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Chlamydophila abortus Vaccine Study and Disease Surveillance on Palestinian Farms in the Bethlehem Region

By

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***Chlamydophila abortus* Vaccine Study and Disease Surveillance on Palestinian Farms in the Bethlehem Region**

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Mohammad Yousef Izzat Manasrah

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"*Chlamydophila abortus* Vaccine Study and Disease Surveillance on Palestinian Farms in the Bethlehem Region"

by (Mohammad Yousef Izzat Manasrah)

ABSTRACT

Ovine enzootic abortion (OEA) in sheep and goats is common in Palestine and causes losses to farming communities worldwide. OEA is caused by *Chlamydophila abortus*, and can be controlled by vaccination and good farm management practices. Vaccination campaigns have been conducted in Palestine, and yet some farmers and veterinarians expressed dissatisfaction with the poor results.

The aim of this study was to assess the safety, suitability and efficacy of the vaccine itself, by conducting a case-control study of vaccination on 5 farms in the Bethlehem region of the West Bank.

In addition, the farm management practices on 20 farms suffering abortions in the Bethlehem region were assessed using a questionnaire designed for this study. The responses were cross-referenced to diagnostic results for infection by *Chlamydophila abortus* and other relevant organisms.

Indirect evidence for the vaccine's ability to protect against infection comes from the survey, which revealed that abortions due to *Chlamydophila abortus* occurred with a frequency of 53% on 15 un-vaccinated farms, while no *Chlamydophila abortus* was diagnosed on 5 previously vaccinated farms. Furthermore, in the vaccine trial, no abortions occurred on vaccinated farms, while abortions were widespread on an infected control farm. This correlation between previous vaccination and lack of *Chlamydophila abortus* also provides evidence for the safety of the vaccine.

Direct evidence for the effectiveness of the vaccine comes from the vaccine trial: 41% of animals became seropositive for antibodies against the immunogenic protein Momp by the end of the trial. Interestingly, 'Baladi' sheep gave the poorest responses of four breeds of sheep and goats studied.

The farm management survey revealed that *Chlamydophila abortus* was more common on farms with poor farm management practices, such as sharing of animals and pastures and the presence of dogs and cats.

Sequences of two local strains of Chlamydophila abortus were closely related to world reference strains and a vaccine strain used in Palestine.

It is concluded that the vaccine is suitable for use in Palestine and has moderately good efficacy. There is no evidence of it causing abortions. Farm management practices may prove to be as important as the vaccine itself.

دراسة على لقاح بكتيريا *Chlamydophila abortus* ومراقبة المرض في المَزارع الفلسطينية في منطقة بيت لحم .

محمد يوسف عزات مناصرة

مُلخَّص

الإجهاض المستوطن في الأغنام هو مرض منتشر في فلسطين يصيب الضأن والماعز ويسبب خسائر في المجتمعات الزراعية على مستوى العالم. مسبب هذا المرض هي بكتيريا تسمى (كلاميدوفيلا أبورتس – *Chlamydophila abortus*)، يمكن السيطرة عليها من خلال التطعيم وتطبيق الإجراءات السليمة في تربية الحيوانات. وقد تم إجراء عدة حملات للتطعيم ضد هذا المرض في فلسطين، إلا أن بعض الأطباء البيطريين ومربيي الحيوانات أظهروا عدم رضاهم عن نتائج التطعيم.

الهدف من هذه الدراسة هو تقييم فعالية اللقاح وملائمته ودرجة الأمان لدى استخدامه، من خلال إجراء دراسة مقارنة للتطعيم في خمس مزارع للأغنام في منطقة بيت لحم في الضفة الغربية. بالإضافة إلى ذلك، تم تقييم إجراءات التربية والإدارة في عشرين مزرعة تعاني الإجهاض في منطقة بيت لحم باستخدام استبيان صُمِمَ لهذه الدراسة، وتمت الإشارة إلى نتائج هذا الاستبيان وربطها بالنتائج التشخيصية للإصابة بالعدوى البكتيرية *Chlamydophila abortus* في مرضات أخرى.

وفّرَ المسح المرضي المُطبق دليلاً غير مباشر على قدرة اللقاح المستخدم على الحماية من الإصابة بهذه العدوى، حيث تبين أن بكتيريا *Chlamydophila abortus* مسؤولة عن ما نسبته ٥٣% من الاجهاضات في خمس عشرة مزرعة غير مطعمة لهذا المرض، بينما لم يتم تشخيص هذه الإصابة في خمس مزارع تم تطعيمها سابقا. بالإضافة إلى ذلك، لم تُسجّل أي حالة إجهاض في أربع مزارع تم تطعيمها ضمن دراسة مقارنة التطعيم بينما كانت الإجهاضات منتشرة في المزرعة الخامسة المصابة المستخدمة للمقارنة يعطي الارتباط بين التطعيم المُسبق والخلو من هذه البكتيريا دليلاً على درجة الأمان الجيدة لهذا اللقاح.

تقدم تجربة التطعيم إثباتاً مباشراً على فعالية اللقاح الجيدة، حيث أن ما نسبته ١٠٠% من الحيوانات المُطعّمَة أصبحت موجبة مصلياً للأجسام المُضادة للبروتين المناعي (Momp) عند نهاية التجربة. ومن الملفت للانتباه أن سلالة الضأن البلدي أظهرت أضعف استجابة مناعية بين السلالات الأربع التي تم دراستها.

كشف المسح أن الإصابة بهذه البكتيريا كانت أكثر انتشاراً في المزارع ذات الإدارة السيئة مثل التشارك في الحيوانات وأماكن الرعي.

أظهر التسلسل الجزئي للحمض النووي المأخوذ من سلالتين محليتين من بكتيريا *Chlamydophila abortus* ارتباطهما الجيد ببعضها و بسلالة لقاح آخر مُستخدم في فلسطين. يُستنتج أن هذا اللقاح مناسب للاستخدام في فلسطين وأن له فعالية جيدة، وأن لا دليل بأن هذا اللقاح قد يسبب الإجهاض، وأن إجراءات إدارة مزارع الحيوانات قد تكون بمثابة أهمية إعطاء اللقاح.

DECLARATION

I declare that the Master Thesis entitled "dissertation title" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: _____" Mohammad Yousef Izzat Manasrah_"___

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Dedication

I dedicate this work honestly, with what it included of efforts, tiredness, concerns, and creations to all those whom have right on me:

The nearest people and the most deserving for my loyalty, whom directed me to this way, my father and my mother.

My partner in my life, my brother Mohanad.

The honest source of great support and encouragement, my wife.

The new gladness in my life, my daughter Baylasan.

The whole family, my brothers and my sisters.

I give this work sincerely to the people and idea whom enabled my personality to be as like, and also to the mother whom was a victim to her impious sons before her enemies - Palestine.

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Finally, I won't forget to thank indeed my veterinarian colleagues and animal owners who worked with me and helped me in collecting samples for this study.

Abbreviations

BLAST	Basic Local Alignment Search Tool
BRC	Biotechnology Research Center
CVL	Central Veterinary Laboratory
EAE	Enzootic Abortion of Ewes
EB	Elementary Body
ELISA	Enzyme Linked Immuno Sorbent Assay
F	Fetus
IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
LPS	lipopolysaccharide
MEGA	Molecular Evolutionary Genetics Analysis
MOMP	Major Outer Membrane Protein
NCBI	National Center of Biotechnology Information
OEA	Ovine Enzootic Abortion
P	Placenta
PCR	Polymerase Chain Reaction
POMP	Putative Outer Membrane Protein
RB	Reticular Body
S/P	Sample/Positive
SC	Subcutaneous
VS	Vaginal Swab

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Chapter 1

1. Introduction

1.1 The Disease

Ovine enzootic abortion (OEA), enzootic abortion of ewes (EAE), or ovine Chlamydiosis all refer to the same disease, which is caused by a bacterium called *Chlamydophila abortus*. This disease causes various reproductive failures in sheep and goats such as: abortion (expulsion of the fetus prior to the normal end of pregnancy) mainly late in the last 2-3 weeks of pregnancy, dead lambs, premature births, still births (live for not more than 48 h) and the birth of weak lambs with low birth-weight. Furthermore, *Chlamydophila abortus* can cause mummification and maceration in sheep and goats, in addition to resorption of fetuses in sheep flocks (Givens and Marely, 2008).

Worldwide, this disease is responsible for large economic losses through the wastage and loss of lambs and kids, and also through lost milk production. In the UK, for example, it was estimated that about 20-23.8 million pounds sterling were lost each year through OEA (Longbottom et al., 2002a, Trickett, 2013).

This enzootic disease is very resilient and difficult to control with its periodic recurrence in the flock and the longevity of the disease-causing organism in farms and host animal bodies, and is endemic to large parts of the world (Maley et al., 2008, Borel, 2008). This disease is commonly characterized with "storms of abortions" that are cyclical every three or four years within an infected flock (Carter and Wise, 2003).

1.2. Chlamdiaceae Family and Taxonomy

OEA is caused by *Chlamydophila abortus* which is a non-motile, gram negative, pleomorphic, obligate intracellular bacterium of the family *Chlamydiaceae*. This bacterium has a unique life cycle inside the host cells in two different forms: non infective reticular body (RB) and infective elementary body (EB) (Litwin, 1959, Moulder, 1991).

Members of the family *Chlamydiaceae* are responsible for a broad range of illnesses that affect human and animal health such as: abortion, encephalomyelitis, pneumonia, conjunctivitis, arthritis, mastitis, gastroenteritis, respiratory infections, trachoma, psittacosis, metritis and sexually transmitted diseases (Longbottom and Coulter, 2003, Longbottom et al., 2002b).

Formerly, the Family *Chlamydiaceae* had only one genus called *Chlamydia* that contained four species: *Chlamydia trachomatis, Chlamydia psitassi, Chlamydia pneumoniae, and Chlamydia pecorum* (Borel, 2008).

The extensive application of 16S and 23S rRNA sequencing generated many difficult anomalies that led to debate about the merits of the former taxonomic system (Everett and Andersen, 1999) until the family *Chlamydiaceae* was redefined by splitting it into two genera: *Chlamydia* and *Chlamydophila*, which were divided into nine species (Everett and Andersen, 1999, Everett, 2000) as listed below.

The genus *Chlamydia* comprises currently:

- *Chlamydia trachomatis*: occurs in humans and occasionally in koala bears and has many serovars, which cause trachoma, pneumonia, arthritis, inclusion conjunctivitis in neonatals, lymphogranuloma venereum, proctitis, and other genitourinary tract infections.
- *Chlamydia suis* (formerly porcine *Chlamydia trachomatis*): causes enteritis, conjunctivitis, and pneumonia in swine.
- *Chlamydia muridarum* (formerly *Chlamydia trachomatis* of mice): causes respiratory infections in mouse, guinea pig, and hamster.

Whereas *Chlamydophila* comprises:

- *Chlamydophila psittaci*: causes human psittacosis, avian Chlamydiosis, and abortions in bovine
- *Chlamydophila abortus* (formerly *Chlamydia psittaci* serotype 1): causes ovine enzootic abortion in sheep, and Chlamydial abortions in cattle, pigs, and goats.

- *Chlamydophila caviae* (formerly *Chlamydia psittaci* guinea pig strains): causes inclusion conjunctivitis in guinea pigs.
- *Chlamydophila felis* (formerly *chlamydia psittaci,* feline strains): causes conjunctivitis or feline pneumonitis in cats.
- *Chlamydophila pneumonia* (formerly *Chlamydia pneumonia*): causes respiratory infections in humans, horses, koalas, amphibians, and reptiles.
- *Chlamydophila pecorum* (formerly *Chlamydia pecorum*): occurs in sheep, goats, cattle, and pigs and causes pneumonia, diarrhea, enteritis, arthritis, infectious polyarthritis, conjunctivitis, abortions, and sporadic bovine encephalitis (CFSPH and IICAB, 2005).

1.3. Host Range of *Chlamydophila abortus*

This study is concerned with *Chlamydophila abortus*, which has a wide variety of host species, but is most commonly associated with sheep and goats. In addition to sheep and goats it is less commonly found in cattle (Wang et al., 2001, Borel, 2008) as well as pigs, horses, deer, llamas and may infect pregnant women (human). The isolation of *Chlamydophila abortus* from rabbits, guinea pigs, mice, green sea turtles, and snakes has not been correlated with evidence of disease in these organisms (CFSPH and IICAB, 2005).

1.4. Disease Prevalence

Chlamydophila abortus is considered the main cause of reproductive losses in sheep and goat at the global level, with the exception of Australia and New Zealand that are free of disease. In Northern Europe OEA is the major cause of infectious abortions (Stuen and Longbottom, 2011). In the UK, for example, OEA accounted for 44% of diagnosed abortions due to infectious agents between the years 1995 - 2008 (Stuen and Longbottom, 2011, Moredun Research Institute, 2010). Moreover, more than 56% of small ruminant abortions in Spain were caused by this disease (Esnal et al.,

2010), while in Turkey 46.6% of the examined aborting flocks of ewes and dairy cattle were infected with *Chlamydophila abortus* (Gokce et al., 2007). Tunisia is another country that has significant infections with 58% of abortions being associated with *Chlamydophila abortus* (Rekikia et al., 2002) while a previous study reported similarly that this disease was responsible for 60% of abortions (Dlissi et al., 1998). A closer example is Jordan, where a study of 56 flocks (36 Awwasi sheep and 20 local goat flocks) showed that all were positive for *Chlamydophila abortus*, in which seroprevalence for 2705 animals was done by CFT on unvaccinated farms with abortions and stillbirths (Al-Qudah et al., 2004).

In Palestine, there are no previous studies published on *Chlamydophila abortus* prevalence.

1.5. Infection and transmission

The sources of infection and the pathways of transmission are listed below and summarized in Figure 1.1:

1. Chlamydophila abortus can be found in feces, urine, uterine discharges, and less commonly in the milk of mothers that have suffered from abortion. Sheep and goats can also be asymptomatic chronic carriers (CFSPH and IICAB, 2005).
2. Ingestion of infectious agents through feed or water that has been contaminated with abortion materials, uterine discharges, and feces is a known route of infection (Merck Veterinary Manual 9th Edition, 2005).
3. Inhalation of contaminated dust, aerosols or droplets. (CFSPH and IICAB, 2005, Carter and Wise, 2003).
4. Mechanical transmission or venereal transmission of infectious agent in both directions during copulation between males and females (Merck Veterinary Manual 9th Edition, 2005, CFSPH and IICAB, 2005, Livingstone et al., 2008).
5. If rams develop orchitis, their semen becomes infectious during mating (Mearns, 2007).

6. By vertical transmission from mother to lamb or kid. Newborns acquire the infection from their carrier mothers in two ways: either during passage through the birth canal, in which case abortion is likely after maturity in the next year (second gestation) (Pelzer, 2012, Givens and Marely, 2008), or by infection congenitally in-utero, in which case it will abort at its first gestation (Merck Veterinary Manual 9th Edition, 2005).
7. Aborting ewes shed *Chlamydophila abortus* one day before abortion and up to 2-3 weeks after abortion in vaginal secretions, in addition to only 3-4 days before and after ovulation (during cervix opening in estrus). Shedding in goats may start more than 2 weeks before abortion. Shedding in the reproductive secretions of sheep and goats may last for more than 2-3 years in some animals. (Merck Veterinary Manual 9th Edition, 2005, CFSPH and IICAB, 2005).

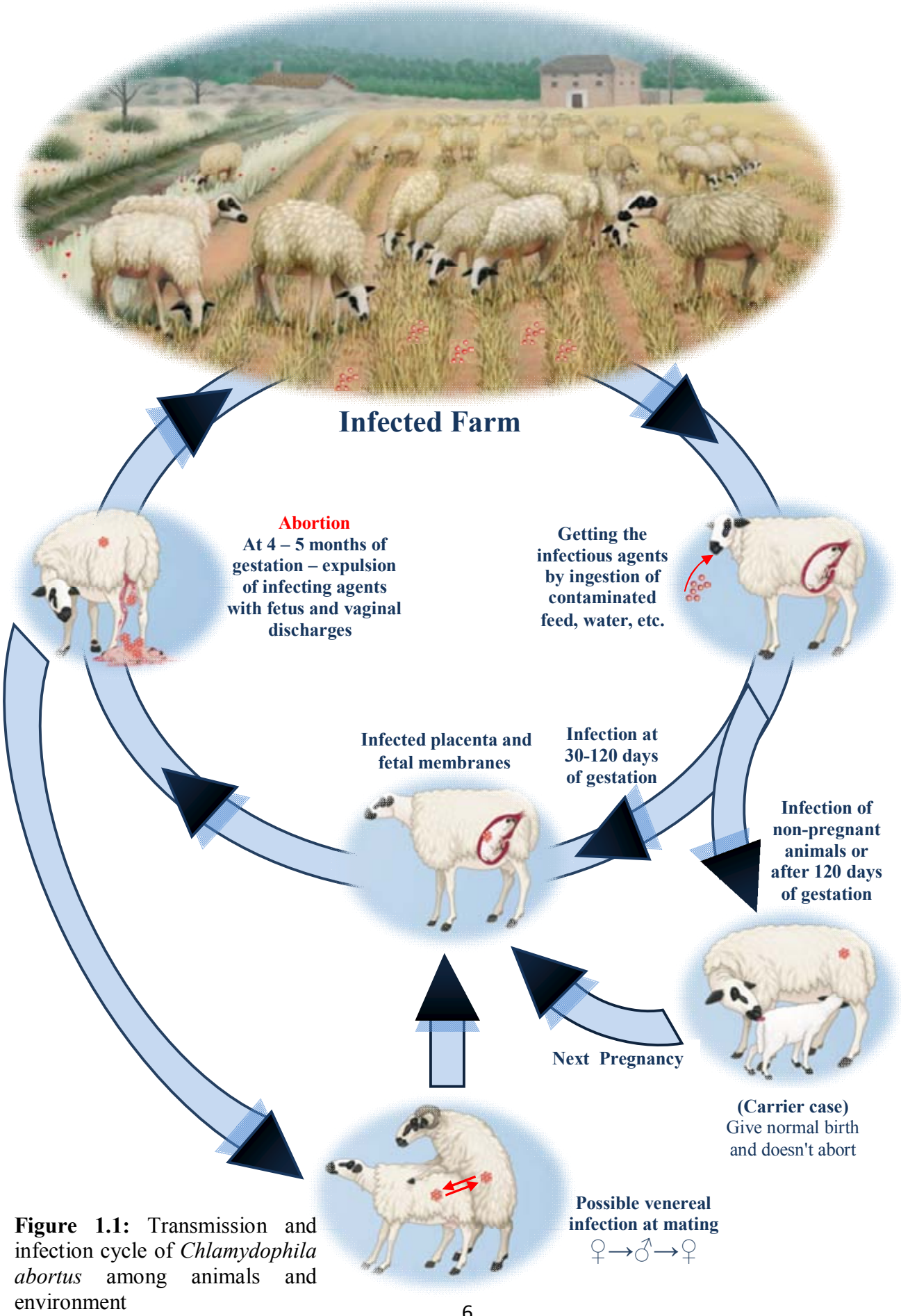

Figure 1.1: Transmission and infection cycle of *Chlamydophila abortus* among animals and environment
(HIPRA Laboratories, 2012a).

1.6. Development and life cycle

Following infection, highly infectious elementary bodies (EB), which are spherical in shape and, about 0.25µm in diameter, enter and localize in the mucosal epithelium or phagocytic cells of the respiratory, gastrointestinal, or genital tract according to the portal of entrance. This bacterium passes through four phases within the host body (Carter and Wise, 2003):

Phase one: considered a dormant phase due to very low metabolic activity in this stage, it describes the elementary bodies (EB) that have adhered to the new host before being engulfed by host cells (especially trophoblasts), as well as during departure from the host cells and release of elementary bodies.

Phase two: once the EBs are phagocytozed by host cells, their metabolic activity is induced within. 12 - 24 hours, and individual membrane-limited inclusions called endosomal vacuoles are formed.

Phase Three: 24-48 hours later, nucleoleoid dispersion occurs and EBs transform into larger, non-infectious reticulate bodies (RB) (about 0.5-0.6µm in diameter) as a result of dispersion. This step is followed by replication and binary fission of RBs 10-15 hours later within their membrane-limited inclusions.

Phase Four: with replication complete, the mature RBs deteriorate back into infectious EBs 20-30 hours after the last replication. After a further 20-30 hours, cell lysis occurs and new highly infectious EBs are released to start a new cycle and infect new host cells.

The elementary bodies that are released after phase 4 may be numbered in the millions and are re-shed in uterine secretions, milk, urine, and also feces in small numbers in case of asymptomatic infection, and in high density in case of symptomatic infection (CFSPH and IICAB, 2005). EBs of *Chlamydophila abortus* can survive and remain infectious for a matter of days during temperate spring weather, but may survive and remain infectious in the environment for several months at or near freezing temperatures (CFSPH and IICAB, 2005, Borel, 2008). Elementary Bodies of Chlamydia can survive in soil and feces for long periods (Carter and Wise, 2003), which guarantees

continuous existence and spreading of pathogen in the environment and among animals and flocks (Lenzko et al., 2011).

1.7. Pathogenesis

Incubation periods can be variable, but abortion usually occurs 5-6 weeks after the infection (Pelzer, 2012). Susceptible animals (sheep and goat) can become infected with *Chlamydophila abortus* at any time during gestation or even before, which results in different outcomes. If the dam was pregnant 30 -120 days at the time of infection, it may abort during this pregnancy, while if not pregnant or the pregnancy had proceeded beyond 120 days at the time of infection, abortion is likely to occur during the subsequent pregnancy (Pelzer, 2012), since the bacterium enters into a latent phase in the first case, (See Figure 1.1, which illustrates transmission and infection pathways of *Chlamydophila abortus*).

Other work suggests that if the pregnant dam become infected after 110 days of gestation (within the last 5 weeks of pregnancy) it is expected to abort in the subsequent but not the current pregnancy (Aitken and Longbottom, 2007).

Chlamydophila abortus spreads within the host through hematogenesis (Givens and Marely, 2008) and prefers to localize in the placenta and more specifically in the trophoblast cells of the fetal chorionic epithelium, and thereafter spreads to the surrounding intercotyledonary membranes and causes characteristic necrotic signs and thick placental lesions, which damage the placenta and cause inflammation that leads to acute placentitis (Aitken, 1993, Buxton et al., 2002). *Chlamydophila abortus* can be detected by PCR, which indicates that EB replication has occurred, from placental tissue taken around 90-95 days into a pregnancy but not before (Buxton et al., 1990b). Chlamydial infection can also end with latency, but can be activated again in stress conditions or in simultaneous infection. In the case of multiplicity of antigenic stimulation, the result will be chronic inflammation in the host tissues, which is mediated by the interlukin-1 and is stimulated by (LPS) that mediates inflammations and scarring (Carter and Wise, 2003).

Virulence factors of pathogenesis:

Virulence is defined as the ability of microorganism or pathogen (bacteria, virus, parasite, etc) to cause a disease in the infected host, where virulence factors are those molecules that are secreted or expressed by the pathogen itself enabling attachment, entrance into its cells, inhibition of host immunity, etc. and play a role in causing disease (Medical Dictionary, 2007) Three main virulence factors are known to play major roles in the pathogenesis of *Chlamydophila abortus* and are described below (Carter and Wise, 2003):

- The lipopolysaccharide genus-specific antigen or complement fixation antigen, is thought to be a virulence factor and to encourage inflammatory reactions in the host, in addition to undermining and escaping from the host defense system.
- The highly conserved Chlamydial protease/or proteasome-like activity factor (CPAF), which enables Chlamydia to escape recognition by T cells through diminishing the presence of host transcription factors that are associated with the production of major histocompatability complex (MHC), consequently the interactions between immune cells (leukocytes and WBCs) mediated by (MHC) are disconnected, and so to give the Chlamydia another chance to live and replicate.
- Type III secretion apparatus, which opens up a hole in the vacuole membrane to facilitate conveyance of pathogenic products into the cytosol of the host cell. An example is the second virulence factor (CPAF), which is thought to be transmitted by this way.

