RESEARCH ARTICLE

DIAGNOSIS OF CHLAMYDOPHILA ABORTUS BY SPECIES–SPECIFIC ELISA TECHNIQUE WITH REAL TIME PCR CONFIRMATION IN CAMELS AND SMALL RUMINANTS IN THE WEST-NORTHERN DESERT OF EGYPT.

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Abstract:
Serological surveys for antibodies of Chlamydophila abortus in camels and small ruminants in the northern-west coast of Egypt were carried out from August 2014 to January 2015; some cases had a history of abortion. A total of 630 blood samples were randomly collected from the jugular vein of camels, sheep and goat. Camels were 228 pregnant female and 48 male with average ages 5:12 years, 224 Ewes and 130 goats with age average 3:5 years old with or without apparent clinical symptoms. One hundred and eighty different swabs (four swabs from each animal were taken fecal, vaginal, nasal and ocular swabs) were collected from 45 female camels, 224 swabs from 56 ewes and 52 swabs from 13 goats and were subjected to serological analysis by IDEXX Enzyme Linked Immunosorobent assay using an ELISA kit (IDEXX Switzerland). All male camel samples were negative while female samples expressed Chlamydophila abortus antibodies with positive percent S/P% equal to 10.87% (30/276).
The seroprevalence in ewes was observed in 3.57% (8/224), while in goats was 15.38% (20/130). Goats have recorded higher antibody titers than ewes, there was a significant relation between age and disease in camels and goats at 0.01 level, and in sheep at 0.05 level. DNA were extracted from swabs of the positive serology animals. Quantification of chlamydiaceae by real time PCR were 81.2% positive in camels, 100% in ewes and 50% in goats.

Key Words:– Chlamydiophilosism, Serology, IDEXX-ELISA, Real time PCR.

Introduction:
Chlamydiophilosism is a major cause of abortion in domestic ruminants. Ovine enzootic abortion, caused by Chlamydia 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 (Papp and Shewen, 1996).

The role of Chlamydiophila abortus in the Ovarianhydrobursitis syndrome in dromedary camels Characterized by fluid accumulation and encapsulation of the ovary. It is manifested by early embryonic death, abortion, (Ali, et al., 2011). The serological prevalence of chlamydiosis in Saudi Arabia is high suggesting that camels might have a role in the epidemiology of this high endemic infection among human population of the Kingdom (Hussein, et al., 2008).

Most infections in sheep and goats are asymptomatic apart from late term abortion and still birth (Jones, Hunt and King, 1997; OIE, 2008). Ovine chlamydiosis, also known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA), is caused by the bacterium Chlamydiophila abortus. Chlamydial abortion typically occurs
in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and inflamed placentas. However, infection can also result in the delivery of full-term stillborn lambs or weak lambs that do not survive longer than 48 hours (OIE, 2012). El Hakim (2008) investigated the forms of Chlamydophila infection in camels and their role in the transmission of this organism to other animals.

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 [Trávniček et al., 2002; Ana et al., 2012]. Recently a more species specific ELISA are commercially available developed by IDEXX laboratories in USA (CHEKIT®-ELISA) for antibodies against Chlamydophila abortus (Samkange et al., 2010; Aljumaah and Hussein 2012; Roukbi et al., 2016). This kit provides a rapid, simple, sensitive and specific method for detecting antibodies against Chlamydophila abortus in serum from ruminants.

The obligate intracellular nature of Chlamydiaceae requiring growth in embryonated eggs or tissue culture for detection make the diagnosis more difficult. Furthermore, there are no reliable biochemical tests to differentiate species, which has complicated research in this field. However, the introduction of molecular methods in the detection of Chlamydiaceae has significantly improved the possibilities for detection. PCR assays have been developed based on the ompA gene, the 16S-23S rRNA operon, and the pmp gene, using traditional and real-time assays (Sachse et al., 2009). In the last years, real-time PCR has become the preferred method in diagnostic laboratories for its rapidity, high throughput and ease of standardization (Nordentoft et al., 2011).

Robertson et al (2009) assessed using the oligonucleotide set 16SG is a robust, simple and rapid technique for differentiation of the nine species of Chlamydiaceae. PCR assays in combination with restriction fragment length polymorphism analysis have been developed with potential to differentiate naturally infected from vaccinated animals (Laroucau et al., 2010 and Wheelhouse et al., 2010).

