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ORIGINAL ARTICLE

Phylogenetic Analysis of Chlamydia abortus Isolated from fetus Aborted ewes of Alborz Province

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ABSTRACT
Chlamydia is an obligate intercellular and Gram negative coccobacilli and one of the most important factors of abortion in ruminants especially in ewes. Purpose of this investigation Phylogenetic Analysis of Chlamydia abortus Isolated from fetus Aborted ewes of Alborz Province. In this study, of 100 aborted fetuses abomasum of 32 sheeps flocks were Alborz Province, DNA was then extracted IGS-Sr- RNA using gene specific primers and PCR amplification was performed,and the positive samples were isolated from 10 randomly for sequencing was sent to Macrogene company in Korea.In this study, in total, 37 samples of 100 aborted foetusis were positive for Chlamydia abortus. After sequencing, recording studied gene in the NCBI gene bank operates BLAST, phylogenetic analysis carried out more than 99% of the positive samples with similar gene sequences in database. The sequencing results that isolates clustered together lik 100% and indicated that isolates LN554882/1,AF051935/1 and CR848038/1 was very similar. The isolates studied, we are in a cluster. As well present investigation indicated that Chlamydia abortus is one of the main reasons of ewes abortion in Alborz province.
Keywords: *Chlamydia abortus, phylogenetic, ewe, Alborz*

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INTRODUCTION

Chlamydia is intracellular micro-organisms that causedifferent diseases in human and animals. According to a classification based on the phylogenic analysis of 16s and 23s r- RNA genes, the family Chlamydiaceae consists of two genus, chlamydia and Chlamydophila, and nine species [1,2].
Enzootic abortion of sheep, caused by *Chlamydia abortus(C.abortus)*, is an infectious disease characterized by placentitis and abortion; It has a negative effect on sheep breeding in many countries.*c.abortus* infection is also observed in goats and cattle and no clinical signs are present in the animals until abortion or delivery; The animals who do not abort deliver very weak lambs the abortion generally occurs in the last 2-3 weeks of pregnancy [3].
It has been reported that the abortion rate in affected herds is low in the first year, The rate reaches or exceeds 30% in the second year and it is of the order of 5-10% in the third year [4].latent infections continuing longer than 3 years have been reported [5]. Pathological changes in the course of *C.abortus* infection can be observed starting on the 90 th day of pregnancy.
Yellowish superficial exudates, red cotyledons and a red thickened inter-cotyledon membrane are described as features of the placenta of sheep aborting in the 18 th week, focal necrosis can be found in the liver of the fetus, as well as small focal areas of necrosis in the lungs, spleen, more rarely also in the brain and lymph nodes [3].
Histological examination of the placenta has shown vasculitis and thrombotic infiltrates of inflammatory cells in the cotyledonary membrane mesenchyme [6].*C.abortus* is also known as the cause zoonotic infection in humans, It is the greatest threat for pregnant women and it causes spontaneous abortion [3].
Direct microscopic examination, pathogen isolation, serological tests (complement fixation test, enzyme-linked immunosorbent assay(ELISA) and immunofluorescence, immunohistochemistry and DNA-based methods (polymerase chain reaction and DNA microarray) are used in routine diagnosis because the pathogen isolation is difficult and time-consuming [7].Cross-reactions between *C.abortus* and Gram-

negative bacteria such as *C.pecorum* and *Acinetobacter* can be seen with CFT, which is recommended by the world Organization for Animal Health(OIE).PCR methods are based on amplification of the chlamydial outer membrane protein genes *OMPA, OPM1*,and *OMP2,* the polymorphic membrane gene *pmp*, genes encoding 16S rRNA and helicase,and the 16S-23S rRNA intergenic interval [8-11].
The purpose of the present study of identification of C. aborus factor in aborted ewes of Alborz province from Iran. Moreover C. abortus will study as one of abortion factors in Alborz province ewes and then we will proceed on phylogenetic analysis and identification of isolated strains through molecular studies and finally we will investigate their similarity and differences with each other and with other strains in the world.

MATERIAL AND METHODS

Samples: our study was carried out in 2014 based on veterinarians and other colleague's reports from ewe's fetus. Sampling was carried out from placenta 100 aborted fetuses of 32 flocks of sheep in Alborz province of Iran. The fetus with its related was selected and the samples were transferred to the laboratory beside ice in sterilized conditions. Then necropsy and sampling was carried out from abomasum of aborted fetus. These samples were kept under suitable conditions then culture tests and PCR reactions were done in *Mabna* laboratory for identification of related factors.
Isolation of the Pathogen.