1.8. Antigenic Nature

The main pathogenic antigens in *Chlamydophila abortus* are:

1. Group specific heat stable libopolysaccharide (LPS): common to all members of *Chlamydiaceae* family and associated with cell wall structure. It is genus-specific antigen and used in CFT for Chlamydia.
2. Major Outer Membrane Protein (MOMP): integrate with the previous antigen (LPS) and forms a complex molecular mosaic that plays an important role in pathogen-host interaction, pathogen protection, and also immunopathology (Lampe et al., 1993). It is the dominant antigen at the surface of infectious EBs. OmpA genes, which encode (MOMP) antigens, contain variable segments termed VS1-VS4 that are different between *Chlamydophila abortus* and *Chlamydophila pecorum* (Kaltenboeck et al., 1993), which can allow a specific detection of the targeted pathogen using its monoclonal antibodies (especially for VS1 and VS2).
3. Putative Outer Membrane Proteins (POMPs): regarded as a type of MOMP and are predicted to be present on the outer membrane of the subtypes of *C.pssitaci* that cause OEA especially strain S26/3 and are encoded by a family of four genes (Longbottom et al., 1998b).
4. Polymorphic Outer Membrane Protein (POMP): is a family of putative outer membrane proteins that plays a role in escaping from host immune defenses.
5. Cystein-rich outer envelope proteins: synthesized as (RB) changed to (EB), they are envelops A and B which are encoded by omp3 and omp2 genes respectively. Both envelops provide a complex structure that increase osmotic stability (Everett and Hatch, 1995).
6. Heat shock proteins (HSPs): present in the outer membrane complex and may cross the cytoplasmic membrane (Raulston, 1995). The major proteins are GroEL, GroEs, Dnak (Peeling and Mabey, 1999), and are conserved in Chlamydia species and play a role in protein folding and degradation as a response to cellular immune activation (Lund, 2001).

7. Glycolipid and Carbohydrate Antigens: comprise a part of the innermost core of the LPS.

1.9. Immunity

Regarding infection with *Chlamydophila abortus*, immunity is usually associated with two major classes of antibodies named IgM and IgG. Presence of one or both in the circulation give direct indicator for status of immune system in the tested animal/s. Immunoglobulin-M (IgM) is the first antibody to be produced after initial exposure to antigen. IgM plays a role in recognizing the antigen which associate in building a stronger and longer immune basis, occurrence of IgM indicates recent infection or uterine infection in case of neonatal births (Wellek et al., 1976, Mifflin, 2004).

Immunoglobulin G (IgG) is the main antibody found in the blood and extracellular tissue. IgG, like IgM, is produced by B cells in the spleen, but is multivalent and binds to the antigen with cross-linking and coating its surface causing immobilization and recognition for pathogen to be engulfed by phagocytic cells. It is considered a secondary immune response and the main part of humoral immunity. This type of antibody arises after a previous antibody (IgM) response, so it is used to identify if the host recognized this pathogen previously (Meulenbroek and Zeijlemaker, 1996, Junqueira et al., 2003). There are several reasons that made IgG favorable and more significant in testing infectious diseases like *Chlamydophila abortus***:** its relative large quantity, excellent specificity against antigens, greater persistence in circulation, it gives an idea about clinical history of animal regarding this pathogen, and whether vaccinated or not (Boenisch, 2009, University of California, 2011). Immunity for OEA can be built up by two ways: natural immunity after infection, and gained immunity after vaccination. For the first class and with natural infection, morbidity rates can reach 30% in pregnant sheep and 60-90% in pregnant goats, and decrease to 5-10% and to 10% in endemically infected flocks of sheep and goat, respectively, with new outbreaks in almost all yearlings and new introduced animals in their first pregnancy. The mortality rate is almost zero since death in dams is very rare (CFSPH and IICAB, 2005, Aitken, 2000, Rodolakis et al., 1998,

Gerber et al., 2007). This great difference between these two strains (sheep and goat) still unjustified (Stamp et al., 1950, Dawson et al., 1986).

Most dams abort only once and gain full immunity after abortion, and this immunity endures for several years (3 years in goat), although they become asymptomatic, chronic carrier, and continue shedding *Chlamydophila abortus* in the surrounding environment (CFSPH and IICAB, 2005).

1.10. Diagnosis and laboratory Inspection

Accurate diagnosis of OEA caused by *Chlamydophila abortus* is considered difficult. This difficulty is due to the intracellular viability, unique cycle of development and infection inside and outside the host, shortage of bacterium number in the uterine shedding over time, short and late antibody responses after abortion, contamination of fetus and fetal membranes with environmental agents, improper or inadequate selection and collection of samples, cross reactions with other related Chlamydial strains or gram negative bacteria, and the absence of predictive signs of abortion before occurring in most abortion cases. That's because most of the obvious normal-birth signs (enlargement, redness, and discharges of vulva, restlessness and other behavioral changes) that usually happen at the last 48 h of normal birth are less obvious or even non-existent at the time of abortion (CFSPH and IICAB, 2005).

Precise diagnosis of *Chlamydophila abortus* requires a complete group of precise procedures; starting with understanding the clinical history of the farm, matching the obvious clinical signs and pathological changes on the tissues (abortion materials and fetuses) with the typical Chlamydial lesions, correct and professional sampling, storage and transportation, and selection of the best test(s).

Building a strong clinical history for a farm suffering from abortions depends on collecting accurate informative data from the animal raiser and that is considered the first step in the right diagnosis process.

As suggested by various studies (Borel, 2008, Pelzer, 2012, Gerber et al., 2007, CFSPH and IICAB, 2005, Merck Veterinary Manual 9th Edition, 2005, Aitken et al., 1990) an investigator should suspect Chlamydial infection in case of :

- The animal herd was subjected to heavy stress condition (e.g. transportation).
- Previous history of abortion with *chlamydophila abortus* or any other pathological causes of abortion (e.g. *Coxiella burnetii*).
- The animal herd was bought newly and not homebred.
- Sharing rams or bucks (herd males) with other animal farms.
- Contaminated source of feed or water.
- Presence of cats or dogs in the farm that may feed on animal carcasses or abortion material.
- Using contaminated tools in case of using artificial insemination or hormonal breeding methods.
- The flock usually grazes in common pastures with other flocks.
- Abortions occur in the late stage of gestation (third trimester)
- Death of newborns within 24 hours after birth (still birth).
- Presence of abortion cases in the farms around the investigative farm.
- Introduction of new females to the origin flock recently.
- High rate of abortions in comparison to the number of flock.
- Using live and/or highly virulent Chlamydial vaccine.

All these actions can be real sources or predispositions of Chlamydial infection. At the same time, many other abortive pathological agents can be transmitted by the same ways and have the same clinical history, which require going further in the analytical protocol.

Clinical signs and pathological changes in the abortion material (Placenta, uterine discharges, and fetus) are also informative tools of diagnosis and/or comparative diagnosis, although the sudden nature of abortion and poor farm management may mean that these materials are not available.

Dams usually show no obvious signs before abortion, and still look healthy after abortion despite the reddish-brown uterine discharges that last for several days later. Some animals shows illness, retained placenta, and metritis after abortion and this is true in sheep more than in goats. Some goats may develop cough, polyarthritis, and keratoconjunctivitis (CFSPH and IICAB, 2005).

Placentitis occurs with thickening lesions of yellowish brown to reddish brown exudates sticking to cotyledons and in the intracotyledonary areas or multifocal necrotic reddish-brown cotyledons (Merck Veterinary Manual 9th Edition, 2005, Carter and Wise, 2003).

Experimentally infected males show orchitis, epdidymitis, seminal vesiculitis, low or no fertility. Freshly aborted fetuses may show small autolysis, and may be stained with reddish-brown exudates from the placenta. Clear or blood-stained edema or blood-stained fluids may be seen in the abdominal and pleural cavities, pinpointed white foci of necrosis on the liver of sheep fetuses. Other signs are petechial on the tongue, buccal cavity, and on the hooves of goat fetuses (CFSPH and IICAB, 2005).

Despite all the above mentioned diagnosis-aiding data, it is still insufficient because it does not differentiate *Chlamydophila abortus* from other infecting agents such as *Coxiella burnetii*. Definitive differential diagnosis can only be accomplished by various laboratory tests depending on the available samples.

1.11. Laboratory Tests

Serological tests are performed on animals using their serum samples for various objectives:

- Performing epidemiological surveys of one disease or more on the animal flocks in particular area or over the all country to identify its occurrence.
- Inspecting and validating the individual status of animals; presence of antibodies with clinical signs in infected, and without clinical signs in carrier, and absence in non-infected nor vaccinated.

- Evaluating both vaccine efficiency and animals immune response after performing vaccination.
- Carrying out differential diagnosis among the clinically resembled diseases.

Despite the multipurpose and easiness of serological diagnostic tests, they have some limitations in testing Chlamydial abortions (*Chlamydophila abortus*). Serological diagnosis enables detecting Chlamydial antibodies in case of infection after 90 days of pregnancy due to the underlying nature of the pathogen that takes a long time in multiplying in the placenta (Buxton et al., 1990a).

Moreover, false positive results are common due to cross reactions with other Chlamydial strains (e.g. *Chlamydophila pecorum* which can infect ruminants and cause several clinical signs) when using non specific antibodies for *Chlamydophila abortus* (Fukushi and Hirai, 1992, Kaltenboeck et al., 1993, Philips and Clarkson, 1995, Anderson et al., 1996).

Serological tests for Chlamydia are available in different natures and variable specificities, two tests are used mainly:

- Complement Fixation Test (CFT): the first described serological test that was based on LPS and was previously the most used test in veterinary laboratories (Stamp et al., 1952). It can show rise in serum antibodies level at the time of abortion and stay as well for 6 weeks at least (Storz, 1971). CFT relies on a common antigen in the *Chlamydiaceae* family which is a heat-resistant lipopolysacharide (Brade et al., 1987), and that makes its specificity low.
- Enzyme linked immunosorbent assay (ELISA): was developed by Salti-Montesanto et al. in 1997, and depends on the binding of specific antibodies against the targeted antigens of the targeted organism, targeted antigens are bound by serum which is detected and categorized by color change to describe the status of the tested animal (Borel, 2008).

1.12. Prevention, Control and Treatment

1.12.1. Primary prevention

Prevention can be accomplished at the first level by establishing a Chlamydia-free flock, starting with pathogen-free mothers as well as males. Animals can be tested for Chlamydia before being introduced into the herd, in addition to finding out their clinical history and origin.

The good management procedures can guarantee the freedom of flocks from Chlamydial abortions without any additional procedures (Lenzko et al., 2011); numerous strict actions have to be practiced:

- Buy and raise females that didn't give births before.
- New animals should be brought from well known sources that are free of *Chlamydophila abortus* when they are tested for Chlamydia.
- No foreign animals (especially females) should be introduced into the flock or farm under any condition.
- No sharing for males (rams and bucks) with other farms.
- Keep feed and water clean all the time and away from contamination sources.
- Animals should graze alone in their specific pastures.
- Owners of other farms and their workers besides animal traders shouldn’t enter the farm and move intensively in it.
- Dogs and cats should be kept away from feed and water.
- Using sterile utensils and clean semen in case of artificial insemination.

1.12.2. Secondary prevention

At the second level, prevention can be accomplished by practicing a complete efficient vaccination protocol using the best choice of vaccine type. The recommended vaccination protocol by the manufacturer should be carried out completely, otherwise, the expected outcome well not obtained.

1.12.3. Vaccination protocol

Vaccination protocol should be practiced exactly as recommended by the manufacturer. It is usually performed twice (6 and 3 weeks) prior to breeding for the first time (replacements), with a booster dose once every 6 – 12 months depending on prevalence and infection if present (HIPRA Laboratories, 2012a).

1.12.4. Vaccine type

Regarding the vaccine choice, molecular studies can determine the vaccine with the most proximity to local field strains genetically.

From another point of view, a decision has to be made in order to use live attenuated or inactivated vaccine according to the health situation of the flock and the degree of disease spreading. Live attenuated vaccines usually give stronger and longer lasting immune responses but with several precautions; they can cause abortion itself in case of depressed immunity level in the herd and even in healthy animals in case of over dose, and are a greater source of zoonotic risk for the people dealing with the animals. Whereas killed or inactivated vaccine are considered safer for animals and handlers, easier in storage and transportation than the live one (Moredun Research Institute, 2010, Boril et al., 2005, Loveren et al., 2001, Gerber et al., 2007).

1.12.5. Control and treatment

In case of infection and abortions, control and treatment measures should be followed, as described below (Pelzer, 2012, Unknown, 2012, CFSPH and IICAB, 2005):

- Removing the abortion materials, fetuses, and contaminated bedding from the barn, burning or disinfection followed by burying them deeply (not less than 1.5 meter), besides cleaning and disinfecting the abortion site and all premises carefully.
- Submission of appropriate samples for laboratory testing under high aseptic condition and with proper tools to detect the exact etiological cause of abortion.

- Segregation and sometimes culling the affected dams and their births (if some are alive) from the entire flock if disease is newly introduced.
- Suspected and aborted animals should be isolated under control for 3 weeks at least in order to reduce disease spreading, and the remaining animals should be moved into another clean place if possible.
- Carrying out a complete vaccination program on all flock animals to reduce incidence and severity even though it well not provide complete protection. (CFSPH and IICAB, 2005, Moredun Research Institute, 2010).
- Treating the aborted animal, their live births, and contact pregnant animals with injectable, long acting oxytetracycline (20mg/kg body weight by IM route) to suppress the organism's multiplication (Mearns, 2007) to reduce the infection severity and/or the abortion losses. This treatment is started 6 and 3 weeks before lambing (Merck Veterinary Manual 9th Edition, 2005) and repeated every ten days or every two weeks until delivery time by subcutaneous route (Stuen and Longbottom, 2011).

  Oral tetracycline (400-500 mg/head/day for 15-21d) in feed and under the veterinary supervision (Pelzer, 2012) has also produced good results in treating and controlling OEA. Other antibiotics such as Macrolides (Erythromycin) and Quinolones can be used also (CFSPH and IICAB, 2005).
- Various antiseptics and detergents can be used in disinfection since *Chlamydophila abortus* is susceptible for (Pelzer, 2012):
  1. Chemical reagents:
     - Ethanol 70%
     - Sodium hypochlorite 1%
     - Dilution of quaternary ammonium compounds 1:1000
     - Formaldehyde
     - Glutaraldehyde

2. *Chlamydophila abortus* can be inactivated also by:
    - Moist heat (121ºC for 15 min at least)
    - Dry heat (160-170ºC for 1 hour at least)
3. *Chlamydophila abortus* is resistant to acidic and alkaline compounds.

It is important to take into consideration that although application of vaccination protocols on infected farms will considerably reduce abortion occurrence, it will not necessarily stop all abortion cases, nor eradicate the disease because of re-shedding of organisms especially at the next birth (Foggie, 1973, Linklater and Dyson, 1979, Mcewen and Foggie, 1954, Rodolakis and Souriau, 1979, Lenzko et al., 2011, Moredun Research Institute, 2010). With returning again to size of infection in UK, a British study suggested that vaccination was still the best choice for controlling OEA even with possible link between disease and live vaccine, it showed that the percentage of abortions in sheep flocks was reduced from 25% to less than 2% after a complete vaccination program (Moredun Research Institute, 2010). On the other hand, although Oxytetracyclines limit the shedding of organisms and minimize the losses, they don't eliminate the organism nor reverse the pathological changes that may occur in the placenta (Stuen and Longbottom, 2011), and as a result the Chlamydial infection cycle will continue among contacting animals.

1.13. Zoonotic risk and public health

Chlamydophila abortus is a real source of zoonotic risk for humans, in that it can be transmitted from animals to humans and result in harm. Infection can occur via ingestion of a contaminated food source, or drinking of non-boiled milk and dairy products from an infected animal source, direct contact of wounds, eyes, mucous membranes, or genitalia with abortion materials (CFSPH and IICAB, 2005, Pospischila et al., 2002).

Although human cases are rare, vulnerable groups include laboratory workers, animal raisers, veterinarians, and pregnant women. *Chlamydophila abortus* can also cause abortion in pregnant

women as a result of contact with infection sources (Aitken, 2000, Buxton, 1986, Jorgensen, 1997), and indeed, 20 abortion cases were recorded and confirmed to be caused by *C. abortus* between 1987 and 2000 (Pospischila et al., 2002). Pregnant women can acquire infection in all stages of pregnancy, and go on to abortion in the 14^{th} -36^{th} week of pregnancy in case of early infection, in addition to septicemia, fatigue, fever, pelvic inflammatory, pneumonia, kidney dysfunction, intravascular coagulation, and hepatitis if not treated. For other people, non-specific symptoms are developed at the beginning, mostly influenza-like symptoms including fever, dizziness, headache, and vomiting. Person to person transmission of *Chlamydophila abortus* is unknown. (CFSPH and IICAB, 2005).

Pregnant women should, therefore, avoid close contact with susceptible animals, and should especially avoid contact with animals suspected of Chlamydia or real abortion cases. Preventive measures should be practiced including good hygiene, wearing protective clothes and gloves, washing hands with water and soap after handling animals or their products, and using disinfectants.

Since the bacterium localizes and multiplies in trophoblast cells, it presents in large quantities in the uterus and placenta of sheep and goats, and spreads into the environment during abortion. Shedding these organisms continues for a few weeks after abortion through vaginal discharges (CFSPH and IICAB, 2005). Moreover, while the aborted animal usually does not abort in the next pregnancy, it goes back again to re-shedding the Chlamydial organisms at birth in the uterine discharges, placenta, fetuses, and coats of lambs (Longbottom and Coulter, 2003), which are considered as heavy and mobile sources of infection and spreading for these infectious organisms to other susceptible animals. That makes controlling disease more difficult if the aborted animals were not isolated.

1.14. In This Study

Various animal diseases affect Palestinian livestock in the West Bank and reduce production leading to severe economical losses for the livestock breeders. Indeed, livestock numbers are decreasing in the West Bank as shown in Table 1.1, and while the effects of Israeli occupation, restrictions and seizure of grazing land are the fundamental problem, losses due to infectious diseases help to make matters much worse.

Table 1.1: Number of sheep and goat in the West Bank during the period 2003-2010 except for year 2009 (not available), source: Palestinian Central Bureau of Statistics (PCBS)

Year / Animal	2003	2004	2005	2006	2007	2008	2009	2010
Sheep	828.678	811.864	803.165	793.874	744.764	688.899	---	503.431
Goat	392.122	398.821	371.198	387.123	343.565	322.082	---	215.774

Palestinian livestock flocks are subjected to a wide range of diseases and health problems; pneumonia, diarrhea, mastitis, skin lesions, infectious viral diseases, abortions and others. While all these health conditions need attention, abortion is one of the most serious health situations and is widespread among Palestinian animal farms. OEA, caused by *Chlamydophila abortus*, is considered by farmers and veterinarians to be the major cause of losses due to abortion in sheep and goats.

Many factors complicate the problem of abortions in sheep and goats such as: lack of statistics and records of abortions and their causes, little scientific investigation of abortion cases, poor farm management, sporadic vaccination programs that are required in control and prevention, lack of farmer knowledge and education about correct handling of the problem.

Although ovine Chlamydiosis is such a prominent disease in Palestine and causes the majority of pathological abortions, no previous scientific studies were published on this disease in Palestine. With no official records for the economical losses due to abortions caused by Chlamydia or other pathological agents, the actual size of the problem and choice of control strategies along with assessment of the success of measures to address it have been subject to some guesswork that hampers efforts to deal with this condition.

In fact, several campaigns were initiated by different organizations and committees (e.g. FAO, Oxfam, Red Cross, etc.) in collaboration with the Ministry of Agriculture to control this disease and stop its spreading. These campaigns have delivered about 630,000 shots of inactivated chlamydia vaccine, with a total cost of about $450,000, to sheep and goat flocks in different areas in the West Bank. Some of these campaigns were directed to the south of this area, because of the higher density of sheep and goat there. Different strategies were practiced during these campaigns; some of them were practiced on mothers and yearlings, but not entire flocks, others targeted infected flocks only, while yet other campaigns targeted specific areas without regard to the infection status. In addition, most of these campaigns administered one shot per animal without complying with manufacturer recommendations for booster shots.

Despite all these efforts, farm owners are still suffering from abortion cases, and some farmers have complained of increasing numbers of abortion and have lost confidence in the safety and efficacy of the vaccines used.

This study reports a partial case control study of vaccination on farms (initially 7 farms, but finally 5 due to farmer drop out) in the south of the West Bank, with serological follow-up and nucleotide sequence comparison between a field strain of *Chlamydophila abortus* and a locally used vaccine.

The following questions about the vaccination for *Chlamydophila abortus* in Palestine are addressed:

- Is the vaccine safe?
- Is the vaccine effective?
- What are the implications of deviating from the manufacturer's recommendations for giving the vaccine?

In addition, as vaccination without good farm management cannot lead to success, an assessment of the scale of the problem of *Chlamydophila abortus*, and assessment of farm management practices related to abortion control has been investigated by correlating diagnostic results reported by veterinarians with results of a questionnaire that was designed specifically for this study.

Chapter 2

2. Study Objectives

2.1. General Objectives

1. To assess the scale of the contribution of *Chlamydophila abortus* to the problem of abortions in sheep and goat farms from the Bethlehem district and the contribution of poor management practices.
2. To assess the suitability, efficacy and safety of vaccines used in the Southern part of the West Bank.

2.2. Specific Objectives

1. To complete a 3 stage vaccine trial over 8 months to assess humoral immunity by ELISA for Momp, which is a major immunogen, and to compare the responses of different breeds of Palestinian sheep and goats in the trial.
2. To conduct a survey of farms where abortions occurred, using a questionnaire about farm management practices, and compare the responses with diagnostic results for *Chlamydophila abortus* and two other infectious agents that are known to cause abortions: *Coxiella burnetii* and *Toxoplasma gondii.*
3. To amplify and sequence local *Chlamydophila abortus* strain(s) and compare with world reference strains and vaccine(s)

Chapter 3

3. Materials and Methods

3.1. Materials

3.1.1. Specimens for PCR Reaction

Three categories of specimens were used to test for the presence of *Chlamydophila abortus* for the abortion cases survey

1) Parts of internal organs of aborted fetuses: liver, heart, lung, rumen, spleen, intestine, and umbilicus.
2) Parts of placental tissue of the mother of the abortus: cotyledons, intercotyledonary areas, and membranes.
3) Abortion discharges, secretions, and blood.

3.1.2. Sampling Equipment

1) Clean nylon packets with double sealing strips to transport and preserve fetuses and placentas.
2) Clean cups with caps for collecting and preserving fetal tissues and placental samples.
3) Sterile plastic stick -cotton swabs (STERILE®) for collecting abortion discharges and blood from the vagina of aborted mothers – vaginal swabs.

3.1.3. Positive Controls

The following positive controls for *Chlamydophila abortus* were generously provided by Dr. Elina Awwad from the Central Veterinary Laboratory (CVL) in Al-Aroub area – Hebron::

1) Extracted DNA sample from inactivated Chlamydial vaccine (ChlamyVax FQ® - *Chlamydophila abortus* + *Coxiella Burnetii*- produced by Merial)
2) Extracted DNA sample from *Chlamydophila psittaci* , used as Chlamydial antigens in CVL.
3) Extracted DNA sample from local field abortion case (from Yatta) that previously gave a positive result for *Chlamydophila abortus* by the (CVL) PCR test.

3.1.4. Oligonucleotide Primers

Two primer sets were used in this study, the first one has been validated in published paper for PCR (Berri et al., 2009) and described in table 3.1. This pair of primers targets the gene *pmp 90/91* which has synonym names like *pmp13G* and *pomp 91A*, this gene is one of four genes family (*pomp91A, pomp90A, pomp 91B, and pomp90B*) that encode a group of proteins called Putative Outer Membrane Proteins (POMPs) which considered a major immunogens in the outer membrane of *Chlamydophila abortus* strain S26/3 (Longbottom et al., 1998a). The second set of primers were used by CVL for their routine diagnosis of Chlamydial species.

Table 3.1: Targeted genes and primers sequences used in detection of *Chlamydophila abortus.*

Targeted genes	Primers direction	Primers names	Primers sequences (5'-3')	Length of amplified PCR products (bp)	Melting temps. (C°)
pmp 90/91	Forward	pmp-F	CTCACCATTGTCTCAGGTGGA	821	64
	Reverse	pmp-R821	ACCGTAATGGGTAGGAGGGGT		66.3
omp2	Forward	F	CAAACTCATCAGACGAG	F-R:582	50
	Reverse	R	CCTTCTTTAAGAGGTTTTACC		55
	Intermedi-ate	AB	TCAGTGCCAATCCGTCGATA	AB-R:330	58

3.1.5. Reagents and Equipment for PCR Reaction

1) Thermocycler PCR machine (Applied Biosystems 2.09®) was used to perform PCR reactions.
2) Commercial master mix for reaction prepared with the following components/ sample reaction:
    - Ultra Pure Water - PCR Grade 100 ml - Fisher biotech /Australia
    - 10X Taq reaction buffer - Mg^{2+} free - Cat.#:37A - Hy-labs.
    - $MgSO_4$ (20 mM) - Cat.#:37B - Hy-labs.
    - dNTPs (10 mM) - Cat.#:RO192 - Fermentas Life Science.
    - Taq DNA polymerase - High Pure (5u/ µl) - Cat.#:HTD0078-Hylabs.
3) Primers:

- Primer Forward (100 pmol/µl in TE buffer) - Hy-labs.
- Primer Reverse (100 pmol/µl in TE buffer) - Hy-labs.

