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Molecular Detection of *Chlamydia abortus* in Rabbits in the Egyptian Desert

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Abstract

Chlamydia abortus (C. abortus) is an obligate intracellular organism that undergoes a dimorphic life cycle within living cells. Little is known about *Chlamydia abortus* in rabbits. The objectives of this study were to detect *C. abortus* in domestic rabbits that were resident under desert conditions by quantitative species-specific real-time PCR (Polymerase Chain Reaction) Kit of C abortus 3-deoxy-D-manno-2-octulosonic acid transferase (kdtA) and by Giemsa staining. Samples were collected from 106 different samples from domestic desert rabbits including, North Sinai – Sahl El Tina (Coordinates: 31.0297°N 32.5658° E), South Sinai (Coordinates: 29.05°N 33.83°E), and the first point of Northwest Coast Amreya (Coordinates: 31.104538°N 29.766226°E), Egypt. Characteristic basophilic chlamydial inclusion bodies were detected in rabbit organs, In all feti and uteruses, 84.6% in intestines, 75% in the spleen, 69.2% in the lung, and 54.54% in the liver.Quantitative real-time PCR (qPCR) showed an exponential increase in *C. abortus* in organs and fecal samples. The significant incidence of *C. abortus* between organ and fecal samples in the Northwest Coast was assessed. *C. abortus* was detected in 88% of organs and 52% of fecal samples. On the other hand, *C. abortus* was recorded significantly among regions: 100% in North Sinai, 74.28% on the northwest coast, and 33.33% in South Sinai. Gabali Sinai and California were the most affected breeds. We concluded that the incidence of *C. abortus* became a reality in domestic rabbits under desert conditions. To our knowledge, this is considered the first molecular evidence of *C. abortus* in domestic desert Egyptian rabbits. Our results support the sustainable achievement in the newly reclaimed desert area of Sinai and the Northwest Coast. Attention should be given to newly disseminated Zoonotic and infectious microbes.

Keywords: *Chlamydia abortus;* Rabbit; Real-time PCR; Giemsa Stain; Desert; Northwest Coast; North Sinai; South Sinai; Zoonosis; Egypt.

Introduction

One of the most important green economies in Egypt is the rabbit industry. The breeding of domestic rabbits for human consumption has a long impact in Egypt; it is considered a promising support and great wealth to rural people.

There are over 300 breeds of rabbits that differ in size, coat color, length of ears, and type of fur. Rabbits are bred for assorted reasons, such as for laboratory animals and as a source of meat, wool, and fur, as well as for pets and exhibition animals [1]. Many breeds have been developed simply by selectively breeding for different physical characteristics. Despite the different breed names and the use of the word *hare* for some breeds, all are derived from *Oryctolagus cuniculus* [2]

Ninety percent of rabbit production is achieved by rural division [3]. However, disease is the main challenge for the rabbit industry. On the other hand, the rabbit industry is threatened by diseases [4].

The *Chlamydiaceae* family comprises a group of Gram-negative, obligate intracellular microorganisms that tend to infect the mucosal area, which can cause diseases in both humans and animals. *Chlamydiaceae* has two main forms: the reticulate body and elementary body forms, representing the stages of replication and infection [5]. Classifications of *Chlamydiaceae* are usually under consideration. Therefore, expansion in the family *Chlamydiaceae* has occurred in recent years. This includes 13 species belonging to the genus *Chlamydia*, namely, *C. trachomatis*, *C.*

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pneumoniae, *C. abortus*, *C. caviae*, *C. felis*, *C. muridarum*, *C. pecorum*, *C. psittaci*, *C. suis*, *C. avium*, *C. gallinacea*, *C. serpentis*, and *C. poikilothermis* [6].

Chlamydiosis is widely distributed around the world. It is important not only because of the economic losses in animal production but also because of the risks posed to humans. The family *Chlamydiaceae* exhibits a large variety of diseases in animals, birds, and humans, including abortion, pneumonia, gastroenteritis, encephalomyelitis conjunctivitis, arthritis, and sexually transmitted diseases. *C. abortus* often affects sheep, goats, and occasionally deer, cattle [7], farmed fur animals [8], and camels [9].*C. abortus is* of great zoonotic importance for women, especially those in contact with animal-infected tissue that remains infected with *C. abortus* [10]. The main *Chlamydiaceae* in the genital tract of cattle were *C. psittaci* and *C. abortus* [11]. Extra gestational *C. abortus* infection for women through pelvic inflammatory diseases was also recorded [12]. The animal diseases caused by *Chlamydiaceae* should be given more attention due to their zoonotic potential [13]. The obligate intracellular *Chlamydiae* has also been associated with cases of abortion in pigs, horses, guinea pigs, and mice [14].