DNA Isolation and PCR:

According to instruction of that we, Qiagen kit used for DNA Extraction DNA was isolated and PCR was done as follows.
ThePCR mixture contained10xPCRbuffer(10mMTris-Hcl,pH9/0,50mMKcl,.0/1%Triton-100,5*ul*,25mM Mgcl2 2.5*ul*, 250*ul* of each deoxynucleotide triphosphates, *Taq* DNA polymerase enzyme(Fermentas),2*ul*; 1*ul* of each primer; and target DNA,5*ul*).The primer pair a 352-bp fragment the IGS-sr- RNA gene region was amplified by using FP(5"-CAAGGTGAGGCTGATGAC-3") and RP(5"-TCGCCTKTCAATGCCAAG-3")(12).
DNA amplification was realized by 5 minute pre heating at 94^{oc} then subject to 40 cycles of , 1 minute denaturation at 94^{oc}, 1 minute Annealing at 50^{oc}, 2minuteExtension at 72^{oc} and a final extension at 72^{oc} for 10 minute.The DNA amplified by PCR was subjected to electrophoresis for 1 hour at 70V in 2% agarose gel , then dyed with ethidiumbromide (0/5ug/ml) and the results evaluated on a UV transilluminator. The 352bp band was shown positive for *C. abortus.*

Sequencing:

Quality of extracted DNAs were observed and confirmed after doing electrophoresis on agarose gel. 37 cases of our samples were positive in PCR test. 10 samples were selected randomly and their sequencing was done with coperation of *Macrogen* Company of Korea. The sequences were blasted in gene bank and phylogenetic analysis was done then isolated factors origin were drawn in the form of a phylogenetic tree and finally they were compared with findings of other countries.

RESULTS

Result's of sequencing form two chains were studied and finally sequence of IGS-sr-RNA with over 352bp was identified for each sample's. BLAST software in which we were used is available on database of Search NCBI(http/www.ncbi.nlm.nih.gov/blast).sowe used it for identification isolations IGS-sr-RNA alignment or identity of all IGS-sr-RNA sequences were analyzed by *Bio edit* and *DNA star* software, then phylogenetic analysis was done phylogenetic tree was drawn. The results of Sequencing and BLAST in NCBI data bank defined similarities and differences between our strains with each other and other strains of the world. A similarity of 100% can be observed among our studied strains and they are at the same branch another percentage Similarities and differences between our studied strains and other strains and Chlamydia species were specified in a tree.
Following figure shows phylogenetic tree of Chlamydia isolated from our study along with the strains from all around the world.

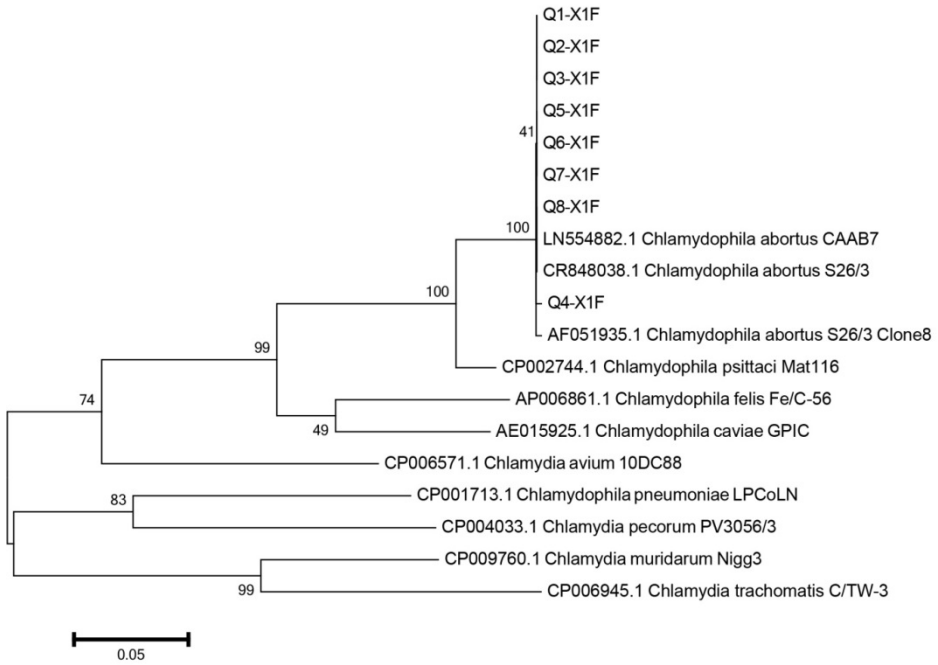

Fig.1: Phylogenetic tree of studied isolations compared with isolations from all around the world

