Antigenic Diversity and Cultural Behavior of Chlamydia Psittaci of Mammalian Origin (Chlamydia Abortion, Elisa, Persistent Infection, Immunofluorescence, Diagnosis).

Jorge Alejandro Perez-martinez
Louisiana State University and Agricultural & Mechanical College

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ANTIGENIC DIVERSITY AND CULTURAL BEHAVIOR OF CHLAMYDIA PSITTACI OF MAMMALIAN ORIGIN

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ANTIGENIC DIVERSITY AND CULTURAL BEHAVIOR OF CHLAMYDIA PSITTACI OF MAMMALIAN ORIGIN

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Medical Sciences with option in Veterinary Microbiology and Parasitology

by

Jorge A. Perez-Martinez
DVM, Universidad Nacional Autonoma de Mexico, 1978
August 1985
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To my children, Oliver and Greta. Thank you for bringing so much happiness into our lives.
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ABSTRACT

ANTIGENIC DIVERSITY AND CULTURAL BEHAVIOR OF CHLAMYDIA PSITTACI OF MAMMALIAN ORIGIN

by

Jorge A. Perez-Martinez

Suspension cultures of L-cells were infected with strains representing different biotypes of Chlamydia (C.) psittaci of bovine, ovine, caprine, equine, feline, porcine and guinea pig origin.

Biotype-1 chlamydiae multiplied to high titers, and high yields of infectivity were obtained in subsequent passages. Chlamydial strains representative of other biotypes did not infect suspension cultures efficiently. Low rates of infection were obtained on the first passage, and the infectivity was lost while attempting subpassages.

L-cells inoculated in suspension with more than one inclusion forming unit/L-cell of the chlamydial strain B577 and allowed to form monolayers, were destroyed within 15 days. However, upon continued incubation, a few surviving cells repopulated the flasks and remained persistently infected. The persistent state was characterized by cycles of repopulation with a low ratio of infected cells, and cycles of extensive cytopathology where > 90% of the cells had inclusions and which could be terminated with penicillin. Immunofluorescence and superinfection during repopulation revealed that the persistently
infected cells adsorb chlamydiae, but their multiplication is arrested. This non-permissive state was terminated by cycloheximide treatment.

The different chlamydial biotypes were immunotyped by an indirect microimmunofluorescence (IMIF) test. Groups of chlamydia-free BALB/c mice received two weekly intravenous inoculations with chicken embryo-propagated, partially purified elementary bodies of each strain. Antisera were collected 4 days after each inoculation, and tested for antichlamydial IgM and IgG antibodies in the IMIF test using cell culture-propagated, partially purified homologous and heterologous antigens. Nine immunotypes of C. psittaci were distinguished, the correlation between immunotypes and biotypes was close, and a pattern of either disease or host specificity was associated with each immunotype.

A modified complement fixation (MoCF) test, an indirect inclusion fluorescence antibody (IIFA) test, and an enzyme-linked immunosorbent assay (ELISA) were developed and evaluated as diagnostic tests for chlamydial abortions in cattle. The pattern of sequential antibody response after experimental chlamydial infection was similar with the three different serological techniques. The MoCF test increased the sensitivity of the standard CF test. However, the IIFA test and ELISA were even more sensitive.
CHAPTER I: INTRODUCTION

The genus Chlamydia (C.) comprises a large group of obligate intracellular pathogens of prokaryotic nature (112). They replicate in the cytoplasm of eukaryotic cells by undergoing a unique developmental cycle (106). The chlamydial development cycle is initiated by the infectious form of the organism, the elementary body (EB), which attaches to the host-cell membrane by means of specific ligand-receptor interactions, and it is internalized by a process of endocytosis. The EB avoids fusion with the lysosomes and reorganizes then into a non-infectious form, the reticulate body (RB), which has specialized metabolic biosynthetic functions for multiplication. The cycle is completed when RBs reorganize into a new progeny of EBs that are released into the extracellular environment as the host cell is lysed.

There are two species of Chlamydia: C. trachomatis which infects only humans (148), and C. psittaci which infects several animal species including man and can cause a wide variety of diseases (171). In addition to their host range, the two species can also be differentiated by the susceptibility to sulfadiazine, the production of glycogen, their DNA composition and the presence of a species specific antigen (112).

I.A. STATEMENT OF RESEARCH PROBLEM.

Different chlamydial strains isolated from domestic mammals are the subject of these studies. These strains, in spite of being classified as one species (C. psittaci), have significant biological, pathogenic and antigenic differences (180). Specific virulence factors have not
yet been identified, but a relationship between antigenic make-up and pathogenic potential appears to exist. Strain specific antigens present on the surface of the EB seem to determine specific mechanisms of parasite-induced endocytosis and might play an important role in determining the host cell range of a given strain. Therefore, further characterization is needed in order to define disease specific antigens and possibly other virulence factors. This knowledge may ultimately lead to an expanded classification scheme. In addition, the diagnosis of the different chlamydia-induced diseases of animals is still a difficult task, and improved laboratory procedures are required for differentiation.

I.B. OBJECTIVES

Following a pertinent literature review, the different chapters of this thesis describe specific experiments that were performed in order to accomplish the following objectives:

1. To explore the usefulness of suspension cultures of L-cells for the in vitro propagation of different strains of C. psittaci of mammalian origin.

2. To develop and characterize an in vitro model of persistence using abortigenic strains of C. psittaci.

3. To study the antigenic diversity of C. psittaci of mammalian origin by means of a highly specific indirect microimmunofluorescence test.

4. To develop and assess the diagnostic value of a modified complement fixation (MoCF) test, an indirect inclusion fluorescence antibody (IIFA) test and an enzyme-linked
immunosorbent assay (ELISA) for the detection of chlamydia-induced abortions in cows.

The aim of these investigations is to get a better understanding of the antigenic properties of different mammalian strains of C. psittaci and their significance for pathogenic potential. The results from the investigations are presented as individual, self-contained chapters which represent manuscripts submitted for publication to different refereed journals. They are entitled:


2. "Persistent infection of L-cells with an ovine abortion strain of Chlamydia psittaci." Accepted for publication in "Infection and Immunity." Co-authored by J. Storz.


II.A. BASIC BIOLOGICAL AND PATHOGENIC PROPERTIES OF THE GENUS CHLAMYDIA.

Chlamydial agents form a large group of organisms that can replicate only in the cytoplasm of living animal cells, a property that resembles the obligate intracellular parasitism of the viruses (12,106). Other chlamydial properties that resemble viral properties are their relatively small size (.2um) and their inability to replicate in interferon-treated cells (28,99,138,139,158). However, in contrast to the viruses, chlamydiae are prokaryotic organisms and their cellular entity is maintained throughout their developmental cycle (105,106).

By virtue of their prokaryotic nature, chlamydiae share some of the basic properties of the eubacteria: they contain both DNA and RNA; they divide by binary fission; they have 70S ribosomes; they possess enzymatic activities and synthesize their own macromolecules; they contain rigid cell walls with an outer membrane similar to that of Gram negative bacteria, and they are susceptible to the action of several antibiotics including tetracycline, chloramphenicol, erythromycin and penicillin (39,80a,105,128,180,181).

In spite of these similarities with the free living bacteria, chlamydiae have important biological properties that distinguish them from all other microorganisms. Their rigid cell walls do not contain muramic acid (7,35,58), which is the hallmark of the typical bacterial cell wall. Instead, strength and rigidity of the cell walls of Chlamydia appear to be conferred by disulfide cross-linked outer membrane proteins (8,70,119,122). Furthermore, the chlamydial envelope
possesses unique morphological features unique to the genus (16,95-98,161).

Chlamydiae are unable to efficiently produce high energy compounds such as ATP and therefore they must derive their metabolic energy from their host (71,107). This property is the principal reason why these organisms cannot multiply extracellularly.

During multiplication the chlamydiae undergo a complicated developmental cycle which has no counterpart (56,106,185). The cytoplasmic inclusions that are present in the infected cells represent membrane-bound microcolonies where the different chlamydial developmental forms are present. The chlamydial developmental cycle begins when the extracellular form of the organism, the elementary body (EB), infects a susceptible host cell by inducing its own uptake through endocytosis (29,47). Preliminary studies suggest that there are specific receptor sites on the host cell for different chlamydial agents (13,72,92,93,112,195). Once inside the host cell, the chlamydiae remain in a phagosome throughout the rest of the developmental cycle. To this effect, phagolysosomal fusion is inhibited by an unknown mechanism that requires the presence of intact chlamydial surface antigens (25,49,50,56,196). The EB reorganizes then into a non-infectious form, the reticulate body (RB), which multiplies actively by binary fission (20,106,167,185). The molecular basis for this differentiation process is not well understood. However, it seems to involve the reduction of disulfide bonds cross-linking the major outer membrane protein and three other minor cysteine-rich membrane proteins present as polymeric complexes in the EB (67,70). After several rounds of division, the RBs reorganize again into a new
progeny of EBs. The monomeric chlamydial outer membrane proteins become disulfide cross-linked again, and the new infective progeny is released as the host cell is lysed (166,186).

The chlamydiae released from infected cells of animals are relatively resistant to the environment. They are resistant to drying and the action of proteolytic enzymes and they can survive in dry feces for months (171). However, the high lipid content of their cell walls makes them quite sensitive to detergents and solvents, and inactivation by a variety of common disinfectants such as formaldehyde or phenol can be readily accomplished (171). Chlamydial agents are also relatively susceptible to thermal inactivation.

In the past, chlamydial agents were named either according to the human diseases that they caused: the psittacosis-lymphogranuloma-trachoma (PLT) group, the trachoma-inclusion conjunctivitis (TRIC) group, the lymphogranuloma venereum (LGV) group; or as Miyagawanella, Bedsonia, Rakeia and by several other terms (60,171). For taxonomic purposes they are all classified now as a single genus, Chlamydia. Furthermore, the uniqueness of their developmental cycle warranted placing the genus (and coordinate family Chlamydiaceae) in a separate order, Chlamydiales (112,179).

All members of the genus Chlamydia share the same morphology and a common lipopolysaccharide antigen (45,46,121,147) which is usually demonstrated by the complement fixation test or by fluorescent antibody (FA) reactions on infected cells and tissues.

Based on the characteristics listed in table 1, two species of Chlamydia are recognized: C. trachomatis and C. psittaci.

Natural C. trachomatis infections occur in man and mouse only, and
all known strains have been subdivided into 3 biovars based on their
natural host, their interaction with cultured cells, and their disease
potential (60,87,108).

*Chlamydia trachomatis* biovar trachoma is associated with the potentially
blinding disease trachoma. In industrialized countries, it is an
important cause of urethritis, cervicitis, salpingitis and infertility
in sexually active adults, and a common cause of conjunctivitis and
pneumonia in neonates from infected mothers (78,144,148,149,151,184).

*Chlamydia trachomatis* biovar LGV is the cause of a relatively uncommon,
world wide systemic disease which is sexually transmitted (60,149).

*Chlamydia trachomatis* biovar mouse pneumonitis is represented by a single
strain associated with respiratory infections of mice (112).

While biovars trachoma and LGV exhibit a high degree of DNA
homology as determined by DNA-DNA hybridization, their homology with
the mouse pneumonitis biovar is far below the 60% homology expected for
members of the same species (111,192). This evidence, together with
more recent antigenic and structural analysis suggest that the mouse
pneumonitis biovar is distantly related to the other two biovars, and
perhaps should be classified as a separate species (34,108,112).

The 2nd species of the genus *Chlamydia*, *Chlamydia psittaci*, exhibits a
much wider range of animal hosts and pathogenic potentials. It can
infect a large number of domestic and wild mammals
(5,65,68,79,101,103,142,171,175,193), a wide range of bird species
(40,62,63,81,126,135,140), and man (9,64,82,132,152,187). In addition,
recent reports have implicated this species as a cause of infection in
frogs (77), and possibly some invertebrate animals (111,128,157).
Clinically inapparent persistent chlamydial infections appear to be the role in these animal populations. However, depending on factors such as virulence of the agent; age, sex, and physiological state of the host; environmental factors and stress, any one or a combination of the following syndromes may develop: polyarthritis, gastroenteritis, conjunctivitis, encephalomyelitis, pneumonia, hepatitis, infertility and abortion (57,77,130,171,175).

No virulence factors have been identified for different strains of _C. psittaci_, and in spite of several attempts to subdivide this highly heterogeneous group of agents, only limited success has been obtained.

Based on inclusion morphology and response to different treatments in cultured cells, at least 8 biovars of _C. psittaci_ can be identified (162). However, the reproducibility of this biotyping assay, in contrast to that of _C. trachomatis_, requires considerable laboratory experience and the process is very laborious.

II.B. INTERACTION OF CHLAMYDIA WITH CULTURED CELLS.

All known strains of _Chlamydia_ grow in the yolk sac of developing chicken embryos. However, when chicken embryos are used it is difficult to analyze the host–parasite interactions at the cellular level, and purification of the organism is tedious (60,107,180). Furthermore, in isolation attempts from certain clinical specimens (i.e. feces, placenta, etc.) the overgrowth of common bacterial contaminants is difficult to control (72).

In recent years, the _in vitro_ conditions for the cultivation of chlamydiae in cell cultures have been significantly improved. While the growth of _C. trachomatis_ in vivo is usually restricted to a few human cell types, several cell lines of both human and animal origin
have been found useful for in vitro cultivation of these agents (60,131). From all the different cell lines tested, McCoy cells (mouse fibroblasts) and a subline of HeLa cells (HeLa 229, from human cervix) were found to be the most uniformly susceptible (60). Similarly, in the case of C. psittaci, several mammalian and avian cell lines have been employed for in vitro investigations (171), but L-cells (mouse fibroblast) clones 929 and 5b are used most frequently.

In general, strains of C. trachomatis biovar LGV and avian isolates of C. psittaci grow relatively well in cell cultures, but strains of C. trachomatis biovars trachoma and mouse pneumonitis, and most strains of C. psittaci of mammalian origin adsorb inefficiently to cultured cells (91,152,162). Therefore, the search for optimal methods for the in vitro propagation of these fastidious strains remains active.

Enhanced chlamydial infectivity for cultured cells can be obtained by aiding the early phases of infection, namely adsorption of the EBs to the host-cell's plasma membrane and the ensuing endocytosis of the attached EBs; and by creating a more favorable intracellular environment for the parasite's multiplication.

The most efficient method for enhancing the infectivity of chlamydiae is centrifugation of the inoculum onto cell monolayers (1,91,163). The actual mechanism by which centrifugation works is not known. Maximum infectivity is obtained using centrifugal forces high enough to sediment chlamydiae (>10,000 xg), but centrifugal forces as low as 70 xg can also enhance chlamydial infectivity significantly (4,44) indicating that centrifugal sedimentation of chlamydial EBs onto cultured cells is not essential. Allan and Pierce (1,4) hypothesized
that during centrifugation morphological changes are induced, both in the membrane and in the cytoskeleton of the host cell, and mediate the exposure of receptors, thus promoting chlamydial adsorption and uptake. On the other hand however, Moulder (114) suggested that during centrifugation the first phase of the infectious process, attachment, is bypassed. This concept was derived from the observation that persistently infected cell cultures on which exogenously added chlamydiae cannot attach can indeed be superinfected with the aid of centrifugation.