3.1.6. Gel Electrophoresis Reagents

Agarose from Sigma Aldrich (Cat# A9539) was used to prepare 1% agarose gels with 1X TBE buffer for running of PCR products.

3.1.7. Vaccine

In this trial, the *Chlamydophila abortus* vaccine that was used was from the same source as that used in previous and ongoing vaccination campaigns under the oversight of the Ministry of Agriculture, who kindly provided vaccine doses as follows:

- Name: (OVIVAC-CS®).
- Produced by: HIPRA laboratories – Spain.
- Administration route: subcutaneous (SC) for sheep and goat, intramuscular (IM) for cattle.
- Dose: 2 ml for sheep and goat, 5 ml for cattle.
- Batch: 22VG-1

This inactivated vaccine was designed to give double protection from abortions caused by *Chlamydophila abortus* and Salmonella in sheep, goats, and cattle (HIPRA Laboratories, 2012a). OVIVAC-CS® vaccine is an injectable suspension that contains both *Chlamydophila abortus* and *Salmonella abortus* in addition to adjuvant.

3.1.8. Blood Collecting and Transportation Requirements

We used the following materials and disposables:

- Sterile disposable syringe 5 and 2.5 ml.
- Sterile disposable needles 18 and 21G.
- Sterile disposable dual sided needle (VACUETTE NEEDLE®)
- Sterile disposable clotting activator tubes (VACUETTE®), 6 and 3 ml size.

- 2 ml eppendorf tubes for serum storage and transportation.
- Blood tube racks.
- Cooling blocks.

3.1.9. Serology Kit

In our serological tracing, we used the same ELISA kit type as used locally by the CVL to diagnose Chlamydial abortions:

- Name: ID Screen® - Chlamydophila abortus Indirect, Multi-species
- Product code: CHLMS-MS ver 0310 GB
- Produced by: ID VET – innovative diagnostic – France.
- Description: Kit for the detection of antibodies directed against *Chlamydophila abortus* in ruminants, swine, and horses.
- Indirect 192-reaction ELISA kit uses a synthetic antigen from a major outer membrane protein (Momp), which is specific for *Chlamydophila abortus* to reduce the non-specific reactions including *Chlamydophila pecorum* (ID VET, ID VET, 2000).

3.2. Methods

3.2.1. Abortions Survey

Abortion samples from twenty sheep and goats farms were collected from Bethlehem area by the researcher and other veterinarians, who responded to requests to follow up abortion cases in the farms to which they attend. The basis for inclusion of farms in the study was the availability of abortion materials combined with the willingness of farmers within the area of investigation to answer questions, and so was essentially random. Samples from these farms were sent by animal owners to CVL for routine testing of pathological abortion causes. These samples were also tested for *Chlamydophila abortus* infection at the Biotechnology Research Center (BRC)/ Palestine Polytechnic University (PPU).

A four page questionnaire was designed for this survey, see appendix A. The choice of questions was informed by the previous veterinary experience of this researcher with common management mistakes of sheep farming in Palestine encountered during his clinical practice, and by the known transmission pathways of this disease. The questionnaires were filled in by the attending veterinarian based on farmer responses to 13 questions about the farm and 8 questions related to abortion history and management. Additional information listing clinical features of aborting animals and diagnostic results were filled, respectively, by the attending veterinarian and the researcher. Animal owners were informed that this data will be used for the purpose of scientific research and analysis only.

3.2.2. Sample Collection and Preservation

Samples were collected at random by tracing abortion cases from different areas in Bethlehem area, and one farm in Jerusalem, see table 3.2. To reduce the risk of zoonosis, protective measures were taken at the sampling site and the laboratory: gloves and lab coats were worn at all times, and soap for hand-washing and disinfectants for decontamination were used during and after sampling according to BRC safety guidelines.

Samples were collected with stick swabs, plastic cups, and plastic packets depending on availability. Fetuses and placentas were discarded quickly by the animal owners in most abortion cases and samples were collected within 24 hours, while vaginal swabs were collected from 0-15 days after abortion since discharges continue to be shed throughout this time (Rekikia et al., 2002). The total numbers of samples collected were: 68 vaginal swabs, internal organs of 3 fetuses, and 2 placentas, these samples were distributed within the survey area as shown in table 3.2.

Samples were stored depending on their nature; vaginal swabs were kept frozen at -20Cº until DNA extraction, fetus tissues and placental parts were kept at -80Cº until becoming well frozen in order to allow freeze-thawing to assist in subsequent crushing of the tissue.

Table 3.2: Areas where abortion samples were collected, with the quantity and type of samples in each area.

No.	Area	Animal	Sample	Number of samples
1.	Bethlehem-Alferdes-1	Sheep	V.S./ P	2 / 1
2.	Bethlehem-Alferdes-2	Sheep & Goat	V.S.	6
3.	Bethlehem-Almasrah-1	Sheep & Goat	V.S.	2
4.	Bethlehem-Almasrah-2	Sheep	V.S.	2
5.	Bethlehem-Alsaff St.	Sheep	V.S.	4
6.	Bethlehem-Alshwawrah1	Sheep	V.S.	5
7.	Bethlehem-Alshwawrah2	Sheep	V.S.	3
8.	Bethlehem-Alubaydia	Sheep	V.S.	3
9.	Bethlehem-Alubayyat	Sheep & Goat	V.S./ F	4 / 1
10.	Bethlehem-Dar Salah-1	Sheep	V.S.	4
11.	Bethlehem-Dar Salah-2	Sheep	V.S.	2
12.	Bethlehem-Khalayel Allouz	Sheep	V.S.	3
13.	Bethlehem-Marah Rabah	Sheep	V.S.	4
14.	Bethlehem-Tekoa'	Sheep	V.S.	3
15.	Bethlehem-Zatarah-1	Sheep	V.S.	3
16.	Bethlehem-Zatarah-2	Sheep	V.S.	3
17.	Bethlehem-Zatarah-3	Goat	V.S./ F	3 / 1
18.	Bethlehem-Zatarah-4	Sheep	V.S.	5
19.	Biet Jala	Sheep	V.S./ P	4 / 1
20.	Jerusalem	Sheep	V.S./ F	3 / 1

V.S.: Vaginal Swabs. F.: Fetal tissues. P.: Placenta.

3.2.3. DNA Extraction

EZ-DNA®- Genomic DNA Isolation Reagent was used to extract DNA form fetal tissues and placenta, while DNA was extracted from the vaginal swabs by using the Qia amp® Viral RNA extraction kit, which performed better than the EZ kit when applied for vaginal swabs and includes a modified procedure suitable for dilute samples of various discrete non-genomic nucleic acids. The final extracted DNA volume was 30µl for each sample of vaginal swabs and 100µl for each sample of tissues.

3.2.4. PCR Assay

Targeted segments were amplified using primers of *pmp90/91* gene (table 3.1) and the reaction mixture components and volumes that are listed in table 3.3. All master mix components, primers, and DNA sample were mixed in PCR reaction tubes. PCR amplification using primers of omp2 gene (table 3.1) was accomplished in CVL.

Table 3.3: Components and volumes of commercial master mix for PCR reaction /sample.

No.	Components	Volumes
1.	Ultra Pure Water (PCR Grade)	14.9 µl
2.	10X Taq reaction buffer (Mg^{2+} free)	2.5 µl
3.	$MgSO_4$ (20 milliMolar)	2.5 µl
4.	dNTPs (10 milliMolar)	0.5 µl
5.	Taq DNA polymerase -High Pure (5u/ µl)	0.1µl
6.	Primer forward (100 pmol/µl in TE buffer)	1 µl
7.	Primer reverse (100 pmol/µl in TE buffer)	1 µl
8.	Sample DNA	2.5 µl
Total reaction volume		**25µl**

Thermocycler conditions were 94Cº for 10 minutes to ensure initial denaturation, followed by 35 cycles of: 1) denaturation at 94Cº for 30 seconds, 2) annealing at 63Cº for 1 minute, and 3) extension at 72Cº for 1 minute. After completion of the last cycle, a final extension at 72Cº for 10 minutes was included.

3.2.5.Gel Electrophoresis and Documentation

Agarose Gels (1% concentration) were prepared with an appropriate number of wells using the materials mentioned in the gel reagents section. 7µl of each sample of PCR products were applied to the wells in addition to 3µl of 100bp ladder (GeneDirex®) in boundary wells and electrophoresed by the electrophoresis system at 120V for 35-50 minutes as required to complete separation of ladder bands.

Agarose gel slabs were visualized by a UV trans-illuminator, and then moved to a gel documentation system to take a photograph of the DNA bands.

3.2.6.DNA Sequencing and Maximum Likelihood Tree Building

Targeted DNA bands of both PCR amplifications were cut out of the gel and treated according to the gel purification protocol (Accuprep® Gel Purification kit) in order to get the amplified targeted DNA purified and get rid of any other unwanted components.

Purified DNA was sent for sequencing, using both forward and reverse primers of the *pmp90/91* and *omp2* genes, at the Bethlehem University Hereditary Laboratory.

Two programs were used to treat acquired sequences, Sequencher program was used to treat and match forward and reverse sequences of same samples, where MEGA-5.02 was used in this study to align derived sequences (sequences from field samples, ChlamyVax vaccine, and positive control (*Chlamydophila psittaci* as Chlamydial antigen in CVL)) with sequences of predominant and well known standard strains of *Chlamydophila abortus* by using similar beginnings and similar ends for all these sequences, and then to consult a maximum likelihood tree - by using boot strap method with number of replications equal 500 and default setting for other variables - for both genes in order to assess the relatedness of local strains of *Chlamydophila abortus* with other strains in the

world, and also to evaluate the likely relative suitability of this vaccine for protecting against local strains. Targeted sequences of the well known standard strains and the outer group were obtained from their complete sequences by using NCBI database for this aim. The outer group was *Chlamydophila pecorum*, since it shares the same genus with *Chlamydophila abortus*.

3.2.7.Animal Groups in Serological Trial

Four breeds of sheep and goat in five groups were subjected to this serological trial as follows:

Group1, healthy Assaf sheep:

- Breed name: Assaf sheep.
- Area: Bethlehem – Wadi Foukeen.
- Number: 14 animals.
- Age: 12-40 month.
- Breed origin: crossbreeding of local Awwasi breed and east Friesian breed.
- Indoor and outdoor feeding.
- Farm history: good health condition, no previous reproduction problems.

Group2, infected Assaf sheep:

- Breed name: Assaf sheep.
- Area: Beit Jala.
- Number: 11 animals.
- Age: 5-50 month.
- Breed origin: crossbreeding of local Awwasi breed and east Friesian breed.
- Indoor and outdoor feeding.
- Farm history: abortions, still births, other reproductive problems, diagnosed to be infected with Chlamydia and Toxoplasma agents by (CVL).

Group3, Awwasi (Baladi) sheep:

- Breed name: Awwasi (Baladi) sheep.
- Area: Bethlehem – Al'abiat.
- Number: 9 animals.
- Age: 12-45 month.
- Breed origin: local breed of sheep in the area, and south west Asia.
- Indoor and outdoor feeding.
- Farm history: good health condition, few reproductive problems; moderate cases of temporary infertility and repeated estrus, no previous testing.

Group4, Shami goat:

- Breed name: Shami goat.
- Area: Bethlehem – Hindaza.
- Number: 10 animals.
- Age: 12-35 month.
- Breed origin: though to originate from Damascus - Syria.
- Indoor and outdoor feeding.
- Farm history: good health condition, no previous reproductive problems.

Group5, Baladi goat:

- Breed name: Baladi goat.
- Area: Hebron – Nuba.
- Number: 7 animals.
- Age: 12-40 month.
- Breed origin: local breed of goat in the area.
- Indoor and outdoor feeding.

- Farm history: good health condition, no previous reproductive problems.

Each animal group was identified for its health history and management condition and examined clinically. We incorporated animals with different reproductive classes in these groups. Individual animals in the groups were identified by plastic ear-tagged numbers and recognized for their health and reproductive status. All these groups were almost under the same management, feeding, and weather conditions. Distinguishing special features of these animal breeds can be done through observing figures 3.1 and 3.2:

Figure 3.1: Investigated sheep farms in the serological trial

A, B: Group 1, healthy Assaf sheep.
C, D: Group 2, infected Assaf sheep.
E, F: Group 3, healthy Awwasi sheep with few reproductive problems.

Figure 3.2: Investigated healthy goat farms in the serological trial
G, H: Group 4, healthy Shami goat.
I, J: Group 5, healthy Baladi goat.

3.2.8.Sample Collection, Preparation, and Storage

Blood samples were collected during this study by following previously designed plan after studying required time for immune response (HIPRA Laboratories, 2012b, Personal communication, 2012). Safety measures were followed in dealing with animals, tools, and samples. Sampling and vaccination dates were recorded on data collection sheets, (see appendix C), which contain animals' identification numbers and antibodies percentage also.

3-5 ml blood samples were collected from the Jugular vein in the neck area at the beginning by double-sided needle and numbered tubes, then by syringe, needle, and numbered tubes.

Generally, the same sampling protocol was applied on all groups, but with minor differences in time of two samplings in Baladi goat group due to difficulties in time, location, and others relating to tools and testing kits. The 1'st sampling was practiced during the month prior to vaccination and directly before the 1'st shot of vaccination, the 2'nd sampling was done three weeks after the 1'st shot of vaccination and directly before the 2'nd shot of vaccination, except for group no.5 in which the 2'nd sampling was one week before the 2'nd shot. The 3'rd sampling was one week after the 2'nd shot of vaccination, followed by the 4'th sampling 5 weeks later except for group no.5 which was 11 week later. The 5'th sampling was done 8 weeks later than the previous one. 9 weeks later and directly before the 3'rd shot of vaccination the 6'th sampling was performed, followed by the final 7'th sampling 4 weeks after the 3'rd shot of vaccination. See figure 3.3.

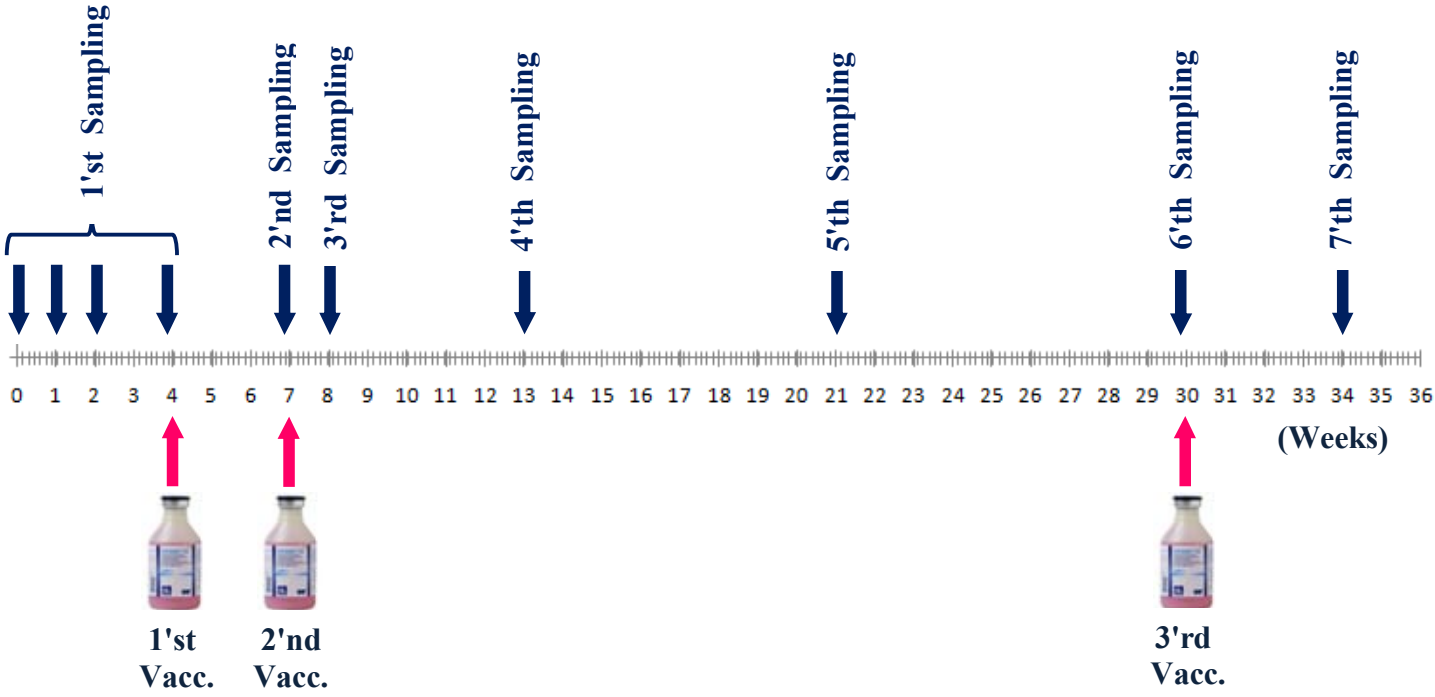

Figure 3.3: Applied time outline of vaccinations and blood samplings. Seventh samplings were applied before and after three shots of vaccinations using the same type of vaccine.

All intravenous blood samples were collected in sterile blood clotting activator tubes that were numbered in to matching with each animal's identification numbers. Blood tubes were positioned in special racks and transported from animal farms at a suitable temperature (20-25 C°) to allow normal clotting and minimize hemolysis. Blood samples were centrifuged within 4 hours of

collection at 3000 g for 10 minutes, serum was decanted and collected in 2 ml eppendrof tubes and stored at -20 Cº until use. Each serum sample was characterized with the number of the animal over number of sampling in addition to the date of sampling.

3.2.9.Vaccination Protocol and Trial

This vaccination trial took into account the vaccination protocol of the vaccine's manufacturer, which varies according to the infection status of the flock and area. The infection pressure has to be determined as high, low, or facing the onset of a Chlamydial outbreak. In the first two conditions (high and low infection pressure) animals with the age of 5-7 months and older are recommended to be vaccinated with an initial shot that should be repeated after 3 weeks, with a third dose to be given 6 or 12 months later for high and low pressure status respectively. Thereafter, a booster dose is recommended every 6 or 12 months for high and low pressures respectively.

For facing the onset of Chlamydial outbreaks, all animals in the flock should be vaccinated directly after the outbreak onset, a second dose is given 3 weeks later, third dose at 6 months later, and a booster dose every 6 or 12 months as the situation requires.

In this vaccination trial, the most intensive program was practiced on all animal farms. After giving the first shot of vaccination, the second shot was given 3 weeks later, and the third shot about 6 months later. See figure 3.3.

Animals in each farm were vaccinated with the recommended dose of 2 ml by SC route of administration, none of the three shots were given to the control animal in each group, and only one shot was given to one-shot control animal in each group. Vaccination shots were given for individual animals that were numbered for careful pursuance of the vaccination protocol, and vaccination dates were recorded on previously designed data sheets.

3.2.10.Measurement of *Chlamydophila abortus* Antibodies in Sheep and Goat Serum by Indirect ELISA

ELISA kit reagents from ID vet (Described in 3.1.9) were removed from storage at +2 - +5 Cº, and brought to room temperature and homogenized by inversion or vortex every time the test was carried out according to the manufacturer's protocol. In brief, serum samples and controls (positive and negative - provided by the kit manufacturer) were added to the reaction wells with all incubations, washing and application of peroxidase conjugated secondary antibodies. After addition incubation with substrate, and stopping of the reaction, an AWARENESS TECHNOLOGYING® ELISA reader was used to measure absorbance at a wavelength of 450 nm.

The test results were validated according to the manufacturer's quality control specification using the following two equations:

1) Mean value of positive controls O.D should be greater than 0.350 ($OD_{PC} > 0.350$).
2) Ratio of the mean O.D values of the positive and negative controls should be greater than 3 ($OD_{PC} / OD_{NC} > 3$). (ID VET, 2000) where,

   OD sample: optical density of sample,

   and

   ODPC: optical density of positive control

Using the validated results, an S/P value (percentage of sample to positive control value) was calculated:

($S/P = OD_{Sample} / OD_{Positive\ Control} \times 100$),

and then interpreted for comparability of vaccine resultant S/P values to those following infection, as directed by the manufacturer (Table 3.4), as the basis of determining seropositivity.

Table 3.4: Diagnostic cut-off values provided by indirect ELISA kit manufacturer (ID vet) for interpretation of percentage of sample to positive control value (S/P):

S/P value	Condition
S/P ≤ 50%	Negative for Chlamydia
50% < S/P < 60%	Doubtful
S/P ≥ 60%	Positive for Chlamydia

3.2.11.Statistical Analysis

Fisher's Exact Test was used by SPSS version 17.0 to examine the relatedness between infection with *Chlamydophila abortus* and each of the investigated actions that were applied by the owners or noticed in their farms. Fisher's Exact Test was used since it deals with small size data and give exact number for P-value when the number of expected repeats is less than 5 in any cell of the table, unlike Pearson Chi-square test that deals with bigger size of data and gives estimated P-value.

In serological trial, S/P values for animals of the 5 groups were compared and represented by Box plot method using SPSS before vaccination and after each of the three shots. Means of S/P values for each group were tested each time to detect the significant changes in these means after each of the three shots using the Wilcoxon Signed-Rank Test which is a non-parametric statistical hypothesis test that is using to compare means of many related samples in case of no normally distributed data, S/P values of lost animals were treated as missing values. Small size of available data make its distribution not normal and since suitability of this test in this study. The overall level of statistical significance was set to 0.05 for all tests.

Chapter 4

4. Results

4.1. Abortions Survey

The survey of abortions on 20 farms was conducted, primarily, to assess the frequency of occurrence of 3 major abortive organisms that beset sheep and goats in Palestine (Table 4.1). In addition, a list of questions was presented to each farmer in the hope of gaining some insights into local farm management practices and possible correlations with the diagnosis of *Chlamydophila abortus* (Table 4.2).

Diagnosis for this survey was done by PCR using DNA extracted from abortion samples by both CVL and BRC (for *Chlamydophila abortus* only) as described in table 4.1. Farms were considered positive for Chlamydophila abortus, *Coxiella burnetii,* or *Toxoplasma gondii* if at least one sample gave a positive result. This farm-based diagnostic approach is the CVL's standard, due to limited availability of diagnostic reagents.

The questionnaire results from the survey are presented below, in table 4.2, along with the numbers of farms in each farmer response category that were diagnosed with *Chlamydophila abortus* from abortion samples. The statistical analyses shown in the final column indicates that the only significant association between farmer response and diagnosis of *Chlamydophila abortus* comes from the presence of 'still births' on the farm (question 15).

Although documented abortions caused by *Chlamydophila abortus* can reach 30% in pregnant sheep (Rodolakis et al., 1998, Aitken, 2000, Gerber et al., 2007), abortion percentages in this investigation ranged from 3.5% in farms that were infected with *Chlamydophila abortus* in previous seasons up to 38% and even 75% in newly infected farms.

In this study, it was found that all abortions where *Chlamydophila abortus* was diagnosed occurred in the third trimester, and that coincides with published data about this disease (Maley et al., 2008, Pelzer, 2012, Borel, 2008). It was recorded that 2 other agents accompanied some abortion cases that occurred in the second trimester, which suggests that abortions in the third trimester allows infection with *Chlamydophila abortus* to be distinguished from infection by *Toxoplasma gondii* and *Coxiella burneti*.

In this survey, 6 farms out of 20 inspected had stillbirths, 5 of them were positive for *Chlamydophila abortus* and the other one remained without diagnosis, which shows that pathology of disease in Palestine is similar to that reported previously (Merck Veterinary Manual 9th Edition, 2005, Aitken, 2000, Buxton and Henderson, 1999) and therefore, stillbirths are one of the prominent signs of infection with *Chlamydophila abortus*, which is also found to be significantly related (P-value =0.018).

Table 4.1: PCR results for samples that were collected from twenty farms suffered from abortions, results were obtained by BRC (for *Chlamydophila abortus* only) and also by CVL for all three Pathogens.

