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DIAGNOSTIC METHODS FOR DETECTION OF Q FEVER IN RUMINANTS

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Introduction. Q fever is a zoonosis caused by *Coxiella* (*C.*) *burnetii*, an intracellular bacterium highly resistant to environmental conditions (Rousset et al., 2007). In ruminants, Q fever has been associated with variable frequencies of abortion (Arricau-Bouvery et al, 2005). Cattle and small ruminants, when infected, shed the bacteria via vaginal excretion and the placenta during parturition as well as in milk, faeces and urine (Muskens et al., 2011; Muskens et al., 2012, Pirtchard et al., 2011). Diagnosis of Q fever based on clinical symptoms or post-mortem examination is very difficult or almost impossible due to unspecific or missing symptoms or lesions caused by this disease. Q fever has been reported in nearly all parts of the world, including Poland, where the first outbreak was detected in 1956. Since then several outbreaks of Q fever in livestock have been described, particularly in the south-eastern region of the country, where this disease is assessed to be endemic. Since Q fever outbreaks have been recorded in recent years, effective diagnosis is vital for effective eradication and control measures.

Routine diagnosis of Q fever in aborted ruminants is generally performed by the detection of bacteria in stained smears or impressions of placentas, combined with serological investigations of adult animals using enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT) or indirect immunofluorescence assay (IFA) (Rousset et al., 2007). Serological methods are of limited validity because they often fail to detect *C. burnetii* shedding animals and show different sensitivities (Kittelberger et al., 2009; Rousset et al., 2009). Moreover, serological diagnosis of Q fever in the early stage of infection can be unsuccessful due to the time-frame of seroconversion spanning 3-4 weeks post infection (Howe et al., 2009). Detection of the etiological agent by PCR or cell culture requires biological material such as placenta, genital swabs or samples from aborted foetuses (liver, lung or abomasum contents). PCR assays provide results within hours rather than weeks as in the case of cell culture methods. The recent definition of the European Food and Safety Authority (EFSA) gives guidelines for Q fever clinical signs, confirmation of pathogen and positive serological results (Sidi-Boumedine, 2010). However, little is known about the correlation and the significance of the results of different laboratory methods. Thus, the aim of the study was to compare and evaluate the significance and the level of agreement between five different diagnostic methods ELISA, CFT, conventional PCR, real-time PCR and cell culture used for detection of *C. burnetii* infections in cattle, sheep and goats.

Materials and methods. Serological tests were carried out on 2,251 serum samples from ruminants originating from different regions of Poland, collected in the years 2007-2011. Of the tested sera 1,270 were taken from goats, 831 from cattle, and 150 from sheep. The samples were taken during monitoring studies in Poland. Most samples were collected from herds where abortions and reproductive disorders had been observed, and the remaining part was taken randomly. The samples were collected from 45 individual farms localised in sixteen Polish districts. Detailed information on the localisations of sampling is presented in Figure 1. The tests were carried out on biological material collected from the same animals. In total, 668 placentas, 1,277 vaginal swabs and 306 specimens of the internal organs of aborted foetuses were examined. Each sample collected from the same individual was tested with an appropriate diagnostic system, i.e. each serum in ELISA and CFT, each placenta, aborted foetus and swab in conventional PCR, real time PCR and cell culture.

For the serological studies, ELISA (Checkit Q fever IDEXX Laboratories) was performed according to the manufacturer's instructions. The sera, as well as positive and negative controls tested in a dilution at 1:400 in duplicate. The ELISA test detects the antibodies against *C. burnetii* in serum the ruminants cattle, sheep and goats. Following the manufacturer's instruction, the value %OD was calculated as (OD sample-ODneg)/(ODpos-ODneg) × 100 after averaging the duplicate values. Sera were considered to be negative when %OD < 30, dubious when % OD ≥30 and % OD ≤ 40, positive when %OD > 40.

