instead of sample tissue was used and processed in the same way as the other samples. The positive control of the test was genomic DNA isolated along with pathogen DNA.

Identification of *Chlamydia abortus* using PCR

Detection of *C. abortus* DNA was carried out with a species-specific real-time PCR for *C. abortus* using primers CpaOMP1-F, CpaOMP2-R and the probe CpaOMP1-S targeting the *omp*A gene (Pantchev et al., 2010). The threshold value (Ct) was calculated automatically with the 7500 Real Time PCR System (Applied Biosystems, Singapore). The positive control (*C. abortus* DNA) was provided by the Institute for Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, Slovenia.

MLVA genotyping of clinical samples

Genotyping of *C. abortus* DNA from clinical samples was conducted using the MLVA method based on 5 loci (ChlaAB_300, ChlaAB_457, ChlaAB_581, ChlaAB_620 and ChlaAB_914), as previously described by Laroucau et al. (2009). Three positive controls of *C. abortus* DNA (AB7, POS and S26/3), representing different genotypes, were provided by the Bacterial Zoonoses Unit, French, ANSES, Maisons-Alfort, France.

Analysis of MLVA-5 data

Band sizes from MLVA-5 results were translated into numbers of individual repeats and the results were presented in the form of 5-digit numerical codes based on the reference.

Results

Molecular identification of *C. abortus*

Over the three-year period, *C. abortus* was detected on eight sheep farms in six counties and on two goat farms in Split-Dalmatia County (Table 2; Figure 1). Of the total 427 samples, *C. abortus* was detected in 36 samples (8.4%) (Tables 1 and 2). Predominantly, *C. abortus* was confirmed from vaginal swabs (16 ewes and 2 goats), from stomach contents (7 ewes and 1 goat), placentas (3 ewes) and foetal livers (3 ewes and 3 goats). The results indicated a higher level of positivity for sheep placentas 17.6% (3/17) and ewe vaginal swabs 14.9% (16/107), than for goat vaginal swabs 2.4% (2/84), and ewe and goat foetal contents 6.7% (14/210).

Genotyping of sheep and goat samples using MLVA on 5 loci

Among the 36 positive *C. abortus* samples, genotyping using the MLVA-5 method was successful for 33 samples (92%). The MLVA-5 method allowed the assignment of a unique 5-digit code to all tested DNA samples (Table 3) corresponding to the genotype [2].

Discussion

C. abortus is an economically important livestock pathogen, particularly for sheep and goats. Infection causes abortion and premature birth or weak offspring. In small

ruminants, the disease is known as ovine enzootic abortion (OEA), enzootic abortion of ewes (EAE) or ovine chlamydiosis. The disease is widespread in European small ruminant farms and in the countries neighbouring Croatia' (Kreizinger et al., 2015; Krkalić et al., 2015). *C. abortus* infection was confirmed in two sheep flocks in continental Croatia and in one sheep flock in Bosnia and Herzegovina (BiH) in the lambing period in 2012–2013 using molecular methods (Špičić et al., 2015). An infection on a goat farm in neighbouring BiH was confirmed in the same time (Krkalić et al., 2015). In Hungary, *C. abortus* was detected in ovine abortion cases by qPCR (Ct: 26.61-35.89) and identified by sequencing (76.2%, 16/21) and in two goat cases (40%, 2/5) (Ct: 34.73 and 41.72) (Kreizinger et al., 2015). A seroprevalence study in Croatia from 2015 to 2017 indicated that chlamydial infections are widespread throughout Croatian sheep and goat farms. Positive sheep and goat reactors were found in 10 continental and coastal counties (Špičić et al., 2017). Seropositive animals could be found at a low rate due to delayed antibody response, two or more weeks after abortion. Differences between individual serological and molecular results were also observed in a study of small ruminant abortions in Algeria (Merdja et al., 2015).

In the three-year period, *C. abortus* was detected on eight sheep farms. It was also confirmed on two goat farms for the first time using species-specific real-time PCR for agent identification. This shows an overall low-frequency transmitting population. Infection was detected in three sheep farms over two consecutive years. Unfortunately, there is no information as to whether the required control measures were applied at the affected farm. *C. abortus* was confirmed in 8.4% of samples. Sheep placentas (17.6%), sheep vaginal swabs (14.9%), goat vaginal swabs (2.4%), sheep and goat foetal livers and stomach contents (6.7%) tested positive for *C. abortus*. It is known that after abortion, *Chlamydiaceae* do not persist in vaginal mucus for a long time, so testing of various samples raises sensitivity significantly. According to the applied genotyping method, the present study detected a genotype [2] MLVA pattern identical to the AB7 reference strain (ANSES, France), identical for all Croatian samples throughout the study period. The same genotype was also found for 115 strains isolated from sheep, goats, cattle and springbok in France, Germany, Greece, Italy, Germany, Namibia, Tunisia and Algeria (Laroucau et al., 2009; Merdja et al., 2015).