Traditional staining is considered an especially important and valuable evaluation in the diagnosis of fastidious organisms, and it is the first step of diagnosis in modestly equipped laboratories [15].

Conventional and real-time polymerase chain reaction, DNA microarray-based detection techniques, and DNA sequencing are highly recommended for the identification of the genetic material of microbes because of their quick, sensitive, and specific diagnosis [16]. Reproducible and accurate quantitative real-time PCR is an essential tool in *Chlamydia* diagnosis [17]. PCR or Giemsa staining methods could be used to aid in the diagnosis of adult inclusion conjunctivitis caused by *Chlamydia trachomatis*[18].

Our previous studies [19] revealed the existence of *Chlamydiaceae* among desert ruminants on the northwest coast of Egypt. This motivated us to detect their effect in rabbit regions in the same places regarding the zoonotic impact on humans and other animals.

To date, there is no information about the molecular evidence of *C. abortus* in rabbits, either in agricultural or newly reclaimed desert regions among rabbit production divisions in Egypt. The present study aimed to detect *C. abortus* in rabbits by characteristic methods in some desert regions in Egypt.

Material and Methods

The Regions of the study were Amreya -King Marut and South Sinai (Ras-Sidr) belonged to the Desert Research Centre station in Egypt. North Sinai (Sahl-El Tina) rabbits were privately owned by individuals, fecal samples were collected from their rabbits during desert research center coverage's veterinary services for this region. Alexandia-road-Farm 1 rabbits were also privately owned by individuals. The samples were directly taken from the owners.

A total of 106 samples were collected from rabbits that were resident in the Northern-West Coast of Alexandria- Amreya -King Marut, North Sinai, and South Sinai in Egypt. (Fig.1). These samples were collected within the intervals between 2019 and 2020. The sample distribution in (Table 1) is as follows: Amreya included (52) organs from dead rabbits, 2 males and 11 females aged 4 to 5 months, and two adult aborted females. They have included: ten livers, 14 lungs, 11 spleens, 14 intestines, 2 feti, and 1 uterus. Ten fecal samples of 150 g each were also collected from Alexandria from the same station.

Ten fecal samples of 150 g each were collected from South Sinai (Ras Sedr). Six fecal samples of 150 g each were obtained from North Sinai (Sahl- El Tina). Alexandia-road-Farm1 includes 20 cervical swabs both after abortion and after birth, and 4 eye swabs were included.

Giemsa stain

Giemsa staining was performed according to Mittal et al. (1993); and Rohde et al. (1975) [20, 21], and impression smears were made from organs and cervical swabs. They were dried by air and left for fixation for 5 min. The fixed films were placed in an airtight staining jar containing freshly prepared diluted Giemsa stain. One volume of stock stain and 49 volumes of neutral distilled water "each slide was arranged in such a way to avoid precipitation of the stain". The jar was sealed and incubated overnight at 37°C. The slides were removed, washed thoroughly in distilled water, dried between blotting papers, differentiated in absolute alcohol, given a final rinse in distilled water, and allowed to dry. Stained slides were examined under a microscope using an oil immersion (100×) lens. Elementary bodies- stained reddish purple, and the initial bodies are basophilic, staining blue.

DNA extraction and real-time PCR

DNA extraction

Using a QIAamp DNA Mini Kit QIAGEN (cat. no.157055236, Germantown, USA), DNA was extracted from vaginal swabs, fecal samples, eye swabs, and positively stained infected organs after aseptic grinding as previously mentioned [6]. A

nanodrop spectrophotometer was used to measure the concentration and purity at an optical density of 260/280.

Detection of C. abortus by Real Time PCR

A Genesig® RealTime PCR Detection England. Kit predesigned and per-validated by Primerdesign Ltd, Southampton, UK .WWW.genesig.com. Path-*C.abortus*. 3-deoxy-D-manno-2-octulosonic acid transferase (kdtA) (JN213863-64646, Primerdesign, Southampton, UK) was used to quantify *C. abortus*, Kit include probe and *C. abortus* positive control. CFX Connectome Real-Time System was used to perform qPCR (cat. no. 1855200, Bio-Rad, Dubai, United Arab Emirates).