Many other procedures have also been used to enhance the entry and multiplication of chlamydiae in cell cultures (44,88,134,150,162,183); the use of DEAE-dextran and cycloheximide being the most useful and best characterized procedures.

DEAE-dextran is a polycation which can be used to neutralize negative charges on the host-cell's surface to enhance the infectivity of chlamydial strains of the biovar trachoma (87,91) and many strains of C. psittaci of mammalian origin (162). It has no effect on the infectivity of LGV strains or strains of C. psittaci of avian origin. This kind of treatment is often used when the more cumbersome centrifugation procedures are to be avoided (89).

Cycloheximide is a glutarimide antibiotic which inhibits protein synthesis in eucaryotic cells but does not affect the metabolism of prokaryotic organisms (51). The addition of cycloheximide in the growth medium of cell cultures infected with chlamydiae consistently enhances inclusion formation by both chlamydial species (69,44,134,163). It is believed that cycloheximide enhances infectivity by increasing the soluble pool of amino acids in the
host-cell's cytoplasm and by reducing cellular competition for some metabolites, particularly amino acids, thus increasing their availability for chlamydial multiplication (3,69). The use of cycloheximide can be combined with centrifugation or with DEAE-dextran treatments to further increase the rates of chlamydial multiplication (42,134,172).

II.C. CHLAMYDIAL ANTIGENS

Chlamydial agents are antigenically complex organisms. Their genome of 3.6 to 6.6 x 10^8 Daltons is relatively small compared to most procaryotes. Nevertheless, if the entire genomic DNA were genetic, they have the capacity to code theoretically for 4 to 8 x 10^2 proteins of an average molecular weight of 40 Kd each, all of which may be potential antigens (112). In spite of this complexity, the following taxonomically important antigens have been characterized in detail.


All members of the genus Chlamydia share a common heat stable glycolipid antigen with a molecular weight of 7 Kd that is associated with the outer membrane of their cell wall and is present throughout the developmental cycle (45,46,90,112,121,147,188). The antigen can be extracted from chlamydial EBs by treatment with sodium dodecyl sulfate, sodium deoxycholate, acid and alkali, and after boiling, with ether, chloroform or phenol.

The structure and biological activities of this glycolipid antigen strongly resemble those of the inner core of enterobacterial lipopolysaccharide (LPS). It is active in the Limulus amoebocyte lysate assay and in the rabbit pyrogen test, both of which are tests
for the lipid A portion of LPS (121). Furthermore, it has been reported
to cross react with the LPS of some strains of Acinetobacter
calcoaceticus (var. antitratus) and with Salmonella sp of the rough Re
chemotype (17,18,121).

Recent chemical and antigenic analysis of the chlamydial glycolipid
antigen using monoclonal antibodies have revealed the presence of
typical LPS constituents (i.e. D-glucosamine, long chain hydroxy fatty
acids, 2-keto-3-deoxyoctonic acid (KDO), and phosphate), and the
presence of 2 immunologically active domains (epitopes), one of which
cross reacts with the LPS of the aforementioned Gram negative bacteria
and one which is unique to the genus Chlamydia (19,30,121,123,124). In
addition, it was shown that the chlamydial antigen exhibits a third
antigenic component which cross reacts with free enterobacterial lipid
A. However, as in enterobacterial LPS, the lipid A antigenicity of
Chlamydia is cryptic and is only exposed after acid hydrolysis.

Based on that evidence, Nurminen et al. (123) suggested that the
chlamydial glycolipid antigen is an LPS made up of a lipid A structure
similar to that of enterobacterial LPS. It is thought to possess 3 KDO
residues in contrast to only 2 KDO residues in Re mutants of
Salmonella spp.

The biological function of the chlamydial LPS remains to be
determined, but the conserved nature of the chlamydia-specific
LPS-epitope in an otherwise antigenically diverse genus suggests that
this epitope may play an essential role for the parasite's
intracellular existence and that it may be important from the point of
view of the molecular mechanism of pathogenesis of the genus Chlamydia
(108,117).
An additional uncharacterized heat-labile, genus-specific chlamydial antigen has been reported (see below) (33).

II.C.2 Species-specific Antigens.

In addition to the genus-specific antigens, chlamydiae also have species-specific antigens. These are antigens shared in common by all members of a chlamydial species.

Caldwell et al. (33,34) applied crossed immunoelectrophoresis to triton X-100 chlamydial extracts, and were able to resolve 19 and 16 different antigens from *C. trachomatis* (strain LGV-434) and *C. psittaci* (strain meningopenumonitis) respectively. These antigens were significantly different in the two chlamydial species since only one reciprocal cross reacting component was observed, and appeared to be the 2nd genus-specific antigen mentioned above. However, since the 18 antigens obtained from the LGV strain cross reacted extensively with antiserum against a strain of *C. trachomatis* biovar trachoma, these antigens were assumed to be species-specific. The apparent protein nature and the dependance on non-ionic detergents for maintaining solubility suggests that these antigens are predominantly membrane antigens (147).

Later studies showed that one of the 18 *C. trachomatis* antigens was consistently precipitated by sera of patients suffering from a variety of proven *C. trachomatis* infections (32). This immunodominant *C. trachomatis*-specific antigen was purified and was identified as a heat-sensitive protein with a subunit molecular weight of 155 Kd. Recent findings using monoclonal antibodies suggest that this antigen may not be a monomeric protein, but a tetrameric aggregate of the 40 Kd chlamydial major outer membrane protein (165) (see below).
II.C.3. **Serotype-specific Antigens.**

Serotype-specific antigens are shared only by closely related strains of the same chlamydbial species. These antigens are usually detected by microimmunofluorescence, and this test has become the standard serotyping procedure for *C. trachomatis*, allowing the differentiation of 15 serotypes (60,147,191). In this system, unknown strains are typed by testing antiserum prepared by intravenous immunization of mice against prototype antigens in an indirect immunofluorescence test.

Serotypes belonging to the trachoma biovar are designated by the letters A through K plus Ba. These serotypes tend to cluster according to geographical distribution and pathogenic potential (60). Thus, ocular isolates from trachoma-endemic areas are mainly A through C, while ocular and genital non-LGV isolates from non-trachoma areas are mainly D through G. LGV serotypes are designated as L1, L2 and L3.

The type-specific antigens form a continuous spectrum of cross reactivities across both the trachoma and LGV biovars, but the highest titers are obtained with homologous antigens, and the pattern of heterologous cross reactions is definitive for each serotype (60,191). The specific immunological activities reside mainly, but not exclusively, in the exposed surfaces of the major outer membrane protein (MOMP) of the chlamydial envelope (37).

Antigenic and structural analysis by peptide mapping of purified MOMPs from one strain of *C. psittaci* and five strains of *C. trachomatis* showed that the MOMPs of the two chlamydial species are antigenically different (31,37). Furthermore, monoclonal antibodies together with polyclonal sera elicited by sodium dodecyl sulfate-denatured MOMPs
identified species-, subspecies-, and serotype-specific epitopes, indicating that the chlamydial MOMPs are complex antigens which possess constant and variable regions (37,41,165).

Additional serotype-specific membrane proteins with a MW of 30 Kd have been described for serotypes A, B and C of \textit{C. trachomatis} (75).

The biological function of the type-specific surface antigens described is unknown, but they seem to represent virulence factors that are functional in the early events of the infectious process. They appear to modulate the critical events of attachment, parasite-induced endocytosis, inhibition of phagolysosomal fusion, toxicity, and the host immune responses that contribute to immunity and pathogenicity (36,66,116,147,164).

Corresponding antigenic and structural analysis of different strains of \textit{C. psittaci} have not been done, but it is likely that their outer membrane proteins are just as antigenically heterogeneous as those of \textit{C. trachomatis}.

In the case of \textit{C. psittaci} isolates from cattle and sheep, the plaque reduction test provided evidence that specific antigens might be disease-specific virulence factors (145,146). With this system, different strains were subdivided into two broad immunotypes. Immunotype 1 included strains associated with abortions, enteritis and subclinical intestinal infections. Immunotype 2 was represented by strains associated with conjunctivitis, polyarthritis, and sporadic encephalomyelitis.

Because the reaction of all the strains within each immunotype was not identical, a postulate for further subtypes is justified. Also, antisera against these strains did not neutralize the infectivity of
several strains of \textit{C. psittaci} of avian origin.

II.D. EPIZOOTIOLOGICAL ASPECTS OF CHLAMYDIAL INFECTIONS OF DOMESTIC MAMMALS.

Because chlamydiae can propagate only within living eukaryotic cells, their habitats are necessarily the tissues of susceptible animal hosts. In the case of ruminants, the intestinal tract appears to be a natural habitat for some of these agents (129,169). Persistent intestinal chlamydial infections in which a well balanced host-parasite relationship exist are common in cattle and sheep. Fecal shedding by carrier animals followed by inhalation or ingestion of infective particles by a new host represent an important mode of transmission and perpetuation of chlamydiae in these animal species. The chlamydial strains involved in inapparent intestinal infections belong to the immunotype 1. Consequently, these infections also play an important role in the pathogenesis of ruminant pneumonia and abortion under conditions where the host-parasite balance shifts in favor of the organism (175). However, different chlamydial strains exhibit different degrees of virulence for the same host. \textit{Chlamydia psittaci} of immunotype 2 are more virulent and invasive than immunotype 1. It is not known whether intestinal or other types of subclinical infections exist with all chlamydial strains of cattle and sheep. This type of question cannot be answered with the seroepidemiological methods currently available for studying \textit{C. psittaci} infections. The most commonly used serological test for the detection of chlamydial infections of animals is the complement fixation (CF) test which lacks the sensitivity necessary to detect many apparently healthy carriers and does not have the specificity required to distinguish infections.
caused by different immunotypes of chlamydiae (175). Therefore, the reservoir and mode of perpetuation of some naturally occurring bovine and ovine chlamydial infections remain uncertain. In the case of other animal species, persistent chlamydial infections occur not only in the intestinal tract but also in the stomach, lungs, and uterus and in cells of the reticuloendothelial system (171). It was proposed that a true state of latency exists in some of these cases.

_C. psittaci_ has relatively little host specificity and interspecies transmission can occur. Chlamydial infections of feral animals including several rodent species, oppossums, koalas, seals, and birds are not uncommon (128,171,175). The chlamydiae found in cattle and sheep are antigenically different from several strains of avian origin (145,146). For this reason, it is unlikely that birds play an important role in the transmission of chlamydial infections in ruminants. However, the significance of the chlamydial infections found in the other wild species mentioned above and in other bird species in relation to the epidemiology of ruminant chlamydiosis has not been fully investigated.

The possibility of vector transmission has been proposed, and chlamydiae have been isolated from ticks, insects and arachnids associated with cattle and poultry (128). Again, the role of these vectors, particularly the tick, and the significance of these isolations needs to be investigated further.

Chlamydia-induced diseases in man and animals are characterized either by local mucous-membrane infections, or by a pathogenesis involving chlamydemia and ensuing systemic infection. Once the
clinical signs develop, transmission can occur through contaminated aerosols, urine, ocular discharges, vaginal secretions, and seminal fluids, depending on the particular syndrome present. Transmission through contaminated semen used in artificial insemination has also been demonstrated (175).

From a public health point of view, mammalian strains of C. psittaci in general do not seem to play a significant role in producing human disease. Nevertheless, a few well documented cases of human infection acquired by contact with infected cattle or sheep are known and laboratory workers have been accidentally infected during the course of experimental investigations (9, 82, 187). These infections may be considered an occupational hazard.

II.E. DIAGNOSIS OF ANIMAL CHLAMYDIAL INFECTIONS.

The principles of diagnosing chlamydial infections are common to other microbial infections. Only a presumptive diagnosis may be made on the basis of clinical signs. A definite diagnosis must involve the direct visualization of the agent in the infected tissues, a demonstration of a significant rise of antibody titers to chlamydial antigens, or the isolation and identification of chlamydiae from discharges, blood, feces or postmortem tissues of affected animals.

II.D.1. Direct Examination of Tissues

The demonstration of either chlamydial inclusions in Giemsa stained exfoliative cytological preparations or conglomerates of elementary bodies in Gimenez (59) stained smears prepared from infected tissues are useful in the diagnosis of chlamydial keratoconjunctivitis, polyarthritis-serositis, and placental infections associated with
abortion. Giemsa stained chlamydial inclusions on infected cells are basophilic and appear as purple-blue, while extracellular elementary bodies stain red with the method of Gimenez. Positive results obtained by these procedures allow for a rapid diagnosis, but it must be kept in mind that their sensitivity is low and failure to demonstrate the agent by these means does not necessarily rule out a chlamydial infection. The usefulness of the Giemsa and Gimenez stains in sections of formalin-fixed paraffin-embedded tissues is limited. Slightly better results can be obtained by staining thin sections (2-4 \( \mu \)m) of tissues preserved in Bouin's fixative and embedded in plastic rather than in paraffin (171).

Fluorescent antibody techniques with chlamydial hyperimmune serum have been successfully employed for detecting chlamydial inclusions in intestinal sections of experimentally infected calves (52). However, these techniques have not been routinely applied in the diagnosis of naturally occurring chlamydial infections of ruminants.

II.D.2. Serology

The serological test most widely used for the diagnosis of \( C. \) psittaci infections is the CF test. This test is genus-specific and therefore the antigens can be prepared from any chlamydial strain (175).

The CF test is most useful in the diagnosis of chlamydial infections involving systemic phases such as encephalomyelitis, pneumonia, polyarthritis-serositis, and abortion. In these instances, a four fold increase in antibody titers of sera collected during the early and late stages of the disease is regarded as significant. However, the CF test is relatively insensitive in detecting superficial
mucous membrane infections such as keratoconjunctivitis, enteritis and localized genital infections.

Several investigators using the CF test for the diagnosis of viral infections have found that it can be significantly improved by supplementing the guinea pig complement with fresh normal calf serum (14,15).

In prevalence studies, the presence of CF antibodies at a dilution $\geq 1:8$ is indicative of a previous stimulation of the immune system by chlamydial antigens, but it is not possible to tell whether the animals are currently infected or not, and the antibodies measured by this test do not play any role in resistance to reinfection (175). It has been repeatedly shown that individual animals bearing clinically inapparent intestinal infections can be superinfected, and pneumonia or abortion can be induced with the strains they are shedding, regardless of their initial CF antibody titer (168). Under these conditions, the superinfected animals develop a measureable increase in serum antibodies.