**Aim of work:-**

Evaluation of the use of IDEXX-ELISA and real time PCR in the diagnosis of chlamydophilosis in camels and small ruminants.

**Materials & Methods:-**

**Animals:-**

1- Camels: This study was done on 276 camels (228 pregnant female and 48 male) aged from 5:12 years old. Samples were collected from Matrouh governorate and Marut – Research station that belong to Desert Research Center in Al-Amrea Alexandria (Table 1).

2- Small ruminants: Samples were collected from 224 Ewes and 130 goats with age ranged from 3:5 years old with or without apparent clinical symptoms. All samples of small ruminants were from Marut – Research station. Small ruminants in the station were in contact with camels. (Table 1).

**Table (1):** Distribution of blood samples, age and region of camel, sheep and goat

<table>
<thead>
<tr>
<th>Animal</th>
<th>Blood samples</th>
<th>Region</th>
<th>Marut</th>
<th>Matrouh</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>♀</td>
<td>♂</td>
</tr>
<tr>
<td>Camel</td>
<td>276</td>
<td></td>
<td>141</td>
<td>3</td>
</tr>
<tr>
<td>Ewes</td>
<td>224</td>
<td></td>
<td>224</td>
<td>Nil</td>
</tr>
<tr>
<td>Goats</td>
<td>130</td>
<td></td>
<td>130</td>
<td>Nil</td>
</tr>
</tbody>
</table>

**Sampling:-**

Samples were collected in the time from August, 2014 to January, 2015. Blood samples were collected in sterile tubes and the tubes were left in a slant position for coagulation and serum separation. The samples were transported as early as possible to the laboratory. The coagulated blood samples were centrifuged at 3000 rpm for 5 minutes. The sera were separated in sterile labeled capped vials and kept at -20ºC until used for serological examination.
Swabs were prepared according to OIETerrestrial Manual(2012). Each swab was collected in approximately 2-3cm sucrose/phosphate/glutamate (SPG transport medium). SPG medium consists of sucrose 74.6 g/l, KH2PO4 0.512 g/l, K2HPO 1.237 g/l, L-glutamic acid 0.721 g/l, streptomycin 0.05 g/l. The pH of the medium was adjusted at 7.2- 7.4. Samples were clarified by centrifugation at 3000 rpm for 15 min. The supernatant was stored at -20ºC until used for DNA extraction.

**Serological identification:**
Total antibodies for *Chlamydophila abortus* were tested by IDEXX Enzyme Linked Immunosorbent assay using ELISA kit (IDEXX SwitzerlandLotC681) according to the manufacturer instructions. Samples giving %OD of >40% were considered positive, as described by [Aljumaah and Hussein(2012)](#).

**Calculation:**
The OD of the positive control (PCx) and the OD of the samples (Sample A450) were corrected by subtracting the OD of the negative control (NCx) one. The percent of positivity of the samples were calculated with the formula:

\[
\text{S/ P \%} = \left( \frac{\text{Sample A450} - \text{NCx}}{\text{PCx} - \text{NCx}} \right) \times 100
\]

The percent of sample positivity S/ P % interpretation is considered negative if the percent was <30%, suspect at ≥30% to <40% and positive at ≥ 40%.

**Isolation of genomic DNA from *Chlamydophila abortus***:
Genomic DNA was extracted from the swabs by DNeasy Blood & Tissue Kit (50) from QIAGEN Catalog no. 69504. The purified DNA had an OD A260/A280 ratio between 1.7 and 1.9. DNA concentration was measured by Nanodrop 2000 spectrophotometer from Thermo scientific.

Quantification of chlamydiaceae in seropositive genomic DNA were examined using real time PCR detection kit for Chlamydiaceae using Primerdesigngenesig® Advanced Kit. serial number JN1 16493-36149 (PrimerDesign Ltd., Southampton, UK) as manufacturer instructions[Primer design (2015a)](#). The amplification was carried out utilizing oasig TM 2x qPCRMasterMix. serial number JN1 16494-36149 [Primer design (2015b)](#) in total final volum 15ul (oasigTM 2x qPCRMasterMix 10ul, Chlamydia primer/probe mix1 μl, Internal extraction control primer/probe mix1μl, RNAse/DNase free water 3μl). The amplification program was 95ºC for 2 min, then denaturation step at 95ºC for 10 sec and 50 cycles for data collection at 60ºC for 60 sec. Fluorogenic data for the control DNA were collected during this step through the FAM and VIC channels, Real-Time PCR Instrument Applied Biosystems 7900.