Oasig 2X qPCR Master Mix (serial number JN116454-36149, Primerdesign, Southampton, UK) was consistent with WWW.genesig.com. Analysis was carried out at the Biotechnology Lab at the Cairo University Research Park, Faculty of Agriculture, (CURP). Egypt.

A reaction mix was prepared for each DNA sample according to the detection Kit: Sufficient reactions for positive and negative controls were included. Final volume of 15 µl that 10µl qPCR Master Mix, 1µl of *C abortus* primer/probe mix, 1µl internal extraction control primer/probe mix, according to genesig Advanced kit handbook HB10.03.10 Primer design (2017) [22] 1µl RNase/DNase free water. 15µl of each mix was pipetted into individual wells after the qPCR experimental plate was set up. Five µl of DNA template of each sample pipetted into each. For negative control wells; 5µl was used of RNase/DNase free water. The final volume in each well was 20µl.

Chlamydia abortus Standard curve preparations

The standard curve was included for quantitative analysis parallel with tested samples; 90µl of template preparation buffer was pipetted into 5 tubes and labeled 2-6 2). Ten microliters (µl) of positive control template were added to tube 2. The mixture was then vortexed thoroughly. After changing the pipette tip, 10 µl from tube 2 was transferred to tube 3. This process of vortexing was repeated to complete the dilution series through to tube 6. Positive control copy numbers of standard curves were 2 x 10^5, 2 x 10^4, 2 x 10^3, 2 x 10^2, 20, and 2 per µl in tubes 1 to 6 respectively.

qPCR amplification protocol

Enzyme activation was 2 min at 95^{o}C, followed by 50 cycles of denaturation for 10 s at 95^{o}C, and data collection for 60 s at 60 ^{o}C. Fluorogenic data collection was at FAM channels at this stage.

Results interpretation

Positive quantitative results were calculated as copy numbers at Cq value ≥30.

Statistical analysis

ANOVA single factor was analyzed between means of positive percentage in Giemsa-stained organs. An automated Real-Time System did linear regression. ANOVA single factors were used to analyze the relation between real-time PCR positive samples.

Results

Giemsa stain

The presence of suspected chlamydial agents in diverse types of organs was examined by Giemsa staining as follows: 54.54% (6/11) of livers, 69.2% (9/13) of lungs, 84.6% (11/13) of intestines, 75% (9/12) of spleen, 100% (2/2) of feti, and 100% (1/1) of uterus. ANOVA single factors between means of positive percent organs were significant (*P value* =0.000151, *F*=34.83992, *F crit*=4.964603- F> F crit and P value≤0.05 significance) (Fig. 2,3).

Quantitative real-time PCR

The one hundred percent specificity of the *C. abortus* kit was determined according to the manual of the gensig kit. The increase in *C. abortus* genes was calculated automatically through linear regressions in all samples. *C. abortus* detection in Ras sidr fecal samples was 33.3% (3/9) with ranges Cq values (Cq=34, 36, 38), Sahle -El-Tina was 100% (6/6), ranged Cq values (Cq=33.06, 37.17) and Marute was 40% (4/10), ranged Cq values (Cq=33.16, 34.97, 36.07, 38.14).

On the other hand, *C. abortus* genes were detected in all Feti, uterus, livers, lungs, and spleens (However, (6/9) 66.7% positivity in rabbit intestines. Cq values ranged between 31.5 and 38.5 All cervical swabs were negative by qPCR.

ANOVA single factor revealed statistically significant differences between fecal and organ mean values from Alexandria, with a P value of 0.013882, as they were collected from the same station in King-Marut. Additionally, there were statistically significant differences between the mean values of *Chlamydia abortus* among the different regions in this study. The P value was 0.003412. (TABLE 2).

The prevalence of *C. abortus* was 88% in organs and 52% in fecal samples. (Fig. 5). On the other hand, *C. abortus* recorded 74.28% on the northwest coast, 33.33% in South Sinai, and 100% in North Sinai. (Fig. 6). The P value was Significance (0.015798).

