CHARACTERIZATION OF GENOMIC AND PLASMID DNA OF *Chlamydia psittaci* ISOLATES

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "**CHARACTERIZATION OF GENOMIC AND PLASMID DNA OF *Chlamydia psittaci* ISOLATES**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy
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CERTIFICATE

Certified that the thesis, entitled **"CHARACTERIZATION OF GENOMIC AND PLASMID DNA OF *Chlamydia psittaci* ISOLATES"**, is a record of research work done independently by **Mrs. Sreeja R. Nair** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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We, the undersigned members of the Advisory Committee of **Mrs. Sreeja R. Nair**, a candidate for the degree **of Master of Veterinary Science in Microbiology**, agree that the thesis entitled **"CHARACTERIZATION OF GENOMIC AND PLASMID DNA OF *Chlamydia psittaci* ISOLATES"**, may be submitted by **Mrs. Sreeja R. Nair**, in partial fulfilment of the requirement for the degree.

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SREEJA R. NAIR

Dedicated to
My Family

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Introduction

1. INTRODUCTION

Disease is one of the major hurdles in livestock development programmes. Many age-old infectious diseases affecting the animals and causing heavy economic loss have been successfully controlled by adopting suitable prophylactic measures. In spite of this, production loss due to morbidity interferes with attainment of anticipated output in livestock industry. Among such diseases "Chlamydiosis" stands as one important entity.

Chlamydiae are obligate intracellular prokaryotic pathogens of animals birds and man, exhibiting a wide variety of clinical symptoms. The diverse diseases caused by these organisms are having both public health importance and economic significance.

In animals, abortion is the most common pathological effect caused by *Chlamydia psittaci.* Abortion occurs in late pregnancy as a consequence of placental necrosis due to colonization of bacteria in the placenta. Ovine abortifacient *C. psittaci* has an affinity for human placenta and thus it is a specific hazard in pregnant women. Isolation of chlamydial species was reported from cases of urogenital infections, polyarthritis, encephalomyelitis, enteritis and pneumonia, though laboratory investigations indicated only casual association of these organisms in these conditions.

Until the twentieth century, based on phenotypic, morphologic and ecologic traits, *Chlamydia* was classified as a single genus in the family, *Chlamydiaceae* of the eubacterial order *Chlamydiales*. Now based on the established taxonomic criteria, including the analyses of ribosomal and coding genes, there is clear evidence for nine different species in the family *Chlamydiaceae* in two different genera, namely *Chlamydia* and *Chlamydophila*. Thus, the erstwhile *Chlamydia psittaci* causing abortion in animals are now reclassified as *Chlamydophila abortus* – a species with the ability to colonize the placenta and primarily be associated with cases of abortion and weak neonates.

Among the several infectious diseases which include epizootic and sporadic infection in ewes and other animals, chlamydiosis has been reported as an important disease from many parts of the world including India. The reporting of this disease in animals in India had been limited to the states other than Kerala. But there were few solitary reports (Francis, 1988; Sulochana, 1994 and Mani, 2001) about the occurrence of this disease in this state, without much indepth studies on the chlamydial species associated with this condition and characterization.

Realising the ability of *C. psittaci* (proposed *Chlamydophila abortus*) in the causation of a wide spectrum of diseases, the very need for sensitive reliable and definitive laboratory diagnostic techniques is badly felt. Early diagnosis of Chlamydiosis is mainly based on direct demonstration of the

organism in clinical materials and confirmatory diagnosis is through cultural recovery of Chlamydia in cell culture and embryonated eggs. The *C. psittaci* causing abortion and other forms of diseases are shown to exist in antigenically distinct forms by biotyping, serotyping and the molecular characterization. Thus, the isolates of *C. psittaci* from a species or a locality cannot be easily differentiated based on the usual diagnostic methods employed for the purpose. But to understand the epidemiology and epizootiology of chlamydiosis in animals and to discern the phylogenetic relationship between the species, sophisticated molecular characterization techniques are essential.

Molecular characterization techniques mainly focus on DNA/RNA based differentiation to discriminate the strains. The fundamental nature of these type of analyses reveal precise information since it is the genetic difference that determines strain variation.

