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Detection of Circulating Immunoglobulins in Serum of Sheep Naturally Infected with *Chlamydia Trachomatis* and *Listeria Monocytogenes* in Two Egyptian Provinces.

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Key words

C. trachomatis, L. monocytogenes, ELISA

ABSTRACT:

Chlamydia trachomatis and *Listeria monocytogenes* are important reproductive tract pathogens in a wide variety of animals including sheep. In humans, chronic or repeated infection of the female genital tract with *Chlamydia trachomatis* has been identified as a significant factor in the development of occlusive infertility or increased risk of ectopic pregnancy. The aim of the current study is to detect circulating immunoglobulins in serum of sheep naturally infected with *Chlamydia trachomatis* and *Listeria monocytogenes* in two Egyptian provinces. A total of 82 serum samples from 4 sheep flocks in two Egyptian provinces were examined with enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies of *Chlamydia trachomatis* and *Listeria monocytogenes*. The results of ELISA revealed the detection of antibodies against the two pathogens at a very high percentage (98.78% and 100% for *Chlamydia trachomatis* and *Listeria monocytogenes*, respectively) despite of absence of elevated abortion rates in the examined sheep flocks. It could be concluded that chlamydial and listerial infections occur frequently in Egyptian sheep flocks, even in the absence of elevated abortion rates and there is possible risk to pregnant women in rural areas where chlamydial and listerial infections in farm animals are widespread. Also, ewes that experienced *Chlamydia trachomatis* or *Listeria monocytogenes* induced abortion provide a unique opportunity to study the host: parasite dynamic as it relates to persistent infection. This natural model of persistent infection may, in some ways, be superior to more contrived models in which the chlamydial or listerial isolate is not a normal reproductive pathogen of the study animal. Thus, the study of persistent Chlamydial or Listerial infection in sheep may be used for the benefit of both human and veterinary medicine.

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1. INTRODUCTION

The Chlamydiae are obligate intracellular parasites of eukaryotic cell found in the leucocytes and consist of minute particles. Chlamydiae are non-motile, coccoid, ranging from 0.2 to 1.5um. Chlamydia trachomatis is composed of two biovars; the lymphogranuloma venereum agents and the ocular genital serotype, which may be distinguished by well-described seroprevalent with distinctive antigens in their outer membrane proteins. On the other hand, Listeria monocytogenes is an emerging bacterial foodborne pathogen responsible for listeriosis, an illness characterized by meningitis, encephalitis, and septicaemia. Less commonly, infection can result in cutaneous lesions and flu-like symptoms (Okoror et al. 2007 and Wesely, 1999). Chlamydiae and L. monocytogenes are important reproductive tract pathogens in a wide variety of animals. In humans, chronic or repeated infection of the female genital tract with C. trachomatis has been identified as a significant factor in the development of occlusive infertility or increased risk of ectopic pregnancy. Moreover, L. monocytogenes can cause bacteraemia, and stillbirth or premature birth of the fetus in pregnant women (Baud et al. 2008). The spectrum of reproductive disease recognized in sheep to be caused by Chlamydia or L. monocytogenes has been primarily restricted to pregnant animals because the

organism was clearly identified as a major cause of infectious abortion. However, following pregnancy failure, a chronic chlamydial infection can become established in the reproductive tracts of experimentally infected ewes (Papp and Shewen, 1997).

Persistent infection of the ewe's reproductive tract may eventually result in pathology, similar to that observed in women infected with C. trachomatis, thus decreasing the breeding life of affected ewes. Furthermore, ewes that experienced Chalmydia induced abortion provide a unique opportunity to study the host: parasite dynamic as it relates to persistent infection. This natural model of persistent infection may, in some ways, be superior to more contrived models in which the chlamydial isolate is not a normal reproductive pathogen of the study animal. Thus, the study of persistent chlamydial infection in sheep may be used for the benefit of both human and veterinary medicine (Papp and Shewen, 1997).

Listeria infection during pregnancy can cause miscarriage or premature birth. If the child is infected in the woman or during delivery this may lead to septicaemia and meningitis, which has a high fatality rate. There can also be long-term effects in many organs, including the airways, eyes and nervous system (Roberts and Wiedemann, 2003).