Discussion

To date, little is known about *Chlamydia abortus* in rabbits. This is considered the first molecular evidence and incidence of *C. abortus* in rabbits. Our concern is about the vital role of infectious diseases through *C. abortus* in rabbits and their spread to the neighboring animals, which in turn constitutes a

danger to the environment and humans. It is known that the newly reclaimed desert areas must be prepared for any sudden change in the epidemiological map of this disease. Recent climate changes in desert areas should be taken into consideration by researchers. This may be achieved through rapid and accurate diagnosis of the disease, especially if it is a microbe of a specific nature, such as *C. abortus*.

For several years, the unavailability of commercially sensitive and species-specific tests for both humans and animals yielded the certainty for excessive diagnostic research to detect an important zoonotic pathogen, chlamydial infection because of their wide distribution and their infectious cycle. [23]

Recent reports established the importance of confirming the diagnosis of *C. abortus* depending on specific PCR-established testing. That is because it is a zoonotic impacts that cause severe infections during pregnancy and can be life-threatening to the mother and her fetus. [24]

In the present study, screening of *C. abortus* in vaginal swabs, fecal samples, and dead organs was carried out. Samples were collected from different rabbit fields that were housed under desert conditions. All dead rabbits were resident in Amria station and have recorded substantial positive inclusion bodies by the Giemsa stain technique.

The present study showed a significant relation between *C. abortus* incidence by qPCR in organs and fecal samples along the same region in the Amria-Alexandria Northwest coast, which should be taken into consideration. The same results were also observed strongly among the North, South Sinai, and Northwest coast regions, with an increased prevalence in North Sinai, followed by the Northwest coast, and South Sinai which had the lowest prevalence. Therefore, the climatic conditions and movements of the wind in the northern site of Egypt might have a role in the dissemination. [25-26]

The prevalence of *C. abortus* was increased in organs compared with fecal samples, supporting the idea of an increased incidence of intracellular *Chlamydia* sp. in the internal organs of diseased animals. These results correspond with the multifactorial process of *Chlamydia sp.* infection that can be divided into distinct stages. Adhesion of the infectious form of the elementary body (EB) to the host cell is a crucial step in the process of infection. [27]

Some breed types of rabbits (New Zealand, Papion, Dutch Hollander, V-line, and Flander), may have a role in obviously, negative Giemsa staining and quantitative real-time PCR in cervical and eye swabs in Alexandria –road Farm1. Compared with California rabbits on the Northwest coast and Sinai Gabali rabbits, this situation revealed markedly C. abortus abundant genes. These findings are congruent with several studies supporting that the highest body mass and weight gain is characterized by the Vesselina breed however the lowest in the Californian breed's animals. [28].

A need for additional investigations considering that environmental transmission poses a major risk to sensitive patients, including pregnant women and people with impaired immune systems, as well as other animals, including wildlife [29].

C. abortus is considered a neglected disease, little information about its role in rabbit disease, and the upgrowing chlamydial classifications may cause great interference in its diagnosis. These findings met with Nowland et al. [2], who demonstrated that chlamydiosis is uncommon in rabbits and that *C. psittaci* is considered an etiologic agent in domestic birds.[30] There was some limited information on the reported presence of *Chlamydia* in rabbits in China, the seroprevalence of *Chlamydia* species in domestic rabbits was discussed [31]. Our findings are consistent with the OIE guidelines [32] and public health.

Conclusion

We concluded that the incidence of *C. abortus* became real among desert regions, although *C. abortus* was considered a neglected microbe in domestic rabbits. Our present study strongly supports the potential role of *C. abortus* in the occurrence of some diseases that could be under consideration. *C. abortus* could be included with pathogens that endangered rabbit production. Rabbits might have a significant zoonotic impact on *C. abortus* spread. More upcoming studies are our goals for this microbe.

Author's contributions

Sahar A. Allam is the owner of the research idea, sampling collection, staining technique, and real-time PCR. Writing the draft and final manuscript, statistical analysis, and assessment of the results and discussion.

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Funding statement

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Declaration of Conflict of Interest

The author declares that there is no conflict of interest.

Ethical of approval

This study follows the ethics guidelines of the Research Ethics Committee Desert Research Center, Egypt, and complied with relevant Egyptian legislation (Approval No. AH/NO. 2019- 8-5).