The CFT was carried out in agreement with the Manual of Diagnostic Tests and Vaccines for Terrestrials Animals (Anon, 2010 a). The CFT assay was performed using commercially available reagents (Institut Virion/Serion GmbH Germany, Institute of Biotechnology, Sera and Vaccines Biomed S.A Poland). The *C. burnetii* phase 1 and 2 antigens were used. The temperature of inactivation of sera was different for the cattle and small ruminants: 57±1°C and 62±1°C, respectively. According to the OIE guidelines samples revealing CFT titres <10 were considered negative and titres ≥10 positive.

Total genomic DNA was extracted from biological materials using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Conventional PCR and real-time PCR assays targeting the IS*1111* gene of *C. burnetii* were performed following the protocols previously described by Panning et al. (2008). Conventional PCR was done in a final volume of 50 µl of reaction mixture containing: 5 µl 10 x PCR buffer, 2 µl of 50 mM $MgCl_2$, 1.0 µl of 2mM dNTP, 1.25µl of 10 pmol forward and reverse primer, 0.1 µl of 5 U/µl thermostable polymerase DNA, 37.40 µl of sterile water and 2 µl of DNA solution. The following conditions were applied: 40 cycles, initial denaturation at 96°C for 60 s and then 40 cycles, denaturation at 96°C for 60 s, annealing at 65°C for 60 s, elongation at 72°C for 60 s, final elongation at 72°C for 60 s. Amplification was carried out in the Tepersonal thermocycler (Whatman Biometra, Germany). PCR results were analysed by

electrophoresis of 8 µl PCR product in 1% agarose gel in 1 x TAE buffer, and made visible by UV light after straining with ethidium bromide (Invitrogen, Germany). The molecular weight of the obtained product was determined on the basis of the molecular weight marker GeneRuler ^{TM} 100 bp DNA Ladder (Fermentas, Lithuania) and a positive control (Genecam, Germany). A sample was considered positive when amplified DNA of the same size as the positive control DNA (448 bp), was present in the PCR reaction.

Real-time PCR was performed in a total volume of 25µl, containing: 2.5 µl of 10 x PCR buffer, 2.0 µl of 50mM $MgCl_2$, 2.0 µl of 10 mM dNTP , 2.0 µl of 10 pmol forward and reverse primer, 0.75 µl of 10 pmol FAM/TAMRA dual labelled probe (FAM-TCATCAAGGCACCAATGGTGGCCA-TAMRA) (in deviation to the published minor groove binder probe; Panning et al. 2008), 8.5 µl sterile water, 0.25 µl of 5 U/µl thermostable polymerase DNA (Invitrogen, Germany), and 5µl of extracted DNA. Cycling conditions in a 7500 fast real-time PCR system (Applied Biosystems, USA) were as follows: 94°C for 2 min, and 45 cycles of 94°C for 15 s 60°C for 30 s. The reagents for PCR and real-time PCR were purchased from Invitrogen, Germany; primers and probes from Genecam, Germany. As positive control *C. burnetii* DNA (Genecam, Germany) and negative control (water) were used. Sample values falling below cycle threshold (Ct) of 38 were considered positive.

Isolation of *C. burnetii* was performed using Vero cells (ATCC® CCL-81™). The Vero cells line was kept at 37±1 °C with 5%CO_2 in MEM (Eagle's Minimum Essential Medium – Sigma-Aldrich, USA) supplemented by 10 % foetal bovine serum (FBS, Sigma-Aldrich, Poland). Antibiotic and antimycotic solutions (contains 10.000 units/ml pencillin G, 10 mg/ml streptomycin sulphate, 25µg/ml amphotericin B (Sigma-Aldrich, USA) were added at the beginning of the culture to a final concentration of 1%.