The fact that there are scarcity of data regarding the relations between C. trachomatis, L. monocytogenes and infertility cases in Egypt. Therefore, this study was aimed in the main to elucidate the seroprevalence of C. trachomatis and L. monocytogenes infection among sheep, Moreover, C. trachomatis and L. monocytogenes are potentially zoonotic agents raise questions about the significance of this reservoir for animal and human health and underline the necessity for regular monitoring (Lenzko et al. 2011).

2. MATERIALS AND METHODS:

2.1. Collection of blood Samples:

A total of 82 blood samples were collected from 4 sheep flocks in two Egyptian provinces (Menofia and Kafr El-Shiekh) throughout the period extended from September 2012 to March 2013. Each animal was identified according to age, locality, time of sampling and presence or absence of previous history of abortion. Samples were collected from the selected animal, left tightly closed in the refrigerator overnight at 4 $^{\circ}$C, and then centrifuged at 1500 rpm for 15 minutes. Sera were obtained by using sterile Pasteur pipettes, labeled and stored at -20 $^{\circ}$C until examined for presence of C. trachomatis and *L. monocytogenes* antibodies by ELISA.

2.2. Enzyme Linked Immunosorbent assay (ELISA) for the detection of *Chlamydia trachomatis* in ovine´s serum samples. (Catalogue No. 201-07-0049 SunRed®).

(Anusz et al., 1990).

It was used for in vitro diagnosis of *C. trachomatis*. It was an enzyme immunoassay for the determination of *C. trachomatis* in ovine serum, blood plasma and other related fluids and performed according to the instructions of the manufacturer company**.**

Assay procedure:

Preparing reagents, samples and standards:

Standard solution:

- The test supplies one original standard reagent 50 µl which can be diluted according to the instruction manual
- The quantity of the plates depends on the quantities of to-be-tested samples and standards.
- It is suggested to duplicate each standard and blank well.

Inject samples:

- Blank well: do not add samples and CT-antibody labeled with biotin, streptavidin-HRP, only chromagen solution A and B and stop solution are allowed other preparation are the same.
- Standard well: add standard 50 µl, streptavidin-HRP 50 µl (since the standard already has combined biotin antibody, it is not necessary to add the antibody).
- To the test wells: add samples 40 µl and then add both CT-antibody 10 µl and streptavidin-HRP 50

µl. then seal the sealing membrane and gently shaking, incubated 60 minutes at 37c.

- Confection: dilute 30 times the 30x washing concentrate with distilled water as standby.
- Washing: remove the membrane carefully, and then drain the liquid, shake away the remaining water.

2- Add chromagen solution A 50 µl, then chromagen solution B 50 µl to each well. Gently mixed Incubate again at (37°C) in the dark for 10 minutes followed by Adding 50 µl stop reagent then carry out the photometric measurement at 450 nm.

3- According to standards concentration and the corresponding OD values, calculate out the standard curve linear regression equation, and then apply the OD values of the sample on the regression equation to calculate the corresponding sample concentration or by using software programs.

Sensitivity, assay range:

Sensitivity: 18.553ng/L (the sensitivity of the assay was defined as the lowest protein concentration that could be differentiated from zero. It is determined by sub-tracking two standards deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.)

Assay range: 20 ng/L -7200 ng/L.

2.3. Enzyme Linked Immunosorbent assay (ELISA) for the detection of *Listeria monocytogenes* in ovine´s serum samples (Catalogue No. 201-12-2067 SunRed®): (Anusz et al., 1990).

The test was used for in vitro diagnosis of *L. monocytogenes*. It was an enzyme immunoassay for the determination of *L. monocytogenes* in ovine serum, blood plasma and other related fluids and performed according to instructions of the manufacturer company.

Assay procedure:

Preparing reagents, samples and standards:

1. **Standard solution:**

2. The test supplies one original standard reagent 50 µl which can be diluted according to the instruction manual.

3. The quantity of the plates depends on the quantities of to-be-tested samples and standards.
   It is suggested to duplicate each standard and blank well.