Comparison of the genomic DNA of *C. psittaci* isolates of diverse origin by Restriction Endonuclease Analysis (REA) has been accomplished as an approved technique by McClenaghan *et al.* (1984) and Rodolakis and Souriau (1992). It had also been claimed that compilation of the genomic profiles could indicate the source/route of the incriminating bacteria in the causation of the disease.

Plasmid profiling has also attracted a disproportionate degree of research interest because plasmids carry genes which determine many

interesting features displayed by bacteria like resistance to antibiotics, virulence, exotic metabolic activity, production of enterotoxins, production of restriction and modifying enzymes, symbiosis etc. Plasmid profiling helps to differentiate strains from one another and has proven to be a very discriminating tool.

There have been reports for and against, about the presence of plasmids in various strains of *C. psittaci.* These reports further clairfy the absence of extra chromosomal DNA in the *C. psittaci* isolates causing abortion (the proposed *C. abortus*) (Lovett *et al.,* 1980; McClenaghan *et al.,* 1988; Thomas *et al.,* 1997; Everett, 2000). Hence demonstration of plasmid may be considered as of taxonomic value in subgrouping mammalian members of *C. psittaci.*

The Department of Microbiology, had isolated and partially characterized *C. psittaci* from cases of abortion. To understand and highlight the epidemiology of this infection, further characterization with regard to the homology/heterogenity of these isolates has to be established. This information will definitely help in chalking out suitable control measures including development of vaccine against chlamydiosis.

In this context, the present study was designed to compare the genomic DNA of the local isolates of *C. psittaci,* employing Restriction Endonuclease Analysis (REA) in relation to a reference isolate obtained from Himachal Pradesh.

An attempt was also made to elucidate the presence of extra chromosomal DNA in all the strains of *C. psittaci* employed in the study.

Parameters included in this study were:

1. Characterization of genomic DNA of *C. psittaci* local isolates of animal origin by restriction enzyme analysis, in comparison to isolate obtained from Himachal Pradesh.

2. Comparative plasmid profile of the isolates.

Review of Literature

2. REVIEW OF LITERATURE

In recent years, interest in the family *Chlamydiaceae* has increased because of the wide variety of human and animal diseases caused by this obligate intracellular bacteria. In animals, one of the most common diseases encountered with this bacterium is abortion resulting from the placental infection with *Chlamydia psittaci.*

2.1 Taxonomic status

Investigation of Chlamydia started in 1907 by Halberstaedter and Von Prowazek who found characteristic intracytoplasmic microorganisms within a vacuole in the cells of conjunctival scrapings from human patients with trachoma. The organism was considered as a protozoa and was conferred the name *Chlamydozoaceae.*

In 1930, the causative agent of psittacosis was isolated from human beings and affected birds (Coles, 1930) and at the same time, organism resembling the one causing psittacosis, namely lymphogranuloma venereum (LGV) was isolated in humans (Hellerstorm and Wassen, 1930).

Lillie (1930) named the etiological agent of pneumonia in the owners of exotic psittacine birds as *Rickettsia psittaci.*

Meyer (1960) suggested the name *Bedsonia* for all the members of the psittacosis lymphogranuloma venereum group in memory of Sir Samuel Bedson, who first described the developmental cycle of these organisms.

Moulder (1966) proposed that the organism was bacteria, considering their unique developmental cycle that completely differed from viral replication mechanism.

Page (1966) studied all the organisms of psittacosis – lymphogranuloma venereum trachoma group and assigned them into one genus *Chlamydia,* recognising their similarities in morphology, mode of replication and the antigenically similar lipopolysaccharide moieties in the cell wall of these organisms.

Chlamydiae differ from other gram-negative bacteria by their morphology, developmental cycle and common group antigens. Because of these characteristics, they were placed in a separate order *Chlamydiales* which contains the family *chlamydiaceae* and one genus *chlamydia* with three species, *Chlamydia trachomatis, Chlamydia psittaci* and *Chlamydia pneumoniae* (Storz and Page, 1971; Grayston *et al.*, 1989).