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		100.0	100.0	99.8	100.0	100.0	100.0	100.0	100.0	100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	1	Q1-X1F
2	0.0		100.0	99.8	100.0	100.0	100.0	100.0	100.0	100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	2	Q2-X1F
3	0.0	0.0		99.8	100.0	100.0	100.0	100.0	100.0	100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	3	Q3-X1F
4	0.2	0.2	0.2		99.8	99.8	99.8	99.8	99.8	99.8	99.5	94.8	84.1	83.1	78.2	77.2	74.8	75.2	74.8	4	Q4-X1F
5	0.0	0.0	0.0	0.2		100.0	100.0	100.0	100.0	100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	5	Q5-X1F
6	0.0	0.0	0.0	0.2	0.0		100.0	100.0	100.0	100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	6	Q6-X1F
7	0.0	0.0	0.0	0.2	0.0	0.0		100.0	100.0	100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	7	Q7-X1F
8	0.0	0.0	0.0	0.2	0.0	0.0	0.0		100.0	100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	8	Q8-X1F
9	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0		100.0	99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	9	LN554882_Chlamydophila_abortus_CAAB7
10	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0		99.8	95.0	84.1	83.3	78.2	77.0	74.6	74.9	74.8	10	CR848038_Chlamydophila_abortus_S263
11	0.2	0.2	0.2	0.5	0.2	0.2	0.2	0.2	0.2	0.2		95.0	84.1	83.3	78.2	76.8	74.4	74.9	74.6	11	AF051935_Chlamydophila_abortus_S263_Clo
12	5.2	5.2	5.2	5.4	5.2	5.2	5.2	5.2	5.2	5.2	5.2		85.5	85.1	79.6	76.5	76.2	75.4	75.1	12	CP002744_Chlamydophila_psittaci_Mat116
13	18.2	18.2	18.2	18.2	18.2	18.2	18.2	18.2	18.2	18.2	18.2	16.4		87.6	79.8	75.4	76.6	76.8	74.6	13	AP006861_Chlamydophila_felis_FeC-56
14	19.5	19.5	19.5	19.8	19.5	19.5	19.5	19.5	19.5	19.5	19.5	17.0	13.6		79.9	74.6	76.9	76.4	74.5	14	AE015925_Chlamydophila_caviae_GPIC
15	26.1	26.1	26.1	26.1	26.1	26.1	26.1	26.1	26.1	26.1	26.1	24.2	23.9	23.9		77.0	76.4	77.1	76.0	15	CP006571_Chlamydia_avium_10DC88
16	28.2	28.2	28.2	27.8	28.2	28.2	28.2	28.2	28.2	28.2	28.5	28.7	30.5	31.9	28.0		74.7	79.2	75.8	16	CP001713_Chlamydophila_pneumoniae_LPCoL
17	31.9	31.9	31.9	31.5	31.9	31.9	31.9	31.9	31.9	31.9	32.3	29.3	28.2	28.1	28.6	31.4		76.8	82.4	17	CP009760_Chlamydia_muridarum_Nigg3
18	31.1	31.1	31.1	30.7	31.1	31.1	31.1	31.1	31.1	31.1	31.1	30.3	28.2	29.1	27.6	24.6	28.0		72.9	18	CP004033_Chlamydia_pecorum_PV30563
19	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.4	31.8	30.9	31.5	31.8	29.4	29.7	20.7	34.2		19	CP006945_Chlamydia_trachomatis_CTW-3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Tab.1: The percentage of similarities and differences between in sequences isolated from our studied strains and other strains and Chlamydia species Isolated from all around the world.

DISCUSSION:

Due to serious technical problems in isolation of disease factors caused by Chlamydia in recent decades, major studies have been done in this field. After popularity of molecular methods about identification of microbial disease in one or two recent decades, some studies have been done on detection of bacteria in different disease. Nucleic acids are universal in cellular biology and nucleotide bases of this molecule aren't influenced by culture media. Nucleic acid, analysis provides base of identification methods which can be varied from laboratory to an other laboratory.

Genotypic data (i.e. genome sequence) are preference to phenotypic data: easy accessibility, higher reliability and more accurate interpretation of results and more useful and more extensive information compared to phenotypic information.