	Description of Animals		**Positive for:**		
No.	**Area**	**Animal**	***Chlamydophila abortus***	***Coxiella burnetii***	***Toxoplasma gondii***
1.	Bethlehem-Alferdes-1	Sheep		Yes	
2.	Bethlehem-Alferdes-2	Sheep & Goat	No	No	No
3.	Bethlehem-Almasrah-1	Sheep & Goat	Yes		
4.	Bethlehem-Almasrah-2	Sheep	Yes		
5.	Bethlehem-Alsaff St.	Sheep	Yes		
6.	Bethlehem-Alshwawrah-1	Sheep	No	No	No
7.	Bethlehem-Alshwawrah-2	Sheep	No	No	No
8.	Bethlehem-Alubaydia	Sheep		Yes	Yes
9.	Bethlehem-Alubayyat	Sheep & Goat	Yes	Yes	
10.	Bethlehem-Dar Salah-1	Sheep		Yes	
11.	Bethlehem-Dar Salah-2	Sheep	No	No	No
12.	Bethlehem-Khalayel Allouz	Sheep	No	No	No
13.	Bethlehem-Marah Rabah	Sheep			Yes
14.	Bethlehem-Tekoa'	Sheep			Yes
15.	Bethlehem-Zatarah-1	Sheep	Yes		
16.	Bethlehem-Zatarah-2	Sheep	Yes		
17.	Bethlehem-Zatarah-3	Goat		Yes	
18.	Bethlehem-Zatarah-4	Sheep	Yes		
19.	Biet Jala	Sheep	Yes		Yes
20.	Jerusalem	Sheep	No	No	No

Table 4.2: Questionnaire Findings presented by numbers and percentages of interested farms to the total number of interrogated farms (20 farms), in addition to statistical analysis for positive farms to *Chlamydophila abortus* using Fisher's Exact Test.

No.	Subject of interest	Farmer responses; numbers followed by percentages	Positive for *Chlamydophila abortus* / N° farms	Fisher's Exact Test for association with *Chlamydophila abortus* / P - value
1.	Types of aborted animals in the farm.	- Sheep only 12/20 (60%) - Goat only 1/20 (5%) - Mixed 7/20 (35%)	5 0 3	1
2.	Previous health problems?	- Yes [1] 10/20 (50%) - No 10/20 (50%)	6 2	0.170
3.	Previous abortions?	- Yes 6/20 (30%) - No 14/20 (70%)	2 6	1
4.	Source of the flock.	- Homebred 10/20 (50%) - Bought in 10/20 (50%)	4 4	1
5.	Sharing males with other farms?	- Yes 9/20 (45%) - No 11/20 (55%)	6 2	0.065
6.	Vaccination for Chlamydia?	- Yes 5/20 (25%) - No 15/20 (75%)	0 8	0.055
7.	Presence of cats and/or dogs in the farm?	- Yes 11/20 (55%) - No 9/20 (45%)	4 4	1
8.	Breeding method.	-Natural breeding 7/20 (35%) -Synchronized breeding 13/20 (65%)	2 6	0.642
9.	Sharing pastures with other flocks?	- Yes 14/20 (70%) - No 6/20 (30%)	5 3	0.642
10.	Level of farmer education.	-Literate 16/20 (80%) -Illiterate 4/20 (20%)	6 2	1
11.	Farmer knowledge about abortions.[2]	-None 15/20 (75%) -Partial 5/20 (25%)	4 4	0.109
12.	Abortion percentage.	In all farms (2% - 75%)	(3.5% - 75%)	---

Table 4.2 continued

No.	Subject of interest	Farmer responses; numbers followed by percentages	Positive for *Chlamydophila abortus* / N° farms	Fisher's Exact Test for association with *Chlamydophila abortus* / P - value
13.	Stage of pregnancy at abortion.	-2nd trimester 2/20 (10%) -3rd trimester 18/20 (90%)	0 8	0.495
14.	Disposing of abortion materials and fetuses.[3]	- Left on ground 15/20 (75%) - Given to dogs 5/20 (25%)	6 2	1
15.	Still births (death of lambs 24 hours after birth).	- Yes 6/20 (30%) - No 14/20 (70%)	5 3	0.018 (Sig.)
16.	Adding new animals to the farm lately?	- Yes 5/20 (25%) - No 15/20 (75%)	1 7	0.603
17.	Other abortion cases in the area?	- Yes 12/20 (60%) - No 8/20 (40%)	7 1	0.070

[1] : All 10 farms had suffered from pneumonia and mastitis.
[2] : There was a third option asking for good knowledge of abortions with 0 positive responses.
[3] : There was a third option asking for Buried in the ground with 0 positive responses.
- (statistical significance was set to 0.05) in all tests, which were 2-sided.

4.2. PCR Amplification and Sequencing

The amplified PCR product using the primers for the *pmp90/91* gene resulted in amplicons matching the expected 821 nt for a *Chlamydophila abortus* template, where amplified PCR products with primers for the *omp2* gene resulted in amplicons matching the expected 528 nt for forward and reverse primers and 330 nt for intermediate and reverse primers. Two local Palestinian sequences of one sample were generated for the *pmp90/91* gene, and also four sequences of two samples for the *omp2* gene. All used sequences are listed in appendix B. Figure 4.1 shows a schematic drawing of the amplified and sequenced targeted areas in both genes. Figure 4.2 shows representative results of PCR amplification.

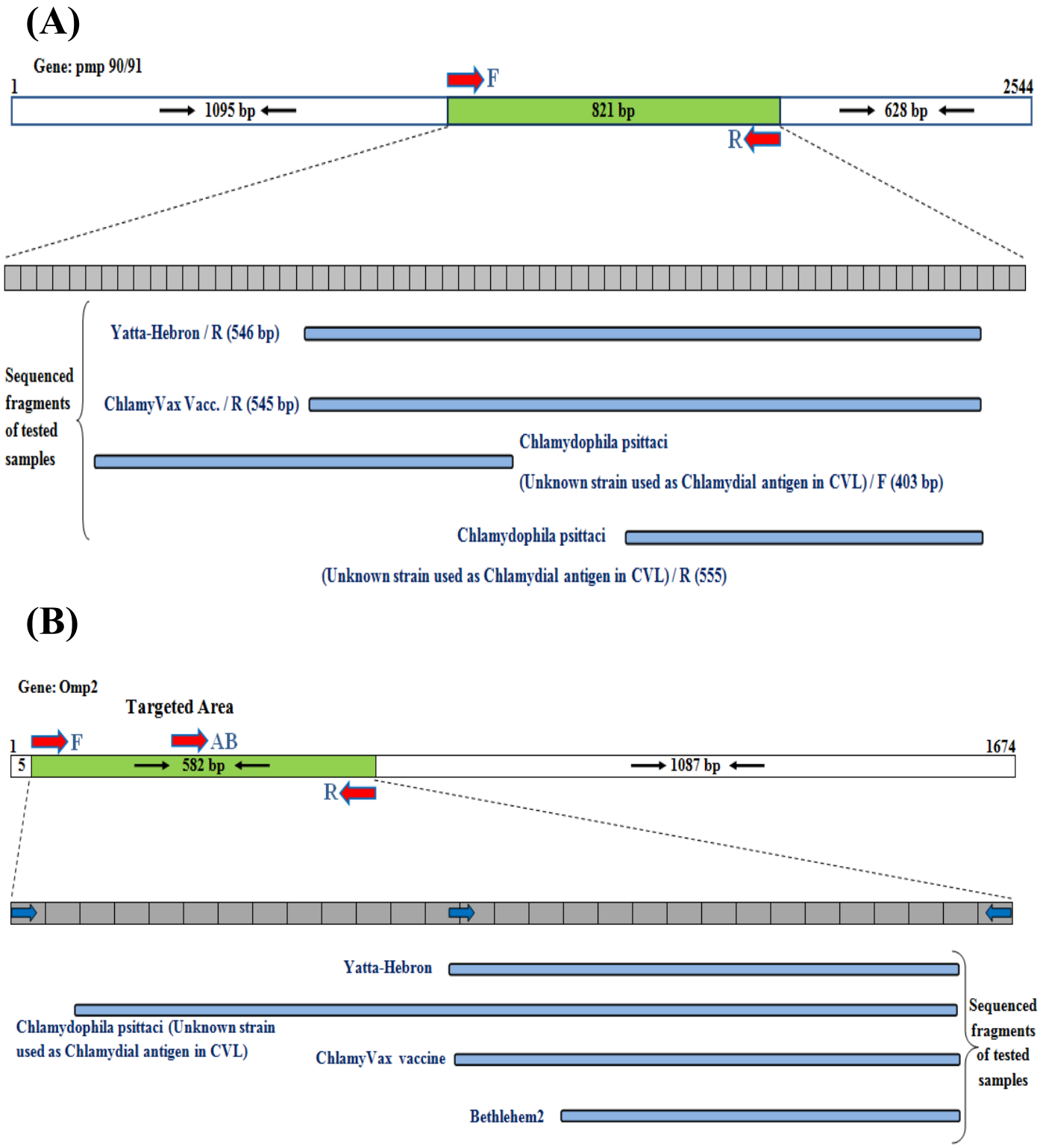

Figure 4.1: Schematic drawing of the amplified targeted areas for which nucleotide sequence data were generated from the *pmp90/91* gene template using a forward and reverse primer (A), and the *omp2* gene template using forward, reverse and intermediate primers (B). For both A and B, the top bar represents the target gene with the amplified region (shaded green), and below that the actual lengths of nucleotide sequence data generated within the amplicon are shown (shaded blue) against a zoomed in representation of the amplicon (shaded grey).

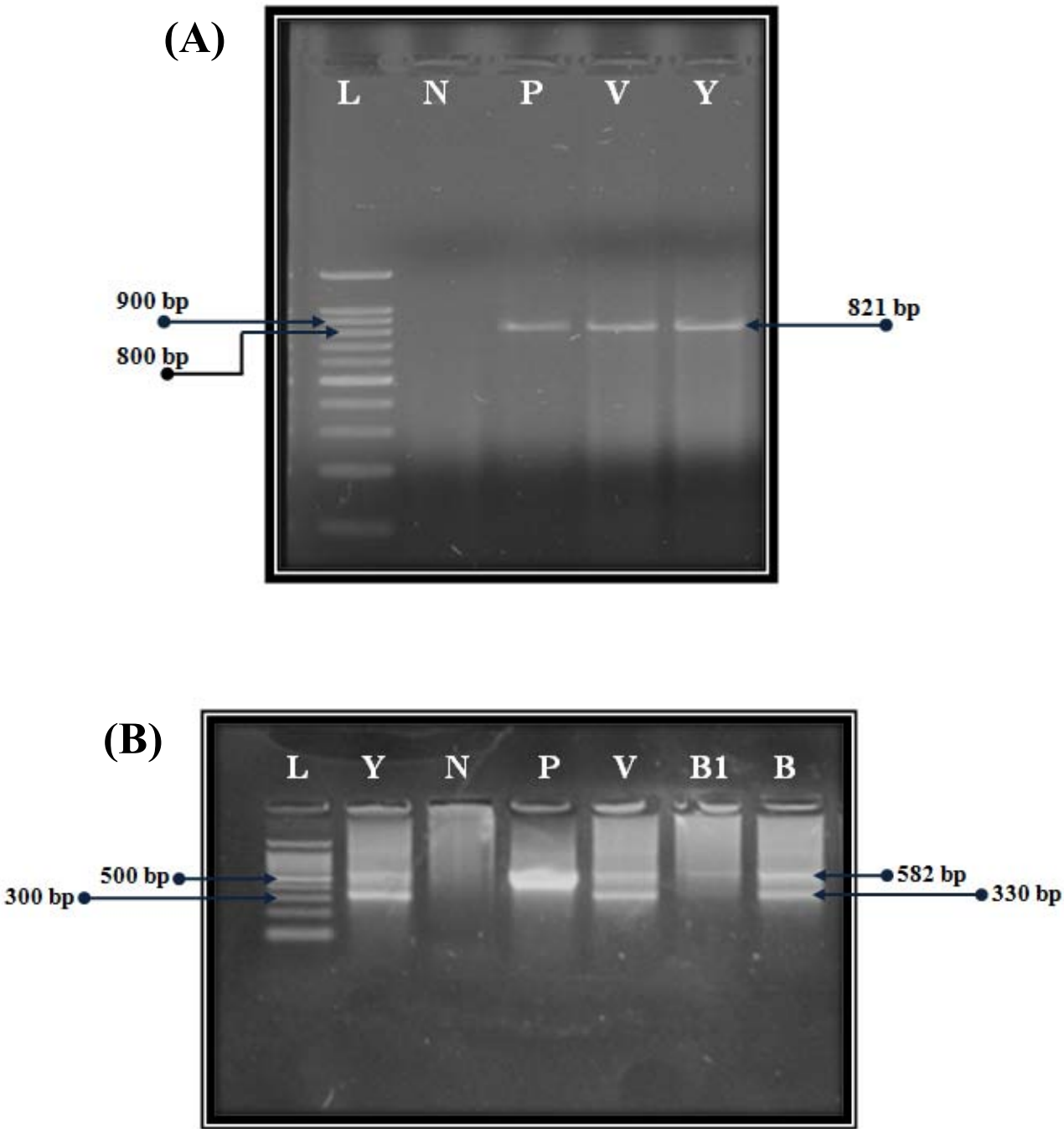

Figure 4.2: Results of amplified PCR amplicons for DNA samples of *Chlamydophila abortus*. A: targeted *pmp90/91* gene, B: targeted *omp2* gene, L: ladder, N: negative control, P: positive control, Y: local abortion case - Yatta / Hebron, V: ChlamyVax vaccine, B: abortion case - Bethlehem (sequenced), B1: local abortion case - Bethlehem/Al-Saff St. (not sequenced).

4.3. Building Maximum Likelihood trees (Dendrogramic trees)

Two trees were generated as one tree for each gene by using the previously described acquired sequences and parallel sequences of reference strains of *Chlamydophila abortus*.

Figure 4.3 shows the maximum likelihood trees for both genes. Clades are separated clearly with high value of boot straps (60 and more) in both trees with the name of each sequenced sample on the top of each branch.

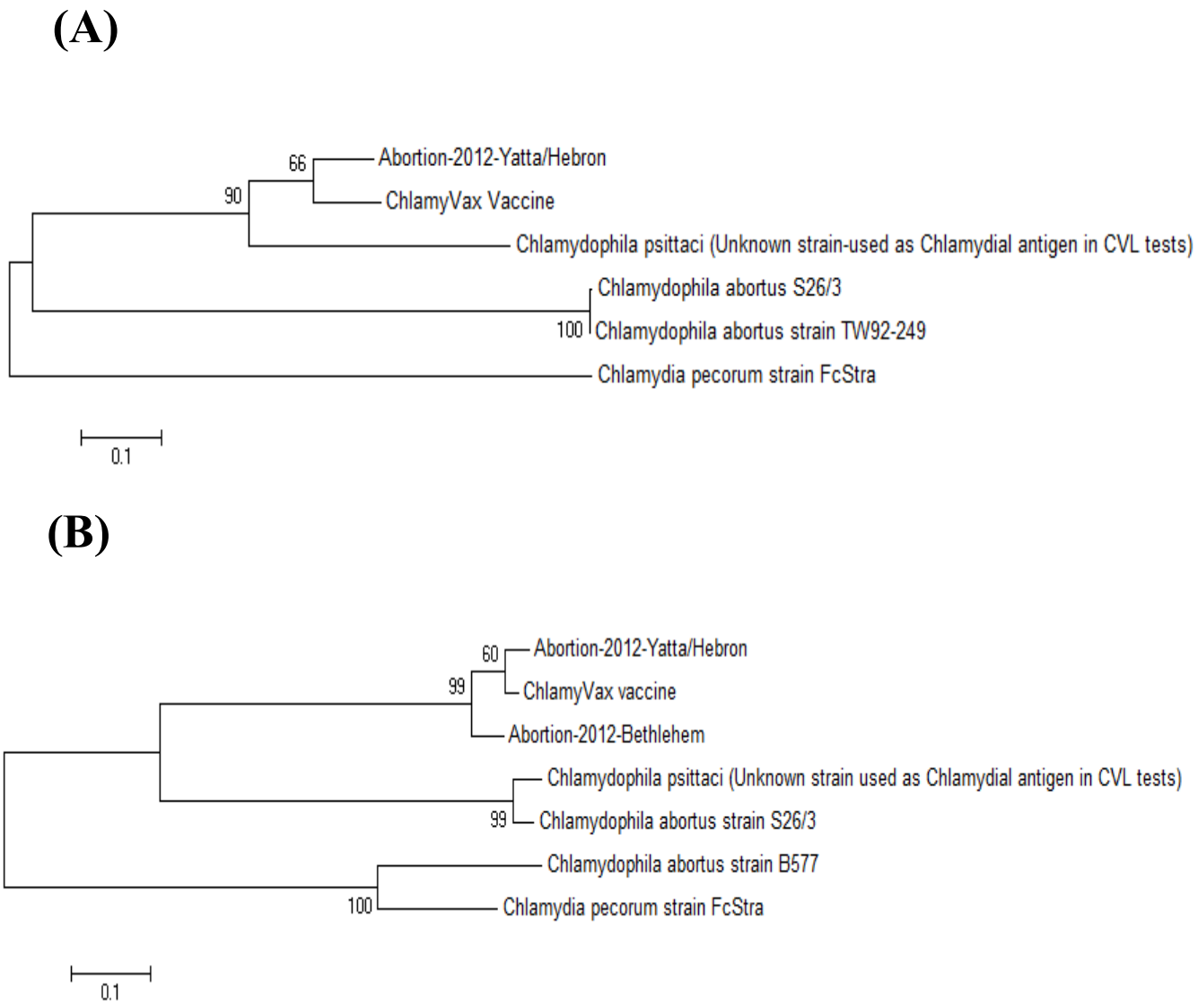

Figure 4.3: Maximum likelihood tree of *Chlamydophila abortus* sequenced samples for: A: *pmp90/91* gene and B: *omp2* gene. *Chlamydophila pecorum* was used as an out-group for both trees. Sequence data were only available for the *omp2* gene of local isolate (Abortion-2012-Bethlehem), while for two reference strains (TW92-249 and B577) data were available for one gene only, *pmp90/91* and *omp2* respectively. The ChlamyVax vaccine represents DNA isolated from a live vaccine used as a positive control in an attempt to extract OVIVAC-CS DNA from the vaccine used in the vaccine trial.

4.4. Serological Trial (ELISA)

Serum samples were assayed by ELISA, as described in section 3.2.10, and for each group and for each animal in the study, values of optical density, antibody percentages (S/P values), dates of vaccination, dates and numbers of sampling, numbers of animals, and their reproductive status, were all recorded in tables in appendix C.

In order to appreciate the whole course of the humoral responses over the 8 months of the trial on an animal by animal basis for each farm, the S/P values or antibody-level percentages for individual animals in each group are presented as linear colored graphs pointed with the levels of antibodies to show the elevation and depression in response to Momp antigen in correlation with vaccination along with time.

Antibody percentage values for each breed at the beginning, two and three weeks after the first vaccination, one week after the second vaccination, and four weeks after the third vaccination are shown in Whisker box plots to allow easier comparison between breeds.

It is obvious from these results that the highest humoral immune response in the non-infected and vaccinated animals was in Baladi goat number-61 with S/P value = 138.4% and OD = 2.749, followed by Assaf sheep number-44 with S/P value = 138% and OD = 2.368; a higher humoral immune response in the infected and vaccinated animals was in Assaf sheep number-100 with S/P value = 148.4% and OD = 2.546 after complete vaccination program. The lowest immune response in the vaccinated animals after complete three shots of vaccination was in Shami goat number-76 with S/P value = 38.8% and OD = 0.667. A lower value was in Baladi sheep animal number-87 with S/P value = 16.5% and OD = 0.335 but after only two months of the first sole shot.

Table 4.3 shows statistical calculation of the alterations in the means of antibody levels of different animal groups. It shows that healthy Assaf sheep responded with a significant increase (P-value = 0.043) in S/P antibody level after the first shot of vaccination, and maintained this significant increase over the subsequent shots. Whereas Infected Assaf sheep did not respond with a significant increase in S/P antibody level until after three shots of vaccination due to the high

levels of antibody in some sheep prior to the first shot of vaccine. Baladi sheep showed no significant increase after only two shots of vaccination. Shami goat exhibit slow immune responses beside late significant increases in the mean value after the third shot of vaccination, whereas Baladi goat showed faster immune response than Shami goat after only one week of the second shot but without significant increase.

Table 4.3: Means of antibody levels of different animal breeds at start, 2 and 3 weeks after the first vaccination, 1 week after the second vaccination, and 4 weeks after the third vaccination, in addition to statistical analysis using Wilcoxon Signed Ranks test.

Group no.	Animal groups	Mean of antibody levels before vaccination	Antibody levels after 1'st shot		Antibody levels after 2'nd shot		Antibody levels after 3'rd shot	
			Mean	P-value	Mean	P-value	Mean	P-value
1	Healthy Assaf Sheep	12.13	65.6	0.043	58.02	0.285	75.64	0.138
2	Infected Assaf Sheep	49.37	56.45	0.735	52.76	0.917	81.83	0.046
3	Baladi (Awwasi) Sheep	29.11	27.90	0.753	29.12	0.500	--	--
4	Shami Goat	14.50	23.96	0.080	21.98	0.500	57.82	0.043
5	Baladi Goat	14.05	17.22	0.068	75.86	0.109	105.3	--

- Shaded blocks in P-value columns imply for significant increase in the mean value in compare with previous mean value.
- Statistical significant was set to 0.05.

4.4.1. Healthy Assaf Sheep

This farm was selected on the basis of its evaluation as being free of *Chlamydophila abortus* before the first shot of vaccine was given, and this status was unchanged throughout the course of the trial. The same held true for all other farms that were evaluated as being free of *Chlamydophila abortus* before the first shot of vaccine. The mean antibody level in Assaf sheep sera prior to vaccination was 12.13%, and three weeks after the first shot of vaccination had increased to 65.6% (n=12), see figure 5.2 that exhibits curves of antibody levels before and after different vaccinations. The increase in antibody level after first vaccination was significant increase (P-value = 0.043), from which it is inferred that a productive humoral immune response had been achieved. Furthermore, in 4 of the animals (41, 44, 47 and 49) their serum antibody level had become comparable to that found after a natural infection, and for all the remaining animals the antibody levels had increased, although this increase was only marginal in 2 of the animals (42 and 45).

The Assaf-sheep breed came from crossbreeding of local Awwasi and east Friesian breeds, which is thought to have resulted in better features of immunity, production, and body conditions. One week after the second vaccination, some animals had maintained the same serum antibody level while others had a lower level; the mean level was 58.02%. This reduction may be due to a short-term neutralization effect occurring between antibodies of the first shot and antigens of the second shot. Five weeks later, after enough time for a robust immune reaction to be expected, six of seven vaccinated animals had responded with higher antibody levels, which declined thereafter. 4 weeks after the third shot of vaccination, the mean antibody level rose to 75.64%, see figure 5.3 to evaluate changes in antibody levels in comparison to each others and positive cut off value (60%) in different stages. These results indicate that the third shot of vaccine has an important role in eliciting a greater humoral immune response in Assaf sheep, while this breed also responded well (better in fact than the other breeds discussed hereafter) to the first and second shot.

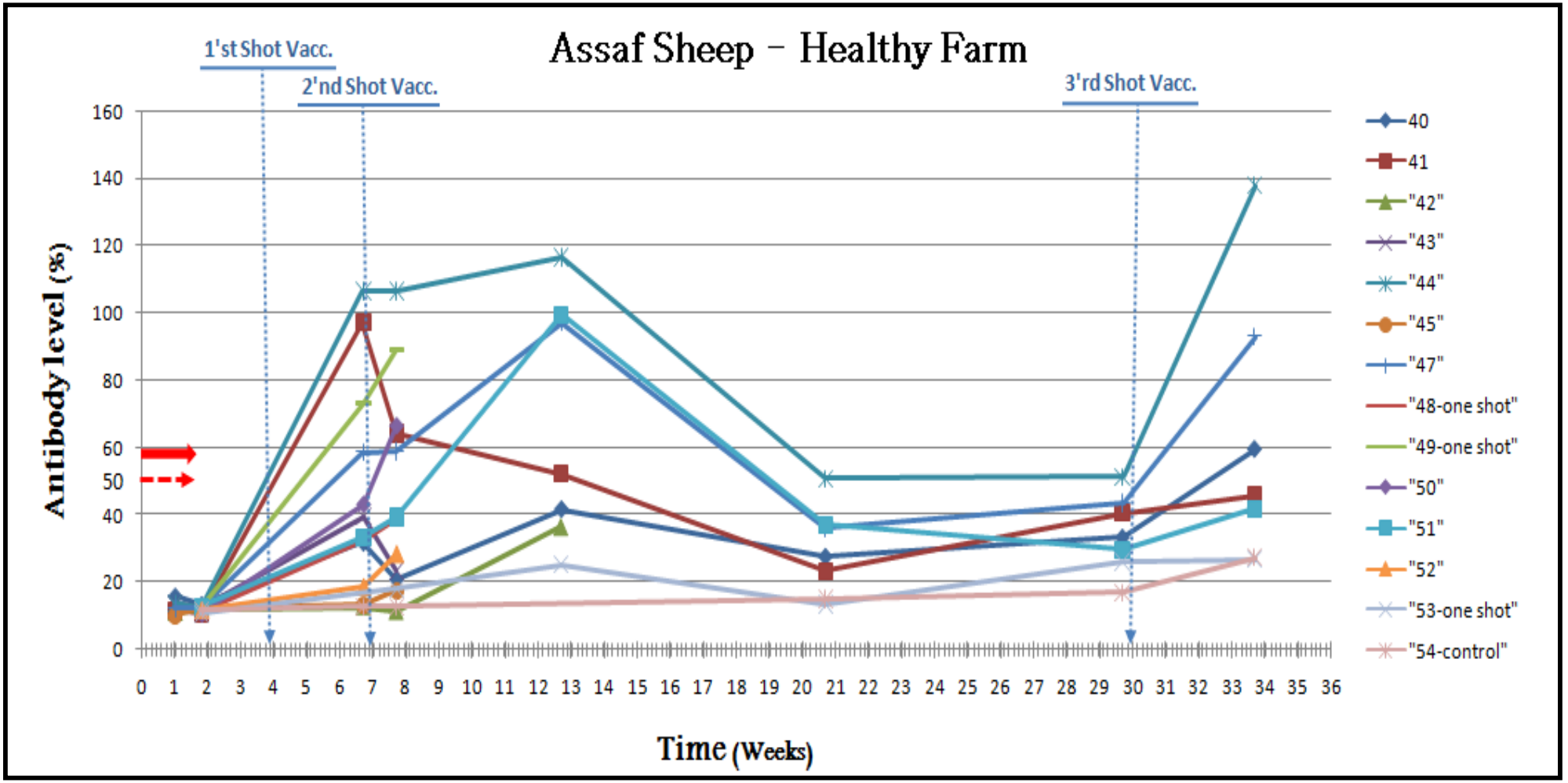

Figure 4.4: Antibody levels for individual animals in Healthy Assaf Sheep farm. Elevations and depressions in anti-Chlamydial antibody levels for individual animals are shown in colored curves along study time, times of vaccinations are also presented, points on curves show the exact time of samplings, constant arrow shows positive cutoff value, and interrupted arrow shows doubtful cutoff value.