Fukushi and Hirai (1992) proposed the fourth species *C. pecorum* on the basis of the results of genetic analysis of chlamydia. Strains were isolated from cattle and sheep with various diseases including sporadic encephalitis, infectious polyarthritis, pneumonia and diarrhoea. The levels of DNA-DNA

homology between *C. pecorum* and strains of *C. psittaci, C. pneumoniae* and *C. trachomatis* were less than 10 per cent. The *C. pecorum* strains were distinguished from *C. psittaci* strains by the results of immunological assays.

Comparison of the OmpI gene of the isolates from genital infection of Koala with isolates from other animal species revealed that variation in DNA sequence did exist, suggesting that a reclassification of the organism to other species or sub-species of *C. psittaci* based on host origin was essential (Martin and Gross, 1997).

Analysis of the 16S and 23S rRNA gene sequence revealed that the order *Chlamydiales* contained four distinct groups at the family level and that within the family *Chlamydiaceae*, were two distinct genera which branched into nine separate species (Everett and Anderson, 1999).

The order *Chlamydiales* contains family *Chlamydiaceae, Parachlamydiaceae, Simkaniaceae* and *Waddleaceae*. The family *Chlamydiaceae* consist of two genera, *Chlamydia and Chlamydophila*. The genus Chlamydia possess three species viz., *C. trachomatis, C. suis and C. muridarum*. The genus chlamydophila possess six species, viz., *C. psittaci, C. pneumoniae, C. pecorum, C. felis, C. caviae* and *C. abortus* (Everett, 2000).

2.2 Isolation of Chlamydia

Chlamydial organisms are obligate intracellular pathogens which do not grow on artificial media. Hence isolation is attempted using living media like embryonated chicken eggs, laboratory animals and cell cultures.

2.2.1 Isolation using chick embryo (CE)

Rake *et al.* (1940) first identified that the most favourable culture medium for cultivation and isolation of chlamydia is the yolk sac of developing chick embryo.

Though embryonated chicken eggs were reported as a universal system for growing chlamydia, variations in growth characters were reported by Tang *et al.* (1957) and Storz (1971).

Incubation of eggs at 37°C to 39°C with 60 per cent humidity was found to be better for the growth of chlamydial organisms (Page, 1971).

The chlamydial strains infected and multiplied in the endodermal cells of the yolksac of six to eight day-old developing chicken embryo (Duguid *et al.*, 1978).

Collier (1984) observed that chick embryo died three to fourteen days after inoculation. The death pattern was characteristic for a given chlamydial strain adapted to the yolksac of chick embryo.

Idtse (1984) reported that at least two or three passages of the chlamydial strain in chicken embryo was required for obtaining sufficient concentration of the organism.

Page (1984) suggested that the chlamydia multiplying in the lining cells of the yolksac produced vascular congestion and haemorrhages in the membrane.

Krishna and Rajya (1985) observed that the harvested, infected yolksacs of different isolates of *C. psittaci* stored at –70°C maintained their viability upto nine to ten months, while the yolksac preserved at –20°C retained viability only upto five to six months.

Francis (1988) observed that the chick embryo that died due to chlamydial infection might be deep red with patchy haemorrhages on the skin, cyanotic legs and toes.

Sreeramalu *et al.* (1989) observed that *C. psittaci* inoculated through yolksac route of chick embryo produced oedema and congestion of yolksac membranes, and cherry red diffuse haemorrhage all through the embryo during initial passages. From the fifth passage onwards the yolksac was thin and yolk was watery.

Batta *et al.* (1997) reported that the *C. psittaci* isolated from pneumonic cases caused regular death of the chick embryo from third passage onwards and death of the embryo was more within four to eight days.

Mani (2001) observed that if the clinical material was having high concentration of organism, isolation through chick embryo was possible in first passage itself and those found negative in the primary passage had not turned out positive in the subsequent passages.

