



## RESEARCH ARTICLE

### Development and Evaluation of an ELISA Assay for Quantitation of *Chlamydia abortus* Pmp18 Antibodies in Pigs

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#### ABSTRACT

*Chlamydia abortus* (*C. abortus*) is a major cause of abortion in sheep and goats with pigs and cattle also being affected. As several infectious agents are known to cause late-term abortions in small ruminants, good detection methods to identify *C. abortus* infection are desired in order to develop a rapid course of action and prevention strategies. The objective of this study was to determine if an indirect ELISA assay utilizing the 65kD N-terminal fragment of the recombinant *C. abortus* Pmp18 (Pmp18-N) protein would function as an improved alternative to the complement fixation test (CFT) for detection of *C. abortus*-specific antibody in pigs. The test was assessed with serum samples from a panel of 58 experimentally infected and 24 uninfected specific pathogen-free (SPF) pigs as well as 495 clinical samples obtained from farm animals. Results were compared with those obtained by the CFT. Compared to the commercial CFT using MOMP antigen, the sensitivity and specificity of the test was 98.2 and 87.5%, respectively while the concordance was 91.7%. No cross reaction with sera positive for other abortion-associated pathogens was found, including classical swine fever virus, porcine reproductive and respiratory syndrome virus, porcine pseudorabies virus, Japanese encephalitis virus, *Brucella suis* and avian *Chlamydia psittaci*. Of the 495 clinical samples analyzed, 12.1% were positive with the Pmp18-N compared to 11.1% with the commercial CFT kit. Interestingly, the Pmp18-N based test detected 0.75, 15.3 and 20.0% positivity in boars, fattening pigs and sows, respectively. Taken together, serological diagnosis based on Pmp18-N was shown to be rapid, sensitive and specific in detecting *C. abortus*-specific antibody.

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#### INTRODUCTION

Chlamydiae are obligate intracellular bacteria that cause serious diseases in humans and animals (Corsaro and Greub, 2006). Chlamydiaceae have been associated with pig pneumonia, polyarthritis, conjunctivitis, reproductive disorders and abortion (Busch *et al.*, 2000). Many of these infections are asymptomatic and so go undiagnosed. Infections are associated with *Chlamydia suis*, *C. abortus*, *Chlamydia pecorum* (*C. pecorum*) and *C. psittaci* (*C. psittaci*). Moreover, *C. abortus*, the causative agent of enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA), also commonly causes abortion in swine, goats, and sheep, resulting in reproductive losses worldwide (Bagdonas *et al.*, 2005; Rodolakis and Yousef

Mohamad, 2010). In a previous report, the prevalence of *C. abortus* in pig herds based on the indirect haemagglutination inhibition (IHI) test ranged from 11 to 80% across regions in China (Qiu *et al.*, 2003). a zoonotic risk to pregnant women and several cases of human chlamydial abortion have been reported (Bagdonas *et al.*, 2005). Therefore, it is important to diagnose *C. abortus* infection promptly and precisely. The complement fixation test (CFT) has been the most widely used assay for the serological diagnosis of OEA and the one recommended by the OIE. However, the CFT lacks specificity as it is based on the heat-resistant LPS, which is common to all chlamydial species (Mitov *et al.*, 2003). The indirect hemagglutination assay (IHA) kit is the only commercial kit available for *Chlamydia* surveillance in

China. Unfortunately, this assay lacks specificity and contributes to high false-positive chlamydial infections due to its required use of whole chlamydial elementary bodies (EB) (Jiang *et al.*, 2013; Burgasova OA *et al.*, 2010). Moreover, the actual frequency of Chlamydia infections is still unclear leading to delays in the application of efficient animal control strategies and places farm workers at high risk. In fact, several laboratory-based assays have been developed, including indirect ELISAs using whole chlamydial EBs or extracts (Buendía *et al.*, 2001). With the introduction of molecular techniques, more defined assays were developed based on the recombinant LPS antigen, MOMP (Borel *et al.*, 2002; Hoelzle *et al.*, 2004; Rahman *et al.*, 2015) and the 80–90 kDa Pmp (Buendía *et al.*, 2001; Livingstone *et al.*, 2005). POMP90 proteins have been suggested to be important serodiagnostic antigen candidates because they have been shown to react strongly in immunoblotting experiments with sera from sheep infected with *C. abortus* (Wheelhouse *et al.*, 2012), but not with sera from sheep infected with *C. pecorum*. In addition, Southern blotting failed to detect any *C. abortus* Pmp gene-related sequences in various *C. pecorum* subtypes, indicating probably low degrees of homology of the sequences between species (Becker and Hegemann, 2014). In a more recent comparison with six serological assays, including the Pourquier and CHEKIT tests, the two rPmp90 ELISAs were found to be the most sensitive and specific (Wilson *et al.*, 2009). Overall, the rPmp90-3 ELISA performed the best, with 96.8% sensitivity and no cross-reaction with sera from animals infected with *C. pecorum* or from EAE-free flocks. Antibodies to the Pmp family have been shown to appear earlier than anti-MOMP antibodies after experimental infection of pregnant ewes or after immunization of mice (Livingstone *et al.*, 2005). This observation suggested that *C. abortus*-infected animals could be detected serologically in the early infection stage before the manifestation of the disease. Moreover, Pmp-based tests can be applied to other species because the N-terminal part of the Pmps has been shown to share common epitopes with *C. psittaci*, and as such, the application of the Pmp-based assays to avian serology and human psittacosis might be interesting in the future (Vretou *et al.*, 2003). Over the last few years, novel assays have been developed that have met the requirements for the specific serological diagnosis of *C. abortus*. Additionally, the specific detection of *C. abortus* antibodies and the serological differentiation of vaccinated and infected animals are urgently needed.