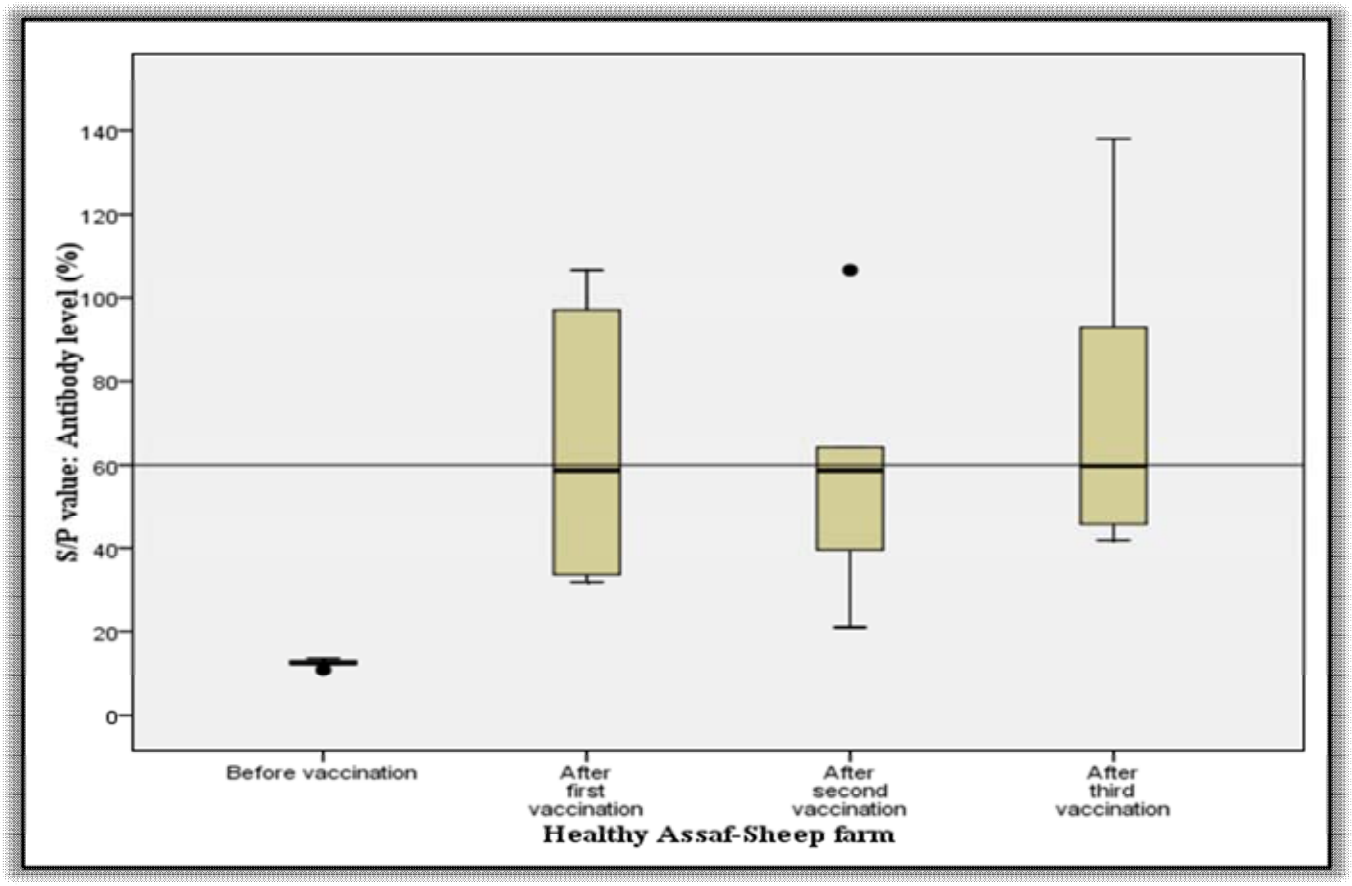

Figure 4.5: Antibody levels of animals in healthy Assaf-sheep farm at the time of samplings viewed in box plot method. Plots show distribution of antibody levels changed around the positive cut off value (60%) each time. Significant increase recorded after the first shot. Values of antibody levels were obtained by indirect ELISA methods. Number of animals was: 6, 5, 5, 5 respectively.

4.4.2. Infected Assaf Sheep

This breed is the same breed as the previous one, but with a history of *Chlamydophila abortus* abortions. In addition to abortions, this farm also suffered from other reproductive problems: infertility, pseudo-pregnancy, stillbirths, and weak lambs. It was chosen, therefore, as a clear example of a Palestinian farm beset by OEA. Animals of this farm showed high antibody levels at the beginning, before vaccination, with a combined mean of 49.37%, and even the lowest antibody levels on the farm were still higher than those of any animal from the uninfected farm, which indicates that all these animals are to varying extents carriers of *Chlamydophila abortus,* figure 5.4 shows curves of antibody levels before and after vaccinations.

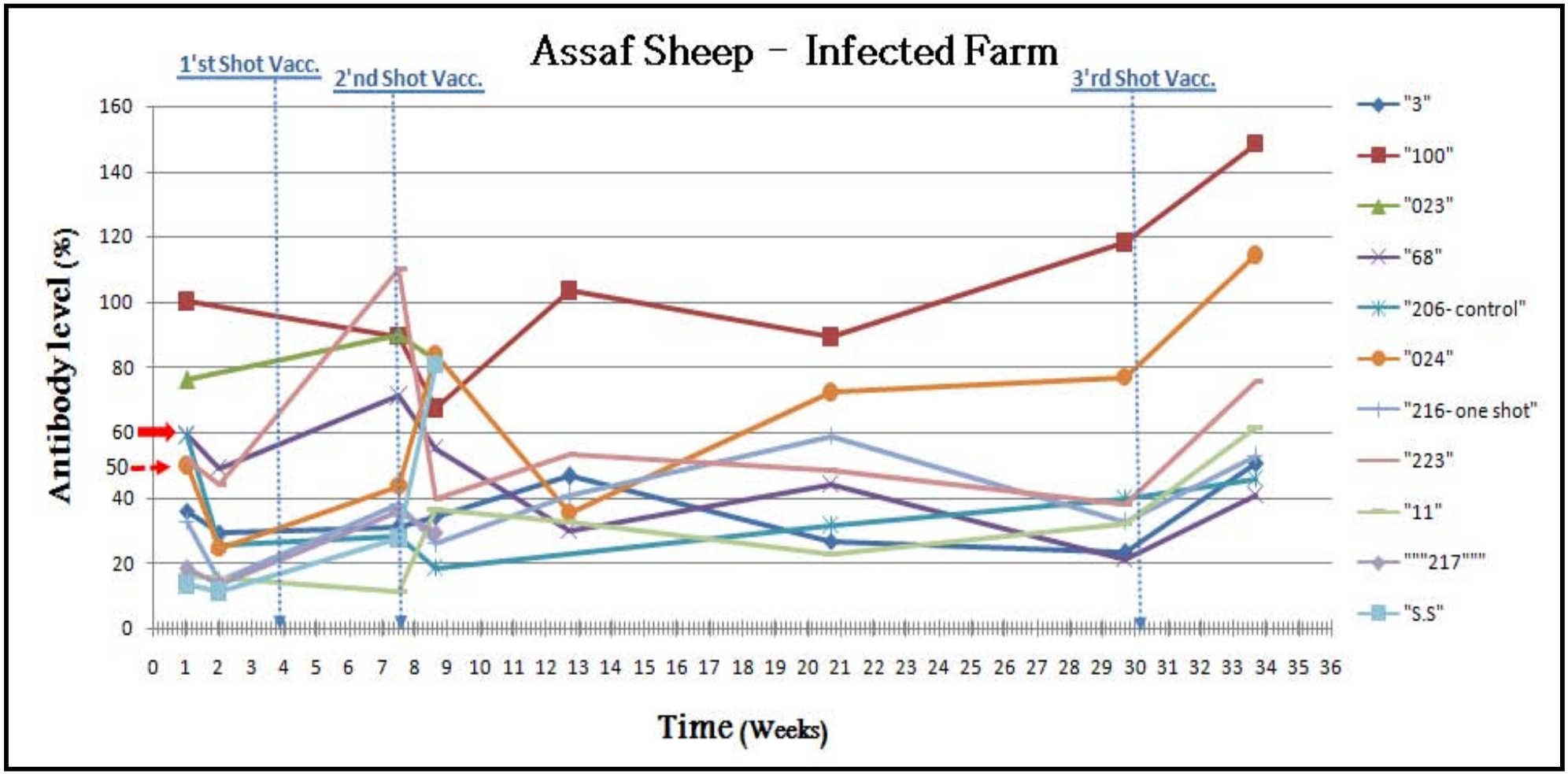

Figure 4.6: Antibody levels for individual animals in Infected Assaf Sheep farm. Levels started with the values and large fluctuation in anti-Chlamydial antibody levels for individual animals are exhibited in colored curves along study time. Times of vaccinations are also presented, points on curves show the exact time of samplings, constant arrow shows positive cutoff value, and interrupted arrow shows doubtful cutoff value.

The 5 ewes with antibody levels of 50% and above (Figure 5.4) all suffered abortion (animals 23, 24, 100 and 223) or delivered 'weak' lambs (animal 68). Of the 4 animals listed above that aborted, 3 did so during the trial and 1 (animal 100) had aborted during its third trimester 2 months prior to inception of the trial (Appendix C). This later animal's serum antibody level was the highest at the beginning of the trial, and remained so till the end. It was infertile throughout the trial. High antibody level for *Chlamydophila abortus* during more than eight months points to the presence of a constant source of Chlamydial antigens, which would prevent normal reproductive activities because of the continuing presence of *Chlamydophila abortus* bacterium in her reproductive tract in high quantities. Sheep no.68 had the third highest level of Chlamydial antibodies (59.2%) at the beginning of the trial and had suffered a previous abortion with *Chlamydophila abortus.* It was pregnant at the start of study, and gave birth to two weak lambs soon after the second shot of vaccination; giving birth to weak lambs is another known effect of *Chlamydophila abortus (Mohale, 2013, Redden, 2013).* Despite its high antibody level at the start of the trial, it had the lowest level of all animals at the end of the trial, and this ambiguous result makes an interesting correlate with the intermediate symptoms of its weak births, rather than outright abortions. While sheep no. 100 appeared to have suffered infertility and consistently high antibody levels throughout the trial, sheep no. 68 was recovering from its previous infection, likely as a carrier animal. The trial was designed to be long enough to allow observation of ewes becoming pregnant after the start of the trial. Indeed, the time between the 2nd and 3rd vaccinations was approximately equal to the 145 day gestation of healthy ewes, and animal '24' (listed above) did become pregnant and it aborted its fetus during the trial.

Of the 5 ewes whose serum antibody levels were below 50% at inception, 2 were pregnant (animals 3 and 11), and did not suffer abortions.

The infected farm of Assaf sheep showed greater fluctuations than the apparently 'clean' uninfected farm in antibody levels for *Chlamydophila abortus.* This may be due to the cyclical

nature of bacterial shedding during estrus of previously aborted animals (Gerber et al., 2007), figure 5.5 shows the non-uniform pattern of fluctuating in antibody means after vaccinations.

One reason to study and compare infected and uninfected farms during the vaccine trial was to attempt to distinguish between vaccinated and infected animals. The vaccination campaigns in Palestine are ongoing and the same ELISA used in this trial to study a specific humoral immune response is used by the governmental laboratory (CVL) to help determine if animals are infected with *Chlamydophila abortus.* This ELISA does not distinguish between vaccinated or infected sheep, but over the course of a trial a new dimension is added, that of time, and this may allow some distinction between the two. Comparing the two farms a distinction was indeed evident. While the sheep of the infected farm maintained relatively unchanging antibody levels throughout the trial, albeit with a lot of fluctuation as mentioned above, the uninfected farm was characterized by a rise, and then more importantly a fall, in antibody levels before levels began rising again after the third shot of vaccine. It is therefore considered possible that re-testing, after not more than 14 weeks (inferred from Figure 5.2) post vaccination date, for animals testing with moderate to high antibody levels could distinguish between an initial result being due to the vaccination or previous infection. Vaccination with a booster shot at the time of re-sampling followed by yet another sample test by ELISA later may help to confirm this result. This could be helpful where farmers are seeking to maintain a farm free of *Chlamydophila abortus.*

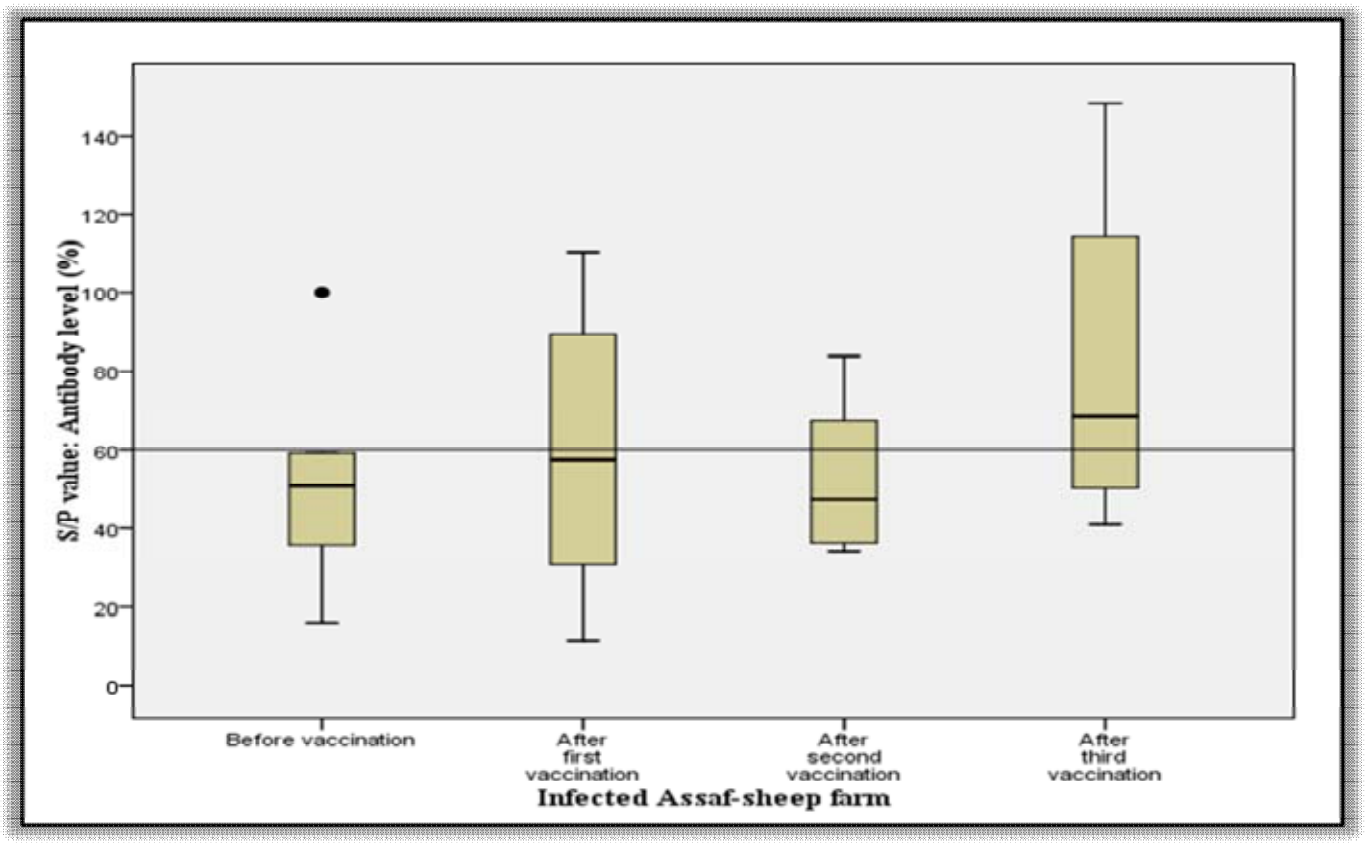

Figure 4.7: Antibody levels of animals in infected Assaf-sheep farm at the time of samplings viewed in box plot method. Plots show fluctuant distribution of antibody levels under positive cut off value (60%) until third vaccination where significant increase was recorded. Values of antibody levels were obtained by indirect ELISA methods. Number of animals was: 7, 7, 6, 6 respectively.

4.4.3. Baladi (Awwasi) Sheep

This breed is thought to be the local sheep breed in our area and south west Asia (Iraq, Syria, Lebanon, Jordan, Palestine, north of Saudi Arabia, and Turkey) (Epstein, 1982). It is characterized morphologically by a fatty-tail and a brownish head. It shows good tolerance to hard weather conditions and good resistance against diseases, but is less productive, however, than other sheep breeds. In this farm of Baladi sheep, all initial serum levels for *Chlamydophila abortus* were below the threshold of 'doubtful positive', as defined by the ELISA kit manufacturer, but it is noteworthy that 1 ram (no. 81) and 1 ewe (no. 83) fell within the upper region of the 'negative' spectrum: 46.3% and 43.6% respectively (the mean of antibody levels was 29.11%), see figure 5.6 that shows antibody levels of animals in this farm during study. While all other Baladi sheep ewes in the study were reproductively normal, this ewe suffered from repetitive estrus and was temporarily infertile at the beginning of the study, and this indicates that even negative results

from the ELISA kit are informative. While the kit is used routinely as a simple yes or diagnostic test, greater scrutiny of the ELISA result data, especially when connected to clinical history, could help veterinarians to make more informed decisions than they do based on a simple yes/no output result from the CVL.

Throughout the trial period, the mean antibody level only once exceeded the initial level, and then only marginally so with a value of 34.1%, see figure 5.7 also to realize the antibody levels in compare to positive cut off value. This very modest effect was measured six weeks after the second shot, whereupon the trial was terminated by the farmer who reported deaths and emaciation caused by a bout of diarrhea and anorexia on his farm. Baladi sheep demonstrated a much lower humoral immune response than that of Assaf sheep over the same trial period, and it is possible that this represents a real difference in the pattern of immunity between these breeds, although it may be that the bout of diarrhea that led to premature termination of the trial may have also depressed the animals' immune system.

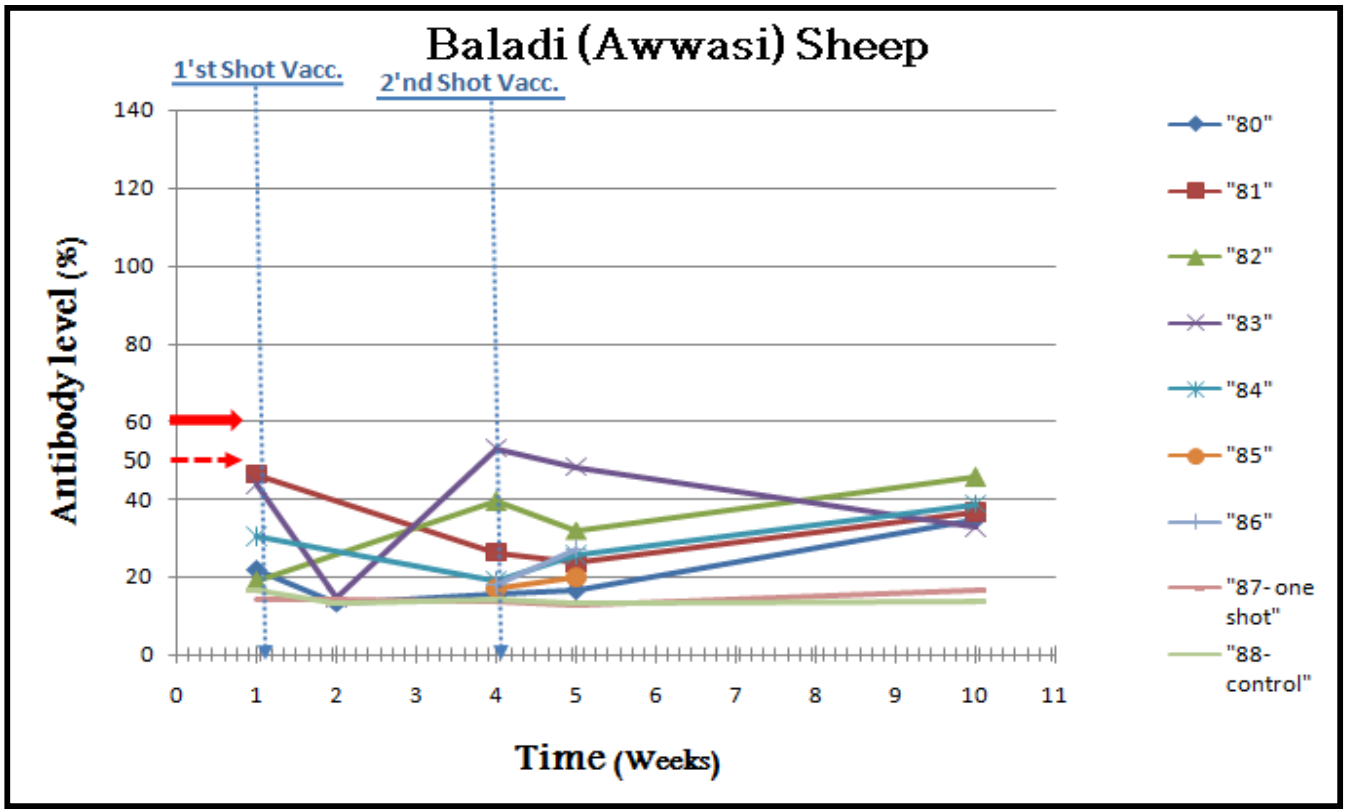

Figure 4.8: Antibody levels for individual animals in Baladi (Awwasi) Sheep farm. Only two shots were applied and no value exceeded positive cut off value. Times of vaccinations and exact time of samplings were shown. Immune response of this breed become much lesser than that of similar breed (Assaf-sheep).
value.