Several modern serological tests have been and continue to be developed for the diagnosis of C. trachomatis infections in man. From these newly emerging serological tests, the enzyme-linked immunosorbent assay (ELISA) and the indirect inclusion fluorescent antibody (IIFA) test seem to be most promising as routine serodiagnostic tests. Their sensitivity appears to be better than that of the CF test and they facilitate determination of the immunoglobulin class present in the serum and local secretions (175). Although these tests have been almost exclusively applied to the titration of human and avian sera, they could also be used for the screening of sera from other animal
species.

In the ELISA systems that have been developed, the antigen is either a deoxycholate extract of chicken embryo-grown chlamydiae or partially purified cell culture-grown EB suspensions. The deoxycholate extracted antigen is identical to that used in the CF test, but the EB suspensions contain not only the genus specific but also serotype specific antigens. The main advantage of the ELISAs seem to be their spectroscopic objectivity, their relative simplicity, and the ease for testing numerous serum samples simultaneously.

In the IIFA test, whole cells infected with a chlamydial agent provide the antigen (133). Both genus- and serotype-specific antigens are present. The difference in sensitivity between the CF test and the IIFA test or the ELISA seems to depend in part, on their ability to detect both genus- and serotype- specific antibodies, although the type specific ones can only be detected in a homologous system (84,133).

II.D.3. Isolation of the Causative Agent

Isolation of chlamydiae from clinical specimens is not only used for confirmation of a clinical diagnosis but also to determine epidemiological patterns of the different serotypes involved in the various clinical syndromes.

Until recently, the most commonly used procedure for the isolation of C. psittaci from mammals was the inoculation of susceptible chicken embryos. However, newer cell culture techniques applied to the isolation of chlamydiae from ruminants have proven to be more sensitive than the standard chicken embryo methods (172). Results are obtained within shorter periods of time, a relatively large number of samples can be handled simultaneously, and the presence of bacterial
contaminants in some of the clinical specimens does not interfere with the positive results. The single most important step in the cell culture procedures involved the enhancement of chlamydial adsorption to the cultured cells by means of centrifugation.

Because infection with some strains of chlamydiae in the absence of clinical signs is so widespread, the investigator should be aware of the possibility that the diseased host's lesions might have been caused by an entirely different agent and that the chlamydial isolate obtained from the clinical specimen may have been incidental to the disease encountered (128). Alternatively, chlamydial strains of low virulence may predispose the host to infection by secondary pathogens. Significant bacterial ecologic changes in the intestines of calves experimentally inoculated with immunotype 1 of C. psittaci have been reported (175). Chlamydial infections complicated with Pasteurella spp were observed naturally, and multiple infections with chlamydiae and viruses have been repeatedly encountered during the course of the bovine pneumonic syndrome known as enzootic pneumonia, and in intestinal infections of calves (175,176).
### Table II.1. Differentiation of the Species of the Genus Chlamydia.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C. trachomatis</th>
<th>C. psittaci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Hosts</td>
<td>Man, mice</td>
<td>Mammals (including man),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>birds, frogs,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>invertebrates</td>
</tr>
<tr>
<td>Biotypes</td>
<td>3</td>
<td>At least 8</td>
</tr>
<tr>
<td>Serotypes</td>
<td>15</td>
<td>Not known. Probably several.</td>
</tr>
<tr>
<td>Glycogen deposition</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>in inclusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptibility</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>to sulfadiazine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See text for specific references
CHAPTER III: PROPAGATION OF OVINE AND BOVINE ABORTION STRAINS OF

CHLAMYDIA PSITTACI IN SUSPENSION CULTURES OF L-CELLS

III.A. INTRODUCTION

The genus Chlamydia comprises a large group of obligate intracellular bacteria which are distinguished from all other prokaryotic organisms on the basis of a unique developmental cycle (106,179). Two species of Chlamydia are recognized: C. trachomatis, which infects human beings and mice only; and C. psittaci, which infects an ever increasing number of mammals including man; birds; and even cold blooded animals (77,112).

The large number of chlamydial strains included in the species C. psittaci represent a heterogeneous collection of organisms which exhibit significant biological, pathogenic and antigenic differences (24,83,127,131,145,146,162). More information needs to be gathered before differentiation or even further speciation of members of this species can be justified. Optimal methods for their in vitro cultivation and quantitation are important for such investigations.

The methods that are currently available for the propagation of these strains are essentially the inoculation of developing chicken embryos and the centrifuge-assisted infection of cell monolayers (172). These two procedures are useful techniques from a diagnostic point of view, but they still have limitations as models for the study of host-parasite interactions at the cellular level, and for the production of high yields of chlamydiae required for structural and biochemical analysis, as well as for vaccine production. The chicken embryo represents a complex multicellular host and has obvious
limitations. In the case of centrifuge-assisted infection of cells, important factors like temperature and pH are sometimes difficult to control, and infection of large numbers of cells is cumbersome.

The purpose of the present investigations was to explore the propagation of chlamydial strains associated with abortions and other clinical conditions, using suspension cultures of L-cells.

III.B. MATERIALS AND METHODS

III.B.1. Growth of L-Cells - Mouse L-cells (clone L5b) were grown in suspension in Waymouth medium or medium 199 containing 12 mM sodium bicarbonate, 200 μg of streptomycin and 75 μg of vancomycin per ml; and 5% fetal calf serum. The initial cell density was 4-5 x 10^5 cells per ml, and the cultures were stirred constantly at 37°C at a rate fast enough to avoid attachment of the cells to the spinner culture flasks. When the viable cell density, determined by trypan blue exclusion, approached 1.5 x 10^6 cells per ml, the cells were centrifuged at 500 x g for 5 min and resuspended in fresh medium at the initial density.

III.B.2. Chlamydial Strains - Two different abortigenic strains of C. psittaci (biotype 1) originally isolated and maintained in chicken embryos were used in these experiments: the B577 strain (passage 33) isolated from an aborted lamb (169) and the EBA-59-795 strain (passage 22) isolated from an aborted calf (177). Other chlamydial strains used were the LW613 of bovine polyarthritis (biotype 2); the 66-P-130, from intestinal infection of calves (biotype 3); the L71 of porcine polyarthritis (biotype 4); the S45, from intestinal infections of swine (biotype 5); the feline pneumonitis vaccine strain (Fromm Laboratory, Grafton, Wis.) (biotype 7); and the strain of guinea pig inclusion
conjunctivitis (biotype 8) (162).

III.B.3. Infection of L-cells - Yolk sacs harvested from infected chicken embryos were used as the primary source of infection for suspension cultures. They were homogenized in 2 mls of sucrose-phosphate-buffer (SPB) (16) containing 2% fetal calf serum, and centrifuged at 200 x g for 5 min. at 4°C. One ml. collected from the interphase was mixed with 1 x 10^8 cells suspended in SPB and stirred constantly for 2 h at 37°C. At the end of this adsorption period, the cells were washed in SPB and resuspended at the same density in growth medium containing 2 µg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml. The cells were maintained in suspension at 37°C, and chlamydiae were harvested after 48 h as summarized in Fig. III.1 (69).

Once the first cell-propagated chlamydial yields were obtained, they were used for subsequent passages in suspension cultures using multiplicities of infection (MOI) of 1 to 2 chlamydial inclusion forming units (IFU).

III.B.4. Titration of Chlamydial Infectivity - One tenth of a ml of infectious cell-propagated chlamydiae was added to each of two flasks containing 1 x 10^7 L-cells in 10 ml of SPB. The flasks were shaken in an orbital shaker at 100 strokes per min. for 1.5 h at 37°C. Small aliquots of cells (1-2 x 10^5 cells) were then seeded in flat bottom 3-dram shell vials (Kimble Products, Toledo, Ohio) which were fitted with a glass coverslip (18 mm in diameter) and contained 1 ml of growth medium plus 2 µg of cycloheximide per ml. (Fig. III.2). After 24 h of incubation at 37°C in 5% CO₂, the coverslips were Giemsa-stained, and the number of chlamydial IFU per ml. of the original preparation was determined by counting the number of
chlamydial inclusions in 400 cells and applying the following formula:

\[
\text{No. of IFU per ml} = \text{Avg. No. of inclusions per L-cell} \times 10^7 \text{ cells} \times 10
\]

III.B.5. Efficiency of Chlamydial Adsorption at Different Temperatures - A cell-propagated chlamydial inoculum was used to infect cells in suspension at two different temperatures. During the first adsorption period, the cells were maintained at 4°C and then pelleted by low speed centrifugation. The adsorbing medium containing the inoculum was saved, and the cell pellet was resuspended in growth medium and seeded into coverslip in vials. The saved adsorbing medium was then retitrated at 37°C using a new population of cells. Control values for the evaluation of this experiment were obtained by assaying at 37°C a different aliquot of the original inoculum containing the same number of chlamydial IFU.

III.B.6. Comparative Infectivity under Different Conditions of Adsorption - The infectivity titers obtained when adsorption took place in suspension were compared to the titers obtained under stationary and centrifuge-assisted conditions. L-cells from actively replicating suspension cultures were seeded on 60 mm Petri dishes at a density of 3 \( \times 10^6 \) cells per dish and incubated for 18 h at 37°C in 5% CO\(_2\) to allow the formation of monolayers. At the end of this incubation period, the average number of viable cells per dish was re-calculated, the monolayers were washed once with Dulbecco's phosphate buffer, and they were then infected with known multiplicities of infection. For the stationary infection, each of 3 dishes (approximately 4 \( \times 10^6 \) cells per dish) received 1 ml of an inoculum containing 5 \( \times 10^6 \) chlamydial IFU per ml (MOI = 1.25), 1 \( \times 10^6 \) IFU/ml (MOI = 0.25) and
0.1 x 10^6 IFU/ml (MOI = 0.025) respectively, for 1.5 h at 37°C. For
the centrifuge-assisted infection, each of 3 dishes received 3 mls of
inoculum containing 1.66 x 10^6 IFU/ml (MOI = 1.25), 0.33 x 10^6
IFU/ml (MOI = 0.25) and 0.03 x 10^6 IFU/ml (MOI = 0.025) respectively
for 30 min. using a centrifugal force of 2000 x g at 37°C.

Since the titers are easier to read in coverslips lightly seeded
with cells than in complete monolayers, the monolayers of cells used
for stationary and centrifuge-assisted infections were detached with a
solution of 0.1% of 1:250 trypsin (Difco, Detroit, Mich.) and 0.4% EDTA
2.5 h after the adsorption phase, and cells were seeded onto coverslips
in vials at convenient densities as described above (Fig. III.3). None
of these vials received cycloheximide in the culture medium. The rates
of infection were determined 24 h post-infection after Giemsa-staining
the coverslips.

Suspension cultures infected with 1, 0.5, 0.1, and 0.01 chlamydial
IFU/L-cell served as control values in the comparative evaluation of
the different conditions of adsorption. In order to rule out any
differences in titer due to the post-adsorption trypsin treatment of
the monolayers used in the stationary and centrifuge-assisted
infections, the suspended cells were allowed to attach to Petri dishes
at the end of the adsorption period, and were detached with
trypsin-EDTA 2.5 h later (Fig. III.3).

III.C. RESULTS

III.C.1. Growth and Infection of L-cells - The growth curves of
non-infected L-cells in suspension cultures in two different culture
media are illustrated in Fig. III.4. Higher viable cell counts were
obtained in Waymouth's medium than in medium 199, therefore, the former was chosen for the routine propagation of the cells.

Suspension cultures of L-cells inoculated with the chicken embryo-adapted abortion strains were readily infected and yielded large numbers of infectious chlamydiae. The high infectivity levels were maintained during several subpassages in suspension cultures. Typically, a suspension of $1 \times 10^8$ cells ($1 \times 10^6$ cells/ml) infected with an MOI of 1-2 chlamydial IFU/L-cell, followed by cycloheximide treatment, yielded titers in the range of $1.5 - 2.0 \times 10^9$ chlamydial IFU. Differences were not observed in the chlamydia-host cell interaction between the two abortion strains, and only the B577 strain was subsequently used for the experiments described below.

Suspension cultures inoculated with the other chlamydial strains exhibited low rates of infection on the first passage, and the infectivity was lost during the second passage (Table III.1). Consequently, experiments involving these strains were discontinued.

III.C.2. Reproducibility of Infectivity Titration Assay - The IFU assay developed for determining chlamydial infectivity was reproducible over a range of dilutions. The Giemsa-stained chlamydial inclusions were small 24 h post-infection (Fig. III.5), but they could be distinguished easily and allowed accurate quantitative determinations. Suspension cultures exposed to known multiplicities of infection gave infectivity titers, expressed as numbers of inclusions/100 cells, which paralleled the expected theoretical values (Table III.2). At an MOI of 1.0, almost 80% of the cells were infected; at an MOI of 0.5, about 50% of the cells were infected; at an MOI of 0.1, about 10% of the cells
were infected; and at an MOI of 0.01, 1% of the cells were infected.

Using MOI's of 5 to 10 IFU/L-cell, more than 95% of the cells became infected, and conditions for a one-step chlamydial growth experiment were obtained (Fig. III.6). Under these conditions, an extensive cytocidal action was observed in the absence of cycloheximide. The non-infected control cells divided logarithmically, but the viability of the infected cells began to decrease as early as 1.5 hrs. post-infection, and continued to decrease significantly during the 3-day period of observation.

III.C.3. Infectivity Titers Observed under Different Conditions of Adsorption - The chlamydial infectivity titers obtained when adsorption took place at 4°C represented approximately 70% of the infectivity observed in the controls. The residual infectivity was detected after retitration of the inoculum at 37°C (Table III.3). A total infectivity of 110% was obtained, probably because the total length of adsorption for the inoculum was twice that of the control.

Results of the infectivity titers obtained after stationary or centrifuge-assisted adsorption are summarized in Table III.4 and shown graphically in Fig. III.7. The control titers observed when adsorption took place in suspension had a linear response as anticipated even though the values did not correspond to the MOI used due to the absence of cycloheximide in the culture medium. Essentially, differences were not observed when the cells were infected in suspension or under stationary conditions, but at least a two fold increase in titer was observed with the centrifuge-assisted infection.
III.D. DISCUSSION

Suspension cultures of L-cells were useful for the in vitro propagation of two abortion chlamydial strains. This method of propagation facilitated the study of host-parasite interactions under different conditions and accurate infectivity titrations could be performed. Chlamydial strains of biotype 1 multiplied to high titers in the first passage, and high levels of chlamydial infectivity yields were obtained in subsequent passages in suspension cultures. Our findings contrast previous reports involving the same strains but different cultural conditions, where repeated subpassages were needed before the strains became fully "cell-adapted" (10,11).

The efficiency with which the B577 and the EBA-59-795 chlamydial strains infected the L-cells resembles the efficient parasite-specified endocytosis observed with the 6BC laboratory strain of C. psittaci (29). Similarly, the process of cellular uptake is also preceded by an attachment step which can be demonstrated by adsorption at 4°C (26).