Recent genomic analysis revealed that polymorphic membrane proteins (Pmps) of *C. abortus* strain S26/3 has variation in the loci encoding a family of outer membrane proteins (Thomson *et al.*, 2005). The expression of Pmps has been identified in all pathogenic chlamydial species (Crane *et al.*, 2006; Kiselev *et al.*, 2007; Wehrl *et al.*, 2004). Little is known about the potential importance of the Pmps in the virulence and pathogenesis of *C. abortus* and their potential in the development of vaccine and diagnostic reagents. In our previous study, an N terminal fragment of Pmp18 of *C. abortus* was reported to react positively with pig and sheep sera and negatively with poultry serum. In the present study, we developed an indirect ELISA assay based on the N-terminal fragment of Pmp18 and compared its potential application in the serodiagnosis of *C. abortus* with the commercial kit (ID-VET, Montpellier, France).

## MATERIALS AND METHODS

**Chlamydia stock and serum samples:** The *C. abortus* CP 12 used in this study is a laboratory strain originally isolated from an aborted piglet [Institute of Veterinary Drug Control (IVDC), Beijing, China]. The inclusion forming units (IFU) of *C. abortus* strain CP12 was determined as  $1 \times 10^7$  IFU/ml in McCoy cells (Ling *et al.*, 2011). The sera of classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine pseudorabies virus (PRV), Japanese encephalitis virus (JEV) and *Brucella suis* (*B. Suis*) were purchased from IVDC. In addition positive sera for *C. psittaci* were obtained from SPF chickens during routine testing.

**Purification of recombinant Pmp18-N terminal:** The recombinant Pmp18-N was purified by the Ni-NTA Purification System (Invitrogen, California, USA). Endotoxin was removed using Detoxi-Gel™ (Thermo, Illinois, USA). Concentration of the purified protein was calculated using the Pierce™ BCA Protein Assay Kit (Thermo, Illinois), diluted in 10mM Tris-HCl (pH 8.0) to a final concentration of 1 mg/ml and aliquots (0.1 mg/ml) were stored at -80 °C. Protein expression was detected by SDS-PAGE, visualized using Coomassie Brilliant Blue and immunoblotting analysis using purified rabbit anti-Pmp18 polyclonal antibody.

**Sensitivity and specificity test by Pmp18-N ELISA:** A total of 58 serum samples were collected from pigs experimentally infected with *C. abortus* strain CP12 on day 21 post-infection and 24 control samples were obtained from specific pathogen free (SPF) piglets. The blood samples were checked by CFT (IVDC, Beijing, China) as previously described (Vretou *et al.*, 2003).

**Optimization of Pmp18-N ELISA** Ninety six-well plates (Nunc, Denmark) were coated with 4µg/ml of recombinant Pmp18-N protein in 100µl of PBS at 4°C overnight. Subsequently, the wells were washed 4 times with PBST and blocked for 2h at 37°C. After another wash, 100µl of a 1:100 dilution of the serum was added to each well and reacted for 1h at 37°C. The plates were again washed and incubated with 100µl HRP-IgG (EarthOx, USA) in PBS at 37°C for 1h and again washed. Finally, 100 µl of write out TMB in full (TMB) (Qiagen, Stuttgart, Germany) was added at 37°C for 10 min, and the reaction was stopped with 2M H<sub>2</sub>SO<sub>4</sub>. The optical density (OD<sub>450</sub>) was read using a universal Microplate Reader (Thermo Life Sciences, Shanghai, China). In addition, serial dilutions of HRP-IgG were added to the plates for optimizing conjugate concentration. Optimization of the blocking buffer was determined with 5% skim milk, 8% skim milk, 0.5% BSA, 1% BSA, 10% fetal calf serum and 10% horse serum, respectively. Finally, the exposure time of the TMB was tested. Absorbance values were determined to be positive if the OD value exceeded the mean value plus three times the standard deviation obtained with control negative sera.