Further research based on molecular methods such MLST or whole genome sequencing, could be conducted for

Table 1. Specimens collected in abortion cases in domestic ruminants over a three-year period.

Year	2015		2016		2017		Total
Origin / Specimen	Sheep	Goat	Sheep	Goat	Sheep	Goat	
Vaginal swab	28	7	40	31	39	46	191
Placenta	6	2	2	1	9	4	24
Foetus (liver and/or stomach content)	48	16	54	36	34	22	210
Milk	0	0	0	0	0	2	2
Total	82	25	96	68	82	74	427

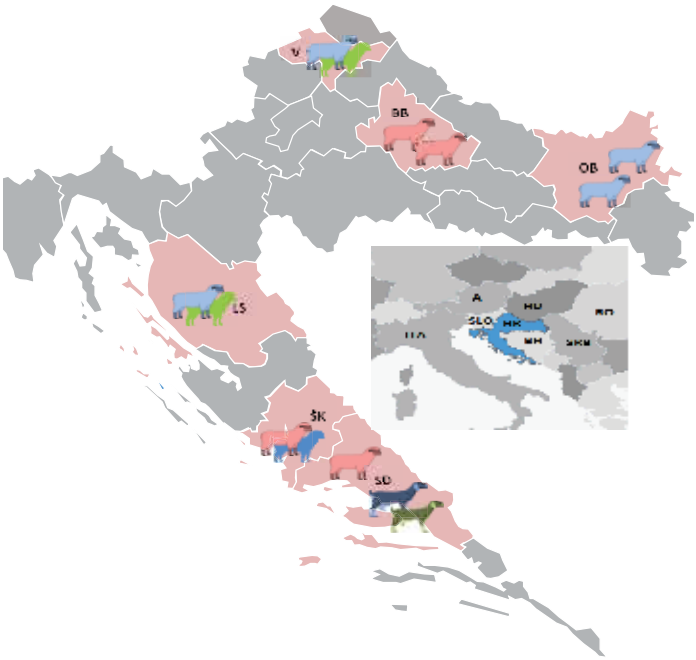

Figure 1. Geographical distribution of *C. abortus* infection cases in small ruminants over a three-year period.
Legend: red sheep figures represent one positive flock in 2015; blue sheep and goat figures represent one positive flock in 2016; green sheep and goat figures represent one positive flock in 2017; sheep silhouettes in Varaždin (V) and Lika-Senj (LS) Counties represent a positive flock in two consecutive years (2016 and 2017); sheep silhouettes in Šibenik-Knin (SK) County represent a positive flock in two consecutive years (2015 and 2016); LS- Lika-Senj County; BB- Bjelovar-Bilogora County; OB- Osijek-Baranja County; SD- Split-Dalmatia County.

Table 2. *C. abortus* positive samples according to year and place of sampling

Year / County	Year 2015	Year 2016		Year 2017	
	Sheep	Sheep	Goat	Sheep	Goat
Bjelovar-Bilogora	9 VS/1 farm; 1 P/1 farm	/	/	/	/
Lika-Senj	/	1 VS, 1 L/1 farm	/	1 VS, 1 L, 1 SC/1 farm	
Osijek-Baranja	/	1 SC/1 farm	/	/	/
Split-Dalmatia	1 SC/1 farm	/	2 VS/ 1farm	/	3 L, 1 SC /1 farm
Šibenik-Knin	2 P/1 farm	1 SC/1 farm	/	/	/
Varaždin	/	1 VS/1 farm, 3 VS, 3 SC, 2 L/1 farm	/	1 VS /1 farm	/
Total	13 samples 4 farms	13 samples 5 farms	2 samples 1 farm	4 samples 2 farms	4 samples 1 farm