The sample preparation depended on the type of biological material. Tissue samples were cut into small pieces and homogenized in 500-1000 µl of sterile PBS. Swab samples were placed in 2mL vials (Eppendorf, Germany) with 1mL of sterile PBS (Biomed, Poland) and incubated at 37±1°C for one hour. Aliquots of 200µl of sample were transferred to two 25cm^2 flasks with a confluent monolayer of Vero cells. The medium was changed weekly. Formation of vacuoles was checked regularly and tested for growth of *C. burnetii* at two week intervals, by real-time PCR. The used culture method is an own validated method.

Pearson's chi-square test was used to compare the results obtained using the 5 methods: ELISA, CFT, conventional PCR, real time PCR and isolation of the pathogen agent in cell culture.

Furthermore, correlation coefficients were calculated for all the methods used. All analyses were conducted using the program STATISTICA version 10 (Software StatSoft, Inc. For the purpose of this study, the following guidelines for interpreting the degree of correlations were used: r = 0 to 0,09: no or negligible r = 0.1 to 0.29: weak relationship; r = 0.3 to 0.49: moderate; r = 0.5 to 0.69: strong; r = 0.7 to 0.99 = very strong; r = 1: full (Stanisz, 2006). For the purpose of proper estimation of correlations, doubtful results in ELISA were excluded from the calculations.

Results. The results obtained using the five different methods are presented in tables 1-3. The χ^2 test confirmed that in most cases (with the exception of ELISA vs. cell culture for aborted foetuses) the results obtained by means of different methods correlated with each other ($P<0.05$), but the correlations had different values. The values of the correlation coefficient (r) for two compared serological methods was 0.43-0.45 and it showed a moderate degree of correlation. Table 4-5 shows the values of the correlation coefficient (r) for the other methods. The highest correlation coefficient was observed in the case of real time PCR and conventional PCR, but it was higher for placenta and swab samples (r=0.86-0.87) in comparison with the samples from aborted foetuses (r=0.76). However, based on the established criteria, all results were classified into the group of "very strong relationship". The comparison between the methods used to detect the infectious agent (cell culture isolation, real time PCR and conventional PCR) showed a moderate degree of correlation (r=0.31-0.42). The relationship between (r)PCRs and ELISA was moderate (r=0.37-0.48), but it was the lowest when the comparison was made between the test results of samples isolated from placentas. No correlation or a weak to moderate relationship was observed when the comparison was made between cell culture isolation and all the other methods. In the majority of cases (excluding the ELISA test described above) the CFT was weakly or negligibly correlated with other methods.

Table 1 – Comparison of the results obtained by ELISA and CFT used for sera samples, and by PCR, real-time PCR and cell culture used for detection of *C. burnetii* in placenta samples (the table shows number of positive, negative or doubtful samples)

placenta/sera*		ELISA*			CFT *		PCR		real-time PCR		cell culture	
		positive	negative	doubtful	positive	negative	positive	negative	positive	negative	positive	negative
ELISA*	positive	-	-	-	57	142	59	140	72	127	25	174
	negative	-	-	-	5	441	17	429	24	422	1	445
	doubtful	-	-	-	0	23	13	10	16	7	5	18
CFT*	positive	57	5	0	-	-	20	42	23	39	15	47
	negative	142	441	23	-	-	69	537	89	517	16	590
PCR	positive	59	17	13	20	69	-	-	89	0	23	66
	negative	140	429	10	42	537	-	-	23	556	8	571

real-time PCR	positive	72	24	16	23	89	89	23	-	-	27	85
	negative	127	422	7	39	517	0	556	-	-	4	552
cell culture	positive	25	1	5	15	16	23	8	27	4	-	-
	negative	174	445	18	47	590	66	571	85	552	-	-