The fact that the other chlamydial strains tested under the same conditions did not infect suspension cultures efficiently reinforces the previous observations that different biotypes of C. psittaci interact differently with cultured cells (18,162,163), and that different factors probably influence entry into the host as suggested for different C. trachomatis biotypes (91). Optimal methods for the propagation of those strains are still needed, and will probably have to await a better understanding of their unique properties.

The laboratory isolation and propagation of mammalian strains of C. psittaci is still a difficult task (44,74). Several treatments have
been used to promote chlamydial uptake and inclusion formation by cultured host cells (44,162), but it is generally accepted that the combination of centrifugation and cycloheximide gives the best results (80,162,163). The suspension culture method was not applied to the isolation of strains from clinical specimens. Nevertheless, centrifuge-assisted infections gave consistently higher titers with all strains studied, regardless of their degree of cell adaptation. This indicates that centrifuging the inoculum onto cell monolayers should remain the method of choice in isolation attempts. The suspension cultures appear to be most useful for the subsequent propagation of abortigenic strains, allowing the production of large amounts of chlamydial antigens required for antigenic and seroepidemiological studies, as well as for vaccine preparation.
<table>
<thead>
<tr>
<th>Chlamydiastrain</th>
<th>Biotype*</th>
<th>1stpassage†</th>
<th>2ndpassage‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-577</td>
<td>1</td>
<td>70§</td>
<td>&gt;95</td>
</tr>
<tr>
<td>EBA-59-795</td>
<td>1</td>
<td>60</td>
<td>&gt;95</td>
</tr>
<tr>
<td>LW-613</td>
<td>2</td>
<td>35</td>
<td>&lt;1</td>
</tr>
<tr>
<td>66-P-130</td>
<td>3</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>L-71</td>
<td>4</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>S-45</td>
<td>5</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>Feline Pneumonitis</td>
<td>7</td>
<td>50</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inclusion-conjunctivitis</td>
<td>8</td>
<td>1</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Biotype determined by Spears and Storz (1979).

† Freshly harvested yolk sacs of chicken embryos infected with the different chlamydial strains were used for the inoculation of suspension cultures of L-cells, as described in the text.

‡L-cell suspension cultures were infected with the chlamydial yield harvested from the 1st passage.

§ Percentage of L-cells with chlamydial inclusions 48 h p.i., determined by Giemsa stain.

ND = 2nd passage was not done.
### TABLE III.2 Relationship Between Number of Chlamydial Inclusions and Multiplicities of Infection

<table>
<thead>
<tr>
<th>Dilution of inoculum</th>
<th>Volume of inoculum</th>
<th>Theoretical MOI (IFU/L-cell)</th>
<th>Observed Titer (IFU/100 cells)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^0$</td>
<td>1.0 ml</td>
<td>1</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>45</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>1.0</td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>1.0</td>
<td>0.01</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Avg. for 400 cells
TABLE III.3 Relative Efficiency of Chlamydial Adsorption at Two Different Temperatures.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Temperature of Adsorption</th>
<th>Inclusions/100 cells*</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37°C</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>Test inoculum†</td>
<td>4°C</td>
<td>41</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>26</td>
<td>40</td>
</tr>
</tbody>
</table>

* Average of at least 200 cells
† The inoculum was titrated first at 4°C and then retitrated at 37°C as described in the text.
<table>
<thead>
<tr>
<th>MOI (IFU/L-cell)</th>
<th>Suspension</th>
<th>Stationary</th>
<th>Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td></td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>0.025</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0.025</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>0.01</td>
<td></td>
<td></td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Avg. no. of inclusions/100 cells for 300 cells.
Figure III.1. Procedure for harvesting chlamydia-infected suspension cell cultures.
Figure III.2. Procedure used for titrating chlamydial infectivity in suspension cell cultures.
Figure III.3. Procedures used to compare the infectivity of *C. psittaci* B577 under 3 different conditions of adsorption.
Figure III.4. Growth curves of non-infected L-cells suspended in Waymouth's medium or in medium 199.
Figure III.5. Chlamydial inclusions 24 h post-inoculation of L-cells in suspension, with C.psittaci B577. Giemsa stain, magnification x 400.
Figure III.6. Cytocidal effect of *C. psittaci* B577 for suspension L-cell cultures infected with 10 chlamydial IFUs/L-cell.
Figure III.7. Infectivity titers obtained with *C. psittaci* B577 under 3 different conditions of adsorption.
CHAPTER IV: PERSISTENT INFECTION OF L-CELLS WITH AN OVINE ABORTION STRAIN OF CHLAMYDIA PSITTACI

IV.A. INTRODUCTION

Chlamydial agents are obligate intracellular parasites of prokaryotic nature. They multiply within the cytoplasm of eukaryotic cells where they undergo a unique developmental cycle (56,106,185). These organisms are widely distributed in nature, and their importance as agents of disease both in man and in domestic animals is well documented (148,170,175).

Chlamydial agents that infect domestic mammals can induce a large variety of clinical conditions such as abortion and other genital infections, enteritis, polyarthritis, conjunctivitis, encephalitis and pneumonia (175). An important state of the host-parasite interaction in some of these chlamydial infections is the persistent, clinically inapparent intestinal infection, which plays a significant role in spreading the organism within animal populations (53,129,136,159,168,169). Very little is known about the mechanisms of persistence at the cellular level in the infected animals, and simpler models are desirable for detailed study.

A few cell culture models of Chlamydia psittaci (C. psittaci) persistence have been described (2,6,27,69,125). However, in most instances, the cultures were deprived of important metabolites for chlamydial multiplication, and the persistent state was lost after correction of the nutrient deficiency (2,6,69). Recently, Moulder et al. (113) described a persistent infection of L-cells with the 6BC strain of C. psittaci in which a hypothetical cryptic body played a key
role in perpetuating the infection for indefinite periods of time. Because different strains of \textit{C. psittaci} behave differently in cultured cells (162,163), chlamydiae of different pathogenic properties may establish persistent infections by unique mechanisms. The objective of this investigation was to develop \textit{in vitro} methods of persistence with abortigenic and arthropathogenic chlamydial strains of ovine and bovine origin, using the approach of Moulder et al. (113).

IV.B. MATERIALS AND METHODS

IV.B.1. Growth of Normal L-cells. The clone 5b of L-cells (mouse fibroblasts) was kindly provided by Dr. J. Moulder of the University of Chicago. The cells were grown and maintained as suspension cultures as described by Hatch (69) in medium 199 supplemented with 5\% fetal calf serum and containing 12 mM sodium bicarbonate, 200 \textmu g of streptomycin sulfate and 75 \textmu g of Vancomycin per ml (growth medium).

IV.B.2. Propagation of Chlamydiae. The R577 strain of \textit{C. psittaci}, originally isolated from an aborted lamb (169), was used in these investigations. This strain had been passaged 33 times in the yolk sacs of chicken embryos before it was adapted to multiply in suspension cultures of L-cells as described previously (see Chapter 3). L-cell-propagated chlamydial stocks were obtained by harvesting infected suspension cultures 48 h post-inoculation as described by Hatch (69). The LW613 chlamydial strain, originally isolated from a calf suffering from polyarthritis (178) was used in limited trials. This strain was in the 18th chicken embryo passage, and we did not succeed in adapting it to multiply in suspension L-cell cultures.

IV.B.3. Titration of Chlamydial Infectivity. To determine the
titers of B577 chlamydial infectivity, normal L-cells were infected in suspension and seeded onto coverslip vials (163) at convenient densities (4-8 x 10^4 cells/cm²). The cells were Giemsa-stained 24 h later, and the titer of the original inoculum was expressed as chlamydial inclusion forming units (IFU) per ml. Infectivity titers of the LW613 strain in L-cells were not determined.

IV.B.4. Establishment and Maintenance of L-cells Persistently Infected with C. psittaci B577. L-cells persistently infected with the B577 strain of C. psittaci were obtained using the method described by Moulder et al. (113). Briefly, suspension cultures were infected with a MOI of 5-10 chlamydial IFU per L-cell, seeded into 25 cm² flasks (2 x 10^6 cells per flask) to allow the formation of monolayers, and incubated at 37°C in 5% CO₂ until >90% of the cells were destroyed through cytocidal chlamydial multiplication (wipe-out). At this point, the flasks were rinsed twice with Earle's balanced salt solution and reincubated in fresh growth medium to allow regrowth of surviving L-cells. Developing colonies were dispersed with 0.5 ml of a solution of 0.1% trypsin (1:250 trypsin, Difco, Detroit, Mich.) and 0.4% EDTA in Earle's balanced salt solution and reincubated in the same flask. Upon regrowth to confluency, the cells were trypsinized as described above and seeded at a 1:5 ratio in new flasks. Daughter flasks were divided in a similar manner at intervals of 5-7 days until the cells were wiped-out again and a new repopulation cycle began.

Attempts to establish persistent infections with the LW613 strain were done by infecting monolayers of L-cells with suspensions of freshly-harvested yolk sacs of infected chicken embryos. Infection of the monolayers was enhanced using centrifugation and cycloheximide
treatments (163).

IV.B.5. Determination of Rates of Infection in L-cells
Persistently Infected with C. psittaci B577. The percentage of persistently infected cells bearing chlamydial inclusions at any given time was determined by Giemsa-staining small aliquots of cells. Alternatively, the percentage of persistently infected cells containing chlamydial antigens was determined by an indirect fluorescent antibody (FA) reaction. Aliquots of cells seeded onto 4-chambered slides (Lab-Tek, Miles Lab., Naperville, Ill.) were treated with a hyperimmune rabbit serum (anti C. psittaci B577) and a commercial FITC-labelled goat anti-rabbit gamma globulines conjugate (U.S. Biochemical Corp., Cleveland, Ohio).

IV.B.6. Treatment of Persistently Infected L-cells with Penicillin. L-cells persistently infected with B577 chlamydiae were seeded onto six 25 cm² flasks. One flask served as a non-treated control, and the remaining flasks received 100 IU of penicillin G per ml. of medium for 1, 2, 4, 8 and 16 days respectively. The cells were maintained as described above until the monolayer was wiped-out, or for a maximum of two months.

IV.B.7. Treatment of Persistently Infected L-cells with Cycloheximide and Centrifugation. L-cells persistently infected with B577 chlamydiae were seeded onto 24 coverslip vials (162) at a density of 5 x 10⁴ cells per vial and incubated at 37°C in 5% CO₂. Twenty-four hours later the vials were divided into 4 groups of 6 vials each. The first group served as a non-treated control. The second group was centrifuged at 2000 x g for 30 minutes at 37°C. Vials from the third group received each 1 ml of growth medium with 4 ug of
cycloheximide per ml., and the fourth group was centrifuged and treated with cycloheximide as described for groups two and three respectively. The coverslips of 2 vials of each group were Giemsa-stained at 24, 48 and 72 h post-treatment, and the percentage of cells with inclusions was determined.

IV.B.8. Stationary and Centrifuge-assisted Superinfection of L-cells Persistently Infected with C. psittaci B577. Persistently infected L-cells were seeded into 25 mm Petri-dishes at a density of 2 x 10^5 cells per dish and incubated at 37°C in 5% CO₂. Twenty-four hours later, the dishes were divided into different groups (2 dishes per treatment) and superinfected with an MOI of 3-5 IFU/L-cell of wild type B577 chlamydiae. For the stationary superinfection, the inoculum (1 ml) was allowed to adsorb for 1.5 h at 37°C, while adsorption took place at 2000 x g for 30 min. at 37°C in the centrifuge-assisted superinfection. After adsorption, the cultures were washed once with Dulbecco's phosphate buffer solution, and fresh growth medium was added. All samples were Giemsa-stained 48 h post-superinfection, and the number of chlamydial inclusions per 100 cells were determined.

IV.B.9. Cycloheximide Treatment of Persistently Infected L-cells After Stationary and Centrifuge-assisted Superinfection. Petri-dishes were seeded with persistently infected L-cells as described in the previous section. The cells were superinfected with a MOI of 0.25-0.5 IFU/L-cell of wild type C. psittaci B577 and 2 ug of cycloheximide per ml were added in the growth medium. All dishes were Giemsa-stained 48 h later, and the percentage of cells with inclusions was measured.

IV.B.10. Infectivity of Wild Type C. psittaci B577 for Normal
L-cells and L-cells Spontaneously Cured from a Persistent Infection.

Normal L-5b cells and L-cells spontaneously cured from a persistent infection were seeded into 25 mm Petri-dishes and then infected through stationary or centrifuge-assisted adsorption with and without cycloheximide, using an inoculum of wild type C. psittaci B577 adjusted to infect >50% of the cells.

IV.B.11. Assay for Interferon Activity in Supernatants from Cell Cultures Persistently Infected with C. psittaci B577. The alpha-interferon activity of pooled cell culture fluids collected from persistently infected L-cells during the repopulation stage was assayed using normal L-cells and vesicular stomatitis virus, as described by Havell and Vilcek (73).

IV.B.12. Statistical Analysis. The statistical significance of the infectivity levels obtained in persistently infected and normal L-cells, after different treatments, was assessed with a paired Student's t test (159).

IV.C. RESULTS

IV.C.1. Establishment of Persistent Infection of L-cells with C. psittaci B577. In a typical experiment, suspended cells were infected with B577 chlamydiae using a MOI of 5-10 IFU/L-cell and then seeded in 25 cm² flasks for the formation of monolayers. About ten days after infection, the monolayers were destroyed as a result of a typical chlamydial cytocidal infection. However a few surviving cells started to repopulate the flasks upon continued incubation in fresh medium and remained persistently infected. The persistent infection was characterized by periods of cellular repopulation and periods of
extensive cell destruction, a process which Moulder et. al. (113) referred to as a wipe-out. This series of events occurred at regular intervals of approximately 30 days over a 167-day period of observation (Fig. IV.1).

The L-cells monolayers infected with LW613 strain developed extensive cytopathic changes after a first round of centrifuge- and cycloheximide-enhanced chlamydial replication. However, after removal of the cycloheximide five days post-infection, the cells regrew to confluency and the infection disappeared soon thereafter. Consequently, experiments using the LW613 strain were discontinued at this point.

The conditions which determine the maintenance of the B577 persistent state were further defined through the kinetics of chlamydial inclusion formation and infectivity shortly before a wipe-out. Initially, only a small percentage of cells had inclusions, but with time the percentage of cells with inclusions increased to a point where practically 100% of the cells had inclusions. Simultaneously, both the cell-associated and the extracellular chlamydial infectivity increased and the wipe-out occurred (Fig. IV.2). The steady increase in the number of persistently infected cells bearing chlamydial inclusions was also monitored with phase microscopy in unstained, live cultures. Figure IV.3A represents a small colony of L-cells shortly after a wipe-out. The cells are repopulating the flask. The number of apparently normal cells is increasing, and only a few cells have chlamydial inclusions. The L-cells continue growing (Fig. IV.3B), and the number of cells with inclusions starts to increase. Finally, most cells develop inclusions, (Fig. IV.3C), and
extensive cytopathic action leads to a new wipe-out.