2.2.2 Isolation using laboratory animals

2.2.2.1 Isolation using mice

Storz (1971) reported that most chlamydial agents could infect mice aged three weeks after intra nasal instillation. This method was useful for propagation and isolation of chlamydial strains of human and avian origin. Attempts to isolate chlamydial agents from field material of other animal species by intranasal inoculation gave negative result.

Cottral (1978) cautioned the use of mouse for isolation of chlamydial agents as the species was demonstrated to get latently infected.

Anderson and Baxter (1986) observed that abortion isolates of *C. psittaci* inoculated into mouse foot pad produced significant spleenic enlargement, but was not a satisfactory method for testing the virulence.

Francis (1988) used mice aged three to six weeks and six to eight weeks for pathogenicity studies. He observed that mortality was more in younger group. Lesions detected at necropsy included a stringy yellowish fibrinous exudate on the peritoneal surface, severe congestion of the visual organs and extensive pneumonia.

Rodolakis *et al.* (1989) attempted experimental infection of mice to reveal variation between isolates from sheep. The chlamydia strains were inoculated into the foot pad of mice and these were killed six to nine days after infection. Strains isolated from chlamydial abortions colonized the spleen and were termed invasive. The fecal isolates that had not left the infection site were termed non invasive. In pregnant mice invasive group produced placental and foetal infection.

Biolatti *et al.* (1991) reported that mice that had been experimentally infected with undiluted yolk sac suspension of ovine abortion isolates showed clinical signs 45 h after infection. They had a rough coat and were not taking feed and cornered in the cage. Those that had been given diluted yolksac suspension of 1 in 10, had not produced any clinical signs. But in both groups, there was spleenic hyperplasia and pneumonia.

Ward and Ridgway (1998) stated that mouse could be infected through various routes, but intravenous inoculation resulted in rapid death within hours to few days.

Mani (2001) used mice of three to four weeks for pathogenicity study of three different *C. psittaci* isolates. He observed that the intra peritoneal inoculation of the caprine strain caused death of mice12 to 15 days post inoculation (PI) whereas no mortality were caused by bovine strain. He opined that chlamydial isolates from ruminants were of low pathogenicity for mice.

2.2.2.2 Isolation using guinea pigs

Guinea pigs were reported to naturally harbour chlamydial agents and hence the use of these animals for isolation attempts must be done with caution (Murray, 1964).

Guinea pigs infected intraperitoneally with chlamydial agent developed severe fibrinous peritonitis with fever, weakness, progressive emaciation and possibly death within seven to fourteen days. The necropsy findings included splenomegaly, hepatomegaly, pulmonary consolidation with a fibrinous yellow grey mat-like covering of the visceral organs (Storz, 1971).

Guinea pigs were the laboratory animals of choice for bovine and ovine chlamydial strains because these animals were more prone to pick up infection even with lesser number of chlamydiae, compared to chicken embryos (Cottral, 1978).

Page (1981) reported that certain strains of *C. psittaci* which were capable of producing death or lesion in mice might not produce infection in guinea pigs and vice versa.

Francis (1998) used guinea pigs for isolation of *C. psittaci*. Out of the eight isolates, six produced an acute infection resulting in death of the animal after 12 to 15 days PI. The remaining two produced latent infection and extremely few organisms could be isolated from them.

Mani (2001) used guinea pigs for isolation and pathogenicity studies. He observed that the two bovine strains were not virulent enough to produce mortality in guinea pigs, while the caprine strain was moderately virulent.

2.2.3 Isolation using cell culture

The uniformity in susceptibility of cultured cells have potential advantage over the chick embryo technique for the isolation and propagation of the chlamydial agent, although the chick embryo yolksac is a more reliable medium for primary isolation. Several cell lines support growth of a variety of strains of chlamydia. Investigators have individual preference as to which cell line and which conditions produce the best result.

Gorden and Quan (1965) described a tissue culture method for the isolation of agents causing trachoma.

Piraino (1969) reported that chick embryo fibroblast primary cell culture could be used for isolation of *C. psittaci.*

Page (1981) reported that both L929 and Mc Coy cell line had rapidly growing cells that supported growth of most chlamydial strains.