**Statistical analysis:**
Three tests were carried out for statistical analysis. The Anova test, homogenicity variance test were carried out on each animal case to test the homogenicity and significance between ELISA results and age. UNIANOVA test was carried only on camel samples to compare between ELISA results in each region in comparison with age.

**Results:**
Four swabs were collected from each animal (fecal, vaginal, nasal and eye swabs). The total number of swabs were 180 different swabs collected from 45 female camels, 224 swabs from 56 ewes and 52 swabs from 13 goats.

In this study 276 camels were tested by IDEXX ELISA, all male samples were negative while female samples expressed *Chlamydophila abortus* antibodies with positive percent S/P% equal 10.87%(30/276) along broad range of age between 5 to 12 years old. Samples from Matrouh exhibited 12.5% (18/144) while samples from Marut represented 9.09% (12/132) Table (4). The IgG titers ranged from 40 to 60% with 18 samples suspected for infection with S/P value between 30 and 40%.

The seroprevalence in ewes was observed in 3.57% (8/224), these ewes having an IgG titers ranging from 40 to 49.3%. The seroprevalence in goats was observed in 15.38% (20/130), they had an IgG titers ranging from 41.481 to 52.628% Table (4). It was observed that neither ewes nor goats were considered to have suspected infection with *Chlamydophila abortus*. Goats recorded higher antibodies titers than ewes in Matrouh Table (4).
Statistical analysis for camel and small ruminants results ELISA:

The relation between ELISA results and age was significant by homogeneity test in each camel, sheep and goat Table (2). ANOVA test revealed that there was a significant relation between age and disease in camels and goats at 0.01 level, and in sheep at .05 level respectively. The relation in camels was significant at .01 and the value of F = 19.972** and in goats was also significant at .01 and the value of F = 19.521**, that means a presence of relation between disease and age in camels and goats. While in sheep it was insignificant with F =.495 which means no relation between age and disease in sheep.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Levene Statistic</th>
<th>df1</th>
<th>df2</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camel</td>
<td>28.828</td>
<td>3</td>
<td>272</td>
<td>.000</td>
</tr>
<tr>
<td>Sheep</td>
<td>3.897</td>
<td>2</td>
<td>221</td>
<td>.022</td>
</tr>
<tr>
<td>Goat</td>
<td>71.797</td>
<td>1</td>
<td>128</td>
<td>.000</td>
</tr>
</tbody>
</table>

Table (3):- Test of homogenicity of variance between the tested animals

<table>
<thead>
<tr>
<th>factor</th>
<th>Type III Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>7307.195</td>
<td>5</td>
<td>1461.439</td>
<td>13.774</td>
<td>.000</td>
</tr>
<tr>
<td>Intercept</td>
<td>22953.810</td>
<td>1</td>
<td>22953.810</td>
<td>216.339</td>
<td>.000</td>
</tr>
<tr>
<td>age</td>
<td>7110.411</td>
<td>3</td>
<td>2370.137</td>
<td>22.338</td>
<td>.000</td>
</tr>
<tr>
<td>region</td>
<td>250.935</td>
<td>1</td>
<td>250.935</td>
<td>2.365</td>
<td>.125</td>
</tr>
<tr>
<td>age * region</td>
<td>576.499</td>
<td>1</td>
<td>576.499</td>
<td>5.433</td>
<td>.020</td>
</tr>
<tr>
<td>Error</td>
<td>28647.353</td>
<td>270</td>
<td>106.101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>59165.450</td>
<td>276</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>35954.548</td>
<td>275</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Quantification of chlamydiaceae by real time PCR:

Eight camels, two ewes and 2 goats were selected which showed positive serology test. Four swabs were collected from each animal (fecal, eye, nasal and vaginal swabs). DNA was extracted from 32 swabs from camels and 16 swabs from ewes and goats. The DNA concentrations ranged from 1.1 to 33.1 ng/ml.

