

Comparative Evaluation of Indirect ELISA, CF Test and PCR for Diagnosis of Ovine Enzootic Abortion (*Ovine chlamydophilosis*)

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Abstract: *Chlamydophila (c) abortus* is the most important causative agent of the ovine chlamydophilosis, Complement fixation (CF) test is a good screening serological test, but it can be complicated by false positive reactions with others species of chlamydophila as *C. pecorum* and with some Gram negative bacteria. Diagnosis can be improved by using of indirect (I) ELISA. Moderate agreement (kappa-0.6) between CF test and iELISA was recommended in this study. Blood serum samples from 50 ewes with reproductive disorders and 50 apparently healthy pregnant ewes were investigated by CF test and iELISA to detect IgG against *C. abortus*. Sensitivity and specificity of iELISA (90% and 96% respectively) used in this study was found to be higher than CF test (70% and 88% respectively). To confirm the serological results and to evaluate iELISA, five tissue of foeti from aborted ewes with (+ve) CFT and (+ve) iELISA, also five tissue samples of foeti from aborted ewes with negative (-ve) CFT and (+ve) iELISA were subjected to polymerase chain reaction (PCR). All samples revealed positive results for *C. abortus* at 119 bp. Therefore, PCR is proved to be specific and accurate technique in the diagnosis of ovine enzootic Chlamydial abortion.

Key words: Ovine enzootic abortion • Chlamydophilosis • ELISA • CFT • PCR

INTRODUCTION

Chlamydophilosis is a major cause of abortion in domestic ruminants. ovine enzootic abortion, caused by *chlamydophila abortus* (Formerly *Chlamydia psittaci* serotype 1) is believed to be responsible for 20 to 50% of all spontaneous abortion, may impair the overall reproductive performance [1- 4]. Most infections in sheep and goats are asymptomatic apart from late term abortion and still birth [5, 6].

CFT is the most widely used test and is still recommended by the office international des Epizooties (OIE) ([http : www.oie.int](http://www.oie.int)) the animal with bad physical and health condition also, antigenic cross reactivity between *C. abortus* and *C. pecorum* as well as with Some Gram-negative bacteria (e.g. *Acinebacter*) Can give false positive CF test results [7].

Compared with the CFT, the iELISA technique is more sensitive and specific for detection of antibodies against chlamydias [8-11]. Several ELISA methods have been developed in order to improve diagnosis of

chlamydophilosis, these include ELISA using purified whole elementary bodies, lipopolysaccharide or more specific assays based on the *C. abortus* major outer membrane protein or a polymorphic outer membrane protein [12-17].

PCR is one of the most modern advanced techniques used for accurate diagnosis of the causative agent [19, 20]. PCR analysis using fresh or frozen samples, formalin fixed and paraffin-embedded samples and on archival material [21].

This study was carried out to evaluate PCR, iELISA, CFT for the diagnosis of ovine enzootic chlamydial abortion (ovine chlamydophilosis).

MATERIALS AND METHODS

Animals: This study was performed on 50 ewes with reproductive disorders and 50 apparently healthy pregnant ewes located at south Sinai-Ras-Sedr research station belong to Desert Research Center. History and clinical examination of animal were recorded.

Blood samples were collected from aborted ewes, ewes lambed weak Unthrifty lamb or still birth, infertility or any reproductive disorders (4 weeks post abortion or parturition) as well as from apparently healthy pregnant ewes for detection of IgG antibodies against *C.abortus* using iELISA and CFT.

CFT was performed according to OIE [7] and Travnicsek [22] Antisera: Reference antisera for Chlamydia (*Chlamydia psittaci* CFT reagents, "seiken", were obtained from Denka Co. Tokyo. Japan. Antisera were used for detection of Chlamydia antibodies in the suspected materials.

Reference Chlamydial Antigen: Obtained from Denka seiken Co., Tokyo, Japan. It was used in serological detection of antibodies.

Complement: Freeze dried preparation of preserved guinea pig serum welcome Co. was used in complement fixation technique. Significant titre $\geq 1/32$ [7].

iELISA for Enzootic Abortion: iELISA kit was obtained from cypress langdorp, Beleguim and the assay was performed according to manufacturer's instruction. and the plate was read in ELISA reader at optical density 450 min.

Cut-Off = mean of negative + 3 standard deviation

Statistical Analysis: Standard procedures were used to calculate the sensitivity and specificity (Free software win EPIS cope 2-0 [http : ///www.clive.ed.ac.UK](http://www.clive.ed.ac.UK)) and were compared by measuring agreement between tests (kappa) according to martin *et al.* [23] and Anthony *et al.* [24].