Johnson *et al.* (1983) described Mc Coy cell line to be more sensitive for isolation of *C. psittaci* from abortion cases.

Anderson and Baxter. (1986) preferred BHK 21 cells for the isolation of ovine abortion isolates.

Timms *et al.* (1988) compared the sensitivity of DNA spot hybridization and direct immunofluorescence staining with cell culture for diagnosis of *C. psittaci* of avian origin. They observed that cell culture was by far the most sensitive technique for detecting chlamydial infection in low levels.

Cell lines that supported growth of the organism included L 929, (Perez-Martinez and Storz, 1985, Everett *et al.* 1991, Kaltenboeck *et al.* 1992) Mc Coy (Gatel *et al.* 1989, Holland *et al.,* 1990, Denamur *et al.*1991, Rodolakis and Souriau, 1992; Amin and Wilsmore 1994), HeLa (Caldwell *et al* 1981; Cox *et al.* 1988; Campbell *et al.*, 1987), BHK 21 (McClenaghan *et al.*, 1984, Anderson, 1987), Vero (Anderson, 1991) and BGM cell lines (Meissler and Kraus 1986, Vanrompay *et al.*, 1992).

Vanrompay *et al.* (1992) compared the sensitivity of three different cell cultures viz., BGM, Mc Coy and Vero cell lines. They reported that BGM cell line was the most sensitive, followed by Vero and Mc Coy cell lines.

2.2.4 Enhancement of infectivity of chlamydia in cell culture

Infectivity of the cultured cells can be enhanced by several methods.

Spears and Storz (1979) enhanced the infectivity of *C. psittaci* for L 929 cells by cycloheximide treatment @ 0.5 microgram per millilitre of maintenance medium. They observed a three fold increase of inclusions and

noticed that infectivity could also increased by treating cells with diethyl amino ethyl dextran (DEAE-D).

Treatment with cytochalasin B (Evans and Taylor-Robinson, 1979), cyclic nucleotides and prostaglandins (Ward and Salari, 1980) and colchicine (Dennis and Storz, 1982) could enhance the infectivity of chamydiae in cell culture.

Lee and Moulder (1981) used Mc Coy cell line for the growth of chlamydiae. To increase the infectivity, the cell monolayer inoculated with chlamydial inoculum was centrifuged.

The success of chlamydial culture depended on the type and age of the host cell used and on the quality control and expertise of the laboratory culturing the organism (Vanrompay *et al.*, 1992).

Amin and Wilsmore (1997) studied the effect of placental extract and erythritol on growth of *C. psittaci* in Mc Coy cells. They observed that erythritol enhanced the infectivity of chlamydiae for Mc Coy cells while placental extract depressed their growth in this cell line.

2.2.5 Growth characteristics of *C. psittaci* in infected cell culture

Spears and Storz (1979) reported that the inclusions appeared in L 929 cells infected with *C. psittaci* 30 h PI as round or oval bodies. The inclusions produced slight or no change to the host cell until late infection.

Page (1981) reported that in infected cell cultures cytopathic changes in the form of rounding, swelling and detachment of infected cells occurred after two to six days of incubation.

Johnson (1984) observed that faecal isolates produced diffuse inclusions by 32 h, whereas placental isolates produced inclusions by 42 h.

Anderson and Baxter (1986) observed large, compact, dark staining inclusions 48 h PI for abortion isolates of *C. psittaci* in cell culture, while the arthritis isolates produced diffuse light staining inclusions from 24 h PI onwards, which later became dark and larger.

Persistent infection of Mc Coy cell line with *C. psittaci* virulent and avirulent strains produced different cytopathic changes. The virulence of virulent strain was not attenuated even after six months of culture, but after two or three months, avirulent strains became invasive for mice and abortive for ewes. The invasive strains of chlamydia caused destruction of monolayer with gigantic chlamydial inclusions (Rodolakis *et al.*, 1989).

Bollo *et al.* (1992) detected intracytoplasmic chlamydial inclusions and scattered elementary bodies in infected McCoy cell line using monoclonal antibodies.