Quantification of chlamydiaceae by real time PCR in camels revealed 81.2% (26/32) positivity. Members of family chlamydiaceae were detected in all collected swabs as follows 62.5% (5/8) in vaginal swabs, 87.5% (7/8) in eye, nasal and fecal swabs. The cycle threshold (Ct values) of the tested real time PCR ranged from 26.98 to 46.84% Table (5), Figure (1). Camel samples with 60% ELISA titre from Matrouh region did not show any members of family chlamydiaceae in their body secretion. Camels with antibody titers 43.5% from Marut showed negative vaginal RT-PCR while positive RT-PCR were recorded from other secretions with high Ct values (28.5% nasal, 27.2% fecal and 29.1% eye). It was also noted that all fecal swabs had average Ct value 26.98%, 27.18, 28.60% and 29.4% which means high level of infection with members of chlamydiaceae.

All swabs from Ewes were positive with Ct values range from 26.55% to 45%, while goats were varied between negative and positive with Ct value range 36 to 48%. Positivity was 100% for ewes and 50% for goats as shown in table (5).

Table (4):- Animal and region prevalence of *Chlamydia abortus* with S/P% IDEXX ELISA of positive animals as a representative model with their quantification of real time PCR from its swabs.
Figure (1):- Standard curve of Chlamydiaceae showing the Ct values of the samples

Discussion:-
Extremely little is known about Chlamyophila abortus epidemiological diseases. El Hakim (2008), Hussein et al. (2008) and Ali et al., (2011) discussed the role of Chlamyophila abortus in camels in Saudi Arabia. To the best of our knowledge this is the first serological evidence of camel chlamydiophilosis in the desert of Egypt.

In this study, anti-ruminant conjugate for camel and small ruminants were used for detecting Chlamyophila abortus by IDEXX ELISA. Only 10.87% (30/276) of the tested camels were positive for C. abortus,
all of them were females. Samples from Matrouh exhibited 12.5% (18/144), while those from Marut presented 9.09% (12/132). On the other hand, the percent was 3.57% (8/22) in ewes and 15.38% (20/130) in goats.

In 1978, antichlamydial antibodies were detected in 11% of the serum of the camels in a study conducted by Schmatz et al., (1978) in Egypt. Elzline and Elhafi, (2016) recorded seroprevalence of chlamydiosis in Libyan camels with 12.25%, the prevalence was 2 folds higher in females than males.

Ali et al., (2011) reported that 86.3% of female camels with ovarionhydropermsitis syndrome accompanied by elevated Chlamydophila abortus antibodies in Saudi Arabia. The overall prevalence of chlamydophilia in Saudi Arabia was 19.4%. (Hussein, et al., 2008) mentioned that nearly 95% of all adult camels aged ≥4 years and 70% of them being at least 8 years old, all seropositive animals were clinically normal at the time of sampling. In United Arab of Emirates, Wernery and Wernery (1990) detected antibodies in the sera of both breeding and racing camels, respective prevalence rates of 24 and 15% respectively.

In this study, most positive samples were female camels having age 6:7 years and some cases were 7 to 12 years. The highest percentage of antibody titer were 60 % recorded in Matrouh region in a case with age 7 years old. The tested camels were pregnant with some clinical signs as eye and nasal secretions in some cases with antibody titer (S/P = 30%). No abortion was recorded in most camels examined in this study except for two cases. This finding was congruent with El Hakim (2008) who reported the presence of C. abortus and/or C. pecorum in examined camels with no abortion but with some respiratory signs. Camels infected with Chlamydophila abortus (even in absence of clinical signs) are considered as a reservoir for the infection of contact animals. Twomeyet al., (2006) studied the role of Chlamydia spp. in upper respiratory tract infections and detected C. abortus and C. pecorum as the main cause of bovine upper respiratory tract disease outbreak.

Lenzko, et al (2011) suggested that a flock is considered sero-positive if at least one animal was positive in ELISA.

In the present study, antibodies were detected in the serum of females but not males. Similar results were recorded by Husseinetal. (2008). While Kauffoldet al. (2007) and Teankum et al. (2007) reported that male camels may be incriminated in transmitting the chlamydial infection. Chlamydiae infect male genital organs of ruminants and cause prostatitis and epididymitis in men (Wagenlehner et al., 2006).