**Concordance of Pmp18-N ELISA and rMOMP based kit:** A total of 79 sera were used to evaluate the specificity and sensitivity of the Pmp18-N ELISA, including 55 *C. abortus* positive and 24 negative samples obtained from

experimentally infected and control pigs. Presence of *C. abortus* antibodies was determined using CFT test kit and the ID-VET commercial kit was used for determining the concordance.

**Detecting clinical samples using Pmp18-N ELISA:** A total of 495 serum samples were tested for *C. abortus* infection using the Pmp18-N ELISA assay and the ID-VET commercial kit. The clinical samples were randomly collected from 122 weaned piglets, 144 fattening pigs, 162 sows and 67 boars.

## RESULTS

**Purification of N terminal fragment of Pmp18-N:** The purified recombinant Pmp18-N terminal was analyzed by Western blot assay with *C. abortus* positive serum. The pig antiserum reacted specifically with the N-terminal fragment of Pmp18 as indicated by a specific band at an approximate molecular mass of 65 kDa in the Western blot assay (Fig. 1).

**Optimizing Pmp18-N ELISA assay:** A checkerboard titration was used to determine the optimal dilutions of antigens, antibodies, blocking buffers and HRP-IgG antibodies. The following references were determined post optimization, such as 0.5 µg/ml for the coating antigen, 100 µl/well (1:400) for the tested serum (Table 1), 100 µl/well (1:20000) are best for HRP-goat anti-swine IgG (Table 2), 5% skim milk are best for blocking buffer (Table 3) and 10 min for color development in the dark and OD reading at 450nm after stopping the reaction (Table 4). Additionally, 24 negative sera were used to determine cut-off value. The average OD value of the samples was 0.252 with a standard deviation of 0.086 and a cut-off OD value of 0.51 (mean±SD). OD values equal to or above the 0.51 cut-off value were considered to be positive while OD values below 0.51 were considered negative in this assay.

**The sensitivity and specificity of Pmp18-N ELISA:** Serum analysis with the pmp18-N ELISA, showed that 54 of the 55 positive serum samples tested were confirmed positive while one tested negative resulting in a sensitivity of 98.2%. In contrast the CFT showed a sensitivity of 90.9% (50 of 54). In addition, 21 of the 24 negative SPF pig samples were confirmed negative by the Pmp18-N ELISA giving a specificity of 87.5% compared to 91.6% using the CFT (Table 5).

**Cross reaction of recombinant Pmp18-N ELISA:** No cross reaction was observed between the Pmp18-N and positive antibodies against CSFV, PRRSV, PRV, JEV, *C. psittaci* and *B. Suis*. Moreover, antibodies to *C. psittaci* did not recognize Pmp18-N (Table 6).

**Concordance between Pmp18-N ELISA and the commercial kit:** Forty-five out of 49 positive sera identified by Pmp18-N ELISA were confirmed to be positive while 5 samples were negative using commercial kit. On the contrary, 22 out of 24 negative sera were confirmed using the commercial ELISA kit (Table 7). Hence, the concordance was 91.7% in terms of two assays.

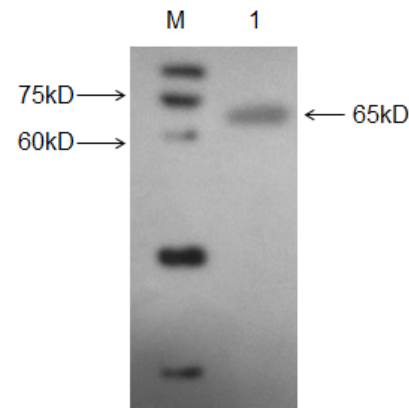


Fig. 1: Identification of the Pmp 18-N terminal by Western blot

Table 1: Optimization of antigen concentrations and sera dilutions

Sera dilutions	Antigen concentrations (µg/mL)*				
	0.5	1.0	2.0	4.0	
OD values					
1:50	<i>C. abortus</i> +	1.53	1.48	1.547	1.452
	<i>C. abortus</i> -	0.422	0.432	0.426	0.408
1:100	<i>C. abortus</i> +	1.468	1.493	1.407	1.348
	<i>C. abortus</i> -	0.298	0.303	0.294	0.291
1:200	<i>C. abortus</i> +	1.355	1.418	1.336	1.291
	<i>C. abortus</i> -	0.229	0.216	0.216	0.212
1:400	<i>C. abortus</i> +	1.273	1.271	1.195	1.212
	<i>C. abortus</i> -	0.166	0.179	0.182	0.182