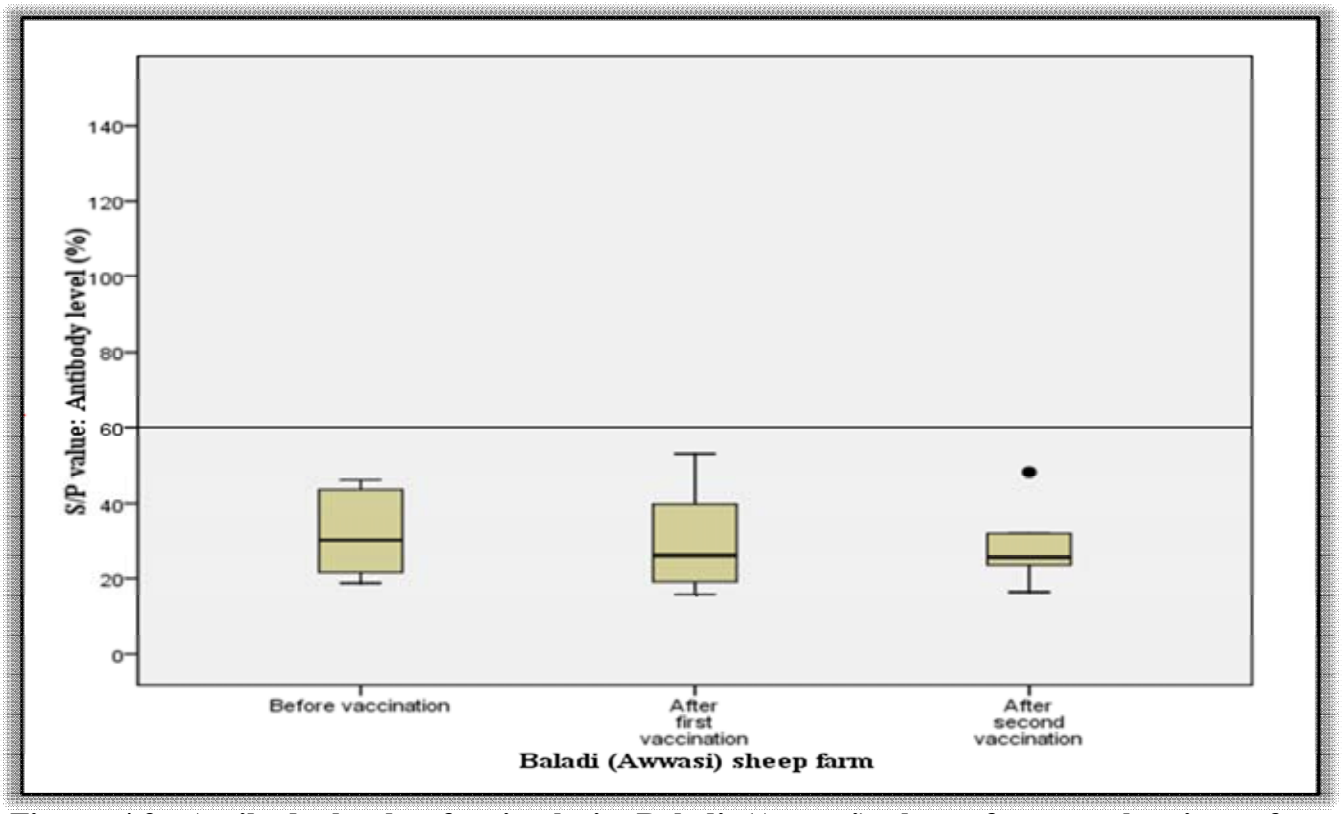

Figure 4.9: Antibody levels of animals in Baladi (Awwasi) sheep farm at the time of samplings viewed in box plot method. No significant increase was recorded after two shots of vaccination. Number of animals was: 6, 5, 5, 5 respectively.

4.4.4. Shami Goat

This breed is thought to originate from Damascus in Syria and is morphologically most easily distinguished from local Baladi breed by the prominent curved shape of the front of the head. This breed is usually less tolerant of etiological diseases and other disease conditions than is the Baladi breed. This group of animals had a good health status in general and was not reported to have faced prior *Chlamydophila abortus* infection . Of all breeds studied, Shami goat gave the poorest humoral immune response. Initially, animals showed a low antibody level with mean = 14.50%, which rose to 23.96% three weeks after the first vaccination and decreased to 21.98% one week after the second vaccination. After that, four of six vaccinated animals showed slight elevation five weeks later, and five of six vaccinated animals showed another decline after eight weeks. Despite the slowness of the humoral response to develop, all goats that received 3 shots of vaccine had distinctly higher antibody levels at week 31 than week 1: four weeks after the third shot of vaccination, the mean antibody level was 57.82%, figure 5.8 illustrates these changes in curves. This consistency of elevation was uncommon among other breeds and is not explained upon to our knowledge.

These results show that three shots at least were needed to develop a significant antibody level in this breed of goat, and none of the previous two shots was able to do that alone, figure 5.9 shows the increase in antibody levels at samplings.

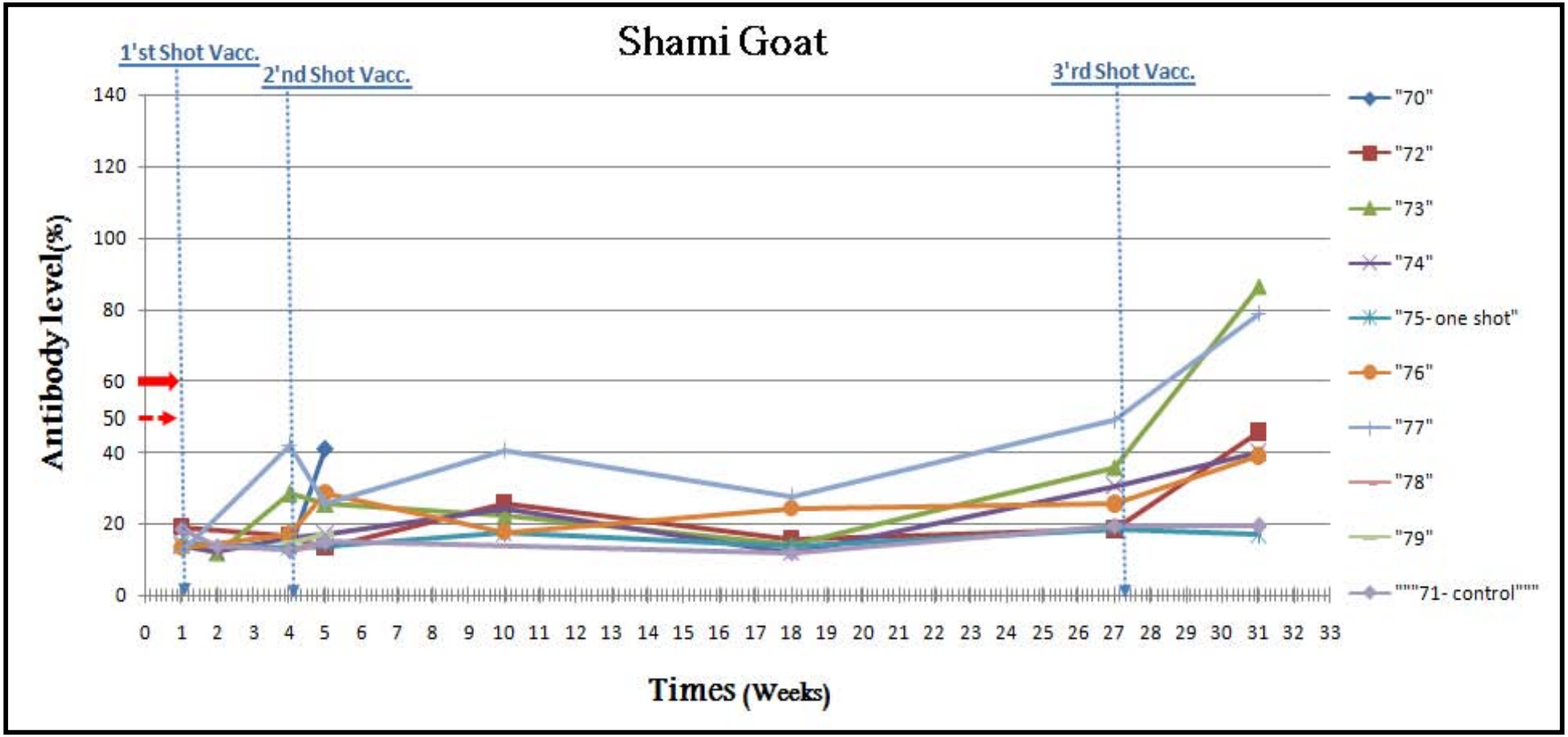

Figure 4.10: Antibody levels for individual animals in Shami goat farm. Antibody levels still beneath the positive cut off value until the third shot after which mean increased well. Colored curves for individuals and times of vaccinations are also presented. Points on curves show the exact time of samplings, constant arrow shows positive cutoff value, and interrupted arrow shows doubtful cutoff value.

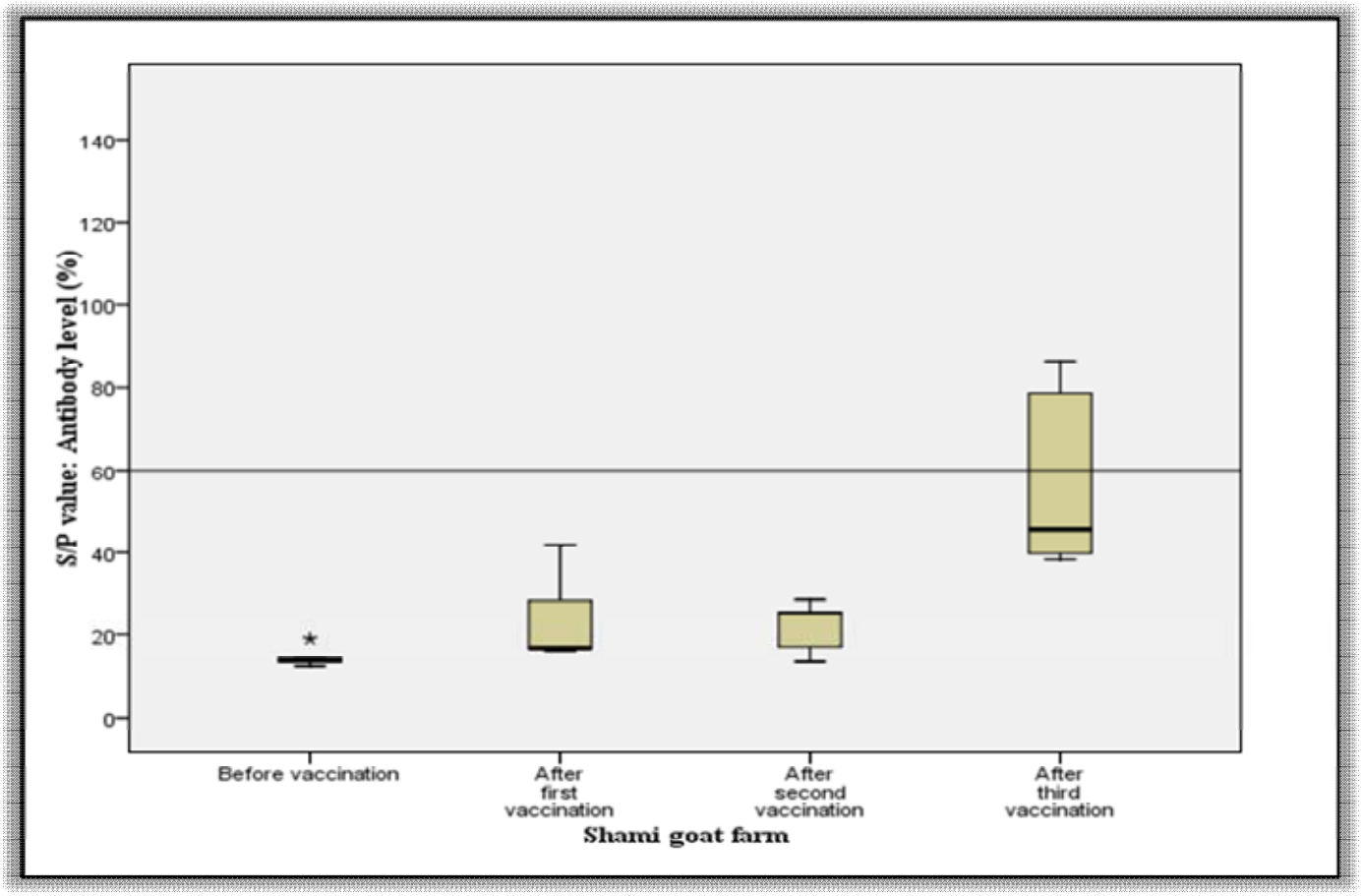

Figure 4.11: Antibody levels of animals in Shami goat farm at the time of samplings viewed in box plot method. Significant increase was recorded after the third shot of vaccination although mean did not exceed the positive cut off value, while slight changes were recorded before that point. Number of animals was: 6, 5, 5, 5 respectively.

4.4.5. Baladi Goat

This breed is thought to be the local indigenous goat breed in our area, and farmers consider it to offer better resistance to diseases and to be more tolerant of difficult weather conditions than Shami goats. Because of its good accommodation with local conditions and good productive features, this breed is widely distributed in Palestine and is much more common than the Shami breed. In this trial, Baladi goats displayed a generally higher and faster humoral immune response than Baladi goats. In the beginning and before the first vaccination, all animals except one showed low antibody levels (less than 15%), but one goat (62) had a high antibody level (57.6%) and was excluded from statistical calculations, The mean increased to 17.22% after the first vaccination. One week after the second vaccination, all vaccinated animals had a higher immune response, and a non-significant increase with antibody level mean = 75.86% due to low number of samples. Unlike other groups of animals, sampling was done eleven weeks later and antibody levels were

found to decrease except for animal no. 62, which kept the same antibody level after the second vaccination. Two animals died after this point (animals 56 and 59), because of pregnancy toxemia at their final stage of pregnancy as described and diagnosed by the supervisor veterinarian. Four weeks after the third shot of vaccination, one goat (no. 61) showed a great humoral immune response (47.3% - 105.3%), while goat no. 62 showed slight elevation from already very high level (105.3% - 108.9%). It is likely that goat no. 62, had been previously infected before the beginning of the trial and showing normal reproductive activity and no signs of Chlamydial infection could be considered as either an asymptomatic carrier or had acquired a strong humoral immunity from previous contact with *Chlamydophila abortus* (Gerber et al., 2007), see figure 5.10.

Baladi goat breed required two shots to develop detectable but not significant antibody level increases, see figure 5.11, and this was faster than Shami goat which required three shots, which suggests a better humoral immune response given by Baladi goat than Shami goat.

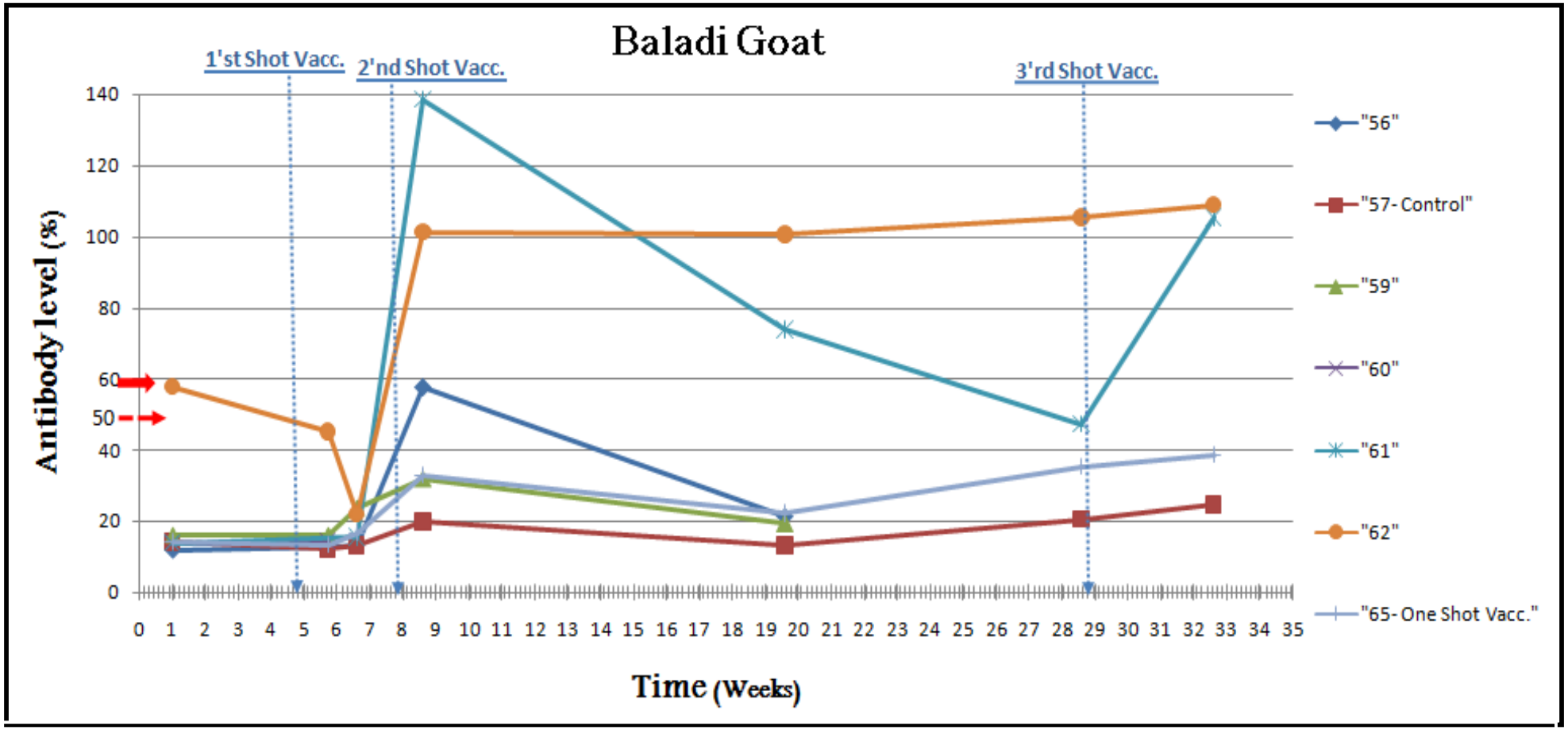

Figure 4.12: Antibody levels for individual animals in Baladi goat farm. Antibody levels showed good immune response directly after the second shot of vaccination. Two animals (59 and 56) were lost before the third shot, and one animal (62) was excluded from statistical analysis because of high level at the beginning. Colored curves for individuals and times of vaccinations are presented, Points on curves show the exact time of samplings, constant arrow shows positive cutoff value, and interrupted arrow shows doubtful cutoff value.

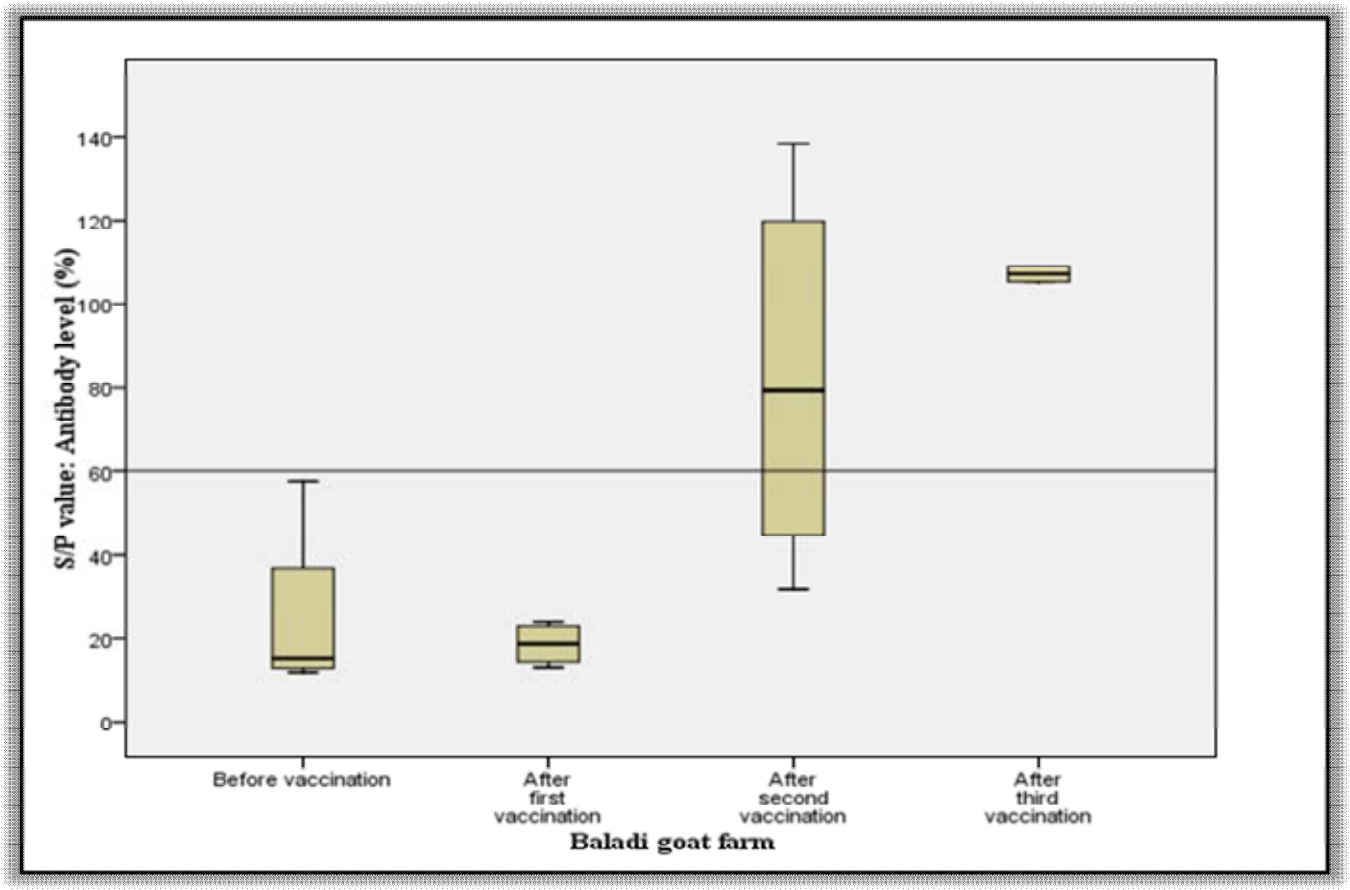

Figure 4.13: Antibody levels of animals in Baladi goat farm at the time of samplings viewed in box plot method. No significant increase was recorded after any of the three shots although mean increased directly after the second shot and exhibit good response. This thought to be because of low number of animals that were tested. Number of animals was: 4, 3, 3, 1 respectively.

Chapter 5

5. Discussion

5.1. Abortions Survey

The survey aimed primarily to determine the incidence of *Chlamydophila abortus* on local farms in the Bethlehem area as well as looking at 2 other infectious agents and relevant farm management practices via a questionnaire that was divided into four sections. The first one requested data in relation to the owner, farm position, type and number of farm's animals. The second section asked for flock history, to reveal any previous health problems and other common related information. The third section concerned the recent abortion case/s. The last section was designed to record number and type of each collected sample from individual animals and the result of laboratory testing from the Central Veterinary Laboratory, and finally if there were any prominent post mortem signs of necropsy.

Questionnaire data was received for only 20 farms for which samples were sent for diagnostic testing. This number was too small for Chi squared tests of association, and in fact the only questionnaire result that did show an association, with the more rigorous Fischer Exact test, was the co-occurrence of *Chlamydophila abortus* on the farms where still-births were reported (where the new-born lamb dies within 24 hours of birth). Data collection was hampered by the availability of diagnostic data and non-compliance of farmers with the lengthy questionnaire process, and future work should seek to reduce the number and complexity of questions to increase the responsiveness of farmers. Another possible explanation for lack of association is that in the absence of control and testing, *Chlamydophila abortus* has become so widely spread that a moderate increase in risk from any given poor management practice is masked by other confounding factors. In the following paragraphs, attention is drawn to a few observations that could help to guide future studies.

8 farms out of 20 were positive for *Chlamydophila abortus* (40%), which was the most common of 3 major abortive pathogens: the occurrence of *Coxiella burnetti* and *Toxoplasma gondii* was 25% and 20%, respectively.

Only 1 of the 20 farms surveyed was solely a goat farm, and when it is considered that participation of farms in the survey was based on the occurrence of abortions, this suggests that Palestinian sheep were more affected than goats, which coincides with previous findings that sheep are more commonly diagnosed with *Chlamydophila abortus* than are goats (Carter and Wise, 2003, Aitken and Longbottom, 2007).

The questionnaire asked about previous health problems, which revealed that 50% of the studied farms experienced pneumonia and/or mastitis, which indicates that these diseases are widespread and require attention by national veterinary authorities.

Animals were brought in from outside sources in half of the inspected farms (50%), and none of them were tested for infectious agents before introducing them to the owner farm. Such a practice is generally considered risky (OIE Terrestrial Manual, 2012, Pelzer, 2012, CFSPH and IICAB, 2005).

Infected shared males are thought to play the role of a mobile infecting source for every female animal they mate with, and venereal transmission of *Chlamydophila abortus* has been suggested by previous studies (Mearns, 2007, Livingstone et al., 2008, CFSPH and IICAB, 2005, Merck Veterinary Manual 9th Edition, 2005).

This study showed that 66.7% of the farms that shared males were infected with *Chlamydophila abortus*. Nevertheless, statistical analysis did not show a relation between infection and this management mistake (P-value =0.065).

It is surprising that despite many *Chlamydophila abortus* vaccination campaigns in Palestine over recent years, only 5 out of the 20 farms of this study had vaccinated their animals against *Chlamydophila abortus*. This indicates that recent vaccination campaigns failed to administer enough doses of vaccine to provide adequate coverage.