Results showed that 8/224 (3.57%) of ewes and 20/130 (15.38%) of goats were positive by ELISA, these results were congruent with Stone, et al., (2012) who recorded prevalence of Chlamydia abortus in small ruminants with 6.0% using IDEXX-ELISA in Grineda India. Aljumaah and Hussein (2012) also recorded the serological prevalence of Chlamydia abortus in Riyadh region, Saudi Arabia using CHEKIT ELISA with 7.52% in sheep, 34.50% in goats. In addition, Samkange et al., (2010) recorded 25% positivity at farm level and 8% at animal level using IDEXX ELISA in Namibia.

Lenzko, et al., 2011 illustrated the seropositive elevation of Chlamydia abortus in sheep in Germany by IDEXX ELISA was 10% at flock level in the absence of elevated abortion rate. Aljumaah and Hussein (2012) reported the serological prevalence of Chlamydia abortus in sheep (7.52%) and goats (34.50%) in Riyadh region, Saudi Arabia, using the CHEKIT enzyme-linked immunosorbent assay (ELISA). In addition, Roukbi et al., (2016) reported elevated seroprevalence rates in sheep and goat stations were 8.8 and 6.8%, respectively in Syria. Farhadet al., (2016) examined dairy goats, in Iraq, and they recorded 11.9% seropositive for C. abortus antibodies.

In this study camels that showed seropositive Chlamydia abortus were further confirmed with Chlamydiaceae real time PCR. RT-PCR is a fast and robust technique, allowing the user to obtain confident and unequivocal results with the additional possibility to quantify the initial amount of target DNA present in a sample.

Nordentoft et al., (2011) evolved a real time screening assay for discriminating chlamydiaceae members that assay was more sensitive than traditional microscopical examination of stained tissue smears.

The present finding that positive chlamydiaceae was 81.2% in camels, positivity was 100% for ewes and 50% for goats, however these animals were mostly apparent healthy. Lenzko, et al., (2011) found that unvaccinated flocks were considered antigen positive if at least one animal was tested positive for chlamydial DNA Chlamydiaceae-specific real-time or conventional PCR.
Although all animals were apparently healthy with no history of abortion, members of family chlamydiaceae were detected in all collected camel swabs as follows 62.5% (5/8) for vaginal, 87.5% (7/8) for nasal, 87.5% (7/8) for fecal and 87.5% (7/8) for eye. On the other hand, members of family chlamydiaceae were detected in all collected vaginal swabs, these data are congruent with data of Lenzko, et al (2011).

Camels apparent healthy or had symptoms sharing the expression of chlamydiaceae in their secretions with cycle threshold (Ct) values ranged from 27.18 to 46.84% are considered as a reservoir for chlamydiaeae.

In the present study, all fecal swabs with average Ct value 29.4% that means high level of member chlamydiaceae female camels as intestinal carriers. Allam, (2006) revealed that chlamydophila abortus (formerly Chlamydia psittaci serotype1) was an intestinal carried by healthy sheep and aborted goat that was isolated by chicken embryo and conventional PCR. The animal expressed antibodies against Chlamydophila abortus that detected by Complement Fixation Test (CFT) and Indirect ELISA.

Wafaa et al., (2007)detected Chlamydophilapsittaci in birds using PCR, although serologically positive birds did not show any clinical symptoms of disease, but they were in contact with sheep and goat that showed previous abortion and were positive for C.abortus.

Salwa et al., (2007)suggested that there was some transmission of Chlamydia species occurring between domestic and wild ruminants. Similar finding by Wafaa et al., (2007)but forChlamydophilapsittaci who recommended that chickens become asymptomatic carrier with other animal species in shedding of Chlamydophilapsittaci in their feces and respiratory discharges.

Pregnant women are particularly susceptible. Pregnant women, especially those who live in rural areas, should generally be aware of the risks of zoonotic diseases (Meijer et al., 2004). Waldereat., (2003) demonstrated the first documented case of an extragestational infection with Chlamydophila abortus in human that the pathogen was identified in a patient with severe pelvic inflammatory disease (PID).

In conclusion:-

Camels play an important role in the Chlamydophila abortus transmission to other animals, so it might play an important role in the epidemiology of chlamydiophilosis.

The IDEXX-ELISA is a recommended method for detection of Chlamydophila abortus IgG

Further studies should, be carried out to expand our knowledge regarding the prevalence, distribution and epizootiology of chlamydiophilosis to investigate more about the organism and their geographical distribution.

Real time PCR is a recommended method for identification because the special nature of Chlamydiaceae which need a specific and robust technique especially in veterinary laboratory as broad sample range.

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