IV.C.2. Effect of Penicillin G on the Onset of a Wipe-out in L-cells Persistently Infected with C. psittaci B577. In order to determine for how long the wipe-out could be delayed before the persistent state was lost, we took advantage of the chlamydiostatic effect of penicillin. Cells were treated with 100 IU of penicillin G per ml. of medium for 1, 2, 4, 8 and 16 days (Fig. IV.4). One day of penicillin treatment had no effect on the onset of the wipe-out when compared with the non-treated controls. Treatment for 2 days delayed the wipe-out 5 days, while treatments of 4 and 8 days the persistent state was lost, and the cultures were cured from the chlamydial infection.

To explain the results obtained after 2, 4 and particularly 8 days of penicillin treatment, where the number of cells with inclusions declined to barely detectable levels and reappeared soon after removal of the penicillin, we hypothesized that a chlamydial variant better suited for coexistence with the L-cells had been selected during establishment of the persistent infection. This hypothesis was tested by studying the adsorbing properties of the chlamydial strain after 7.5 months of persistence (Table IV.1). Wild type chlamydiae gave higher infectivity titers when the adsorbing medium was sucrose-phosphate buffer (16) rather than growth medium, but significantly higher titers were observed in presence of the growth medium when the persistent B577 strain was used. Accordingly, the persistent mutant strain could spread out and kill L-cell monolayers more efficiently than the wild type (Table IV.2).

IV.C.3. Immunofluorescent Reaction of L-cells Persistently
Infected with *C. psittaci* B577. To study the repopulation stage of the persistent infection, indirect fluorescent antibody test capable of detecting all developmental stages of chlamydiae was used. In some instances, only typical chlamydial inclusions were observed (Fig. IV.5A), and the percentage of infected cells correlated with that obtained with the Giemsa stain. On other occasions, the FA test revealed an additional component. Cells with typical chlamydial inclusions were found, but various numbers of cells with particulate chlamydial antigens (presumably elementary bodies) on the cell surface were also present (Fig. IV.5B). This pattern of fluorescence did not correlate with the Giemsa stain and could be observed for several days, long before the wipe-out occurred.

IV.C.4. Effect of Cycloheximide and Centrifugation on L-cells Persistently Infected with *C. psittaci* B577. The observations made with the FA test suggested that during repopulation, the L-cells go through a period of resistance in which they can adsorb elementary bodies, but do not appear to permit chlamydial multiplication and formation of inclusions.

This hypothesis was tested by manipulating the cultures in ways which are normally used to enhance chlamydial infectivity. We studied the effect of cycloheximide and centrifugation on the percentage of cells with inclusions.

In the experiment illustrated in Fig. IV.6, about 15% of the cells had inclusions as determined by the Giemsa stain, but 80% of the cells were FA positive when both cells with inclusions and cells with surface fluorescence were accounted for. No significant differences (P>0.05) were found between the control cells and the cells that were
centrifuged. However, when the cells were treated with cycloheximide alone or in combination with centrifugation, statistically significant differences (P<0.05 and P<0.005 respectively) were observed (Fig. IV.6 and Table IV.3). In particular, the combined treatment boosted the percentage of cells with inclusions to about 80%, which is the percentage of L-cells that were positive by immunofluorescence at the beginning of the experiment.

**IV.C.5. Superinfection of L-cells Persistently Infected with C. psittaci B577.** Results of the experiment outlined in Fig. IV.6 indicated that the persistently infected cells appear to go through a period of partial non-permissiveness which can be altered by manipulation with cycloheximide and centrifugation. To further assess the extent of the non-permissive state, monolayers of persistently infected cells were superinfected with known MOI of wild type C. psittaci B577. The results of these experiments are summarized in Tables IV.4 and IV.5. Persistently infected cells superinfected in the absence of cycloheximide (Table IV.4) were significantly less susceptible when compared to the normal L-cell population. When cycloheximide was added to the growth medium, differences in susceptibility were not observed (Table IV.5).

**IV.C.6. Susceptibility of Normal L-cells and L-cells Spontaneously Cured from a Persistent Infection to C. psittaci B577.** In order to determine whether the differences in susceptibility between the persistently infected and the normal L-cell populations were due to the selection of a more resistant cell type during establishment of the persistent infection, the susceptibility of normal L-5b and spontaneously cured L-cells was compared. As indicated in Table IV.6,
their susceptibility was not significantly different under four conditions of infection.

IV.C.7. Interferon Activity in Supernatants of Cell Cultures Persistently Infected with C. psittaci B577. Pooled samples of cell culture fluids collected from persistently infected cells in which a small percentage of cells had chlamydial inclusions were devoid of antiviral activity for vesicular stomatitis virus propagated in normal L-5b cells.

IV.D. DISCUSSION Persistent infections were established in vitro with the abortigenic chlamydial strain B577, but not with the arthropathogenic strain LW613. This observation may be relevant from the point of view of chlamydia-host interactions in naturally-occurring infections of animals. The B577 strain is classified as a biotype 1 of C. psittaci, and is indistinguishable from other strains commonly isolated from cattle and sheep with clinically inapparent, persistent intestinal infections (162,169). In contrast, the LW613 strain is a biotype 2 of C. psittaci, and has only been isolated from animals suffering from polyarthritis, conjunctivitis, or meningoencephalomyelitis (162,178). Naturally occurring persistent infections with biotype 2 have not been documented.

The B577 persistent state is characterized by cycles of low cytopathic expression and cycles of high cytopathic expression. These cycles seem to be determined, at least in part, by temporary changes in the susceptibility of L-cells to chlamydial infection, and by the selection of a chlamydial mutant better adapted to the cultural
conditions.

Immunofluorescence studies, together with centrifugation and cycloheximide treatments, were instrumental in showing that the persistently infected cultures go through periods of resistance in which infectious elementary bodies may adsorb, but productive infection does not occur. These changes in susceptibility are not absolute, but relative to the susceptibility of normal L-cells and can be abolished by cycloheximide treatment, a situation similar to that described for L-cells treated with alpha interferon and infected with C. trachomatis (138). Efforts to detect an antiviral activity indicative of interferon alpha in supernatants collected from persistently infected cell cultures were unsuccessful. Consequently, the factors responsible for changing the susceptibility of persistently infected cultures remain unknown at this point. In contrast to the observations of Moulder (110), in which the onset of a wipe-out is suppressed by replacing the medium 199 by Earle's minimal essential medium (MEM), the behavior of cultures persistently infected with the B577 strain was not affected by MEM (data not shown).

In the model of persistence developed using the 6BC avian strain of C. psittaci (113), the behavior of the persistently infected L-cell population is explained by assuming that every cell is "cryptically infected" and that the cryptic infection, among other properties, makes the cultures completely resistant to superinfection with exogenous chlamydiae. In our study, morphological evidence for the presence of a chlamydial "cryptic body" was not obtained. Chlamydial agents are prokaryotic organisms which, in spite of multiplication by a unique developmental cycle, maintain their cellular entity throughout the
entire infectious process (106,185). Theoretically, the presence of a "cryptic body" would have been revealed by the specific and sensitive fluorescent antibody test employed. Additional evidence against the presence of a cryptic body in our system is provided by the fact that penicillin-sensitive chlamydial infectivity was required for maintenance of the persistent state. Also, persistently infected cultures could be cured from the chlamydial infection simply by repeated subculture of the cells at very low cell densities (100 cells per 60 mm Petri dish) (Fig. IV.7), indicating that not every cell in the population is infected.

L-cells persistently infected with the B577 strain are useful for the production of large quantities of chlamydial antigens required for seroepidemiological studies using inclusion immunofluorescence and ELISA tests (See Chapter VI); they have potential as a possible tool for attenuating chlamydial virulence, and they are useful in vitro models for studying chlamydia-host cell interactions.
TABLE IV.1 - Infectivity of Wild Type (WB577) and Persistent (PB577) C. psittaci for I-cells Suspended either in Medium 199 or Sucrose-phosphate-buffer (SPB)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Passage</th>
<th>Inoculum size (ml)</th>
<th>Relative Inclusions/100 cells</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>WB577</td>
<td>2</td>
<td>1.0</td>
<td>106±5.8*</td>
<td>P&lt;.005</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>58±1.2</td>
<td>8±1.3</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>PB577</td>
<td>7.5 mo1</td>
<td>1.0</td>
<td>69±3.9</td>
<td>P&lt;.005</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>23±1.0</td>
<td>60±0.75</td>
<td>P&lt;.001</td>
</tr>
</tbody>
</table>

1 months of persistence

* mean ± SE for 4 x 100 cells
**TABLE IV.2 - Comparative Efficiency of Secondary Cycles of Infection (in L-Cells) Between Wild Type and Persistent Chlamydia psittaci**

<table>
<thead>
<tr>
<th>Strain</th>
<th>3</th>
<th>1</th>
<th>.5</th>
<th>.1</th>
<th>.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>WB577</td>
<td>9*</td>
<td>9</td>
<td>13</td>
<td>19</td>
<td>ND**</td>
</tr>
<tr>
<td>PB577</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>14</td>
</tr>
</tbody>
</table>

* days needed to lyse > 95% of the cells

** Not determined
TABLE IV.3 - Effect of Cycloheximide and Centrifugation on L-cells with Persistent Chlamydial Infection

<table>
<thead>
<tr>
<th>Hours Post-treatment</th>
<th>% of cells with chlamydial inclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>16±1.5*</td>
</tr>
<tr>
<td>48</td>
<td>9±1.7</td>
</tr>
<tr>
<td>72</td>
<td>5±0.8</td>
</tr>
</tbody>
</table>

* Mean ± SE for 5 x 100 cells

1 = No significant difference (P>0.05)

2 = Significant difference (P<0.05)

3 = Significant difference (P<0.005)
TABLE IV.4 - Stationary and Centrifuge-Assisted
Superinfection of L-Cells Persistently Infected with
Chlamydia psittaci

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Statistical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(MOI = 3-5)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>No</td>
</tr>
<tr>
<td>No Treatment</td>
<td>51</td>
</tr>
<tr>
<td>Centrifugation Only</td>
<td>ND</td>
</tr>
<tr>
<td>Stationary Infection</td>
<td>-</td>
</tr>
<tr>
<td>Centrifuge-Assisted Infection</td>
<td>-</td>
</tr>
</tbody>
</table>

PL-5b= Persistently Infected L-cells
NL-5b= Normal L-5b cells (MOI controls)

1 Inclusions per 100 cells at 48 h
2 Mean ± SE for 4 X 100 Cells

* 50-70% of the cells lysed at 48 h

ND= Not Determined
TABLE IV.5 - Stationary and Centrifuge-Assisted Superinfection of L-Cells Persistently Infected with Chlamydia psittaci, Followed by Cycloheximide Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NO</th>
<th>PL-5b</th>
<th>NL-5b</th>
<th>(T test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment</td>
<td>31</td>
<td>19±2.92</td>
<td>22±2.2</td>
<td>P&gt;0.5</td>
</tr>
<tr>
<td>Cycloheximide Only</td>
<td>6</td>
<td>23</td>
<td>80±1.8</td>
<td>89±2.0</td>
</tr>
<tr>
<td>Cyclo. Plus Centrifugation</td>
<td>23</td>
<td>80±1.8</td>
<td>89±2.0</td>
<td></td>
</tr>
</tbody>
</table>

PL-5b= Persistently infected L-cells
NL-5b= Normal L-5b cells (MOI controls)
1 % of cells with inclusions at 48 h
2 Mean ± SE for 4 X 100 Cells
TABLE IV.6 - Infectivity of Wild C. psittaci for Normal L-5b Cells and L-cells Spontaneously cured from a Persistent Chlamydial Infection

<table>
<thead>
<tr>
<th>Type of Cycloheximide Adsorption Treatment</th>
<th>Inclusions/100 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal L-cells</td>
</tr>
<tr>
<td></td>
<td>Stationary no</td>
</tr>
<tr>
<td></td>
<td>Centrifugation no</td>
</tr>
<tr>
<td></td>
<td>Stationary yes</td>
</tr>
<tr>
<td></td>
<td>Centrifugation yes</td>
</tr>
</tbody>
</table>

* mean ± SE for 3 x 100 cells.

1 = no significant difference for all treatments (P>0.05)
Chlamydial strain: *Chlamydia psittaci* B577 (ovine abortion)
Initial MOI = 10
Period of observation: 167 days

<table>
<thead>
<tr>
<th>P*</th>
<th>wp 1</th>
<th>wp 2</th>
<th>wp 3</th>
<th>wp 4</th>
<th>wp 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>43</td>
<td>73</td>
<td>106</td>
<td>139</td>
</tr>
</tbody>
</table>

Days after initial productive infection

* Persistent infection begins
Avg. no. days between wipe-outs = 31 days; Range = 28–34 days

Figure IV.1. Frequency of cytolytic episodes or wipe-outs in L-cells persistently infected with *C. psittaci* B577.
Figure IV.2. Kinetics of inclusion formation and chlamydial infectivity titers during 6 days before a wipe-out, in L-cells persistently infected with *C. psittaci* B577.
Figure IV.3. Phase contrast photomicrographs of sequential changes in L-cells persistently infected with C. psittaci B577. Top: Cells at the beginning of the repopulation cycle (day 10). Center: Same population of cells 10 days later. The cells have increased in number, and the percentage of cells with chlamydial inclusions is higher. Bottom: Confluent monolayer showing extensive cytopathic changes as the result of a dramatic increase in the percentage of cells with inclusions (day 28, two days before a wipe-out). Magnification, x 250
Figure IV.4. Effect of penicillin G treatment on the onset of a wipe-out
in L-cells persistently infected with C. psittaci B577.
Figure IV.5. Differential immunofluorescent reaction of L-cells persistently infected with *C. psittaci* B577. Top: Cells with typical intracytoplasmic chlamydial inclusions exhibit specific fluorescence. Bottom: Large numbers of cells with a pattern of fluorescence suggestive of chlamydial elementary bodies attached to the cell surface are evident. Magnification, x 400.
Figure IV.6. Effect of centrifugation and/or cycloheximide treatments on the number of persistently infected L-cells showing chlamydial inclusions.
Figure IV.7. Relationship between repeated subculture at low cell densities (100 cells/60 mm Petri dish) and time at which the wipe-out occurs in L-cells with persistent chlamydial infection.
CHAPTER V: ANTIGENIC DIVERSITY OF CHLAMYDIA PSITTACI OF MAMMALIAN ORIGIN DETERMINED BY MICROIMMUNOFLUORESCENCE

V.A. INTRODUCTION

The genus Chlamydia includes a large group of pathogens that can multiply only within the cytoplasm of living animal cells \(^{(108,111)}\). Based on their susceptibility to sulfadiazine, the production of glycogen, and the presence of a species-specific antigen, two species are currently distinguished \(^{(33,34,112)}\). Further subdivisions within each species are based on properties that do not define the species.