Table 2 – Comparison of the results obtained by ELISA and CFT used for sera samples, and by PCR, real-time PCR and cell culture used for detection of *C. burnetii* in samples from aborted foetus (the table shows number of positive, negative or doubtful samples)

aborted foetus/sera*		**ELISA***			**CFT***		**PCR**		**real-time PCR**		**cell culture**	
		positive	**negative**	**doubtful**	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**
ELISA*	positive	-	-	-	24	43	28	39	36	31	5	62
	negative	-	-	-	6	199	8	197	19	186	5	200
	doubtful	-	-	-	7	27	6	28	8	26	2	32
CFT*	positive	24	6	7	-	-	14	23	18	19	4	33
	negative	43	199	27	-	-	28	241	45	224	8	261
PCR	positive	28	8	6	14	28	-	-	41	1	8	34
	negative	39	197	28	23	241	-	-	22	242	4	260
real-time PCR	positive	36	19	8	18	45	41	22	-	-	10	53
	negative	31	186	26	19	224	1	242	-	-	2	241
cell culture	positive	5	5	2	4	8	8	4	10	2	-	-
	negative	62	200	32	33	261	34	260	53	241	-	-

Table 3 – Comparison of the results obtained by ELISA and CFT used for sera samples, and by PCR, real-time PCR and cell culture used for detection of *C. burnetii* in samples isolated from swabs (the table shows number of positive, negative or doubtful samples)

Swabs/sera*		**ELISA***			**CFT***		**PCR**		**real-time PCR**		**cell culture**	
		positive	**negative**	**doubtful**	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**	**positive**	**negative**
LISA*	positive	-	-	-	33	88	47	74	52	69	10	111
	negative	-	-	-	9	1105	21	1093	38	1076	0	1114
	doubtful	-	-	-	4	38	22	20	25	17	4	38
CFT*	positive	33	9	4	-	-	16	30	22	24	2	44
	negative	88	1105	38	-	-	74	1157	93	1138	12	1219
PCR	positive	47	21	22	16	74	-	-	89	1	14	76
	negative	74	1093	20	30	1157	-	-	26	1161	0	1187
real-time PCR	positive	52	38	25	22	93	89	26	-	-	14	101
	negative	69	1076	17	24	1138	1	1161	-	-	0	1162
cell culture	positive	10	0	4	2	12	14	0	14	0	-	-
	negative	11	1114	38	44	1219	76	1187	101	1162	-	-

Table 4 – Results of statistical analysis of correlations between methods used for detection of *Coxiella burnetii* (PCR, real-time PCR, cell culture)

method	**Placenta (r coefficient)**		**Aborted fetus (r coefficients**		**Swab (r coefficient)**	
	PCR	**rPCR**	**PCR**	**rPCR**	**PCR**	**rPCR**
real-time PCR	0.87	-	0.76	-	0.86	-
cell culture	0.40	0,42	0.31	0.31	0.38	0.33

Table 5 – Results of statistical analysis of correlations between methods used for detection of *Coxiella burnetii* (PCR, real-time PCR, cell culture) against serological methods used for Q fever diagnosis (ELISA, CFT)

method	Placenta (r coefficients)		Aborted fetus (r coefficients)		Swab (r coefficients)	
	ELISA	CFT	ELISA	CFT	ELISA	CFT
PCR	0.37	0.18	0.48	0.26	0.48	0.21
real-time PCR	0.40	0.17	0.48	0.26	0.45	0.26
cell culture	0.29	0.30	0.12*	0.13	0.27	0.06

*statistically insiginificant correlation

Discussion. In the routine veterinary diagnosis of Q fever, which is connected with monitoring investigations of the disease in animals, different kinds of methods are used. The types of assays largely depend on the type and number of samples for investigation, the availability of diagnostic tests in a laboratory, and the size of the herd tested. Despite the growing interest in infections caused by *C. burnetii* in recent years, there is a paucity of data on the comparative evaluation of the methods used in the diagnosis of Q fever. In particular, there is very little data showing comparative studies of serological assays with molecular biology and cell culture techniques. In the present study, we described the diagnostic potential of five different methods (CFT, ELISA, PCR, real-time PCR and culture) used in Q fever diagnosis in ruminants by direct comparison.