4. **Inject samples:**

- Blank well: do not add samples and LM-antibody labeled with biotin, streptavidin-HRP, only chromagen solution A and B and stop solution are allowed other preparation are the same.
- Standard well: add standard 50 µl, streptavidin-HRP 50 µl (since the standard already has combined biotin antibody, it is not necessary to add the antibody).
- To be the test wells: add samples 40 µl and then add both LM-antibody 10 µl and streptavidin-HRP 50 µl. then seal the sealing membrane and gently shaking, incubated 60 minutes at 37c.

5. **Confection**: dilute 30 times the 30x washing concentrate with distilled water as standby.
6. **Washing**: remove the membrane carefully, and then drain the liquid, shake away the remaining water.
7. **Add chromagen solution A** 50 µl, then **chromagen solution B** 50 µl to each well. Gently mixed Incubate again at (37Cº) in the dark for 10 minutes followed by Adding 50 µl stop reagent and finally, carry out the photometric measurement at 450 nm.
8. According to standards concentration and the corresponding OD values, calculate out the standard curve linear regression equation, and then apply the OD values of the sample on the regression equation to calculate the corresponding sample concentration or by using software programs.

Sensitivity, assay range:

Sensitivity: 0.1nnmol/L (the sensitivity of this assay was defined as the lowest protein concentration that could be differentiated from zero. It is determined by sub-tracing two standards' deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.)

Assay range: 0.1 nmol/L - 27nmol/L.

3. RESULTS

Table (1): The results of indirect enzyme linked immunosorbant assay (ELISA) for detection of circulating immunoglobulin in serum of sheep naturally infected with C. trachomatis in two Egyptian provinces

Province	No. of examined sheep	+ve	%	-ve	%	mean absorbance of positive sera ±SD (ng/L)
Menofia	40	39	97.5%	1	2.5%	1889.21±678.7
Kafr ELshiekh	42	42	100	0	0	1767.81±804.13
Total	82	81	98.78	1	1.22	

Determined at 450 nm wave length and cutoff level corresponding to 18.553 ng/L (the lowest protein concentration that could be differentiated from zero).

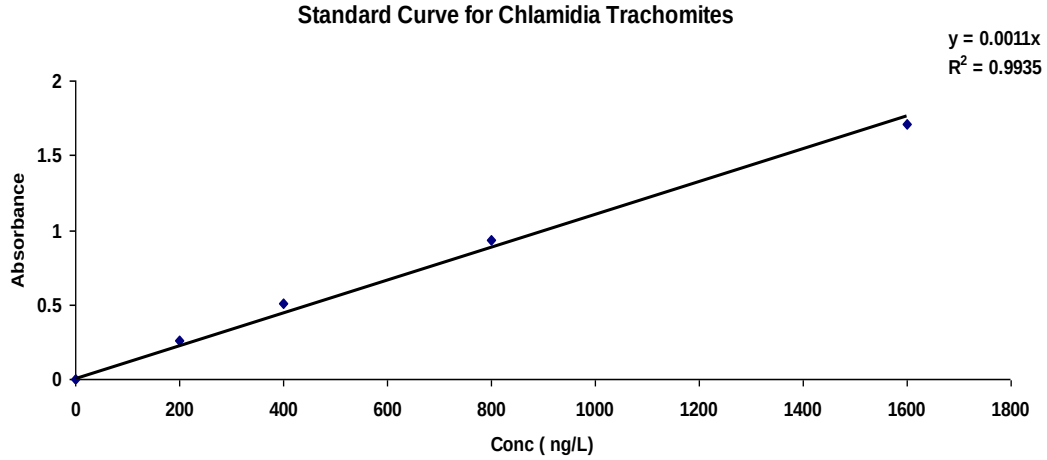

Fig (1): Graph illustrates the standard curve obtained from ovine *C. trachomatis* ELISA test. The concentration of the protein (ngl/L) in each sample was obtained the equation showed on the graph. The equation corresponding to a linear fit of the absorbance.

