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ORIGINAL ARTICLE

Serological and nested PCR survey to determine the occurrence of chlamydia infections in the Polish cattle population

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Abstract

Chlamydia spp. is an obligate intracellular agent that causes chlamydiosis in animals and humans. The aim of the presented study was to investigate the prevalence of *Chlamydia* infection in the Polish cattle population, both asymptomatic and having reproductive disorders. The study was performed on 4,475 serum samples collected from 16 Polish provinces at the turn of 2009–2011. The samples (3,419 from asymptomatic cattle and 1,056 from cattle with reproductive disorders) were tested by complement fixation test (CFT). Moreover, 160 and 201 samples of biological materials from both groups of cattle, respectively, were tested by nested PCR. The results obtained for two tested groups were compared by $\chi 2$ (ch-squared) test, both individually for each region (province), and generally for the whole country. The CFT results showed that the seroprevalence of *Chlamydia* spp. infections in the asymptomatic cattle population was 4.15%, while in the cattle with reproductive disorders – 7.20%. There was a significant statistical difference between compared groups for whole country, but there were no significant differences for individual provinces. The results of PCR showed that *Chlamydia* spp. was present in both asymptomatic cattle and cattle having reproductive disorders. The nested PCR study confirmed the presence of *Chlamydia abortus* and *Chlamydia suis* in the tested samples. The presented study indicates that infections with *Chlamydia* spp. are present among Polish cattle, but the percentage of infected animals is not high.

Key words

cattle, *Chlamydia spp.,* chlamydiosis, complement fixation test, PCR

INTRODUCTION AND OBJECTIVE

Chlamydia are obligate, intracellular, gram-negative bacteria that infect a wide variety of host species, including vertebrates, arthropods, and amoeba [1]. As their general importance is based on two aspects: economic losses to the animal owners and potential zoonotic transmission to humans. The most prominent *Chlamydia* agent in cattle is *Chlamydia abortus* [2]. Moreover, recent evidence suggested that infection of cattle with *Ch. suis* and *Ch. psittaci* is also possible [3, 4]. Chlamydial infection in cattle has been associated with reproductive disorders, including abortion, endometritis, repeat breeding, vaginitis, seminal vesiculitis, weak calves, and perintanal mortality [5]. In cattle, *Chlamydia* such as *Chlamydia pecorum*, *Chlamydia abortus,* and *Chlamydia psittaci,* are also found in connection with infection of the respiratory tract [6]. *Chlamydia abortus* is a recognised cause of epizootic bovine abortion and a cause of bovine infertility worldwide. In bulls, the infection can cause epididymitis, seminal vesiculitis, and testicular atrophy, and affects semen quality. The organism has also been shown to be shed in semen, and multiplication of *Chlamydia abortus via* contaminated semen can result in local infections and inflammatory reactions in the uterus, which can subsequently lead to infertility in heifers [7].

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Despite the serious economic problem connected with *Chlamydia* infections, no studies have been carried out in the cattle population in Poland. The problem of *Chlamydia* infection in cattle is important not only for breeders in Poland, but also for other countries of the European Union as a result of increased trade leading to increased risk of spreading *Chlamydia* infection in the European cattle population. The scale of economic losses caused by the disease is not available in the literature. Therefore, the risk of transmission to humans also increases. Based on the literature data, it is known that zoonotic transmission is possible by contact with infected animals [1]. Cases of transmission of *Chlamydia abortus* from infected animals to humans have been reported [8, 9]. The most common route of transmission is inhalation of infected aerosol from urine, foetal fluids and stools, and also after a short stay in an accommodation where infected animals have been kept. The infection with *Chlamydia* may result in respiratory disorders, and can lead to abortion in the case of pregnant women.

The aim of the presented study was to investigate the prevalence of chlamydial infection in the Polish cattle population, in both asymptomatic cattle and having reproductive disorders.

MATERIALS AND METHODS

The study was performed at the turn of 2009–2011 on 4,475 serum samples collected from cattle in 16 Polish provinces (Voivodeships). The samples came from asymptomatic cattle

(3,419 sera) cattle and having reproductive disorders (1,056 sera), and tested by complement fixation test (CFT). Moreover, 160 samples (vaginal swabs, placenta, and tissues from aborted foetuses) were taken from asymptomatic animals and 201 from cattle with reproductive disorders, and tested by nested PCR.