Legend: VS- vaginal swab; L- liver; SC- stomach content; P- placenta

Table 3. Genotypes found in sheep and goat samples

KEY	YEAR	FARM	ANIMAL	SAMPLE	COUNTRY	COUNTY	CAUSE	ChlaAB_300		ChlaAB_457		ChlaAB_581		ChlaAB_620		ChlaAB_914	
								SIZE (bp)	REPEATED UNIT	SIZE (bp)	REPEATED UNIT	SIZE (bp)	REPEATED UNIT	SIZE (bp)	REPEATED UNIT	SIZE (bp)	REPEATED UNIT
1	2015	A	sheep	fetus	CRO	SD	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
2	2015	K	sheep	placenta	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
3	2015	B	sheep	placenta	CRO	ŠK	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
4	2015	B	sheep	placenta	CRO	ŠK	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
5	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
6	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
7	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
8	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
9	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
10	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
11	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
12	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
13	2015	C	sheep	genital swab	CRO	BB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
14	2016	D	sheep	genital swab	CRO	V	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
15	2016	B	sheep	placenta	CRO	ŠK	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
16	2016	D	sheep	stomach contents	CRO	V	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
17	2016	D	sheep	genital swab	CRO	V	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
18	2016	D	sheep	genital swab	CRO	V	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
19	2016	D	sheep	stomach contents	CRO	V	AB	~ 217	**3**	~ 365	1	~ 145	1	~ 228	2	~ 157	1
20	2016	D	sheep	stomach contents	CRO	V	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
21	2016	E	sheep	genital swab	CRO	V	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
22	2016	F	sheep	stomach contents	CRO	OB	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
23	2016	G	sheep	genital swab	CRO	LS	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
24	2016	G	sheep	fetus - liver	CRO	LS	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
25	2016	G	sheep	fetus - liver	CRO	LS	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
26	2016	I	goat	genital swab	CRO	SD	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
27	2016	I	goat	genital swab	CRO	SD	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
28	2017	D	sheep	genital swab	CRO	V	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
29	2017	J	goat	fetus - liver	CRO	SD	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
30	2017	J	goat	fetus - liver	CRO	SD	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
31	2017	J	goat	fetus - liver	CRO	SD	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
32	2017	G	sheep	genital swab	CRO	LS	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
33	2017	G	sheep	stomach contents	CRO	LS	AB	~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
PC1	ANSES	AB7			France			~ 226	3	~ 365	1	~ 145	1	~ 228	2	~ 157	1
PC2	ANSES	POS			France			~ 223	2	~ 440	2	~ 145	1	~ 220	1	~ 157	1
PC3	ANSES	S26/3			France			~ 226	3	~ 365	1	~ 160	2	~ 240	3	~ 173	2

Legend: bp- base pair; V- Varaždin County, LS- Lika-Senj County, SK- Šibenik-Knin County, BB- Bjelovar-Bilogora County; OB- Osijek-Baranja County; SD- Split-Dalmatia County; PC1- positive control- representing genotype 2 MLVA pattern, PC2- positive control- representing genotype 6 MLVA pattern; PC3- positive control- representing genotype 5 MLVA pattern

better discrimination between the strains isolated in particular geographic locations (Siarkou et al., 2015; Seth-Smith et al., 2017). However, in order to effectively control the disease in the small ruminant national flock, the current abortion control policy should be continued. It is important to insist on timely controls of animal migrations inside and outside the country. This is possible by instructing online open register of infected and disease-free farms.

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Identifikacija i MLVA genotipizacija vrste *Chlamydia abortus* iz slučajeva pobačaja malih preživača u Hrvatskoj

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Osim zoonotskog potencijala, vrsta *Chlamydia* (*C.*) *abortus* je vrlo važna bakterija koja može prouzročiti tešku bolest malih preživača. Dominantni klinički znak bolesti je pobačaj u kasnom stadiju graviditeta sa znatnim ekonomskim učinkom za farme. Tijekom trogodišnjeg razdoblja (2015.-2017.) metodom Real time (RT) PCR na *C. abortus* testiran je 191 vaginalni obrisak, 24 placente, 210 organa fetusa i 2 uzorka mlijeka iz slučajeva pobačaja malih preživača. Pozitivni uzorci ustvrđeni su u 8 farmi ovaca i 2 farme koza, što ukupno predstavlja 8,4 % pozitivnih uzoraka. Ovi uzorci su tipizirani metodom MLVA i identificiran je jedinstveni genotip MLVA (genotip [2]). Dobiveni rezultat ukazuje na visok stupanj podudarnosti sojeva *C. abortus* u nacionalnom stadu ovaca i koza. Ovo istraživanje prvi je opis vrste *C. abortus* kao uzročnika pobačaja u koza u Hrvatskoj. Temeljem ustvrđenog rezultata bilo bi uputno provesti daljnja, detaljnija istraživanja da bi se prepoznalo epidemiološko i ekonomsko značenje infekcija vrstom *C. abortus*. Radi kvalitetnijeg nadzora bolesti potrebno je uspostaviti otvoreni registar gospodarstava s definiranim zdravstvenim statusom za svaku pojedinu farmu.

Ključne riječi: *mali preživači, Chlamydia abortus, klamidioza, enzoootski pobačaj ovaca, Hrvatska*