The statistical analysis showed a moderate correlation between the results of CFT and ELISA, which belong to the most frequently used serological methods. In fact, several previous reports demonstrated a weak sensitivity of CFT compared with other methods (Rousset et al., 2007; Kittelberger et al., 2009, Rousset et al., 2009; Ruiz-Fons et al., 2010; Natale et al., 2012; Field et al. 2000). ELISA shows a higher sensitivity and specificity than CFT (Horigan et al., 2011) and is therefore recommended by the EFSA (Anon, 2010 b).

The serological methods are useful for carrying out preliminary surveys of infection at the herd level but they do not allow for the identification of *C. burnetii* shedding animals. When the positive serological results are found at the herd level the PCR is the method of choice to trace shedders. However, it should not be forgotten that if there is suspicion of infection or shedding of *C. burnetii* despite the absence of serological response the test for pathogen detection (PCR or culture) should be performed. For example, according to the literature data, at least 24 % of seronegative goats shed the bacteria (Rousset et al., 2009). On the other hand, serological testing can generate valuable results in cases of negative results in tests for pathogen detection Guatteoa et al. (2006) demonstrated that persistent shedders cows were usually persistently highly seropositive. The authors concluded that repeated serological testing could be a reliable tool to screen heavy shedders before implementation of PCR assays. However, the PCR assays of sera or blood should be performed in the early stage of *C. burnetii* infection before antibodies are detectable. According to the data published, PCR methods are the most useful in the first two weeks of infection (Fournier et al. 2003). Our results showed that correlation between serological and molecular studies is not higher. The correlation between CFT and classical PCR, or real time PCR was very weak; however, the correlation between the second compared serological method – ELISA and molecular assays was higher (average relationship).

In conclusion, laboratory diagnosis of Q fever should be based on the interpretation of results obtained by different kind of methods both detecting the serological response as well as the presence of pathogen. In addition, the choice of proper laboratory methods depends on numerous factors e.g. case definition, type of available material etc. To sum up the values and results obtained, it can be concluded that the statistical analysis of data from a comparison of the five diagnostic methods has shown that serological, molecular and culture methods can be used in practice for the diagnosis of Q fever. However, their diagnostic potential and the level of correlation between them was variable and it is necessary to use several methods simultaneously, preferably ELISA for serological studies and PCR for pathogen detection.

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IMPACT OF CO-INFECTION WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ON DURATION OF PORCINE CIRCOVIRUS TYPE 2 VIREMIA IN FIELD CONDITIONS

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Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) infections cause serious economic losses to the global swine industry. The study aimed to study of the impact of co-infection with porcine reproductive and respiratory syndrome virus on duration of porcine circovirus type 2 viremia. The experimental study was based on serological ELISA based testing, and PCR assays. Results has been calculated using biostatistical methods. PRRS infection was occurred in 17 from 22 farms. Also the PCV-2 co-infection has been described. Prolonged infection with this PRRSV may affect farm productivity. PRRSV may potentially affect control of PCV infection by immunoprophylaxis. Proper management practices are very important in reducing the impact of PCV2 on the health status of the herd, even in herds where PCV2 immunoprophylaxis is already implemented.

Keywords: porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, prophylaxis, co-infection

Introduction. Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) infections cause serious economic losses to the global swine industry (2, 3, 5). Porcine reproductive and respiratory virus may cause reproductive disorders in sows and respiratory lesions in weaners and fatteners (5). PCV2 causes mainly subclinical infections in nearly all commercial pig herds, but is also associated with a range of different disease syndromes collectively described as Porcine Circovirus (Associated) Disease. Although PCV2 is a main etiological agent of PCVD, multiple other infectious or non-infectious factors are also involved in clinical expression of the disease.

Experimental infection studies proved that PRRSV enhance and prolong PCV2 replication and shedding in co-infected pigs what may result in enhanced respiratory disease and severity of associated lesions (1, 4). Co-infections with those viruses are often observed in swine herds, but little is known on the possible outcome of their synergistic effect in the field.