Serological examinations were performed using CFT, a diagnostic technique recommended by the World Organisation for Animal Health (OIE) [10]. For CFT, Institut Virion/Serion GmbH (Germany) and Sera and Vaccines Manufacturing (Biomed-Kraków, Poland) reagents were used. Before each examination, an intra-laboratory evaluation, including antigen titration against a positive control serum, and checking the activity of other reagents used in the reaction, were carried out to find the actual titre *versus* activity ratio in relation to that declared by the manufacturers. The specific reaction of CFT, its consecutive steps, and result interpretation, were performed according to the Manual of Standards for Diagnostic Tests and Vaccines [10]. The starting dilution of the examined samples was 1:4, and the ending dilution was 1:64. Serum was considered as positive when a partial inhibition of haemolysis was observed at the dilution of 1:32.

DNA extracts from swabs, placenta, and tissues were performed using the commercial QIAamp DNA mini kit (Qiagen), following the manufacturer's instructions. The DNA extracts were tested by nested PCR for *Chlamydia abortus/Chlamydia suis*. The first step was genus-specific amplification where primers 191 CHOMP (5'-GCI YTI TGG GAR TGY GCI AC-3') and CHOMP371 (5'-TTA GAA ICK GAA TTG ICG RTT IAY GTG IGC IGC-3'), were used. The primer set amplified a 576–597 base pair (bp) fragment of the *omp*A gene. For the second amplification, 1 µl of the genus-specific product and primer combination 201 CHOMP (5'-GGI GCW GMI TTC CAA TAY GCI CAR TC) /CHOMP 336 (5'CAAGMTTTTCTGGAYTTMAWYTTG TT-3') and generates the amplicon of 434–455 bp size were used. In order to determine the *Chlamydia* species, 1 µl of the genus-specific product form the first PCR reaction and primer combination 201CHOMP (5'-GGI GCW GMI TTC CAA TAY GCI CAR TC-3')/TRACH269 (5'-ACC ATT TAA CTC CAA TGT ARG GAG TG-3') and second combination of primers CHOM336 (5'CAA GMT TTT CTG GAY TTM AWY TTG TT-3') and 218 PSITT (5'GTA ATT TCI AGC CCA GCA CAA TTY GTG-3') generates the amplicon of 250bp and 389–404bp specific for *Chlamydia suis* and *Chlamydia abortus,* respectively, were used. The final volume of the reaction mixture amounted to 50 µl including 5 µl of 10 x PCR buffer, 2 µl of 50 mM $MgCl_2$, 0.2 µl of 10 mM dNTP, 1 µl of 20 pmol specific primer, 0.2 µl of 5 U/µl thermostable polymerase DNA, 39.6 µl of sterile water, and 1 µl of genomic DNA. The amplification was carried out in an Tepersonal termocycler (Whatman Biometra) using the following cycling parameters: 50 cycles, initial denaturation at 97 °C – 60 s, denaturation at 97 °C – 60 s, annealing at 50 °C – 60 s, elongation at 72 °C – 60 s, final elongation at 72 °C – 60 s. The PCR reactions were analysed by electrophoresis of 8 µl PCR product and 2 µl of Gel Loading Solution (Sigma) through a 1% agarose gel in 1 x TAE buffer, and visualised by staining with ethidinum bromide and ultraviolet transillumination. The molecular weight of the obtained product was determined on the basis of molecular weight marker, which was GeneRuler™100 bp DNA Ladder (Fermentas) and positive control of DNA.

The $\chi 2$ (ch-squared) test with appropriate Yatesa's corrections, V-squared or Fisher's exact test at the level of significance $\alpha=0.05$ were used for statistical analysis. The results obtained for asymptomatic cattle were compared with results for animals with reproductive disorders for individual region of Poland and for the whole country.

RESULTS

Serological results are presented in Tables 1–2 and their statistical analysis in Table 5. Taking into account all CFT results as positive, the seroprevalence of *Chlamydia* spp. infections in the Polish cattle population is not high, and ranges from 1.47% in the Mazovia Province to 7.14% in the Lubuskie Province (average 4.15%). The individual statistical analysis for individual provinces did not show statistically significant differences between two compared groups (asymptomatic cattle and having reproductive disorders). On the other hand, comparison of the results for whole country showed that the value of $\chi 2$ was 16.13, and was highly statistically significant ($p=0.0001$). The cattle with reproductive disorders had an overall percentage of 7.20%, with a range from 4.04% in the Pomerania Province to 16.67% in the Warmia-Mazuria Province. The CFT results were negative, but the presence of antibodies was noted (titre <32) in asymptomatic cattle. The antibodies were found in 0.90% of the cattle in the Świętokrzyskie Province and 41.49% in the Lubelskie Province (average level 23.92%). The similar percentage of animals with the same antibody titres was noted in cattle with reproductive disorders. The mean percentage of samples with the presence of antibodies with titre <32 amounted to 18.47%, and was the highest in the Lower Silesian Province (39.76%).