Since 1965, new molecular methods were developed, gradually in the field of microbiology and new molecular methods were considered as new useful methods of classifying bacteria. Identifying genetical properties and DNA bases, nucleic acid hybridization studies, cellular wall analysis and protein sequencing were used for bacteria reliable phylogenetic classification. These primary molecular methods were not superior for showing bacteria toxonomy and there is no similar result in the cases. The molecule, that is sequence randomly was changed in time passage, is as sumed as a molecular chronometry. Accumulated sequence change equals product of rate (till mutations are stabilized) in the

time (i.e. in the time that changes happened). Biologist can not measure changes , but he can just identify differences between sequences. All sequences have not the same value in determination of phylogenic relation. An effective chronometer should have following characteristics: it should behave like a clock (changes in its sequences should occur randomly as much as possible) and they should have suitable size (the molecules with big size can provide enough information). Why r- RNAs are suitable? r- RNAs are known as the most efficient molecular chronometers because they have higher degree of stable function, they are in at all organisms and they provide conditions which have maximum phylogenetical relation (maximum genetically distances) can be measured. On the other side they have big sizes and wide domains. There are almost 50 spiral stems in second structure of r- RNAs. It might be that the most important reason for using r- RNAs as a chronometers is that they can be sequenced directly[13].

Nowadays molecular methods such as PCR and sequencing are used in most studies to identify Chlamydia, because it is a reliable method compared to most traditional methods such as culturing and staining which were ambiguous in most case. Moreover it can identify species and strains with more similarity.

In a study Kilic et.al in (2013), took vaginal swap from 71 cases of goat and ewe abortions through culturing on 6-8 days embryonated eggs and analyzed with PCR methods that 7 cases of them C. abortus was observed [14].

In an article titled" complete gene sequence of four *Chlamydia psittaci* isolated from mammals such as cow, sheep , pig and human" Gerhard et.al in (2011) tried to determine and name their genome. In this study, *Chlamydia psittaci* isolated from ewe were obtained as C19/98 and pig genome obtained in isolated in (2001) was in the form of 01DC11 and it was 02DC15 in aborted embryo of cow obtained in(2002). Finally it was identified as 08Dc60 in human type obtained from bronchial secretion [15].

In an article titled" comparison of Plasmid *orf3*of*Chlamydia psittaci* and -Chlamydia trachomatis based on RFLP_PCR " published in (2006), it was identified that plasmid *orf3* is different in both presented Chlamydia [16].

Borel *et. al* [12] in 235 cows Chlamydia abortions observed almost 63.4% Histopathological lesions such as Chlamydia necrotizing and placenta specimens and they clearly indicated immunohistochemistry anti gen in one case (0.4%).

Margaret M.C.G.et.al in an article titled " comparison of *Chlamydia psittaci* isolated from animals by DNA Endonuclease analysis observed difference in DNA patterns by comparing DNA isolated from *Chlamydia psittaci* of 8 samples obtained from ewe abortion with of Endonuclease and PCR on polyacrylamide gel [17].

In an article special sequences obtained from helicase strain S26/3 of Chlamydia psittaci, the abortion factor of ewes were checked by PCR it was shown PCR is rapid and sensitive method compared to MC coy cell culture in culture method, This article was investigated by Julie et.al in 2000(18).

Tabatabayi and Nadalian [19] identified Chlamydia factor in follicular Conjunctivitis in ewes of *Somalia* for the first time in Iran.

Tabatabayi reported, *Chlamydia psittaci* from Conjunctivitis of cat's eye for the first time in Iran [20].

In a study titled" molecular investigation of *Chlamydia psittaci* frequency in pigeon droppings of chaharmahal va Bakhtiari Province "Doosti et.al [21] collected 300 sample of pigeon droppings from different towns of the province and extracted their genomic DNA and carried out PCR on *Chlamydia psittaci OMPA gen*e and reported *Chlamydia psittaci* frequency (14.33%).

In a study titled "isolation of *Chlamydia psittaci* from poultries" Madani et.al [22] reported that Conjunctivitis and cloaca of 17 species of birds, MC coy cell culturing were stained by Giemsa to observe inclusion body of Chlamydia and 4 cases of *Chlamydia psittaci* were traced .

Results of present study indicate high and capability of designed experiment for suitable recognition and reproduction of IGS-sr-RNA gene with length of 352bp which identified chlamydophila abortus as one factor of ewe abortion by PCR. Moreover nucleic acids sequencing indicated that studied strains were similar to each other 100% and also they have some similarities and differences with other strains and species recorded in NCBI gene bank.

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