Polymerase Chain Reaction (PCR): From five aborted ewes serologically positive by CFT and iELISA for chlamydophilosis and five aborted ewes serologically Negative by CFT and positive by iELISA for chlamydophilosis, fresh and paraffin embedded tissue samples (placenta, internal organs of aborted foeti as liver, kidney, lung and brain) were subjected to PCR.

DNA Extraction: The genomic DNA was extracted from samples using Dineasy tissue kit (Qiagen co. cat.no.201443)

PCR amplification of chlamydial DNA was performed using oligonucleotide primers chla. 2AF and

chla. 2Br according to Sykes *et al.* [20]. The expected band length is 119 bp.

Primers:

2AF 5-GCTTTTCTAATTTACACC-3

2Br5-ATAGGGTTGAGACTATCCACT-3

Control: Distilled H₂O as negative control and pure DNA of *C.abortus* as positive control.

2 μ l of template were added to each tube containing master mix (Table1) the reaction was subsequently at 95°C for 10 minutes, then for 40 cycles at 95°C for 30 seconds, 50°C for 30 seconds and 72°C for 45 seconds, followed by an additional elongation at 72°C for 10 minutes. Reaction product was visualized by ethidium bromide staining under UV transillumination after electrophoresis on 1.5% agarose gel.

RESULTS

History and clinical manifestation, the most common clinical signs observed in pregnant ewes were abortion at late stage of pregnancy with high rate in the first year while on the other hand still birth, birth of weak unthrifty lambs or infertility were recorded year after year more than abortion.

In the first group, ewes with reproductive disorders (n = 50), sero-positivity was 70% by using CF test, whereas, the same was 90% by using iELISA as shown in Table (2)

In the second group of apparently healthy pregnant ewes (n = 50) a total of 12% seropositive was detected by using CFT, sero investigation in these apparently healthy pregnant ewes was 4% in the same Animal using iELISA as shown in Table (2).

Results of Polymerase Chain Reaction (PCR):

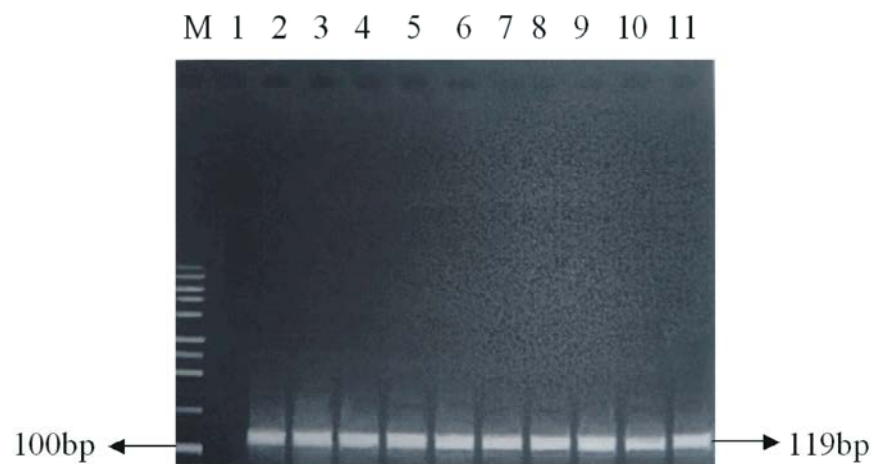
Five collected samples of placenta and foeti of aborted ewes from serologically positive CFT and iELISA cases for *C. abortus* revealed positive results by using PCR at (119 bp) as well as five collected Samples of placenta and foeti of aborted ewes from serologically-ve CFT and +ve iELISA cases for *C.abortus* revealed positive results by using PCR at 119 bp The positive control showed the expected amplification product (119 bp) as shown in photo (1).

Table 1: The amplification conditions, the master mix and primers structure are as follows :

Constituent	Initial concentration	Amount (μ l)	Final concentration
Distilled H ₂ O		13.2	
Buffer	X10	2.0	
Dntps	10mM	0.4	0.2mM
Tag polymerase	5 μ /Ml	0.4	2 μ /ml
Primer 2AF	20 mM	1.0	1 mM
Primer 2Br	20 mM	1.0	1 mM
Total volume		18.0 μ l	

Table 2: Sero investigation of IgG antibodies in ewes with reproductive disorders and apparently healthy pregnant ewes using CFT and iELISA.