Of the 5 farms whose animals were vaccinated, none were positive for *Chlamydophila abortus.* However, statistical analysis did not demonstrate significant (The result was borderline with a P-value = 0.055).

Cats and dogs may serve as reservoirs and play a role in transmitting infectious diseases like Chlamydiosis to animals either (Rand, 2007). Despite such risks, 55% of the farms were co-habited by dogs or cats.

Synchronized breeding methods were more usual than natural methods and neither displayed any association with diagnosis of *Chlamydophila abortus* (P-value =0.642).

Sharing pasture land with sheep from other flocks did not correlate with a greater infection rate than for not sharing pasture (P-value = 0.642).

Practical knowledge about flock history, causes, signs, and prevention of *Chlamydophila abortus* infection have been shown to be very important (Redden, 2013), which leaves room for improvement in the future as 75% of the owners surveyed had no information about abortions and their transmission.

Abortion materials and fetuses are heavy sources of infecting agents and it is strongly recommended to dispose of these by burying or burning (Pelzer, 2012, Redden, 2013). Remarkably, however, not a single farm in the study used this method of disposal. Two equally unhygienic disposal methods were utilized: 75% of the studied farms left these materials on the

ground, and 25% allowed dogs to eat these materials in their farms. No significant relatedness (P-value =1) and no distinction was observed between these methods and presence of *Chlamydophila abortus*. Dogs can be a heavy transmitter for *Chlamydophila abortus* through their bodies, while on the other hand, aborted material left on the ground contaminates feed, water, and grass allowing for uninfected sheep to become infected while eating and drinking (Redden, 2013, Mohale, 2013).

Adding new animals to a farm is a risk when public livestock markets are unregulated and essential laboratory tests are neglected (Figure 5.1.).

Figure 5.1: A livestock market in Bethlehem city shows farmers besides their animals in dispersed clusters where mingling is possible.

5.2. Sequencing Analysis

The Dendrogramic trees for both sequenced genes showed that little genetic difference between local samples and world reference strains downloaded from the NCBI database and even less difference among the local samples.

OVIVAC-CS was used in our vaccine trial and is marketed as a safe, inactivated vaccine (HIPRA Laboratories, 2012b). The most effective method of inactivation of infectious organisms for vaccines occurs by preventing the possibility of replication by destroying nucleic acid. It was therefore of interest to check the quality of DNA that could be extracted from the vaccine, and therefore a DNA extraction procedure for OVIVAC-CS was performed alongside a live vaccine (ChlamyVax) as a positive control. Subsequent PCR and then sequencing was possible for the ChlamyVax control, but not for OVIVAC-CS as expected. The aligned ChlamyVax vaccine sequences are included in the dendrograms (Figure 4.3) and it is interesting to note that these sequences appear closely grouped with the local sequences, which raises possible questions as to the reason for this genetic similarity.

5.3. Serological Trial (ELISA)

This study started with seven farms: four farms of Assaf sheep and one farm of each of Baladi sheep, Shami goat, and Baladi goat. Three of the four Assaf sheep farms were previously diagnosed to be infected with *Chlamydophila abortus*, but two of these three infected farms were excluded after initial sampling, and prior to the first vaccination shot, since the owner of one farm sold his animals and transferred them to another area, while the animals of the second farm were not given numbered ear tagged (data are not shown for these two farms). Ten weeks after the study began, the Baladi sheep farm also had to be excluded since the farm's owner refused to continue under the trial. He wanted to slaughter some animals and sell the others. In addition to the withdrawal of entire farms, some animals were lost from farms that continued to participate in the trial due to slaughter or premature death by diseases unrelated to the subject of this study.

Animals with different reproductive and health classes in these groups were incorporated to correlate serological and infected status with clinical findings and to simulate the real situation of the animal farms.

To our knowledge, no previous research studied the differences in humoral immune response at the level of different breeds, especially those living in our areas. As expected before, it was found that a broad variety in humoral immune response among these breeds. In general Assaf-sheep breed animals showed faster and better immune response than Baladi sheep animals, whereas Baladi goat showed faster and better immune response than Shami-goat with regarding to the whole flocks after complete vaccination program.

At the level of individual breeds and as it was anticipated also, it was found that a very wide range in humoral immune response among different animals in the same breed, each animal breed demonstrated individual animal-specific immunoreactions after complete vaccination program. These results matched with results of (Gerber et al., 2007) that points clearly to individual immunoreactions between sheep can vary considerably. These immunological differences may referred to various endogenous factors like sex, age, function of immune system, and genetic factors, besides exogenous factors like nutrition, stress, and individual exposure for infectious disease (Loveren et al., 2001). Small age can affect maturation of immune system and hence the response of this system. By studying the genetic factors and comparing with immune responses, it has been found that non responders or low responders had higher rates of homozygosity among alleles (Loveren et al., 2001), since similar alleles exhibit lesser diversity in genetic functions. Nutrition as one of the exogenous factors also affect immune response, protein deficiency resulting from malnutrition and anorexia seriously influence immune response, Besides many other reasons that need much investigation at the genetic level. Some infectious viral and bacterial agents can suppress the immune system such as Salmonella, where others may have cross-reacting with the agent provided by the vaccine if the animal has both agents in its body (Loveren et al., 2001).

It is clear from the results that no distinction can be made between infected and vaccinated animals by our ELISA kit, and that supports the results of (Gerber et al., 2007) who showed that the antibody value range in a lately vaccinated flock could be higher than that of a naturally infected flock during the sampling period. This study observation also correlates with (Boril et al., 2005) in which 3 of 3 vaccinated animals gave titers as high as 4 of 4 infected animals. Therefore, it is very important to match the clinical symptoms and disease history with the results of serology testing for *Chlamydophila abortus* infections, especially in case of high antibody level. On the other hand, ELISA is a useful test for surveying infectious diseases such as Chlamydiosis in non-vaccinated animal flocks.

By comparing healthy and infected Assaf sheep farms in this study, it was noticed that infected animals generally maintain high antibody level because of persistent existence of Chlamydial antigens, while vaccinated animals shown increases followed by decreases in antibody levels over time. It may therefore be possible to attempt some distinction between vaccine induced and replicating Chlamydial antigens induced response.

5.3.1. Vaccine Efficacy

The obtained results above gave an indication of good efficacy for the used vaccine, since 41% of the vaccinated animals responded to vaccination and developed a detectable humoral immune level during the course of the trial. OVIVAC-CS is an inactivated vaccine, but it seemed to give a better result than a previous trial of a previous inactivated vaccine (inactivated egg-grown preparation of *Chlamydophila abortus*) where only one sheep out of three develop a detectable antibody response 82 and 98 days after vaccination (Boril et al., 2005).

5.3.2. Vaccine Safety

The safety of OVIVAC-CS vaccine was also was indicated by getting new healthy births from completely vaccinated animals in Healthy Assaf sheep, Shami goat, and Baladi goat farms where birth for vaccinated animals were followed up, and no abortions were noted in the vaccinated animals that were negative for *Chlamydophila abortus* at the start of the trial.

By this study, unsatisfactory results of the various vaccination campaigns applied on the Palestinian flocks can be related to incomplete vaccination protocol, since most vaccinated animals received only one shot and few animals received two shots, and most animals received no shots taking in consideration that infected farms should also be treated with Oxytetracyclines besides vaccination as indicated by some previous studies (Buxton and Henderson, 1999).

An important point is that no vaccine producer promises full protection against *Chlamydophila abortus* (Moredun Research Institute, 2010).Furthermore, vaccination doesn't stop shedding of Chlamydial agents from already infected sheep during lambing (Boril et al., 2005), which certainly means the maintenance of the infection cycle in unprotected animals in the same flock.
The above mentioned points can explain why abortions continued despite the applied vaccination campaigns, and why prospected results were not fully achieved.

Chapter 6

6. Conclusions and Recommendations

Conclusions

- Based on a survey of abortions on 20 farms, *Chlamydophila abortus* infection was the most common of 3 major abortive pathogens: the occurrence of *Chlamydophila abortus* was 40%, while the occurrence of *Coxiella burnetti* and *Toxoplasma gondii* was 25% and 20%, respectively.
- There were no indications that previous or current vaccines posed a safety hazard. The survey of 20 farms showed that *Chlamydophila abortus* was absent from abortion materials where farms had been previously vaccinated, and none of the pregnant ewes that were vaccinated with OVIVAC-CS in this vaccine trial suffered abortions.
- OVIVAC-CS is capable of producing a significant increase in antibody levels for Assaf sheep after the first vaccine shot, while Shami goats did not generate a significant rise in antibody levels until after the third shot. Baladi sheep did not generate a significant response after the first or second shot and the results for Baladi goats were ambivalent. These variations in both the scale and timing of humoral immune responses between different local breeds of sheep and goat, should be taken into consideration in future vaccination programs.
- Analysis of sequencing data from 2 genes revealed that local strains of *Chlamydophila abortus* that were sequenced from DNA extracted from abortion materials in Yatta/Hebron and Bethlehem share considerable genetic similarity to each other and to international reference strains of *Chlamydophila abortus*, which indicates that vaccines that are effective internationally would likely be effective here in Palestine.

Recommendations

- The manufacturer's recommended vaccination protocol should be adhered to, because farmers typically fail to administer recommended booster shots, which this study shows to be important for initiating humoral responses.
- No easy distinction can be made between infected and vaccinated animals using current serological tests, and it is recommended that veterinarians take note of vaccination history when submitting samples for diagnosis by ELISA.
- Poor farm management practices were in evidence on the surveyed farms. The most egregious practice was the complete failure to adequately dispose of aborted fetuses and placental material. Despite being an infection risk, these materials were either left on the ground or fed to dogs in all the farms that were surveyed.

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Appendices

- Appendix A: Questionnaire Form

- Appendix B: Sequences of constructing trees

- Appendix C: Tables of serological trial results in different animal strains

- Appendix D: Output of statistical analysis by SPSS

Abortions in Palestinian Flocks (Sheep & Goat)
(Questionnaire)

This questioner is for the sole purpose of scientific research about abortions in flocks of sheep and goats in Palestine, and the information gained from it will be used as part of a Master degree in Molecular Biotechnology and may be published, without naming individual farms or farmers, in the scientific literature.

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Abu Romman Mountain
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Section A : Farm Data

Farmer Name:……
Address:……
Phone:……
Date:……
Total Flock No.:…… Flock species: a. Sheep No:……
b. Goat No:……

Sheep type: a. Awassi b. Assaf c. Other
Goat type: a. Baladi b. Shami c. Other

Section B: Flock History

1. Did the flock have any previous health problems, diseases? If yes what were they?
   a. Yes b. No

……
……..….

2. Did the flock face abortion problems before? If yes; when, and what was the diagnosis and treatment?
   a. Yes b. No

……
…….

3. What was the percentage of abortions if present?

……

4. What is the flock feed?

……
…….

5. Is the flock homebred or are bought in?
   a. homebred b. bought in

6. Does the farmer share rams/bucks with other farmers?
   a. Yes b. No
7. What is the source of water?
   ……
8. What vaccinations and antibiotics are used?

……
…….

9. Has the flock undergone recent handling or transportation?
   a. Yes b. No
10. Are there cats, dogs, or rodents on the farm?
    a. cats b. dogs c. rodents
11. What is the method used for breeding?
    a. natural breeding b. vaginal sponges and hormone c. artificial insemination (without males)
    d. no breeding occurs
12. Does the farmer graze his animals in common pastures?
    a. Yes b. No
13. What is the level of farmer education, and his Knowledge about abortions?
    a. illiterate b. elementary c. secondary or higher
    d. no knowledge e. intermediate f. good

Section C: Recent Abortion Case(s)

1. How many abortions have occurred? Percentage (%)

……

2. Are the animals that aborted sick? What signs are there?

……
……

3. What prominent signs appeared on aborted fetuses?

……
……

4. At which stage did the abortion(s) occur?

a. First trimester b. Second trimester c. Third trimester

5. How were the aborted material and fetus disposed of?

a. given to dogs b. left on the ground c. buried in the ground

6. Did any newborn animals die within 24 hour after birth?

a. Yes b. No

7. Did the farmer add new animals to the flock recently, when?

a. Yes b. No ……

8. Are there any other abortion cases in the same area?

a. Yes b. No

** For Veterinarians, please fill the information regarding the aborted animals and their samples in **Part A ONLY** in the next page.

Part A											Part B									
						Samples					Diagnosis (For Laboratory Use Only)									
Animal No.	Type		Age (Months)	Sex (M,F)	Abortion Date	Sampling Date	Serum	Placenta	Fetus, Still Birth	Vaginal Swabs	Chlamydophila Abortus		Toxoplasma Gondii		Coxiella Burnetii		Neospora Caninum		Brucella	
	Sheep	Goat									ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR
1																				
2																				
3																				
4																				
5																				
6																				
7																				
8																				
9																				
10																				
11																				
12																				
13																				
14																				
15																				
16																				

Post-Mortem Signs in Fetuses or Still Births:

-
-
-
-
-

November 2011

Appendix B: Sequences of constructing trees.

Sequences of local strains, vaccine, and positive control in CVL (*Chlamydophila psittaci*) with other well known strains of *Chlamydophila abortus* for both genes in addition to outer groups sequences.

1. pmp90/91 gene:

>Yatta sample
GTAAAGTGCCGGTAGAGCCATGAGCTTAGTTATATTGCTGAAAAAGGCTGTGAGCAG
AAAGTGATAGGATACTTCGGAATCATTGCCATTGTCATCTACTATTATTGACAGCATT
GATTGTGACTGGTTTACTTTGAATTGTTGCTTCTACTTTATGAAGCTCTGCCCCGACCC
CCCCCCCTCCCAAGAGGCAACGTTAATATCCAAATTAGTTAGGGTGATGGTTTCTCCA
CCTGGGGAAGGCGTTGGTAACGTAATCCCTAGGCCCATGACAACGGTAGGGCCCTTT
GTTTGTTTGAAGGTTTTCGTTTCTAGAGTGACTCCATCTTTAAGGATTAAAAACCCGG
ACCCGATTTTTAAGGGCTGTTTGAAATCTGATTTCAAATTGGCCGCACCTTTTTTTTAT
TAATCTGATACTCTTTAAACAAAAAAAACAATTTTGCCTGTATAATCAACAGGGTTTT
GTTTATTAAAATTTAAATTACATCAAAATCCTCCTCAAGAAAAAAGGGGCATTAAAA
A

>ChlamuVax Vaccine
GTAAAGTGCCGCTAGAGCCTGAGCTTACTTATATTGCTGTGAAAGGCTGAGAGCAGA
ATGTGTAGGATACTTCGTAGGCATTGCCATTGTCATCGACTATTATTGACGCATTGAT
TGTGACTGGTTTACTTTGAGTTGTTGCTTCTACTTTATGAGGCTCTGGCGCGACCCCCC
CCCCTCCCAATGGGGCAACGTTAATATCCAAATTAATTAGGGTGATGGTTTCTCCACC
TGGGGAAGGCGTTTGTACCGTATCCCCTAGGCCCTTGACACCGGTTGCGCCCTTTGTT
TGCTTGTGGGTTTTGGTTTCTAAAGGGCCTCCTTCTTTAAGGATTAAGCATCCGTATCC
GGTTTTTAAGGAATGTTTGAAATCTGATTCCAGATAGCCCCATCCTTTTTTCAAATCTG
TTAACCTTAAAAAAAAAAAACAATTTTTCCCTGATTACCACCAGGGGTTTTGTTTAAT
TAATATTTAATCAACACATCCTTCTCCAGAAATGGGGGCCATTAAAAAACAACCC

>Positive control - CVL
TGAAAGGCTGAGATGCAGCAAGTATAGGATACTCGTAGGCATTACCGTTGTCATCGA
CTAGATTGACAGCATTGATTGTGACTGTTTTACTTTCAGTTGTTGCTTCGACTTTAGCA
GGAGTAGGAGCGACCCCCCCCCCCCCCCAAGGGGGGGCCTTTAATTCCCAAATTTTTTG
GGGGGGGGGTTTTCCCCCCGGGGAAAGGGGTTGGAAACGGATTTCCAAAACCCATGA
AACCAAACCCGGTTGTTGGGGGAATTTTTTTTGTTTTAAAAGGGACCCCTTTTTTAAG
GCCAAAAAAACCGGGCCCATTTTTTAAAGGGTGTTAGAAAAAAGATGGCAAATTGCC
CCACCCCTTTTTTTTTTTACCGAAAATTTTTCCCCCGGAAAAAAAAAATTTTTCCGTGG
GGAAAAAAAAAAAAACCCCCCTTTAACCTTAAAAAAAAATAAAAACGGGGGAAAAA
CCCCCCCGGGGGAAAAAAAAAGGGGGGGCCAAAAA

>Chlamydophila abortus strain S26/3
GTAAAGCAAGCAGTAGTGAAGCTGAATTAAAAATAGAAAATAATCAAAATCTTGTTT
TCGCAGAAAACTCCTCCTCTTCAAGCGGCGGGGCTATTTATGCTGATAAACTCACCAT
TGTCTCAGGTGGACCTACGTTATTTTCTAATAACTCCGTATCCGCTTCTTCACCTAAAG
GTGGAGCCATTTGCATAAAAGATTCAGGTGGTGAATGTAGCTTAACCGCTGATCTCGG
AGATATCACCTTTGATGGGAACAAAATCATCAAAACTAATGGTGGAAGTCCTACAGT
AACAAGAAATTCCATCGATCTCGGCTCTAGCGGAAAATTTACAAAACTAAATGCTAA
AGAAGGTTTCGGGATTTTCTTCTATGACCCTATTACTGGAGGAGGATCTGATGAATTA
AATATTAATAAACAAGACACTGTTGATTATACAGGCAAGATCGTCTTCTCTGGTGAAA

GATTATCAGATGAAGAAAAAAAGGTTGCGGCCAATCTGAAATCAGATTTCAAACAAC
CCTTAAAAATCGGTTCCGGATCTTTAATCCTTAA

>Chlamydophila abortus strain TW92-249

GTAAAGCAAGCAGTAGTGAAGCTGAATTAAAAATAGAAAGTAATCAAAATCTTGTTT
TCGCAGAAAACTCCTCCTCTTCAAGCGGCGGGGCTATTTATGCTGATAAACTCACCAT
TGTCTCAGGTGGACCTACGTTATTTTCTAATAACTCCGTATCCGCTTCTTCACCTAAAG
GTGGAGCCATTTGCATAAAAGATTCAGGTGGTGAATGTAGCTTAACCGCTGATCTCGG
AGATATCACCTTTGATGGGAACAAAATCATCAAAACTAATGGTGGAAGTCCTACAGT
AACAAGAAATTCCATCGATCTCGGCTCTAGCGGAAAATTTACAAAACTCAATGCTAA
AGAAGGTTTCGGGATTTTCTTCTATGACCCTATTACTGGAGGAGGATCTGATGAATTA
AATATTAATAAACAAGACACTGTTGATTATACAGGCAAGATCGTCTTCTCTGGTGAAA
GATTATCAGATGAAGAAAAAAAGGTTGCGGCCAATCTGAAATCAGATTTCAAACAGC
CCTTAAAAATCGGTTCCGGATCTTTAATCCTTAA

>Chlamydia pecorum strain FcStra
TTAAACTGTAGAGTTTCTCCTGGGCAGAGTTCTTTGATACACCATACGGCTTTGTTAC
AAGAGATTTCTGCACCTGCAGCTTCTAGGATCAATGCAGATCCAGGAAGGGTATCTTC
AACAACAACATTACGGAGAACGAGATCCCCAGGGTTAGATACTGTGATTGTGTATTC
TACAGGTTTGCATACATAAGCCCAATCAACTCCAGATATTGTGACATTGACACAAGGC
TCATTAATTACTGTCATGACGTTTGCAGAACACTTATGGCCTCCACAATAGCTCACTG
TAGCGACATTAGTCACTTGTCCTCTTTTTTGAGGG

2. Omp2 gene:

>Yatta sample
GCCTTTTTACCTTGTCCTAGCAATCAATTGTTCCAGTATTAATTTGCTATCTGAGGTTG
GTGTTGTCGCAGGATACACTGCTGACAAACTCAACTTCGCAAGGAAGTTGTTGAGTG
ATCACAACATTAACGCAATCTTTTTTACCTACAGCAAGAATTTCAATAGGATAAGGAG
ATCCTACTGTTGCATATTCAGGTACAGCTTGGCTAATTTGCCACGTATACAGTCATCG
TTAACACGGACGCAATACATTTTGCCGTAGCAAGATTCTTGTGTAGCATAAACGGATT
GCAACTGACCACC

>ChlamyVax vaccine
GCTTTTTACCTTGTCCTAGCAATCAATTGTCCAGTATTAATTTGCTATCTGAGGTTGGT
GTTGTCGCAGGATCACTGCTGACAAACTCAACTTCGCAAGGAAGTTGTTGAGTGATCA
CAACATTAACGCAATCTTTTTTACCTACAGCAAGAATTTCAATAGGATAAGGAGATCC
TACTGTTGCATATTCCAGGTACAGCTTGGCTAATGTTCCACGTTACAGTCATCAGTTA
ACACGGTACACAGATACATGTATCCCAAGTACCAAAAGACTTGTGTAGTATCGACGG
ATTGCCCCTAAAAAAA

>Bethlehem sample
GCTTTTTTACACTTGCACTAGCAATCAGATTGTTCCTGTTTAATTTGCTATCTCGAGGT
TGGTGTTGTCGCAGGAATCACTGCTGACAAACTCAACTTCGCAGGAAGTTGTTGAGTG
ATCACAACATTAACGCAATCTTTTTTACCTACAGCAAAAATATCACTAGGATAAGGCA
GATCCTACTGTTGCATATTCAGGTACAGCTTGGCTAGGATTCCCCGTTACAGTACATC
AGTTAACACGATACGCAGAGACGGCATCCATAGTCTTAGTTGCTTATT

>Positive control-CVL
GCTTTCTGTGATAAAGAATTTTATCCTTGTGAAGGTGGCCAGTGCCAACCAGTAGACG
CTACACAAGAATCTTGCTACGGCAAAATGTATTGTGTCCGTGTTAACGATGACTGTAA
CGTTGAAATCAGCCAATCTGTACCTGAATATGCAACAGTAGGATCTCCTTATCCTATT
GAAATTCTCGCTGTAGGTAAAAAAGATTGCGTTAATGTTGTGATTACTCAACAGCTTC
CTTGCGAAGTTGAGTTTGTCAGCAGTGATCCTGCGACAACACCAACCTCGGATAGCA
AATTAATACTGGACAATTGATCGTTAGGGCAAGGTAAAAAGCAAATATCTGT

>Chlamydophila abortus strain S26/3
GCTTTCTGTGATAAAGAATTTTATCCTTGCGAAGGTGGTCAGTGCCAATCCGTCGATA
CTACACAAGAATCTTGCTACGGCAAAATGTATTGTGTCCGTGTTAACGATGACTGTAA
CGTGGAAATTAGCCAAGCGTACCTGAATATGCAACAGTAGGATCTCCTTATCCTATTG
AAATTCTTGCTGTAGGTAAAAAAGATTGCGTTAATGTTGTGATCACTCAACAACTTCC
TTGCGAAGTTGAGTTTGTCAGCAGTGATCCTGCGACAACACCAACCTCAGATAGCAA
ATTAATCTGGACAATTGATTGCTTAGGTCAAGGTGAAAAATGCAAAATT

>Chlamydia pecorum strain FcStra
GCTTTTACCAATACTTTAAACTGTAGAGTTTCTCCTGGGCAGAGTTCTTTGATACACCA
TACGGCTTTGTTACAAGAGATTTCTGCACCTGCAGCTTCTAGGATCAATGCAGATCCA
GGAAGGGTATCTTCAACAACAACATTACGGAGAACGAGATCCCCAGGGTTAGATACT
GTGATTGTGTATTCTACAGGTTTGCATACATAAGCCCAATCAACTCCAGATATTGTGA
CATTGACACAAGGCTCATTAATTACTGTCATGACGTTTGCAGAACACTTATGGCCTCC
ACAATAGCTCACTGTAGCGACATTAGTCACTTGTCCTCTTTTTTGAGGG

>Chlamydophila abortus strain B577
GCTTTGTTACAGCAGATTTCAGCTCCCTCGGCTTCTAAAATTGTAGCTCCTGAAGGTA
CGGTATCTTCTACAACGACATCGTAAAGTTTAAGATCCCCTAGGTTGGACACAACGAT
AGTGTATTCTACAGGCTTAATACATAAGACCAGTCAGCTCCAGAGATATTGACTTGTA
CGCAGGGTTCGTTAACTACAGTAGTTACGTTCGCGGAACATTTATGTCCTCCGCAGTA
AGATACGGTAGCTACGTTAGTAATTTTTCCTCTTTTTTGCGGGCAAAACTCCACAGAG
AAGCATTTAGAATCCCCAGGGCGCATATCTCCTAAGTTAAAGGAAAGAA

Table 1: Results of serological trial in group 1: healthy Assaf sheep farm.