*Chlamydia trachomatis* infects only man and mice, and all known strains were subdivided into 3 biotypes based on their natural host and biological properties determining their interaction with cultured cells \(^{(60,111)}\). In addition, this species was also subdivided into 15 immunotypes through strain-specific protein antigens found on the chlamydial envelop and usually demonstrated by indirect immunofluorescence \(^{(60,109)}\).

*Chlamydia psittaci*, the second species of the genus, comprises a large collection of strains and exhibits broad host ranges and pathogenic potentials \(^{(171,175)}\). Several efforts have been made to differentiate this highly heterogeneous species \(^{(24,55,83,102,127,131,143,145,146,162,163)}\), but only limited success has been achieved. Schachter et al. \(^{(145,162)}\) distinguished several isolates of *Chlamydia psittaci* of ovine and bovine origin into 2 broad antigenic groups based on a plaque reduction test, but a few strains of similar origin could not be typed. Antibodies against these strains did not neutralize infectivity of several strains of avian origin. More...
recently, Spears and Storz (162) studied chlamydial strains isolated from a broader range of animal hosts. Based on inclusion morphology and response to different treatments in cultured cells, 8 biotypes of C. psittaci were established, but the usefulness of this classification scheme remained limited because considerable experience is required and the process is very laborious.

The purpose of this investigation was to immunotype different biotypes of C. psittaci of mammalian origin using a modification of Wang's (189) indirect micro-immunofluorescence test (IMIF).

V.B. MATERIALS AND METHODS

V.B.1. Chlamydial Strains. Twenty five strains of C. psittaci representing at least 7 biotypes of bovine, ovine, caprine, porcine, feline and guinea pig origin were used throughout this investigation. The origin of each isolate, together with other relevant information, are summarized in Table V.1.

V.B.2. Chlamydial Antigens for Mouse Inoculations. Chlamydial antigens used for inoculation of mice consisted of partially purified elementary body suspensions. Briefly, the different chlamydial strains were propagated in chicken embryos as described previously (163). Heavily-infected yolk sacs were homogenized in sucrose phosphate buffer (SPB) (16), and the suspension was centrifuged at 200 xg for 5 min at 4°C. The chlamydiae present in the supernatant were pelleted at 12,000 xg for 1 h at 4°C, and resuspended in 0.01M phosphate buffered saline (pH 7.1) (PBS).

Partial purification was accomplished by centrifugation at 40,000 xg for 1 h at 4°C, through a solution of 35% renografin (diatrizoate
meglumine and diatrizate sodium, 76% for injection; E.R. Squibb and Sons, Princeton, N.J.) in PBS (Fig. V.1). The partially purified chlamydial elementary bodies were washed once, and resuspended in a final volume of 10 mls of PBS per yolk sac. Antigens that were not used immediately for mouse inoculations were stored at -70°C.

V.B.3. Chlamydial Antigens for Indirect Microimmunofluorescence. Chlamydial antigens used in the IMIF test consisted of L-cell-propagated, partially purified EB suspensions. Briefly, cultures of L-cells were infected using a modification of the procedure described by Lee (91). Confluent monolayers grown in 60 mm Petri dishes were inoculated with $10^{-2}$ dilutions of clarified yolk sac homogenates, using a centrifugal force of 2,000 xg for 30 min at 37°C (Fig. V.2). The infected cells were incubated at 37°C in culture medium containing 2 ug of cycloheximide per ml, and chlamydiae were harvested 48 to 72h later, after freezing and thawing the monolayers. Partial purification of chlamydial EB was accomplished by the procedure described above. Yields obtained from 24 to 36 Petri dishes were resuspended in 1 to 2 ml of PBS containing 0.12% bovine serum albumin, and sonicated for 15 to 20 seconds at a power setting of 40 using a Branson cell disruptor. All antigens were aliquotted in 0.25 ml amounts, and stored at -70°C.

V.B.4. Production of Antisera in Mice. Different groups of chlamydia-free BALB/c mice (10-20 mice/group) received two weekly intravenous inoculations (0.5 ml/mouse/inoculation) of the chicken embryo-propagated, partially purified chlamydial antigens. Immunoglobulin (Ig) M-rich antisera were collected 4 days after the first inoculation, and IgG-rich antisera were collected 3 to 4 days
after the 2nd inoculation (Fig. V.3). All serum samples were stored at 
-70°C.

V.B.5. Indirect Microimmunofluorescence Test. The IMIF test was 
performed using a modification of the procedure described by Wang 
(189). Briefly, small amounts of each cell-propagated antigen were 
placed on 12-well teflon coated slides (Cell-Line Associates Inc., 
Newfield, N.J.) by means of a 20 ul Eppendorf pipet. The antigens were 
arranged in groups of 4 per well (Fig. V.4). By placing a different 
set of antigens on the top and bottom row of each slide, a total of 8 
different antigens could be tested with one serum on each slide.

Following a period of 30 min for air-drying at room temperature, 
the slides were fixed in cold formaldehyde (4% in PBS pH 7.2) for 5 
min, and 15 ul of the proper antiserum dilution (two fold dilutions 
from 1:8 to 1:256) were used, followed by 15 ul of the working 
conjugate dilution (FITC-conjugated sheep anti-mouse IgG or 
FITC-conjugated goat anti-mouse IgM, U.S. Biochemical Corporation, 
Cleveland, Ohio) in PBS with rhodamine-conjugated bovine albumin (Difco 
Laboratories, Detroit, Michigan) as a counterstain. The microscopic 
examination was performed with a Carl Zeiss epifluorescence microscope 
equipped with a 40 x oil immersion lens. The highest serum dilution 
giving specific fluorescence associated with elementary bodies was 
recorded as the end point titer (Fig. V.5).

V.C. RESULTS

The IgM-specific antiserum response of mice is summarized in Tables 
V.2 and V.3. Extensive cross reactions were observed among chlamydial 
strains of the same biotype, and some cross reactions between different
biotypes occurred. Results obtained with the IgG-specific IMIF test are summarized in Tables V.4 and V.5. In this instance, the specificity of the immune serum reaction within a given biotype was maintained, but the extent of cross reactions between different serotypes increased.

Based on the IgM-rich and IgG-rich antiserum responses of mice, nine immunotypes of *C. psittaci* were distinguished, and an overall pattern of cross reactivities among the different immunotypes identified could be obtained (Table V.6). With this information, additional chlamydial strains of bovine, caprine and equine origin were immunotyped in one-way tests, using antisera from nine strains representing the different immunotypes identified (Table V.7).

With one exception (serotype 6), the different immunotypes of *C. psittaci* identified by the IMIF test were numbered based on the predominant biotype which they represented (Table V.8). Immunotype 1 comprised chlamydial strains previously classified as biotype 1, plus four chlamydial strains of bovine, caprine and equine origin whose biotype had not been previously determined. Immunotypes 2, 3, 5 and 8 included strains of only biotypes 2, 3, 5 and 8 respectively. Immunotype 4 included a chlamydial strain of biotype 4 plus a porcine isolate of unknown biotype. Immunotype 6 was represented by a chlamydial strain of biotype 4, together with one porcine and one bovine isolate, both of undetermined biotype. Immunotype 7 included the feline pneumonitis vaccine strain (biotype 7), and the Z-10 strain reportedly of bovine origin and of undetermined biotype. Immunotype 9 included a single isolate of ovine origin, whose biotype has not been determined.

The yields of EB's obtained from cell cultures infected with the
different chlamydial strains varied considerably. Variations in their degree of cell association were also observed, in spite of being cultivated and partially purified under the same conditions. Consequently, the IMIF reaction was significantly more difficult to read with some antigens than with others. In order to diminish this subjective effect, a 50% difference in titer among strains of the same biotype was not considered significant.

V.D. DISCUSSION

The IMIF test differentiated 25 strains of \textit{C. psittaci} of mammalian origin into 9 immunotypes. An excellent correlation was observed between immunotypes and biotypes, indicating that strains with unique biological properties also have unique antigenic make-ups.

This proposed immunotyping scheme should be expanded to include other chlamydial strains, especially those of avian origin. Although none of the \textit{C. psittaci} immunotypes identified in this study cross reacted significantly with the mouse pneumonitis strain, antigenic relationships with other strains of \textit{C. trachomatis} should also be explored.

Our findings are in agreement with previous reports on the antigenic and genomic relationships of limited numbers of ovine strains of \textit{C. psittaci}. Eb and Orfila (48), using the IMIF test, differentiated 6 ovine abortion strains of similar antigenic make-up from the ovine arthropathogenic strain LW-679. More recently, McClenaghan et al. (102), using DNA restriction endonuclease analysis, identified unique DNA fragments common to 8 ovine abortion chlamydial strains which differed to some extent from those of a single isolate.
obtained from polyarthritic lambs in Scotland.

The different immunotypes identified in our investigation apparently have some degree of disease and host specificity. The intestinal mucosa emerges as a common site of infection. Immunotype 1 includes strains isolated from ruminants affected with abortions, seminal vesiculitis, pneumonia, and clinically inapparent intestinal infections. The pathogenic potential of most of these isolates had been confirmed experimentally (171,175). Two isolates from horses, one from the lung and one from synovial fluid are also identified as immunotype 1. However, their pathogenic properties have not been assessed experimentally. Since none of the other strains classified as immunotype 1 has arthropathogenic properties for cattle or sheep, and since all arthropathogenic strains have uniquely distinguishing features, more equine isolates should be studied to determine whether they are related indeed to strains from ruminants. It could be that the isolate recovered from a foal with polyarthritis was spurious.

Immunotype 2 also includes strains isolated from ruminants, but the disease association of this group of strains is conjunctivitis, polyarthritis, encephalitis, and enteritis, under natural and experimental conditions (171,175).

Immunotypes 3 and 9 are represented by chlamydial isolates which appear to be part of the intestinal flora of cattle and sheep. The pathogenic potential of these isolates has not been explored experimentally.

Immunotypes 4, 5 and 6 represent procine strains associated with polyarthritis or generalized infections, clinically inapparent intestinal infections, and abortion or pneumonia, respectively. A
single isolate from bovine pneumonia is also identified as immunotype 6. The pathogenicity of these strains has not been studied experimentally.

Immunotype 7 includes the chlamydial strain used in the live feline pneumonitis vaccine, and an isolate presumably cultured from a calf with pneumonia (strain Z-10). Whether a common chlamydial antigenic make-up may be associated with respiratory infections in cats and cattle remains to be proven. In some areas of this country it has been common practice to vaccinate cattle with the live feline pneumonitis vaccine (Fromm Lab.). It is not known whether the bovine isolate classified as immunotype 7 comes from a calf of a herd vaccinated with the feline pneumonitis strain or whether it is a naturally occurring pneumopathogenic strain from calves.

While some of the immunotypes identified induced antibodies which reacted only with the homologous strains (immunotypes 5 and 8), most of the other immunotypes induced cross reacting antibodies. Notably, immunotype 2 strains did not seem to induce good antibody responses in mice, and cross reactions of the same magnitude as the homologous low-titered reactions were frequently present with antigens of different immunotypes.

The molecular basis for the specific antigenic relationships observed was not determined. However, Caldwell et al. have demonstrated that a major outer membrane protein (MOMP) of MW of approximately 39.5 kd is responsible for the immunotype specificity observed in the genus Chlamydia (31,35,37). Antibodies against purified MOMP from several strains of C. trachomatis and from the meningopneumonitis strain of C. psittaci, reacted with the same
specificity as whole EB's in the IMIF test (35).

The chlamydial genus-specific lipopolisaccharide (LPS) antigen is also found in the envelop of the EB's (121), but its effect on the antigenic relationships observed in the IMIF test appears to be insignificant. Wang (189) suggested that the early mouse antibodies used in the IMIF test contain not only type-specific, but also genus-specific antibodies. However, the chlamydial LPS does not seem to be readily available on the surface of untreated or paraformaldehyde fixed EB's (165,190).

The findings in our investigation should provide the necessary background for the rational selection of prototype strains of C. psittaci to be used in more refined studies involving the interaction of antibodies with purified chlamydial antigens, and the production of monoclonal antibodies of defined specificity.
Table V.1 - Biotypes of *C. psittaci* of Mammalian Origin Included in a Serotypification Scheme by Indirect Microimmunofluorescence.

<table>
<thead>
<tr>
<th>Chlamydial Strain</th>
<th>Biotype*</th>
<th>Host</th>
<th>Clinical finding</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBA-59-795</td>
<td>1</td>
<td>Cattle</td>
<td>Abortion</td>
<td>177</td>
</tr>
<tr>
<td>EBA-LX-578</td>
<td>1</td>
<td>Cattle</td>
<td>Abortion</td>
<td>Storz²</td>
</tr>
<tr>
<td>LW-508</td>
<td>1</td>
<td>Cattle</td>
<td>Enteritis</td>
<td>Storz²</td>
</tr>
<tr>
<td>SV-139</td>
<td>1</td>
<td>Cattle</td>
<td>Seminal vesiculitis</td>
<td>174</td>
</tr>
<tr>
<td>B-577</td>
<td>1</td>
<td>Sheep</td>
<td>Abortion</td>
<td>169</td>
</tr>
<tr>
<td>Fitz-65</td>
<td>1</td>
<td>Sheep</td>
<td>Abortion</td>
<td>Storz²</td>
</tr>
<tr>
<td>Equine Synovial</td>
<td>ND</td>
<td>Horse</td>
<td>Synovitis</td>
<td>100</td>
</tr>
<tr>
<td>Bovine Pneumonia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-1</td>
<td>ND</td>
<td>Cattle</td>
<td>Pneumonia</td>
<td>Siegfried²</td>
</tr>
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<td>Bovine Pneumonia</td>
<td></td>
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<td></td>
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<td>Siegfried²</td>
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<td>Goat</td>
<td>Abortion</td>
<td>Poston³</td>
</tr>
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<td>Pneumonia</td>
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<tr>
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<td>2</td>
<td>Cattle</td>
<td>Sporadic encephalomyelitis, polyarthritis</td>
<td>Storz²</td>
</tr>
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</table>
Table V.1 - Continued

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<th>Strain</th>
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<th>Clinical finding</th>
<th>Source</th>
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<td>Storz^a</td>
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<tr>
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<td>Cattle</td>
<td>Normal (feces)</td>
<td>176</td>
</tr>
<tr>
<td>JP-I-751</td>
<td>ND</td>
<td>Sheep</td>
<td>Normal (feces)</td>
<td>Perez-Martinez^d</td>
</tr>
<tr>
<td>L-71</td>
<td>4</td>
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<td>Polyarthritis</td>
<td>Kolbl^e</td>
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<td>PR-86</td>
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<td>Generalized infection</td>
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<td>Swine</td>
<td>Abortion</td>
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</tr>
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<td>S45</td>
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<td>Normal (feces)</td>
<td>Kolbl^e</td>
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</tr>
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<td>Cat</td>
<td>Live vaccine</td>
<td>Fromm Lab^f</td>
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<td>Cattle?</td>
<td>Pneumonia?</td>
<td>Fromm Lab^f</td>
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<td>8</td>
<td>Guinea</td>
<td>Conjunctivitis</td>
<td>115</td>
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* = Determined by Spears and Storz (165)

ND = Not determined

^a Isolated in the laboratory of J. Storz
Table V.1 - Continued

b Obtained from B.L. Siegfried, Dept. of Pathobiological Sciences, School of Vet. Medicine, Univ. of Wisconsin, Madison, Wis.
c Isolated by R. Poston, Louisiana State University, Veterinary Diagnostic Laboratory, Baton Rouge, LA.
e Obtained from O. Kolbl, Federal Institute for Viral Diseases of Animals, Vienna, Austria.
f Obtained from Fromm Laboratories, Grafton, Wis.
Table V.2 - Antigenic Relationships of Chlamydial Strains of Biotypes 1 and 7, Mediated by Mouse IgM Antibodies in the Indirect Microimunofluorescence Test.