The most of seropositive asymptomatic animals had a titre of 32. The average percentage of positive samples with antibody titre of 32 was 2.69%, while the average percentage of stronger seropositive animals with titre of 32 was 1.46%. The percentage of seropositive cattle (titre 32) was the highest in the Lubuskie Province (7.14%), while 0.0% of animals was noted in the provinces of Opole, Mazovia, and Silesia. The presence of *Chlamydia* antibodies with titre >32 ranged from 3.41% in the Wielkopolska Province to 0.0% in the Lubuskie, Malopolska, and Warmia-Mazuria Provinces. For comparison, in cattle with reproductive disorders, the titre 32 ranged from 16.67% in the Warmia-Mazuria Province to 0.00% in the Opole Province. The average percentage of animals with titre >32 was 3.03%, and was lower than animals with titre 32 (4.17%).

Based on the presented study, it was possible to observe that the highest level of the *Chlamydia* spp. seropositivity was localised in the more industrialised regions of Poland, which have more intensive levels of agricultural and cattle breeding production (Fig. 1). The highest percentage of positive samples was noted in the Wielkopolska Province (6.82% – asymptomatic cattle; 7.14% – cattle with reproductive disorders), the Podlasie Province (6.75% – asymptomatic cattle; 7.29% – cattle with reproductive disorders), the Małopolska Province (6.67% – asymptomatic cattle; 7.14% – cattle with reproductive disorders), the Warmia-Mazuria Province (5.88% – asymptomatic cattle; 16.67% – cattle with reproductive disorders), the Łódź Province (5.0% – asymptomatic cattle; 7.32% – cattle with reproductive disorders), whereas the lowest in the Mazovia Province (1.47% – asymptomatic cattle; 8.06% – cattle with reproductive disorders), the Lower Silesia (1.53% – asymptomatic cattle; 6.02% – cattle with reproductive disorders).

Table 1. Seroprevalence of *Chlamydia* spp. in the Polish cattle population (asymptomatic animals).

Province	Negative samples		Positive samples		Total	Negative sample	Positive sample
	samples without *Chlamydia* antibodies	samples with presence of *Chlamydia* antibodies	samples with presence of *Chlamydia* antibodies	samples with presence of *Chlamydia* antibodies			
	Titre		Titre				
	-	< 32	32	>32			
Lower Silesia	98 (74.80%)	31(23.66%)	1 (0.76%)	1 (0.76%)	131	129 (98.47%)	2 (1.53%)
Kujawy-Pomerania	750 (77.32%)	220 (21.95%)	21 (2.09%)	11 (1.10%)	1,002	970 (98.80%)	32 (3.20%)
Lubelskie	189 (54.47)	144 (41.49%)	5 (1.44%)	9 (2.59%)	347	333 (96.00%)	14 (4.0%)
Lubuskie	24 (85.71%)	2 (7.14%)	2 (7.14%)	-	28	26 (92.86%)	2 (7.14%)
Łódź	90 (90.00%)	5 (5.00%)	3 (3%)	2 (2.00%)	100	95 (95.00%)	5 (5.00%)
Mazovia	67 (98.53%)	-	-	1 (1.47%)	68	67 (98.53%)	1 (1.47%)
Małopolska	21 (70.00%)	7 (23.33%)	2 (6.66%)	-	30	28 (93.33%)	2 (6.67%)
Opole	25 (78.12)	6 (18.75)	-	1 (3.12%)	32	31 (96.87%)	1 (3.12%)
Podkarpacie	561 (69.90%)	196 (24.4%)	34 (4.20%)	12 (1.50%)	803	757 (94.27%)	46 (5.73%)
Podlasie	85 (80.95%)	13 (12.38%)	5 (4.85%)	2 (1.90%)	105	98 (93.33%)	7 (6.75%)
Pomerania	132 (63.77%)	68 (26.88%)	6 (2.89%)	1 (0.48%)	207	200 (96.17%)	7 (3.38%)
Silesia	58 (46.98%)	20 (16.20%)	-	3 (3.70%)	81	78 (97.3%)	3 (2.70%)
Świętokrzyskie	104 (94.45%)	1 (0.90%)	3 (2.73%)	2 (1.81%)	110	105 (95.46%)	5 (4.54%)
Warmia-Mazuria	31 (91.17%)	1(2.94%)	2 (5.88%)	-	34	32 (94.12)	2 (5.88%)
Wielkopolska	74 (84.09%)	8 (9.09%)	3(3.41%)	3(3.41%)	88	82 (93.18%)	6 (6.82%)
West Pomerania	150 (59.28%)	96 (37.94%)	5 (1.98)	2 (0.79%)	253	246 (97.23%)	7 (2.77%)
Total	2,459 (71.92%)	818 (23.92%)	92 (2.69%)	50 (1.46%)	3,419	3,277(95.85%)	142 (4.15%)