Animals	Number	Tests			
		CFT		iELISA	
		Positive	%	Positive	%
Aborted ewes	28	20	71.4	26	92.8
Ewes with reproductive disorders other than abortion	22	15	68.2	19	86.4
Total ewes with reproductive disorders	50	35	70	45	90
Apparently healthy pregnant ewes	50	6	12	2	4

Photo 1: PCR of (*C. abortus*) DNA from fetu tissues of aborted ewes (M,100 bp ladder marker, lane 3-11 specific *C. abortus* PCR product (119bp detected). Lane 2 positive control and lane 1 negative control

DISCUSSION

Chlamydophilosis characterized by abortion, placentitis and decreased fertility in ovine enzootic abortion (OEA) [25, 26], this disease, common in Europe, North America and some parts of Africa, is a zoonosis and therefore, the agent must be dealt with great care and adequate microbiological precautions and laboratory equipment [13].

Two serological tests indirect immunofluorescence test and iELISA for detection of fetal antibody to *C. abortus* for diagnosis of ovine abortion, an indirect

ELISA (ROMP 91B ELISA) based on recombinant protein fragment of the polymorphic outer membrane protein, POMP 91B of *C. abortus* was developed by Kennedy [27] and used by Longbottom [28]. Indirect ELISA proved more sensitive (84.2%) and specific (98.5%), than CF test in experimentally as well as naturally infected sheep. In this study the results obtained in ewes with reproductive disorders using iELISA showed 90% positivity which is significantly higher than that of CF test (70%). The sensitivity and specificity obtained for indirect ELISA (90% and 96% respectively) as compared to CF test (70% and (88%) was higher. While both these tests

were moderately in agreement (kappa – 0.6) this result was accordance with the previous results of Travnick et al [29] who reported that the results obtained in sheep with reproductive disorders using iELISA showed 63.2% positivity which is significantly higher than that of CF test (45.8%). The sensitivity and specificity obtained for iELISA as compared to CF test was higher while both tests were moderately in agreement (Kappa-0.568).

In this study 4 serum samples gave positive results by CFT in apparently healthy pregnant ewes but these samples gave negative results by iELISA this positive reactors was in parallel with that of OIE [7] recorded Antigenic cross reactivity between *C.abortus* and *C.Pecorum* as well as with some gram-negative bacteria (e.g. *Acinebacter*), can give rise to low false positive CF test results. Furthermore, iELISA was found better at differentiating *C.abortus* from *C. pecorum* in infected animals [28].

An iELISA based on the rOMP90-4 fragment of the outer membrane protein POMP90 is suitable for the diagnosis of ovine enzootic abortion. This iELISA is highly sensitive and specific, showing no cross reactivity with animals infected with *C.pecorum* which gives this test an important advantage over others based on cross-reactive antigens and epitopes, such as CFT and EB-based tests [30].

In the previous study to evaluate the performance of two commercial ELISA, the CHEKIT®-CHLAMYDIA which uses inactivated *C. psittaci* antigen and the *C.abortus* ELISA produced by the Institut pourquier which uses a recombinant fragment of the 80-90 kDa (POMP) protein as improved alternative to CFT for serological diagnosis of OEA. The results indicate that the POMP-based pourquier-ELISA was highly specific since it didn't react with any of the sera from the SPF-lambs experimentally infected with various subtypes of *c. pecorum* Furthermore, it didn't produce any false positive results with reference sera known to be free from OEA or with sera from flocks with no clinical history of abortions in contrast to chekit-ELISA and CFT that recorded by vretou *et al.* [31] and in agreement with Buendia *et al.* [25].

Clinical manifestation are not specific for diagnosis, CFT and iELISA are screening tests may be complicated with false positive in cross reactivity and or in animals with bad physical and health condition OIE [7] so using of molecular biology for confirmation the diagnosis of chlamydophilosis is very important.

All tissue samples of placenta and aborted foeti from ewes with positive CFT and iELISA showed the expected

amplification product specific for *c. abortus* (119bp) and also the results that showed PCR of foeti from ewes with negative CFT and positive indirect ELISA recorded positive PCR, so iELISA is a highly sensitive and specific serologically test for diagnosis of enzootic chlamydial abortion. Also, PCR is a specific, sensitive and rapid accurate technique for diagnosis of ovine enzootic abortion in ewes. These results were in agreement with Nieves Ortega *et al.* [21] Reitt *et al.* [32] Juan Manuel *et al.* [33] and Da Silva *et al.* [34].

CONCLUSION

The results confirmed that infection with *c. abortus* is common pathogens, control programs to eradicate this disease must be and of great importance and should be followed in diagnosis and control strategies as iELISA seems to be suitable for diagnosing abortion due to *C.abortus* and in particular to implement early prophylactic measures as soon as the first cases of abortion occur in order to stop the spread of infection and prevent related economic losses Nevertheless, this serological test must be combined with a more accurate technique for *C.abortus* detection as PCR.

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