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*Cross reaction as percentage of homologous titer (0=No reaction of serum at a 1:8 dilution)

1 Serum titer with homologous antigen, expressed as reciprocal of the dilution
Table V.3 - Antigenic Relationships of Chlamydial Strains of Biotypes 2, 3, 4, 5 and 8, Mediated by Mouse IgM Antibodies in the Indirect Micro-Immunofluorescence Test.

<table>
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<tr>
<th>Antiserum</th>
<th>LW-613</th>
<th>LW-623</th>
<th>LW-679</th>
<th>Fc-Stra</th>
<th>66-P-130</th>
<th>L-71</th>
<th>PR-86</th>
<th>S45</th>
<th>1708</th>
<th>1710S</th>
<th>GP-IC</th>
<th>JP-I-751</th>
<th>Titer(^1)</th>
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<td>0</td>
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<td>0</td>
<td>16</td>
</tr>
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<td>0</td>
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</tr>
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\* Cross reaction as percentage of homologous titer (0=6% or no reaction of serum at 1:8 dilution)

\(^1\) Serum titer with homologous antigen, expressed as reciprocal of the dilution
### Table V.4 - Antigenic Relationships of Chlamydial Strains of Biotypes 1,2,4,6 and 7, Mediated by Mouse IgG Antibodies in the Indirect Microimmunofluorescence Test.

<table>
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<tr>
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<td>PR-86</td>
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<td>0</td>
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</tr>
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<tr>
<td>Z-10</td>
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<td>100</td>
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<tr>
<td>Fel. Pneum.</td>
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<td>12</td>
<td>12</td>
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<td>25</td>
<td>12</td>
<td>100</td>
<td>100</td>
<td>64</td>
</tr>
</tbody>
</table>

* Cross reaction as percentage of homologous titer (0=<6% or no reaction of serum at 1:8 dilution)

1 Serum titer with homologous antigen, expressed as reciprocal of the dilution
Table V.5 - Antigenic Relationships of Chlamydial Strains of Biotypes 2, 3, 4, 5 and 8, Mediated by Mouse IgG Antibodies in the Indirect Microimmunofluorescence Test.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>LW-613</th>
<th>LW-623</th>
<th>LW-679</th>
<th>Fc-Stra</th>
<th>66-P-130</th>
<th>L-71</th>
<th>PR-86</th>
<th>S45</th>
<th>1708</th>
<th>1710S</th>
<th>GP-IC</th>
<th>JP-I-751</th>
<th>Titer^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LW-613</td>
<td>100*</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>200</td>
<td>50</td>
<td>12</td>
<td>200</td>
<td>200</td>
<td>0</td>
<td>200</td>
<td>32</td>
<td></td>
</tr>
<tr>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>64</td>
</tr>
<tr>
<td>LW-679</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>100</td>
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<td>100</td>
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<td>0</td>
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<td>32</td>
</tr>
<tr>
<td>Fc-Stra</td>
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<td>100</td>
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<td>50</td>
<td>25</td>
<td>12</td>
<td>12</td>
<td>50</td>
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<td>128</td>
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<tr>
<td>66-P-130</td>
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<td>L-71</td>
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<td>PR-86</td>
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<td>12</td>
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<tr>
<td>S45</td>
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<td>0</td>
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<td>256</td>
</tr>
<tr>
<td>1710S</td>
<td>6</td>
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<td>12</td>
<td>0</td>
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<td>12</td>
<td>6</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>JP-I-751</td>
<td>12</td>
<td>25</td>
<td>25</td>
<td>12</td>
<td>50</td>
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<td>0</td>
<td>200</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>128</td>
</tr>
</tbody>
</table>

*Cross reaction as percentage of homologous titer (0<6% or no reaction of serum at 1:8 dilution)

^1 Serum titer with homologous antigen, expressed as reciprocal of the dilution
Table V.6 - Summary of Maximum Cross Reactions Observed Among Different Immunotypes of *C. psittaci*, Mediated by Mouse IgG Antibodies in the Indirect Microimmunofluorescence Test.

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
<th>Type 4</th>
<th>Type 5</th>
<th>Type 6</th>
<th>Type 7</th>
<th>Type 8</th>
<th>Type 9</th>
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<td>12</td>
<td>50</td>
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<td>12</td>
</tr>
<tr>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
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<td>100</td>
<td>100</td>
<td></td>
<td>50</td>
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<td>12</td>
<td></td>
<td>12</td>
</tr>
<tr>
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<td>12</td>
</tr>
<tr>
<td>Type 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 6</td>
<td>50</td>
<td>12</td>
<td>12</td>
<td>12</td>
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<td>25</td>
<td></td>
<td></td>
<td>25</td>
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<tr>
<td>Type 7</td>
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<td>100</td>
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<td></td>
</tr>
<tr>
<td>Type 8</td>
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<td>50</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

*Cross reaction as percentage of homologous titer
Table V.7 - Results of a One Way Immunotyping of Four *C. psittaci* Isolates, Mediated by Mouse IgG Antibodies in the Microimmunofluorescence Test.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
<th>Type 4</th>
<th>Type 5</th>
<th>Type 6</th>
<th>Type 7</th>
<th>Type 8</th>
<th>Type 9</th>
<th>Identified</th>
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<tr>
<td>Equine Pneumonia</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Bov. Pneum. L-1</td>
<td>100</td>
<td>0</td>
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<td>12</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Goat Abortion</td>
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<td>12</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*Cross reaction as percentage of the type antiserum titer with its homologous antigen (0=6% or no reaction of serum at 1:8 dilution).*
Table V.8. Correlation Between Immunotypes Identified by Indirect Microimmunofluorescence, and Biotypes of *C. psittaci* of Mammalian Origin

<table>
<thead>
<tr>
<th>IMIF-Immunotype</th>
<th>Chlamydial strain</th>
<th>Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EBA-59-795, EBA-LX-578, LW-508, SV-139, B-577, Fitz-65, Bov. Pneumonia L-1, Goat abortion</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Eq. Pneumonia, Eq. Synovial</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>LW-613, LW-623, LW-679, Fc-Sta</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>66-P-130</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>L-71</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>PR-86</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>S45</td>
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<tr>
<td>6</td>
<td>1710S</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1708, Bov. Pneumonia L-1</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>Fel. Pneumonitis (Fromm vaccine)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Z-10 (Bov. pneumonia?)</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>GP-IC</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>JP-I-751</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not determined
Figure V.1. Procedure used for the production of chicken embryo-propagated chlamydial antigens for mouse inoculation.
Figure V.2. Procedure used for the production of cell culture-propagated chlamydial antigens for indirect microimmunofluorescence.
Figure V.3. Schedule of mouse inoculations and bleedings for the production of chlamydia-specific antisera.
Figure V.4. Arrangement of chlamydial antigens and antiserum dilutions in the indirect microimmunofluorescence test.
Figure V.5. Photomicrograph of chlamydial elementary bodies as seen in the indirect microimmunofluorescence test. Magnification, x 400.
CHAPTER VI: ANTIBODY RESPONSES OF COWS EXPERIMENTALLY INOCULATED WITH ABORTIGENIC CHLAMYDIAE: DEVELOPMENT AND COMPARISON OF A MODIFIED COMPLEMENT FIXATION TEST, AN INDIRECT INCLUSION FLUORESCENCE ANTIBODY TEST, AND AN ENZYME-LINKED IMMUNOSORBENT ASSAY.

VI.A. INTRODUCTION

Chlamydia (C) psittaci is a widely distributed obligate intracellular bacterium which exhibits a broad pathogenic potential. Depending on factors such as virulence of the strain, physiological state of the host, environmental factors and stress, a chlamydial infection may lead to pneumonia, gastroenteritis, conjunctivitis, polyarthritis, encephalomyelitis, abortion and infertility in different animal species (175).

Diagnosis of chlamydial infections of domestic mammals is still a difficult task, and the incidence of these infections is underestimated with the currently applied diagnostic tests.

The clinical signs associated with the different chlamydia-induced diseases are not specific enough for an accurate diagnosis. The direct visualization of the agent in infected tissues represents a relatively rapid but insensitive diagnostic procedure, and its usefulness is restricted to exfoliative cytological preparations of conjunctival or placental samples which often are not available for examination. The isolation of chlamydiae from clinical specimens has been improved by replacing the commonly used chicken embryo techniques by faster and more sensitive cell culture methods (80, Perez-Martinez and Storz, Abstr. 63rd Mtg. CRWAD, Chicago, pp 45), but the process remains technically difficult and relatively few laboratories offer this
For these reasons, the diagnosis of chlamydial infections of cattle, sheep and goats is done mainly by serology. For this purpose, a conventional complement fixation (CF) test employing a genus-specific chlamydial antigen (Ag) is frequently used (118,129,136,175). This serological procedure is useful for the diagnosis of chlamydial diseases with a systemic phase of infection, but not all immunoglobulin (Ig) isotypes can be detected (23,76,104). Furthermore, this test probably does not detect chlamydial infections localized to mucous membranes.

Since the application of modern tests such as immunofluorescence, enzyme-linked immunosorbent assays (ELISA) and radio immunoassays has significantly improved the serodiagnosis of chlamydial infections in man (38,84,94,133,141,155,182,191), birds (54,140,153), and laboratory animals (156), the objective of this study was to develop and assess the diagnostic value of modern tests for bovine chlamydial abortions. For this purpose, a modified complement fixation (MoCF) test, an indirect inclusion fluorescent antibody (IIFA) test, and an ELISA were evaluated by measuring the chlamydia-specific antibody responses of cows experimentally inoculated with different abortigenic strains of C. psittaci.

VI.B. MATERIALS AND METHODS

VI.B.1. Experimental Animals. The chlamydia-specific antibody responses of 11 cows that had previously been experimentally inoculated with different abortigenic strains of C. psittaci was studied using sequential serum samples collected from the time of inoculation and for
up to 380 days later (177). The cows were of different breeds and they were in the 3rd to 8th month of gestation.

Six of the cows (cows 134, 513, 576, 577, Angus and red heifer) were intramuscularly (IM) inoculated, two cows (cows 329 and 2177) were subcutaneously (SC) inoculated, and two other cows (cows 275 and Holstein) were intravenously (IV) inoculated with the ERA-59-795 or the LX-578 chlamydial strains originally isolated from aborted fetuses in California and Utah, respectively (173,177). An additional cow (No. 101) was inoculated IM with a chlamydial strain (M.b.) isolated from cattle with intestinal infections (104a).

VI.B.2. Complement Fixation Tests. The MoCF test was performed in a microtiter system as described by Boulanger et. al. (14), using 2 units of a genus-specific chlamydial antigen (Ag) (kindly provided by Dr. L. Page) (129), and 2 units of guinea pig complement supplemented with 5% normal calf serum free of chlamydia-specific antibodies (Ab). This bovine serum had a 50% hemolysis titer of 320.

The serum-Ag-complement mixture reacted for 18 h at 8°C before the hemolytic system was added. It contained 2 100% hemolytic units of rabbit hemolysin for sheep red blood cells, and 5% sheep red blood cells. End point titers were recorded as the highest 2-fold serum dilutions (0.3 log$_{10}$) with at least a 50% inhibition of hemolysis. The procedures for the standard CF test were identical to those of the MoCF test but without bovine serum supplement. The two tests were performed simultaneously when serum samples were tested with both.

VI.B.3. Indirect Inclusion Fluorescent Antibody Test. The IIFA test was performed in 12-well teflon coated slides (Cell-Line Associates Inc., Newfield, N.J.) previously seeded with L-cells
infected in suspension with the B577 ovine abortion strain of C. psittaci as previously described (See Chapter III). The cells were fixed in cold formaldehyde for 5 min and stored at -70°C until they were used. Serum samples were tested as described elsewhere (133) using a commercial FITC-rabbit anti-bovine IgG (H+L) conjugate (U.S. Biochemical Corporation, Cleveland, Ohio) diluted in phosphate buffered saline (PBS) of pH 7.1, and counterstained with Evans blue. The test was read with a Carl Zeiss epifluorescence microscope using a 40x oil immersion lens. End point titers were recorded as the highest 2-fold serum dilutions (0.3 log10) with which characteristic chlamydial inclusions fluoresced (Fig. VI.1).

VI.B.4. Enzyme Linked Immunosorbent Assay. The ELISA was performed on non-irradiated Immulon I microtiter plates (Dynatech Laboratories Inc., Alexandria, Va.) as recently described in detail (155). A particulated chlamydial antigen at a dilution of 1:200 and duplicates of a single serum dilution of 1:100 were used. The chlamydial Ag was obtained by differential centrifugation after freezing and thawing heavily infected L-cell monolayers persistently infected with the B577 ovine abortion strain of C. psittaci (See Chapter IV). Cell lysates from five 150 cm² tissue culture flasks were centrifuged first at 500 xg for 5 min to sediment the cellular nuclei. The resultant supernatant was centrifuged at 12,000 xg for 1 h to sediment the chlamydiae. The chlamydial pellet was washed twice before it was resuspended in 10 mls of PBS of pH 7.1, using a Branson sonifier. This antigen was aliquoted and stored at -70°C. Cell lysates of non-infected L-cell monolayers were treated the same way and served as L-cell Ag controls.
Microtiter plates coated with the chlamydial and L-cell control Ags were incubated for 18 h at 37°C, treated with formaldehyde for 5 min and stored in a dessicator at 4°C until they were used.

Immunofinity-purified Abs specific for bovine IgG (H+L) and conjugated with horseradish peroxidase (154) were used at a dilution of 1:2000, followed by 2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) (Sigma Chemical Co., St. Louis, Mo.) as a substrate. Sera of the same cow were tested in sequence of bleedings on the same microtiter plate, and the optical density at 410 nm (OD\textsubscript{410}) was recorded through an ELISA reader (Dynatech Laboratories, Inc., Alexandria, Virginia) when a positive standard serum reached a value of 0.800. The net OD value of each serum was determined by subtracting the average (n=2) OD obtained with the L-cell Ag from the average OD (n=2) obtained with the chlamydial Ag. A base line of significance was calculated at an OD=0.100, which represents the average L-cell Ag-reaction of 50 random determinations, plus 2 standard errors of the mean.