Table (2): The results of indirect enzyme linked immunosorbant assay (ELISA) for detection of circulating immunoglobulins in serum of sheep naturally infected with *L. monocytogens* in two Egyptian provinces.

Province	No. of examined sheep	Positive	%	Negative	%	mean absorbance of positive sera ±SD (nmol/L)
Menofia	40	40	100	0	0	7.61±2.67
Kafr ELshiekh	42	42	100	0	0	5.71±2.51
Total	82	82	100	0	0	

Determined at 450 nm wave length and cutoff level 0.1 nmol/L (the lowest protein concentration that could be differentiated from zero).

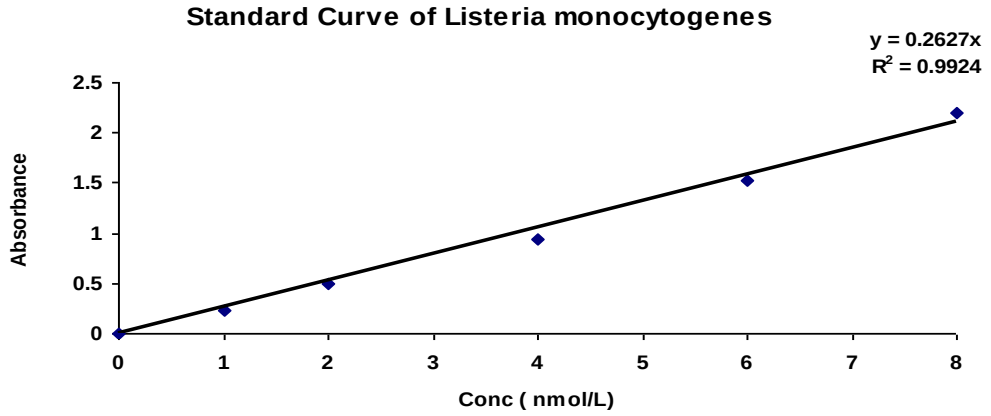

Fig (2): Graph illustrates the standard curve obtained from *listeria monocytogenes* ELISA test. The concentration of the protein (nmol/L) in each sample was obtained the equation showed on the graph. The equation corresponding to a linear fit of the absorbance.

4. DISCUSSION

C. trachomatis and *L. monocytogenes* are two important zoonotic pathogens (Low and Donachie, 1997 and Baud et al. 2008). *C. trachomatis* causes about 40% to approximately 50% non gonococcal urethritis in men, epididymitis, mucopurulent cervicitis, proctitis, urethritis, endometritis, salpingitis, endemic trachoma, inclusion conjunctivitis, newborn pneumonia, later post-partum endometritis, pelvic inflammatory diseases in women and cervical pains. It can also cause post gonococcal urethritis. The pelvic inflammatory diseases often result in infertility and ectopic pregnancy.

The epidemiological situation of ovine chlamydial and listerial infections in continental Africa, especially Egypt is poorly characterized. Using the Menofia and Kafr Elshiekh as a model example, the chlamydial and listerial seroprevalence was estimated in four randomly selected sheep flocks with an average abortion rate lower than 5%.

A total of 82 samples from 4 flocks in two Egyptian governorates (El Menofia and Kafr El-Sheikh) were examined by ELISA for detection of antibodies of *C. trachomatis* and *Listeria monocytogenes*. The recorded result in Tables 1 and 2 revealed that 98.78% and 100% of the tested sheep samples were serologically positive with ongoing infection for *C. trachomatis* and *L. monocytogenes*, respectively although the elevated abortion rates was absent in the examined sheep flocks. Moreover, the results were nearly similar in the two governorates for *C. trachomatis* and *L. monocytogenes* **(**Tables 1 and 2). *C. trachomatis* was detected in 39 out of 40 (97.5%) samples in Menofia governorate with mean absorbance of positive sera of 1889.21±678.7 while it was detected in 42 out 42 (100%) samples in Kafr ElShiekh with mean absorbance of positive sera of 1767.81±804.13. On the other hand, *L. monocytogenes* was detected in 40 out of 40 (100%) samples in Menofia governorate with mean absorbance of positive sera of 7.61±2.67 while it was detected in 42 out 42 (100%) samples in Kafr ElShiekh with mean absorbance of positive sera of 5.71±2.51. Among several authors who studied the infectious causes of abortion in animals. Lenzko et al. (2011) reported that 94% of the tested sheep flocks were serologically positive with chalamydial infection in Germany. Moreover, Husu, (1990) and stated that *L. monocytogenes* was a ubiquitous organism, and regular exposure of animals and humans to this microorganism was very common. Furthermore, many healthy individuals were intestinal carriers (2–6%) and anti-*L. monocytogenes* serum antibody prevalence as high