Table 2. Seroprevalence of *Chlamydia* spp. in the Polish cattle population (reproductive disorders only).

Province	Negative samples		Positive samples		Total	Negative sample	Positive sample
	samples without *Chlamydia* antibodies	samples with presence of *Chlamydia* antibodies	samples with presence of *Chlamydia* antibodies	samples with presence of *Chlamydia* antibodies			
	Titre		Titre				
	-	< 32	32	>32			
Lower Silesia	45 (54.22%)	33 (39.76%)	2 (2.41%)	3 (3.61%)	83	78 (93.98%)	5 (6.02%)
Kujawy-Pomerania	30 (88.24%)	2 (5.88%)	2 (5.88%)	-	34	32 (94.12%)	2 (5.88%)
Lubelskie	60 (61.86%)	30 (30.93%)	3 (3.09%)	4 (4.12%)	97	90 (92.78%)	7 (7.22%)
Lubuskie	15 (93.75%)	-	1 (6.25%)	-	16	15 (93.75%)	1 (6.25%)
Łódź	29 (70.73%)	9 (21.95%)	3 (7.32%)	-	41	38 (92.68%)	3 (7.32%)
Mazovia	56 (90.32%)	1 (1.61%)	3 (4.84%)	2 (3.23%)	62	57 (91.94%)	5 (8.06%)
Małopolska	7 (50.00%)	6 (42.86%)	1 (7.14%)	-	14	13 (92.86%)	1(7.14%)
Opole	13 (81.25%)	2 (12.50%)	-	1 (6.25%)	16	15 (93.75)	1 (6.25%)
Podkarpacie	98 (78.4%)	15 (12%)	7 (5.60%)	5 (4.00%)	125	113 (90.40%)	12 (9.60%)
Podlasie	80 (83.33%)	9 (9.38%)	3 (3.13%)	4 (4.17%)	96	89 (92.71%)	7 (7.29%)
Pomerania	91 (91.92%)	4 (4.04%)	2 (2.02%)	2 (2.02%)	99	95 (95.96%)	4 (4.04%)
Silesia	26 (81.25%)	3 (9.38%)	2 (6.25%)	1 (3.13%)	32	29 (90.63%)	3 (9.38%)
Świętokrzyskie	45 (64.29%)	21 (30%)	2 (2.86%)	2 (2.86%)	70	66 (94.28%)	4 (5.71%)
Warmia-Mazuria	10 (83.33%)	-	2 (16.67%)	-	12	10 (83.33%)	2 (16.67%)
Wielkopolska	140 (66.67%)	55 (26.19%)	9 (4.28%)	6 (2.86%)	210	195 (92.86%)	15 (7.14%)
West Pomerania	40 (81.63%)	5 (10.20%)	2 (4.08%)	2 (4.08%)	49	45 (91.84%)	4 (8.16%)
Total	785 (74.34%)	195 (18.47%)	44 (4.17%)	32 (3.03%)	1056	980 (92.80%)	76 (7.20%)

The results of PCR reaction of asymptomatic cattle are presented in Table 3 and their statistical analysis in Table 6. The results showed that *Chlamydia* spp. organisms were present in the cattle. The percentage of affected animals was significantly lower (6.25%) than in cattle with reproductive disorders. Both species of *Chlamydia suis* and *Chlamydia abortus* were detected in the tested samples. However, the presence of *Chlamydia suis* was not confirmed in placenta and tissues from aborted foetuses.