VI.C. RESULTS

VI.C.1. Modified Versus Standard Complement Fixation Test. The application of the MoCF test to sequential serum samples of 3 different cows (cows 101, 2177 and Angus), resulted in an average (n=23) 4-fold increase in titer and a larger number of pre- and post-inoculation positive samples in comparison to the standard CF test.

The typical response of the IM inoculated cow 101 aborting 45 days post inoculation is represented in Figure VI.3. This cow was seronegative as determined by the standard CF test, and it did not
become positive until after abortion. In contrast, with the modified test, an earlier antibody response was detected, and in fact, the cow had already a detectable titer before the inoculation.

Because of the consistently higher sensitivity of the MoCF test, the standard CF test was not applied to the serum samples collected from the rest of the cows in this comparative evaluation.

VI.C.2. Relationship Between Optical Density and Serum Dilution in the ELISA. ELISA titrations of selected serum samples of known IIFA-titers ranging from 1:8 to 1:4096 showed a statistically significant correlation (p<0.001) between the OD values and the dilution factors, indicating that time consuming serum titrations could be replaced by a single determination at a 1:100 dilution to demonstrate quantitative differences in chlamydia-specific Ab levels (Fig. VI.2).

VI.C.3. Dynamics of the Antibody Responses Determined by the MoCF, IIFA and ELISA Tests.

Six of the 11 cows tested (55%) had detectable pre-inoculation chlamydia-specific Ab titers between 1:8 and 1:16 when tested by the MoCF test. In contrast, 9 of 11 (82%), and 10 of 11 (91%) pre-inoculation samples had detectable Ab titers or significant OD values as determined by the IIFA and the ELISA tests respectively, both of which use conjugates of the same specificity (Table VI.1). The pre-inoculation IIFA titers ranged from 1:8 to 1:64, and the ELISA values (OD_{410}) varied from 0.150 to 0.450. Following the experimental chlamydial inoculations, significant increases in Ab levels were always observed irrespective of the pre-inoculation titer.

The chlamydia-specific reaction of sequential serum samples of cows
experiencing abortion after IM inoculation with either one of the bovine abortion strains or with the intestinal isolate was similar. A typical biphasic Ab response is depicted for the Angus cow, which aborted 52 days post IM inoculation with the LX-578 isolate (Fig. VI.4). A minor antibody rise appeared 20 days post inoculation, and a 2nd major Ab rise appeared 30 days following abortion.

The remaining cows experiencing abortion after SC or IV inoculation with bovine abortion chlamydial strains had changes in antibody levels similar to those described. Figure VI.5 represents the typical biphasic pattern observed in the SC inoculated cow 329, which aborted 56 days post inoculation. The changes characteristic of the IV inoculated cow 375, which aborted 36 days post-inoculation and was challenge-inoculated 92 days later, are represented in figure VI.6. The abortion occurred earlier in this case and the biphasic type of response was not apparent. Maximum Ab levels were observed 69 days post inoculation, and started to decline gradually thereafter. The challenge-inoculation boosted the immune response again, but the increase in Ab levels observed at this time appeared to be less pronounced.

The IV inoculation of a pregnant Holstein cow, which did not experience an abortion, resulted in a single Ab rise with peak values observed 15 days post inoculation (Fig. VI.7). The titers then declined gradually.

Overall, the dynamics of the antibody responses had similar patterns with all three serological tests employed. The titers obtained by the IIFA test were plotted against the ELISA values obtained at a single serum dilution of 1:100, and a statistically significant
correlation value was obtained ($p<0.001$) (Fig. VI.8).

VI.D. DISCUSSION

Findings from this investigation clearly demonstrate that it is possible to improve significantly the serodiagnosis of bovine chlamydial abortions, and possibly other chlamydial infections of cattle. The consistency of a biphasic antibody response observed in the cows which experienced chlamydia-induced abortions indicates that these infections can be diagnosed by testing paired serum samples collected at the time of abortion and 3 weeks later.

It is likely that the high percentage of pre-inoculation seropositive cows detected by the IIFA test and the ELISA was the result of previous exposure to chlamydial antigens. The occurrence of clinically-inapparent persistent intestinal infections with chlamydiae in ruminants has been reported frequently (129,169). Furthermore, the presence of these intestinal infections do not seem to stimulate immunity against experimental challenge, even when the homologous strain is used (169).

The sensitivity of the commonly used standard CF test was notably improved by the addition of bovine complement. This observation is in agreement with several previous reports by other investigators in which the serodiagnosis of different infectious diseases, not only of cattle (15,104) but also other animal species (21), was similarly improved by supplementing the guinea pig complement with complement from the species being tested. The basis for this improvement seems to be the need of species-specific heat-labile complement component Cl for fixation of guinea pig complement by some immunoglobulin (Ig) isotypes
In the case of cattle, it has been shown that while IgG₁ can fix guinea pig complement under conditions of the standard CF test, IgG₂ can only be detected in the supplemented test (23,76,104). The ability of bovine IgM to fix guinea pig complement seems to depend on the nature of the Ag involved (43,76,154).

Preliminary evidence obtained by ELISA using subclass-specific conjugates seems to indicate that the chlamydia-specific antibody fraction of the serum samples tested contains high levels of non-complement binding IgG₂ (Schmeier et. al. Manuscript in preparation). This observation may explain the different results obtained by the standard and the MoCF tests. Furthermore, the good correlation found between the MoCF test and the IgG H+L chain-specific IIFA test and ELISA is most likely based on the predominant reaction of IgG₂.

In spite of the superiority of the MoCF test over the standard CF test, the IIFA test and the ELISA surpassed the reactivity of the MoCF test. The IIFA test and the ELISA detected a larger number of preinoculation seropositive cows than the MoCF test. Relatively higher titers were obtained with the IIFA test, and high OD values were reached with the ELISA using single serum dilutions of 1:100.

The MoCF test described in this study used a boiled chicken embryo-propagated chlamydial Ag, and thus detected mainly genus-specific Abs (46,129,175). In contrast, both the IIFA test and the ELISA used whole C. psittaci organisms containing both genus- and strain-specific Ags (84,133). Since the chlamydial strain used in these two procedures belongs to the same immunotype as the chlamydial strains used to infect the cows tested (See chapter V), it is likely that in addition to the
genus-specific Abs, strain-specific Abs were also detected.

The unsatisfactory results obtained in a previous effort to develop an ELISA for the diagnosis of chlamydial infections in cattle may have derived from the use of Ag preparations and immune reagents of different specificities (86).

The IIFA test and the ELISA seem to be well suited for routine serological diagnosis of chlamydial infections of cattle. The selection of either one of them should depend on the particular needs of a given laboratory. The IIFA test is relatively easy to perform and to interpret, and requires only a microscope fitted for immunofluorescence. On the other hand, the ELISA eliminates the subjective interpretations inherent to immunofluorescence procedures by giving objective spectrophotometric values. Because single serum dilutions give readings reflecting Ab levels, relatively large numbers of serum samples can be tested simultaneously. An additional advantage offered by the IIFA test and the ELISA is the opportunity for determining specific isotype- and subisotype-antibody responses without using time-consuming fractionation procedures.
TABLE VI. 1 - Frequency of Animals Seropositive for Chlamydial Antigens, prior to Experimental Inoculation.

<table>
<thead>
<tr>
<th>Serological test</th>
<th>No. of Positives (%)</th>
<th>Titer</th>
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<tbody>
<tr>
<td>MoCF</td>
<td>6/11 (55)</td>
<td>1:8-1:16</td>
</tr>
<tr>
<td>IIFA</td>
<td>9/11 (82)</td>
<td>1:8-1:64</td>
</tr>
<tr>
<td>ELISA (OD$_{410}$)</td>
<td>10/11 (91)</td>
<td>0.150-0.450</td>
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</table>
Figure VI.1. Photomicrograph of intracytoplasmic chlamydial inclusions as seen in the indirect inclusion fluorescence antibody test. Magnification, x 400.
Cow 101

Complement fixation (CF) □

Modified CF. □

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>IM. inoculation</th>
<th>IM. abortion</th>
</tr>
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<tbody>
<tr>
<td>17</td>
<td>1:32</td>
<td>&lt;1:8</td>
</tr>
<tr>
<td>38</td>
<td>1:16</td>
<td>&lt;1:8</td>
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<tr>
<td>66</td>
<td>1:8</td>
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<td>87</td>
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<td>1:128</td>
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<td>170</td>
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Figure VI.2. Chlamydia-specific antibody response of the IM inoculated cow 101, as determined by the standard and the modified CF tests.
Figure VI.3. Relationship between optical density and serum dilutions in ELISA.
Figure VI.4. Chlamydia-specific antibody response of the IM inoculated Angus cow, as determined by the MoCF test, the IIFA, and the ELISA.
Figure VI.5. Chlamydia-specific antibody response of the SC inoculated cow 329, as determined by the MoCF test, the IIFA test, and the ELISA.
Figure VI.6. Chlamydia-specific antibody response of the IV inoculated cow 375, as determined by the MoCF test, the IIFA test, and the ELISA.
Figure VI.7. Chlamydia-specific antibody response of the IV inoculated Holstein cow, as determined by the MoCF test, the IIFA test, and the ELISA.
Figure VI.8. Correlation between the ELISA and the IIFA test.
CHAPTER VII: SUMMARY

Suspension cultures of L-cells were infected with chlamydial strains associated with bovine and ovine abortion, as well as with strains associated with other clinical conditions. Freshly harvested yolk sacs of infected chicken embryos were used as the primary source of infection. Infectivity of cell propagated chlamydiae was assayed as inclusion forming units per ml. Abortigenic chlamydiae (biotype 1) multiplied to high titers, and high yields of chlamydial infectivity were obtained in subsequent passages. In contrast, chlamydial strains representative of biotypes 2, 3, 4, 5, 7 and 8 did not infect suspension cultures efficiently. Low rates of infection were obtained on the first passage, and the infectivity was lost in the process of attempting subpassages in suspension cultures. Findings from the interaction between chlamydiae and suspended host cells reinforce the concept that strains of \textit{C. psittaci} of specific pathogenic potential interact differently with cultured cells. The suspension culture method, although limited to the propagation of abortigenic chlamydiae, represents a useful technique for the production of large amounts of chlamydial antigens required for vaccine production, and for antigenic and seroepidemiological studies.

L-cells inoculated at multiplicities of infection (MOI) \( \geq 1 \) inclusion forming unit of the abortigenic chlamydial strain B577 were destroyed within 10-15 days. Upon continued incubation in fresh medium, a few surviving cells repopulated the flasks and the reemerging cultures remained persistently infected. The persistent state was characterized by cycles of repopulation with a low ratio of infected
cells, and cycles of extensive cytopathic changes where > 90% of the cells had chlamydial inclusions and which could be delayed or even terminated by penicillin treatment. Immunofluorescence and superinfection during the period of repopulation revealed that the persistently infected cells could adsorb chlamydiae but their multiplication was arrested. This non-permissive state could be terminated by the specific action of cycloheximide. L-cells spontaneously cured from a persistent infection exhibited no change in susceptibility to chlamydiae when compared to normal L-cells. However, chlamydiae derived from L-cells after 7.5 months of persistence destroyed L-cell monolayers more rapidly and at lower MOIs than the wild type. This state of chlamydia-host cell interaction could not be established with the arthropathogenic strain LW613 because chlamydial infectivity was lost following the first cytolysic burst of infection in the cell cultures. The persistence described for the strain B577-L-cell system appears to differ from previously described models involving other chlamydial strains.

Twenty five isolates of C. psittaci representing at least 7 different biotypes of bovine, ovine, caprine, equine, feline, porcine and guinea pig origin were immunotyped by an indirect microimmunofluorescence (IMIF) test. Different groups of chlamydia-free BALB/c mice received two weekly intravenous inoculations with chicken embryo-propagated, partially purified elementary bodies of each strain. Antisera for immunotyping were collected 4 days after the first inoculation and 3 to 4 days after the second inoculation, and tested for antichlamydia IgM and IgG antibodies in the IMIF test using cell culture-propagated, partially purified homologous and
heterologous antigens.

Nine immunotypes of \textit{C. psittaci} were distinguished, the correlation between immunotypes and biotypes was close, and a pattern of either disease or host specificity could be associated with each immunotype. Most immunotypes identified induced cross reacting antibodies against each other, but no significant cross reactions were observed with elementary bodies of the mouse pneumonitis strain of \textit{C. trachomatis}. Findings from this study should provide the necessary background for the rational selection of prototype strains of \textit{C. psittaci} for further antigenic analysis at the molecular level.

Sequential serum samples from 11 cows experimentally inoculated with different abortigenic strains of \textit{C. psittaci} were tested by a modified complement fixation (MoCF) test, an indirect inclusion fluorescence antibody (IIFA) test, and by an enzyme-linked immunosorbent assay (ELISA). Six of 11 cows (55%) had detectable pre-inoculation titers between 1:8 and 1:16 when tested by the MoCF test. In contrast, 9 of 11 (82%) and 10 of 11 (91%) pre-inoculation samples had detectable chlamydia-specific antibodies (Ab) as determined by the IIFA and the ELISA tests respectively. The pre-inoculation IIFA titers ranged from 1:8 to 1:64, and the ELISA values (OD\textsubscript{410}) varied from 0.150 to 0.450.

In all instances, significant increases in Ab levels were detected following experimental inoculation and/or abortion. Overall, the pattern of antibody response was similar with the three different serological techniques and the correlation between the IIFA test and the ELISA was statistically significant ($r=0.65$, $p<0.001$). The MoCF test increased the sensitivity of the standard CF test. However, the
IIFA test and the ELISA were even more sensitive probably because they were able to detect not only genus- but also strain-specific Abs. The IIFA test and the ELISA were easy to perform and to interpret, but ELISA was more objective and simultaneous screening of large numbers of sera was possible. The criteria for adopting either one of these two procedures in the routine serodiagnosis of chlamydial infections of cattle should be determined by the individual needs and facilities of each laboratory involved.
CHAPTER VIII: BIBLIOGRAPHY


120


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<tr>
<td>Veterinary School, University of Mexico, Mexico City</td>
<td>1972-1976</td>
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**WORK EXPERIENCE:**

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Candidate: Jorge A. Perez-Martinez

Major Field: Veterinary Microbiology

Title of Dissertation: Antigenic diversity and cultural behavior of Chlamydia psittaci of mammalian origin.

Approved:

[Signatures of Major Professor and Chairman, Dean of the Graduate School]

EXAMINING COMMITTEE:

[Signatures of committee members]

Date of Examination:

July 10, 1985