TABLE 1. Distribution of the rabbit samples under study

Region	Breed	Sample type	Number of samples	symptoms	Age	Total
Northwest coast Egypt Amreya--Alexandria	California	liver	11	Collected from dead rabbits by diarrhea, and pneumonia	4-5 months (2male-11female)	52
		lung	13			
		Spleen	12			
		intestine	13			
		Feti	2	Abortion	age>a year -Adult	
		uterus	1	Abortion and dead		
		Fecal-samples	10 (150gm-each)	Apparent healthy	Adult female	
Alexandria-road-Farm1	Two NewZealand -Two Papion - Pne Dutch Hollander -One V-line - Four Flander	10-cervical swabs	20 cervical Swabs +4 eye Swabs	Birth, Abortion	adult-females 4,5,5.5,6, 7and 8 months	28
	Ten New Zealand	4-eye swabs, 10 cervical swabs		Apparent healthy	adult-females	
North Sinai-Sahl El Tina	Gabali	Fecal samples	6 (150gm-each)	Apparent healthy	adult-females	16
South Sinai-Ras Sedr	Gabali	Fecal samples	10 (150gm -each)	Apparent healthy	adult-females	
Total			106			

TABLE 2. Detection of *Chlamydia abortus* by real-time PCR in domestic rabbits under desert conditions

*Region	Sample	Total Number	Positive	% Positive
Ras -Sedr	Feces	9	3	33.3%
Sahl –El Tina	Feces	6	6	100%
***Marut**	Feces	10	4	40%
	Feti	2	2	100%
	Uterus	1	1	100%
	Liver	3	3	100%
	Lung	6	6	100%
	Spleen	4	4	100%
	Intestine	9	6	66.7%
Alexandria-road-Farm1	Cervical swab	20	0	0%
	Eye swab	4	0	0%

*ANOVA single factor; F> F crit and P value≤0.05 significance.
*statistical significance between positive values of feces and organs in Marut (P value =0.013882, *F*=13.75029, *F crit*=6.607891).
*statistical significance between positive values among regions (P value =0.003412, F=16.92684, F crit=5.143253).

Fig. 1. Egypt Google Map shows some desert areas under study. The first point of Northwest Coast Amreya (Coordinates: 31.104538°N 29.766226°E), North Sinai – Sahl El Tina (Coordinates: 31.0297°N 32.5658° E, and South Sinai (Coordinates: 29.05°N 33.83°E), Egypt.

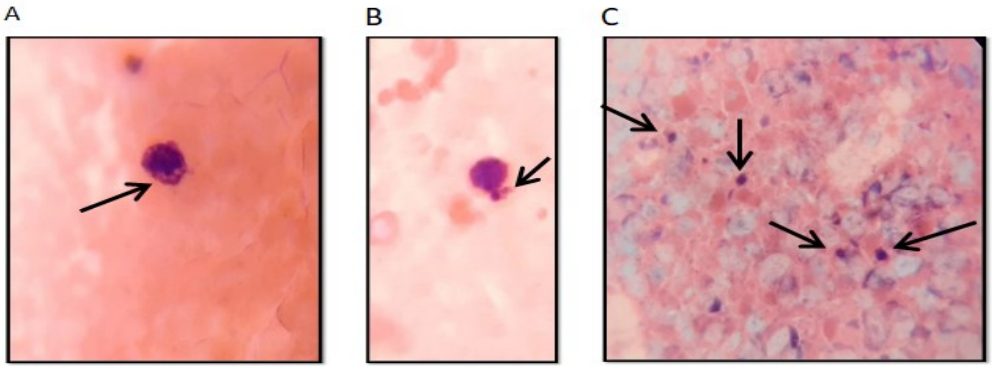

Fig. 2. Basophilic purple color of suspected chlamydial inclusion bodies under oil immersion lens (100×) in rabbit organs. A (lung); B (liver), and C (intestine).

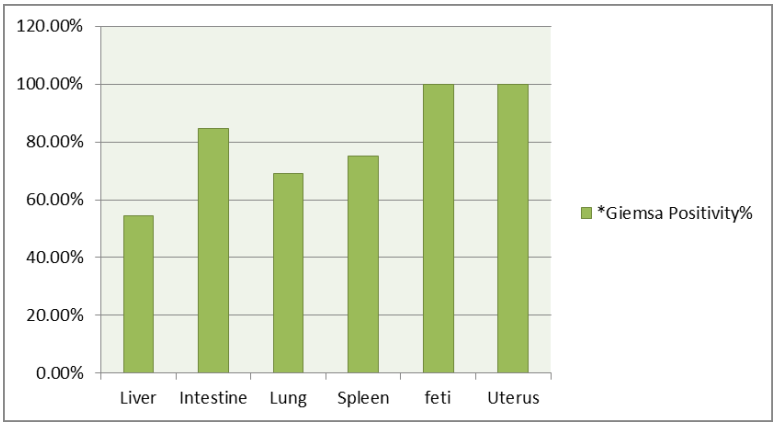

Fig. 3. Positivity of suspected *Chlamydial* agent *by* Giemsa's stain investigation in organs of dead rabbits