The results of molecular examination (PCR) of cattle with reproductive disorders are presented in Table 4, and their statistical analysis in Table 6. The Table 4 indicates that

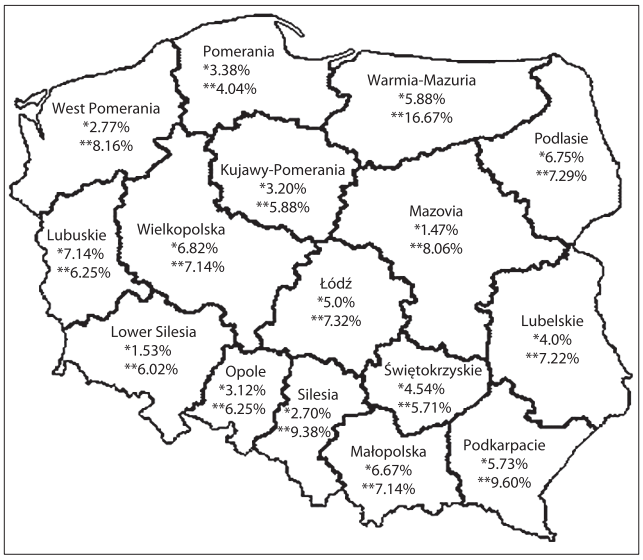

Figure 1. Seroprevalence of *Chlamydia* spp. in asymptomatic cattle with reproductive disorders in different regions of Poland.
* asymptomatic cattle
** cattle with reproductive disorders

Table 3. Molecular examination (nested PCR) of *Chlamydia* in the Polish cattle population (asymptomatic animals).

Type of sample	Total	No. of unaffected animals	No. of affected animals	Detected chlamydial species
vaginal swabs	125	119	5	*Chlamydia abortus*
			1	*Chlamydia suis*
placenta	20	17	3	*Chlamydia abortus*
			0	*Chlamydia suis*
tissues from aborted foetuses	15	14	1	*Chlamydia abortus*
			0	*Chlamydia suis*
Total	160	150	10 (6.25%)	

Table 4. Molecular studies (PCR) of *Chlamydia* in the Polish cattle population (reproductive disorders).

Type of sample	Total	No. of unaffected animals	No. of affected animals	Detected chlamydial species
vaginal swabs	157	142	15	*Chlamydia abortus*
			3	*Chlamydia suis*
placenta	25	19	6	*Chlamydia abortus*
			1	*Chlamydia suis*
tissues from aborted foetuses	19	17	2	*Chlamydia abortus*
			0	*Chlamydia suis*
Total	201	178	23 (11.44%)	

Chlamydia spp. was present in 23 out of 201 tested animals (11.44 per cent). *Chlamydia abortus* was detected by PCR in vaginal swabs, placenta, and tissues from aborted foetuses. The specific sequences of DNA for *Chlamydia suis* were confirmed in three tested vaginal swabs and one placenta. Among 157 tested vaginal swabs, one with the presence of *Chlamydia suis* and *Chlamydia abortus* was found, thus suggesting mixed chlamydial infection.

Statistical analysis results of nested PCR showed that values of $\chi 2$ with appropriate correction were not statistically significant for both comparisons of results categorized by type of sample and altogether.

Table 5. Results of statistical analysis for results of CFT.

Method	CFT	
Province	$\chi 2$	p value
Lower Silesia	1.98*	0.15
Kujawy-Pomerania	0.14*	0.70
Lubelskie	1.07*	0.30
Lubuskie	0.26*	0.61
Łódź	0.02*	0.88
Mazovia	1.88*	0.17
Małopolska	0.34*	0.55
Opole	0.07*	0.79
Podkarpacie	2.76**	0.09
Podlasie	0.03**	0.86
Pomerania	0.00*	0.96
Silesia	0.56*	0.45
Świętokrzyskie	0.00*	1.00
Warmia-Mazuria	0.30*	0.58
Wielkopolska	0.01**	0.92
West Pomerania	2.04*	0.15
all provinces	16.13 *^	0.001^

* $\chi 2$ with Yates correction
** $\chi 2$ with V-quarter correlation
^ significant difference

Table 6. Results of statistical analysis for results of PCR.