\*ELISA plate was coated with 100 µl recombinant Pmp18-N fragment. Positive sera and negative sera were determined by commercial CFT and indirect ELISA kit

Table 2: Determination of the optional dilutions of goat anti-pig IgG-HRP antibodies for Pmp18-N ELISA

	Goat anti-pig IgG-HRP antibody dilutions			
	1:20000	1:30000	1:40000	1:80000
<i>C. abortus</i> +	0.226	1.121	0.642	0.409
<i>C. abortus</i> -	0.074	0.143	0.1	0.075
P/N*	7.839161	6.42	5.453333	3.054054

\* P/N = OD value of positive *C. abortus* sera / OD value of negative *C. abortus* sera.

### Clinical application of recombinant Pmp18-N ELISA:

A total of 459 serum samples from diverse pigs were simultaneously tested for *C. abortus* antibodies using the pmp18-N ELISA and the commercial kits. Sixty of the 459 serum samples (12.1%) were positive for *C. abortus* antibodies. Comparable positive samples were detected by both the Pmp18-N ELISA and the commercial kit, including 22 of 144 samples from fattening pigs (15.2%), 33 of 162 sow samples (20.4%) and 5 out of 67 boar samples (0.75%). No positive antibody was found in 122 weaning pigs using both ELISAs (Table 8).

## DISCUSSION

In the current study, the newly developed indirect ELISA assay based on Pmp18-N was demonstrated to be highly sensitive (98.2%) and specific (87.5%) for detecting *C. abortus* antibodies. The concordance between this assay and the commercial kit based on MOMP ELISA was 91.7%. Furthermore, no cross-reaction was observed between Pmp18-N and positive antibodies against the other pathogens associated with pig abortion. More importantly, less cross reaction was found between antibodies to *C. psittaci* and the *C. abortus* Pmp18-N.

**Table 3:** Optimization of buffers

Buffers	5% skim milk	8% skim milk	0.5%BSA	1%BSA	10% bovine serum	10% horse serum
<i>C. abortus</i> +	1.01	1.181	1.483	1.484	1.453	1.574
<i>C. abortus</i> -	0.136	0.162	0.346	0.463	0.773	0.856
P/N*	7.426471	7.290123	4.286127	3.205184	1.87969	1.838785

\* P/N = OD value of positive *C. abortus* sera /OD value of negative *C. abortus* sera.

**Table 4:** Optimization of reaction time for *C. abortus* positive or negative sera

	Reaction time (min)			
	6	10	15	20
<i>C. abortus</i> +	0.778	1.121	1.343	1.537
<i>C. abortus</i> -	0.109	0.143	0.19	0.235
*P/N	7.137615	7.839161	7.068421	6.540426

\*P/N=*C. abortus* + OD<sub>450</sub> value / *C. abortus* - OD<sub>450</sub> value

**Table 5:** Sensitivities and specificities of serological detection of *C. abortus* antibodies in pigs

Assay	True positives <sup>a</sup> (n=55)		True negatives <sup>b</sup> (n=24)		Sensitivity <sup>c</sup> (%)	Specificity <sup>d</sup> (%)
	positive	negative	positive	negative		
Pmp18-N ELISA	54	1	3	21	98.2	87.5
CFT	50	5	2	22	90.9	91.6

<sup>a</sup>Sera obtained from *C. abortus*-infected pigs were tested by indirect ELISA kit post infection on day 30. <sup>b</sup>Sera collected from specific pathogen free mini-pigs were determined by indirect ELISA kit. <sup>c</sup>Sensitivity=[number of true positives/(number of true positives + number of false negatives)] × 100; <sup>d</sup>Specificity=[number of true negatives/(number of true negatives + number of false positives)] × 100.

**Table 6:** Evaluation of cross reactivity of Pmp18-N ELISA with pig sera positive for other abortion-causing pathogens

Samples	OD values of the positive sera*						
	<i>C. abortus</i> (-)	<i>C. psittaci</i>	CSFV	PRRSV	PPV	JEV	<i>Br. suis</i>
OD values	0.219	0.243	0.272	0.437	0.414	0.453	0.193

Classical swine fever virus (CSFV); Porcine reproductive and respiratory syndrome virus (PRRSV); Porcine pseudorabies virus (PRV); Japanese encephalitis virus (JEV); *Brucella suis* (*Br. Suis*).