Group1: Healthy Assaf Sheep			Date	2-May 2012	7-May 2012	22-May 2012	13-June 2012	13-June 2012	20-June 2012	27-July 2012	27-Sep. 2012	3-Dec. 2012	3-Dec. 2012	2- Jan. 2013
Animal No.	Sex (M,F)	Reproductive status at beginning - later	Action	0 Samp. OD S/P (%)	1'st Samp. OD S/P (%)	1'st shot of Vacc.	2'nd Samp. OD S/P (%)	2'nd shot of Vacc.	3'rd Samp. OD S/P (%)	4'th Samp. OD S/P (%)	5'th Samp. OD S/P (%)	6'th Samp. OD S/P (%)	3'rd shot of Vacc.	7'th Samp. OD S/P (%)
40	F	Not pregnant - get pregnant later		0.580 16	0.470 13.5	Yes	1.042 31.9	Yes	0.686 21	0.898 41.9	0.956 27.8	0.680 33.5*	Yes	1.024 59.7
41	F	Not pregnant - get pregnant later		0.404 11.6	0.376 10.8	Yes	3.172 97.1	Yes	2.097 64.2	1.121 52.3	0.803* 23.4	0.823 40.6	Yes	0.785 45.7
42	F	Pregnant		0.383 11	0.423 12.1	Yes	0.418 12.8	Yes	0.369* 11.3	0.785 36.6	--	--	--	--
43	F	Not pregnant - get pregnant later		0.497 14	0.432 12.4	Yes	1.287 39.4	Yes	0.772 23.6	--	--	--*	--	--
44	F	Not pregnant - get pregnant later		0.483 13.8	0.427 12.2	Yes	3.481 106.6	Yes	3.480 106.6	2.501 116.7	1.743 50.8	1.041* 51.3	Yes	2.368 138
45	F	Pregnant		0.362 10.4	0.427 12.2	Yes	0.453 13.3	Yes	0.572* 17.5	--	--	--	--	--
47	F	Not pregnant - get pregnant later		0.440 12.6	0.437 12.5	Yes	1.918 58.7	Yes	1.925 58.9	2.079 97	1.246* 36.3	0.887 43.7	Yes	1.594 92.9
48	F	Not pregnant - get pregnant later		--	0.394 11.3	Yes	1.052 32.2	No	1.265 38.7	--	--*	--	--	--
49	F	Not pregnant		--	0.432 12.41	Yes	2.397 73.4	No	2.912 89.2	--	--	--	--	--
50	M	The ram of the flock		--	0.371 10.6	Yes	1.412 43.2	Yes	2.174 66.6	--	--	--	--	--
51	F	Give new birth lately - get pregnant later		--	0.453 13	Yes	1.103 33.7	Yes	1.293 39.6	1.921 99.6	1.286* 37.4	0.602 29.7	Yes	0.719 41.9
52	F	Pregnant		--	0.415 11.9	Yes	0.611 18.7	Yes	0.927* 28.4	--	--	--	--	--
53	F	Not pregnant - get pregnant later / one shot control		--	0.379 10.8	Yes	--	No	0.588 18	0.541 25.2	0.470 13.6	0.529* 26.1	No	0.460 26.8
54	F	Not pregnant / Control		--	0.415 11.9	No	0.422 12.9	No	0.420 12.8	--	0.527 15.3	0.346 17	No	0.470 27.4

Table 2: Results of serological trial in group 2: infected Assaf sheep farm.

Group2: Infected Assaf Sheep			Date	30-Apr. 2012	7-May 2012	22-May 2012	18-June 2012	18-June 2012	25-June 2012	27-July 2012	27-Sep. 2012	3-Dec. 2012	3-Dec. 2012	2- Jan. 2013
Animal No.	Sex (M,F)	Reproductive status at beginning - general health condition	Action	0 Samp. OD S/P (%)	1'st Samp. OD S/P(%)	1'st shot of Vacc.	2'nd Samp. OD S/P (%)	2'nd shot of Vacc.	3'rd Samp. OD S/P (%)	4'th Samp. OD S/P (%)	5'th Samp. OD S/P (%)	6'th Samp. OD S/P (%)	3'rd shot of Vacc.	7'th Samp. OD S/P (%)
3	F	Pregnant - good		1.247 35.8	1.022 29.3	Yes	1.003 30.7	Yes	0.818 34.2	1.000 46.6	0.921 26.8	0.470 23.1	Yes	0.863 50.3
11	F	Pregnant - good		0.562 16.1	--	Yes	0.377 11.5	Yes	0.869 36.4	0.695 32.4	0.786 22.9	0.650 32	Yes	1.057 61.6
023	F	Aborted 2 weeks later (positive for Chlamydia) - good		2.645 76	--	Yes	2.777 89.8	Yes	1.960 82.1	--	--	--	--	--
024	F	Not pregnant - good		1.734 49.8	0.849 24.3	Yes	1.352 43.7	Yes	2.006 84	0.757 35.3	2.482 72.3	1.558 76.9	Yes	1.960 114.2
68	F	Pregnant (doubtful for Chlamydia) - good		2.060 59.2 §	1.704 48.9	Yes	2.201 71.2	Yes	1.314 55	0.642 29.9	1.518 44.2	0.432 21.3	Yes	0.704 41
100	F	Aborted 2 months ago (positive for Chlamydia) - good		3.481 100	--	Yes	2.771 89.6	Yes	1.628 67.4	2.213 103.3	3.058 89.1	2.349 118	Yes	2.546 148.4
206	M	The ram of the flock (doubtful for Chlamydia) - good Control		2.052 58.9	0.891 25.6	No	0.876 28.3	No	0.438 18.3	--	1.078 31.4	0.804 39.6	No	0.787 45.8
216	F	Not pregnant - good one shot control		1.143 32.8	0.507 14.5	Yes	1.183 38.2	No	0.622 25.9	0.873 40.7	2.024 58.9	0.666 32.8	No	0.910 53
217	F	Not pregnant (less than 8 m of age) - good		0.631 18.1	0.465 13.3	Yes	1.104 35.7	Yes	0.706 29.5	--	--	--	--	--
223	F	Not pregnant (less than 8 m of age) - good		1.809 51.9	1.539 44.2	Yes	3.411 110.3	Yes	0.975 39.6	1.142 53.3	1.663 48.4	0.772 38.1	Yes	1.296 75.5
S.S	F	Not pregnant - good		0.464 13.3	0.389 11.1	Yes	0.852 27.5	Yes	1.925 80.6	--	--	--	--	--

Table 3: Results of serological trial in group 3: Baladi (Awwasi) sheep farm.

Group3: Baladi (Awwasi) sheep			Date	22-May 2012	22-May 2012	30-May 2012	13-June 2012	13-June 2012	20-June 2012	27-July 2012
Animal No.	Sex (M,F)	Reproductive status at beginning - general health condition	Action	1'st Samp. OD S/P (%)	1'st shot of Vacc.	2'nd Samp. OD S/P (%)	3'rd Samp. OD S/P (%)	2'nd shot of Vacc.	4'th Samp. OD S/P (%)	5'th Samp. OD S/P (%)
80	M	Ram-1 of the flock - good		0.752 21.6	Yes	0.466 13.3	0.513 15.7	Yes	0.536 16.4	0.739 34.5
81	M	Ram-2 of the flock (high level of Chlamydia Ab.) - good		1.247 46.2	Yes	--	0.852 26.1	Yes	0.768 23.5	0.782 36.5
82	M	Ram-3 of the flock - good		0.507 18.8	Yes	--	1.302 39.8	Yes	1.045 32	0.978 45.6
83	F	Repetitive estrus (temporary infertility) - good		1.520 43.6	Yes	0.489 14.1	1.732 53	Yes	1.575 48.2	0.700 32.6
84	F	Pregnant - good		0.815 30.2	Yes	--	0.624 19.1	Yes*	0.835 25.5	0.828 38.6
85	F	Not pregnant - good		--	Yes	--	0.551 16.8	Yes	0.646 19.7	--
86	F	Not pregnant - good		--	Yes	--	0.582 17.8	Yes	0.890 27.2	--
87	F	Not pregnant - good one shot control		0.501 14.3	Yes	0.489 14.1	0.448 13.7	No	0.411 12.5	0.335 16.5
88	F	Not pregnant - good Control		0.580 16.6	No	0.481 13.4	0.459 14	No	0.439 13.4	0.299 13.9

Table 4: Results of serological trial in group 4: Shami goat farm.

Group4: Shami goat			Date	22-May 2012	22-May 2012	30-May 2012	13-June 2012	13-June 2012	20-June 2012	27-July 2012	27-Sep. 2012	3-Dec. 2012	3-Dec. 2012	2- Jan. 2013
Animal No.	Sex (M,F)	Reproductive status at beginning - general health condition	Action	1'st Samp. OD S/P (%)	1'st shot of Vacc.	2'nd Samp. OD S/P (%)	3'rd Samp. OD S/P (%)	2'nd shot of Vacc.	4'th Samp. OD S/P (%)	5'th Samp. OD S/P (%)	6'th Samp. OD S/P (%)	7'th Samp. OD S/P (%)	3'rd shot of Vacc.	8'th Samp. OD S/P (%)
70	F	Pregnant - good			Yes	--	0.409 12.5	Yes	1.331 40.7*	--	--	--	--	--
71	F	Not pregnant and get pregnant later Control- good		0.603 18.6	No	0.457 13.6	0.420 12.8	No	0.490 15	--	0.470 11.8	0.616 19.4*	No	0.627 19.3
72	F	Not pregnant and get pregnant later - good		0.616 19	Yes	--	0.544 16.7	Yes	0.438 13.4	0.547 25.5	0.537 15.6	0.371 18.3*	Yes	0.783 45.6
73	F	Not pregnant and get pregnant later - good		0.501 14.3	Yes	0.418 12	0.926 28.3	Yes	0.833 25.5	0.483 22.5	0.494 14.3	0.727 35.8*	Yes	1.481 86.3
74	F	Not pregnant and get pregnant later - good		0.474 13.6	Yes	0.422 12.1	0.526 16.1	Yes	0.561 17.1	0.523 24.4	0.403 11.7	0.394 30.4*	Yes	0.683 39.8
75	F	Not pregnant One shot control - good		0.506 14.5	Yes	0.463 13.3	--	No	0.444 13.6	0.371 17.3	0.467 13.6	0.377 18.6	No	0.548 16.9
76	F	Pregnant - good		0.458 13.3	Yes	--	0.549 16.8	Yes	0.932 28.5*	0.378 17.6	0.835 24.3	0.516 25.4	Yes	0.667 38.8
77	F	Not pregnant - good		0.423 12.3	Yes	--	1.369 41.9	Yes	0.830 25.4	0.866 40.4	0.946 27.5	0.990 48.8	Yes	1.349 78.6
78	F	Not pregnant and get pregnant later - good			Yes	--	0.404 12.3	Yes	0.482 14.7	--	--	--	--	--
79	F	Not pregnant and get pregnant later - good			Yes	--	0.480 14.7	Yes	0.557 17	--	--	--	--	--

Table 5: Results of serological trial in group 5: Baladi goat farm.

Group4: Baladi goat			Date	8-May 2012	5- June 2012	13-June 2012	19-June 2012	26-June 2012	5-July 2012	27-Sep. 2012	3-Dec. 2012	3-Dec. 2012	2- Jan. 2013
Animal No.	Sex (M,F)	Reproductive status at beginning - general health condition	Action	1'st Samp. OD S/P (%)	1'st shot of Vacc.	2'nd Samp. OD S/P (%)	3'rd Samp. OD S/P (%)	2'nd shot of Vacc.	4'th Samp. OD S/P (%)	5'th Samp. OD S/P (%)	6'th Samp. OD S/P (%)	3'rd shot of Vacc.	7'th Samp. OD S/P (%)
56	F	Not pregnant and get pregnant later - good		0.416 11.9	Yes	0.412 12.6	0.427 13	Yes	1.140 57.4	0.733 21.3	0.504 24.8	Yes	Φ
57	F	Not pregnant and get pregnant later Control- good		0.488 14	No	0.395 12.1	0.413 12.6	No	0.393 19.7	0.449 13	0.418 20.6*	No	0.421 24.5
59	F	Not pregnant and get pregnant later - good		0.569 16.3	Yes	0.526 16.1	0.782 23.9	Yes	0.632 31.8	0.664 19.3	0.442 21.8	Yes	Φ
60	F	Pregnant - good		0.476 13.6	Yes	0.438 14.1	--	Yes	--	--	--	--	--
61	F	Not pregnant and get pregnant later - good		0.483 13.8	Yes	--	0.482 15.5	Yes	2.749 138.4	2.532 73.7	0.960 47.3*	Yes	1.807 105.3
62	F	Pregnant - good		2.005 57.6	Yes	1.474 45.1	0.716 21.9	Yes	2.011 101.3*	3.445 100.4	2.135 105.3	Yes	1.869 108.9
65	F	Pregnant - one shot control good		0.421 14.2	Yes	0.433 13.2	0.539 16.5*	No	0.656 33	0.760 22.1	0.718 35.4	No	0.664 38.7

- OD: Optical Density by ELISA reader
- S/P (%): Percentage of Chlamydial antibodies in Sample to Positive control.
- Dates of getting pregnant were not provided.
- Reproductive status was not followed in infected Assaf-sheep farm
- (*): Animal gave normal healthy birth/s (nearest date).
- (§): Animal was given antibiotic to avoid abortion (Oxytetracycline, Sulphadiazime, and Trimethoprime).
- (Φ): Animal were dead by pregnancy toxemia at last month of pregnancy.

Appendix D: Output of statistical analysis by SPSS.

A. Survey Analysis

1- Abortion By Chlamydia * Animal Type Crosstabulation

Count

		Animal Type		
		Sheep only	Goat only	Total
Abortion By Chlamydia	Yes	5	0	5
	No	7	1	8
Total		12	1	13

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.677[a]	1	.411		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	1.023	1	.312		
Fisher's Exact Test				1.000	.615
Linear-by-Linear Association	.625	1	.429		
N of Valid Cases	13				

a. 3 cells (75.0%) have expected count less than 5. The minimum expected count is .38.

b. Computed only for a 2x2 table

2- Abortion By Chlamydia * Previous Health Problems Crosstabulation

Count

		Previous Health Problems		
		Yes	No	Total
Abortion By Chlamydia	Yes	6	2	8
	No	4	8	12
Total		10	10	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.333[a]	1	.068		
Continuity Correction[b]	1.875	1	.171		
Likelihood Ratio	3.452	1	.063		
Fisher's Exact Test				.170	.085
Linear-by-Linear Association	3.167	1	.075		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.00.

b. Computed only for a 2x2 table

3- Abortion By Chlamydia * Previous Abortions Crosstabulation

Count

		Previous Abortions		
		Yes	No	Total
Abortion By Chlamydia	Yes	2	6	8
	No	4	8	12
Total		6	14	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.159[a]	1	.690		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.161	1	.688		
Fisher's Exact Test				1.000	.545
Linear-by-Linear Association	.151	1	.698		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.40.

b. Computed only for a 2x2 table

4- Abortion By Chlamydia * Source of Flock Crosstabulation

Count

		Source of Flock		
		Homebred	Bought in	Total
Abortion By Chlamydia	Yes	4	4	8
	No	6	6	12
Total		10	10	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.000[a]	1	1.000		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.000	1	1.000		
Fisher's Exact Test				1.000	.675
Linear-by-Linear Association	.000	1	1.000		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.00.

b. Computed only for a 2x2 table

5- Abortion By Chlamydia * Sharing Males Crosstabulation

Count

		Sharing Males		
		Yes	No	Total
Abortion By Chlamydia	Yes	6	2	8
	No	3	9	12
Total		9	11	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.848[a]	1	.028		
Continuity Correction[b]	3.039	1	.081		
Likelihood Ratio	5.032	1	.025		
Fisher's Exact Test				.065	.040
Linear-by-Linear Association	4.606	1	.032		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.60.

b. Computed only for a 2x2 table

6- Abortion By Chlamydia * Vaccination for Chlamydia Crosstabulation

Count

		Vaccination for Chlamydia		
		Yes	No	Total
Abortion By Chlamydia	Yes	0	8	8
	No	5	7	12
Total		5	15	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.444[a]	1	.035		
Continuity Correction[b]	2.500	1	.114		
Likelihood Ratio	6.193	1	.013		
Fisher's Exact Test				.055	.051
Linear-by-Linear Association	4.222	1	.040		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.00.

b. Computed only for a 2x2 table

7- Abortion By Chlamydia * Presence of Cats and Dogs Crosstabulation

Count

		Presence of Cats and Dogs		
		Yes	No	Total
Abortion By Chlamydia	Yes	4	4	8
	No	7	5	12
Total		11	9	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.135[a]	1	.714		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.135	1	.714		
Fisher's Exact Test				1.000	.535
Linear-by-Linear Association	.128	1	.721		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.60.

b. Computed only for a 2x2 table

8- Abortion By Chlamydia * Breeding Method Crosstabulation

Count

		Breeding Method		
		Natural Breeding	By Hormone and Males	Total
Abortion By Chlamydia	Yes	2	6	8
	No	5	7	12
Total		7	13	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.586[a]	1	.444		
Continuity Correction[b]	.082	1	.774		
Likelihood Ratio	.600	1	.439		
Fisher's Exact Test				.642	.392
Linear-by-Linear Association	.557	1	.456		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.80.

b. Computed only for a 2x2 table

9- Abortion By Chlamydia * Shairing pastures Crosstabulation

Count

		Shairing pastures		
		Yes	No	Total
Abortion By Chlamydia	Yes	5	3	8
	No	9	3	12
Total		14	6	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.357[a]	1	.550		
Continuity Correction[b]	.010	1	.921		
Likelihood Ratio	.354	1	.552		
Fisher's Exact Test				.642	.455
Linear-by-Linear Association	.339	1	.560		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.40.

b. Computed only for a 2x2 table

10- Abortion By Chlamydia * Level of Education Crosstabulation

Count

		Level of Education		
		literate	Illiterate	Total
Abortion By Chlamydia	Yes	6	2	8
	No	10	2	12
Total		16	4	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.208[a]	1	.648		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.205	1	.651		
Fisher's Exact Test				1.000	.535
Linear-by-Linear Association	.198	1	.656		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.60.

b. Computed only for a 2x2 table

11- Abortion By Chlamydia * Knowledge with Abortions Crosstabulation

Count

		Knowledge with Abortions		
		No Knowledge	Intermediate	Total
Abortion By Chlamydia	Yes	4	4	8
	No	11	1	12
Total		15	5	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.444[a]	1	.035		
Continuity Correction[b]	2.500	1	.114		
Likelihood Ratio	4.519	1	.034		
Fisher's Exact Test				.109	.058
Linear-by-Linear Association	4.222	1	.040		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.00.

b. Computed only for a 2x2 table

12- Abortion By Chlamydia * Stage of Pregnancy Crosstabulation

Count

		Stage of Pregnancy		
		Second Trimester	Third Trimester	Total
Abortion By Chlamydia	Yes	0	8	8
	No	2	10	12
Total		2	18	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.481[a]	1	.224		
Continuity Correction[b]	.208	1	.648		
Likelihood Ratio	2.190	1	.139		
Fisher's Exact Test				.495	.347
Linear-by-Linear Association	1.407	1	.235		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is .80.

b. Computed only for a 2x2 table

13- Abortion By Chlamydia * Disposing Abortion Materials Crosstabulation

Count

		Disposing Abortion Materials		
		Left on the Ground	Given to Dogs	Total
Abortion By Chlamydia	Yes	6	2	8
	No	9	3	12
Total		15	5	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.000[a]	1	1.000		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.000	1	1.000		
Fisher's Exact Test				1.000	.693
Linear-by-Linear Association	.000	1	1.000		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.00.

b. Computed only for a 2x2 table

14- Abortion By Chlamydia * Still Births Crosstabulation

Count

		Still Births		
		yes	No	Total
Abortion By Chlamydia	Yes	5	3	8
	No	1	11	12
Total		6	14	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.706[a]	1	.010		
Continuity Correction[b]	4.375	1	.036		
Likelihood Ratio	6.965	1	.008		
Fisher's Exact Test				.018	.018
Linear-by-Linear Association	6.371	1	.012		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.40.

b. Computed only for a 2x2 table

15- Abortion By Chlamydia * Adding New Animals Crosstabulation

Count

		Adding New Animals		
		Yes	No	Total
Abortion By Chlamydia	Yes	1	7	8
	No	4	8	12
Total		5	15	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.111[a]	1	.292		
Continuity Correction[b]	.278	1	.598		
Likelihood Ratio	1.189	1	.276		
Fisher's Exact Test				.603	.307
Linear-by-Linear Association	1.056	1	.304		
N of Valid Cases	20				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.00.

b. Computed only for a 2x2 table

16- Abortion By Chlamydia * Presence of Other Abortions Crosstabulation

Count

		Presence of Other Abortions		
		Yes	No	Total
Abortion By Chlamydia	Yes	7	1	8
	No	5	7	12
Total		12	8	20

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.201[a]	1	.040		
Continuity Correction[b]	2.509	1	.113		
Likelihood Ratio	4.592	1	.032		
Fisher's Exact Test				.070	.054
Linear-by-Linear Association	3.991	1	.046		
N of Valid Cases	20				

a. 3 cells (75.0%) have expected count less than 5. The minimum expected count is 3.20.

b. Computed only for a 2x2 table

B. Serological Trial Analysis

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Healthy Assaf Sheep	6	12.1333	1.12368	10.80	13.50
Healthy Assaf Sheep 1'st Vacc.	5	65.6000	34.90759	31.90	106.60
HAS2V	5	58.0200	32.04859	21.00	106.60
HAS3V	5	75.6400	40.23541	41.90	138.00

Test Statistics[c]

	Healthy Assaf Sheep 1'st Vacc. - Healthy Assaf Sheep	HAS2V - Healthy Assaf Sheep 1'st Vacc.	HAS3V - HAS2V
Z	-2.023[a]	-1.069[b]	-1.483[a]
Asymp. Sig. (2-tailed)	.043	.285	.138

a. Based on negative ranks.

b. Based on positive ranks.

c. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Infected Assaf Sheep	7	49.3714	26.55878	16.10	100.00
Infected Assaf Sheep 1'st Vacc.	7	56.4571	35.10816	11.50	110.30
IAS2V	6	52.7667	19.90273	34.20	84.00
IAS3V	6	81.8333	41.44860	41.00	148.40

Test Statistics[c]

	Infected Assaf Sheep 1'st Vacc. - Infected Assaf Sheep	IAS2V - Infected Assaf Sheep 1'st Vacc.	IAS3V - IAS2V
Z	-.338[a]	-.105[b]	-1.992[a]
Asymp. Sig. (2-tailed)	.735	.917	.046

a. Based on negative ranks.

b. Based on positive ranks.

c. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Baladi (Awwasi) Sheep	6	29.1167	13.30600	14.30	46.20
Baladi (Awwasi) Sheep 1'st Vacc.	6	27.9000	15.50987	13.70	53.00
BS2V	5	29.1200	12.02942	16.40	48.20

Test Statistics[b]

	Baladi (Awwasi) Sheep 1'st Vacc. - Baladi (Awwasi) Sheep	BS2V - Baladi (Awwasi) Sheep 1'st Vacc.
Z	-.314[a]	-.674[a]
Asymp. Sig. (2-tailed)	.753	.500

a. Based on positive ranks.

b. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Shami Goat	6	14.5000	2.34009	12.30	19.00
Shami Goat 1'st Vacc.	5	23.9600	11.25202	16.10	41.90
SHG2V	5	21.9800	6.40367	13.40	28.50
SHG3V	5	57.8200	22.79653	38.80	86.30

Test Statistics[c]

	Shami Goat 1'st Vacc. - Shami Goat	SHG2V - Shami Goat 1'st Vacc.	SHG3V - SHG2V
Z	-1.753[a]	-.674[b]	-2.023[a]
Asymp. Sig. (2-tailed)	.080	.500	.043

a. Based on negative ranks.

b. Based on positive ranks.

c. Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Baladi Goat	4	14.0500	1.80462	11.90	16.30
Baladi Goat 1'st Vacc.	4	17.2250	4.68713	13.00	23.90
BG2V	3	75.8667	55.64758	31.80	138.40
BG3V	1	105.3000	.	105.30	105.30

Test Statistics[b]

	Baladi Goat 1'st Vacc. - Baladi Goat	BG2V - Baladi Goat 1'st Vacc.
Z	-1.826[a]	-1.604[a]
Asymp. Sig. (2-tailed)	.068	.109

a. Based on negative ranks.

b. Wilcoxon Signed Ranks Test