as 53% have been reported in humans. Carriage rate for animals was similar to that of humans, with some differences depending on the species and a little higher rate during indoor season, as compared to animals on pasture (Iida et al. 1991).

On the other hand, Yildiz et al. (2009) determined seroprevalence of *Neospora caninum* in dairy cattle with higher abortion rates and co-existence of *N. caninum* with *L. monocytogenes* antibodies and indicated that *N. caninum* seroprevalence was 10.77% (60/557) while the co-existence rate of *N. caninum* seropositivity with *L. monocytogenes* was 42.85% (162/378). Moreover, AbdEl-Malek, et al (2010) examined 28 stool cultures from hospitalized children with diarrheal disease in Assiut Univ. hospital Egypt, 2 (7.14%) were found positive for *Listeria monocytogenes* using polymerase chain reaction (PCR).

Reproductive disease of sheep caused by *Chlamydia spp*. and *L. monocytogenes* has been primarily restricted to pregnant ewes because the organism was clearly identified as a major cause of infectious abortion. However, following pregnancy failure, a chronic chlamydial infection can become established in the reproductive tracts of experimentally infected animals. Persistent infection of the ewe's reproductive tract may eventually result in pathological lesions, similar to that observed in women infected with *C. trachomatis*, thus decreasing the breeding life of affected ewes (Papp and Shewen, 1997).

Infection within flocks of sheep in which chlamydial abortion has occurred may be maintained between lambing seasons by enteric spread from infected ewes to lambs, and subsequently between lambs, to give a persistent but mainly subclinical gut infection. Placental infection, when the ewes mature and become pregnant, might arise by the selection of variants with affinity for the placenta from the pre-existing enteric infection (Johnson et al. 1985).

The public must be aware of the possible risk to pregnant women in rural areas where chlamydial and listerial infections of farm animals are widespread. Women who are, or who may be, pregnant are potentially at risk of acquiring certain infectious diseases from pregnant livestock including sheep. Also, it is important to note that these risks are not only confined to the spring (when the majority of lambs are born), nor are the risks only associated with sheep. Cows and goats that have recently given birth can also carry similar infections (Low and Donachie, 1997 and Baud et al. 2008).

Infection with certain organisms can cause abortion in pregnant sheep, and if a pregnant woman becomes infected with these organisms they may harm her unborn child. The most important organisms are *C. trachomatis* which causes abortion of ewes, *Toxoplasma gondii, L. monocytogenes* and also Q fever which, although less likely to cause harm to the unborn child, may cause infection in the mother (Baud et al. 2008).

Such women should be advised against close contact with animals during their pregnancy and, especially, helping with sheep during the lambing season. If clinically similar cases occur medical attendants should recognize that confirmatory laboratory tests are available, including tests for ELISA to Chlamydia and listeria and, in cases of abortion, the simple microscopic examination of smears of the placenta stained with Giemsa (Low and Donachie, 1997 and Baud et al. 2008).

Based on the obtained results in the current study, it was clear that chlamydial and listerial infections occur frequently in Egyptian sheep flocks, even in the absence of elevated abortion rates.

The fact that *C. trachomatis* or *L. monocytogenes* are potentially zoonotic pathogens, raise questions about the significance of this reservoir for animal and human health and underline the necessity for regular monitoring. Further studies are needed to identify precisely the possible role of *C. trachomatis* or *L. monocytogenes* infections in woman abortion in Egypt.

5. ACKNOWLEDGEMENT

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