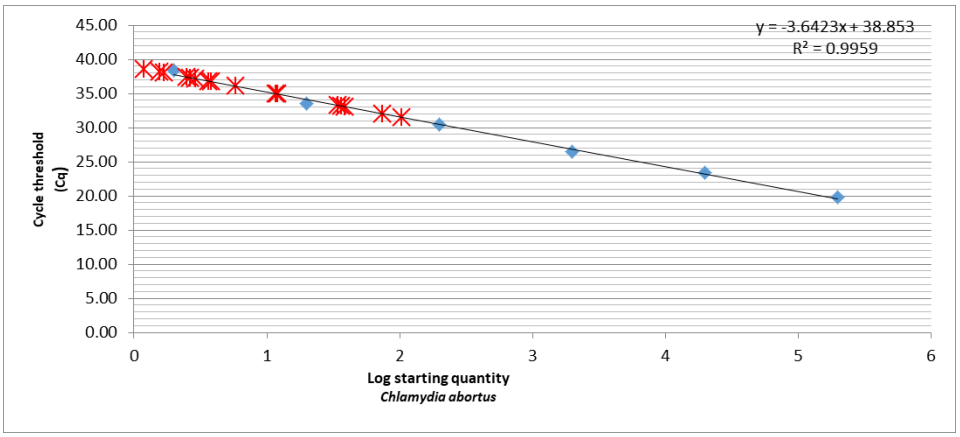

Fig. 4. Standard curve of *Chlamydia abortus* positive control and samples. The cycle threshold (Cq) values concerning the concentration of initial target gene copies obtained by a serial 10-fold dilution of positive control (Kit included) containing 2×10^5, 2×10^4, 2×10^3, $2\times10^2$and 20 copies, and 2 copies/µl of the target gene. The Cq values are inversely proportional to the initial number of the target gene in the sample. The number of copies of the target gene was calculated automatically by Bio-Rad,
Slope= - 3.64, Y-intercept= 38.853, R^2=0.9959. y = -3.6423x + 38.853. Efficiency= 89.1%

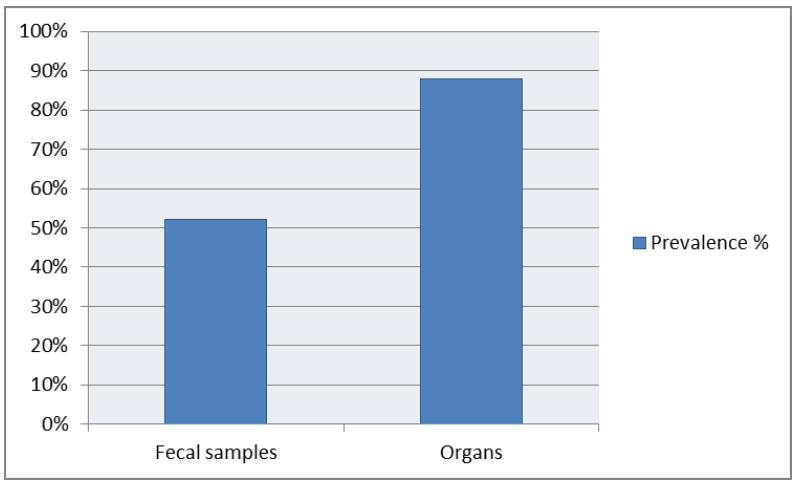

Fig. 5. Prevalence of *Chlamydia abortus* in fecal and organ samples of domestic rabbits under desert conditions