Method	PCR	
tested material	$\chi 2$	p value
vaginal swabs	2.27**	0.13
placenta	0.14*	0.70
aborted foetuses	-	0.590
total samples	2.89 ***	0.89

* $\chi 2$ with Yates correction
** $\chi 2$ with V-quarter correlation
*** Fisher's exact test

DISCUSSION

The serological results showed that cattle revealed positive results of serological survey in all provinces of Poland, and the presence of *Chlamydia* antibodies was noted. These data show that the problem of *Chlamydia* spp. infection in cattle in Poland is not significant, but the pathogen is countrywide, and control investigations have to be performed. These results compare with published data from Poland over the several past years, and indicate that the percentage of seropositive samples in cattle is now lower [11, 12], although the previous studies were performed on a selected population of cattle and not from all provinces. The numbers of samples tested from the provinces varied, but the rate of positivity does not appear to relate directly to the number of samples tested. To our knowledge, the present survey is the first epidemiological evaluation of the prevalence of *Chlamydia* in cattle in the whole country.

Antibodies against *Chlamydia* were found in sera of cattle in several European countries [13, 14, 15, 16]. Most of the surveys were performed by ELISA, making it is very difficult to compare them with the presented results because CFT is less sensitive, but still recommended by the OIE for detecting

antibodies against *Chlamydia*. The complement fixation test detects only IgG1 immunoglobulins persisting for 3–4 weeks after infection, ELISA also detects other antibody subclasses [17]. Moreover, most of the serological tests cannot detect infection with these pathogens in the first phase because, generally, the titres are very low [18].

Pantchev et al. (2010) performed the examinations of cattle in Germany, and they too observed cases of mixed chlamydial infection [4]. Combinations of *Chlamydia abortus* and *Chlamydia psittaci,* or *Chlamydia suis* and less *Chlamydia pecorum* and *Chlamydia suis* in cattle were described. Recent data have demonstrated that subclinical chlamydial infections by both species, *Chlamydia abortus* and *Chlamydia pecorum*, are ubiquitous in cattle and often not detected due to low sensitivity of diagnostic techniques [3, 19]. However, frequently there is no correlation between the results of PCR or real-time PCR detecting the presence of DNA and serological tests determining the level of specific antibodies. In recent studies of bulls, *Chlamydia* antibodies were detected in 50.8%, while the PCR confirmed the presence of *Chlamydia* in semen in 9.2%, preputial washing in 10.7%, and faecal samples in 18.0% of the bulls [14]. PCR-positive but serologically negative bulls might have not exhibited a systemic immune response, possibly due to the obligate intracellular lifestyle of *Chlamydia* that might hide them from the systemic immune response. In contrast, other data has shown that naturally infected calves were in 60% seropositive, while all of them were the shedders of *Chlamydia* [20].

Generally, based on the literature data, it is known that chlamydial infections occur in cattle breeding; however, the epizootic situation varies in different European countries. For example, in Sweden there are 0.4% of seropositive cows, and the performed studies suggest that *Chlamydia abortus* infection is absent or rare, whereas *Chlamydia pecorum* is probably more widespread. The same researcher suggests that *Chlamydia* spp. are not related to reproductive disorders in Swedish cattle [21]. In Italy, serological investigation of cattle in different areas detected seroprevalence for *Chlamydia* ranging from 2 – 28%, but association between seropositivity and abortion has not been examined [17]. In Ireland, the percentage of seropositive cattle in ELISA was 4.44%, in Taiwan – 51.3% for asymptomatic animals, and 71.4% for aborted cows [15, 16].

The occurrence of *Chlamydia abortus* and *Chlamydia suis* in the animals with reproductive disorders can suggest that these pathogens may be one of the many factors responsible for these disorders, and can have an impact on economic losses. Economic losses caused by late-term *Chlamydia abortus* infection, and the subsequent epizootic bovine abortion, are readily apparent. However, infection may result in unrecognised economic losses as the consequence of subclinical infertility [19].

CONCLUSION

The presented study indicates that infections with *Chlamydia* spp. are present in Polish cattle. The seroprevalence study has demonstrated that 4.15% and 7.20% of the Polish asymptomatic cattle population and cattle with reproductive disorders, respectively, is sero-positive to *Chlamydia* spp. Statistical analysis showed that there are significant differences between the two compared groups of cattle for the whole country. In contrast, in individual provinces, no statistical significant differences for the compared groups of cattle were noted. Moreover, the molecular studies show that infection with *Chlamydia abortus* is more widespread than with *Chlamydia suis*. It should be noted that if the *Chlamydia* agent is present in the Polish cattle population, there is a real risk to transmission of the infection to humans, but there is no information in the available literature about the epidemiological situation in humans exposed to chlamydiosis in Poland.

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