**Table 7:** Concordance of Pmp18-N ELISA and MOMP ELISA for the experimentally infected pigs

Pmp18-N ELISA	Assay Commercial MOMP ELISA	
	Positive <sup>a</sup>	Negative <sup>b</sup>
Positives	45	4
Negatives	2	22
Concordance	91.7	

Concordance=(the sum of positive-positive values and negative-negative values)/ the total number of serum samples ×100%; <sup>a</sup>Sera obtained from *C. abortus*-infected pigs were tested with high seropositive samples by CFT post infection on day 30. <sup>b</sup>Sera collected from specific pathogen free pigs were determined by CFT.

Therefore, the indirect ELISA based on Pmp18-N is a promising approach for serological diagnosis of *C. abortus* and it might also be useful for differentiating *C. abortus* from avian chlamydial species.

*Chlamydia* possesses a unique family of autotransporter proteins known as the Polymorphic membrane proteins (Pmps). Although pmp genes vary between *Chlamydia* species, all species encode a single pmpD gene. This Pmp 18 protein is split into an N-terminal signal sequence, a passenger (effector) and a carboxyl terminal β-barrel translocator domain (Thomson *et al.*, 2005; Crane *et al.*, 2006; Sait *et al.*, 2011). Several reports have confirmed that *C. abortus* Pmp18 belongs to the Type V autotransporter protein family using the RT-PCR approach and proteomic techniques.

More important, Pmp18 could be quantified from 36 h post infection and detected at all stages of the

chlamydial life cycle. Using antibodies directed against different regions of Pmp18, it was shown to be proteolytically processed at the cell surface similar to the PmpD of *C. trachomatis* and *C. pneumoniae*. However, patterns of cleavage are species-specific, with low sequence conservation across the genus (Sait *et al.*, 2011; Wheelhouse *et al.*, 2012). Our pioneer study also demonstrated that the N-terminal fragment of Pmp18 was expressed early in the infection and paralleled chlamydial growth [data not show]. Moreover, less cross-reactivity with positive sera of *C. psittaci* was found by Western blot analysis. In this sense, the N terminal of Pmp18 is a promising novel candidate antigen for vaccine development as well as for diagnosis of early infection. Further studies would determine the utility of this ELISA assay for early diagnosis of infection. With regard to application, the commercial MOMP-based ELISA kit was designed for detection of *C. abortus* antibodies in ruminants and can differentiate between *C. abortus* and *C. pecorum* infections. Recent evaluations of an assay based on Pmp90 have reported good specificity (90-100%) and sensitivity (80-93.5%) when compared to other serological tests, including CFT, CHEKIT, cELISA, and rPmp90 tests (McCauley *et al.*, 2007; Wilson *et al.*, 2009). The Pmp18-N-based ELISA assay could serve as an alternative to rMOMP and Pmp90 ELISA kits. Another advantage of this assay is that it can be used for discriminating between *C. abortus* and *C. psittaci* due to its low reactivity with *C. psittaci*. The main reason is that the interspecies heterogeneity of Pmp18 genes in *C. abortus* is low compared to other Pmp genes (Sait *et al.*, 2011).

In our field survey, using the Pmp18-N ELISA assay, the average positive rate was 12.1% out of 459 blood samples, which was significantly lower than the average positivity (25.5%) using the indirect IHA (Tian *et al.*, 2012). This difference may be associated with lack of specificity and cross-reaction due to the use of the whole chlamydial inclusion body in IHA kit.

**Conclusion:** Indirect ELISA based on the N-terminal Pmp18 is a novel serological diagnostic tool for diagnosis of *C. abortus* infection and discrimination of *C. psittaci* in clinical samples.

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**Author's contribution:** CH designed the study. TZ and SL collected and analyzed the samples. QZ and JC performed the statistical analyses. FOE edited the manuscript. All authors have read and approved the final manuscript.

**Table 8:** Serological detection *C. abortus* in pigs using Pmp18-N ELISA assay

Species	No. <sup>A</sup>	Pmp18-N ELISA			Commercial MOMP ELISA		
		Positives	Negatives	Positive (%)	Positives	Negatives	Positive (%)
Weaned pigs	122	0	122	0	0	122	0.0
Fattening pigs	144	22	122	15.2	21	123	14.5
Sow	162	33	129	20.4	31	131	23.6
Boars	67	5	62	0.75	3	64	0.46
Total	495	60	435	12.1	55	440	11.1

<sup>A</sup>Total of 495 blood samples were randomly collected from 4 intensive pig farms.

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