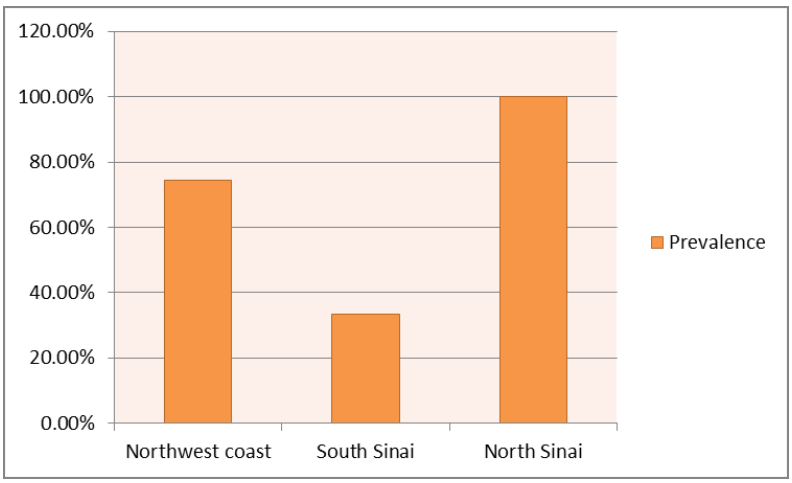

Fig. 6. Prevalence of *Chlamydia abortus* in rabbits in some desert regions in Egypt

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الكشف الجزيئي عن الكلاميديا الاجهاضيه في الأرانب في الصحراء المصرية

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الملخص

الكلاميديا الاجهاضية هى كائن حي ملزم داخل الخلايا تخضع لدورة حياة ثنائية الشكل داخل الخلايا الحية. لا يعرف الكثير عن الكلاميديا الاجهاضية في الأرانب. تتمثل أهداف هذه الدراسة في الكشف عن الكلاميديا الاجهاضية في الأرانب المستأنثة تحت الظروف الصحراوية بواسطة مجموعة تفاعل البلمرة المتسلسل اللحظى الكمى (3-deoxy-D-manno-2-octulosonic acid transferase (kdtA) ، وبواسطة صبغة الجيمسا. تم جمع العينات من 106 عينة مختلفة من الأرانب المستانسة تحت الظروف الصحراوية بما في ذلك شمال سيناء - سهل الطينة (إحداثيات: 31.0297 درجة شمالاً 32.5658 درجة شرقًا) وجنوب سيناء (إحداثيات: 29.05 درجة شمالاً 33.83 درجة شرقًا) والنقطة الأولى من الساحل الشمالي الغربي العامرية (إحداثيات: 31.104538 درجة شمالاً 29.766226 درجة شرقًا) مصر. تم الكشف عن أجسام متضمنة من الكلاميديا القاعدية الصبغه المميزة في أعضاء الأرانب، في جميع الأجنة والرحم، 84.6٪ في الأمعاء، 75٪ في الطحال، 69.2٪ في الرئة، و 54.54٪ في الكبد. أظهر تفاعل البلمرة المتسلسل اللحظى الكمي زيادة في للكلاميديا الاجهاضيه داخل الأعضاء الداخلية وعينات البراز. كان يوجد فروق احصائية ذات دلاله معنوية بين وجود الكلاميديا الاجهاضية فى عينات الأعضاء الداخلية والبراز في منطقة الساحل الشمال الغربي. تم الكشف عن الكلاميديا الاجهاضية لتسود بنسبة 88 ٪ من الأعضاء الداخلية و 52 ٪ من عينات البراز.و من ناحية أخرى، كانت الايجابية الكلية للمناطق كالتالى: 100٪ في شمال سيناء، و 74.28٪ في الساحل الشمالي الغربي، و 33.33٪ في جنوب سيناء. كانت سلالة الارانب الجبلى وكاليفورنيا أكثر السلالات تضررا. تم استنتاج انتشار الكلاميديا الاجهاضية أصبح حقيقة واقعة في الأرانب المستانسة تحت الظروف الصحراوية. على حد علمنا ، يعتبر هذا أول دليل جزيئي على الكلاميديا الاجهاضيه في الأرانب المصرية الصحراوية. تدعم نتائجنا التنمية المستدامه في المنطقة الصحراوية المستصلحة حديثا في سيناء والساحل الشمالي الغربي. وينبغي إيلاء الاهتمام للميكروبات الحيوانية المنشا و المعدية المنتشرة حديثا.

الكلمات الدالة: الكلاميديا الاجهاضية ، الأرانب ، تفاعل البلمرة المتسلسل اللحظى الكمى ، صبغة الجيمسا ، صحراء، شمال سيناء، جنوب سيناء، الساحل الشمالى الغربى، الأمراض الحيوانية المنشأ، مصر.