Griffiths *et al.* (1992) observed that the ovine abortion and intestinal isolates were distinguishable by inclusion development and morphology in cell culture. Abortion isolates developed compact deeply methylene blue

stained inclusions which were visible after 41 h and reached maturity around 72 h, whereas ovine intestinal isolates of *C. psittaci* produced less deeply staining inclusions that were visible after 24 h and matured within 48 h.

Kaltenboeck and Storz (1992) reported that the *C. psittaci* isolated from pigs showed two types of inclusion morphology in cell culture. One type had finely granular inclusions whereas the other had pleomorphic reticulate bodies in inclusions. They further reported that when the cell culture medium containing cycloheximide was inoculated and centrifuged, there was a 1.5 fold increase in the number of inclusions produced.

Philips and Clarkson (1992) observed that abortion and enteric isolates of *C. psittaci* from sheep differed considerably in their growth in ovine fibroblast cell culture. Abortion isolates could be passaged several times and produced large inclusions whereas enteric isolates could not be passaged and produced sparse inclusions.

The ovine abortion and non-abortion isolates of *C. psittaci* could be distinguished by analysing the inclusion morphology. The abortion strains produced compact, helmet-shaped inclusions with well defined margin whereas the inclusions of non-abortion strains were irregular lobed with poorly defined margins and had a granular appearance (Markey *et al.*, 1993).

Storz and Kaltenboeck (1993) reported that the chlamydial infectivity could be determined by counting the inclusion forming units in cell culture 24 to 48 h PI, after staining with Giemsa or Gimenez staining.

Vanrompay *et al.* (1996) on studying the ultrastructural changes of the various serovars of avian *C. psittaci* in BGM cells observed that the most pathogenic serovars replicated faster and produced larger inclusions with numerous infectious organisms. The inclusions were scattered throughout the cytoplasm and induced severe degenerative changes in BGM cells.

Nagal *et al.* (1997) reported that the chlamydial inclusions or elementary bodies in Mc Coy cells were visible after 72 h PI. Inclusions were intracytoplasmic, pink and finely granular ball like structures with bluish green background when stained by modified Gimenez staining.

Escanlente-Ochoa *et al.* (2000) compared L 929 and BGM cell lines for the development of *C. psittaci* inclusions in the presence or absence of cycloheximide and polyethelene glycol. Significant differences were found in growth of the organism in both the cell lines. Higher number of inclusions were observed in L 929 cells, while larger inclusions were found in BGM cells. Free extracellular inclusions were found in both cell lines towards later periods of study. Addition of cycloheximide or polyethelene glycol had no effect on the number of inclusions or their size.

2.2.6 Confirmation of *C. psittaci* in infected cell culture by serology

Several diagnostic methods were reported for the detection of chlamydia in infected cell cultures. The most specific and sensitive method was reported to be detection of chlamydia antigen in cycloheximide treated stationary Mc Coy cells using fluorescent antibody technique (Stamm *et al.*, 1983; Thomas *et al.*, 1997).

Immunoperoxidase staining was used to detect chlamydial antigen in mouse L cells infected with *C. psittaci* (Shatkin *et al.*, 1976).

Page (1981) reported the use of fluorescein-conjugated antiserum preparation for specific identification of chlamydiae in tissues of infected animals and in cell culture.

Anderson (1987) reported the use of indirect immunofluorescence as a method to detect *C. psittaci* in embryonic bovine tracheal cells.

Griffiths *et al.* (1992) and Vanrompay *et al.* (1994) reported the use of indirect immunofluorescence for the detection of *C. psittaci* antigens in infected Mc Coy cells.

Nagal *et al.* (1997) reported that the indirect immunofluoresence with the group specific monoclonal antibody could result in detection of intracytoplasmic inclusions.

2.3 Characterization of *C. psittaci* isolate

2.3.1 Biotyping

The species of *C. psittaci* consists of several biotypes which cause specific infection in divergent species of animals. Studies have indicated that these agents have distinct serologic, biologic and pathologic properties.

Spears and Storz (1979) subdivided *C. psittaci* based on their biological characteristics into eight biotypes. For this the criteria adopted were the morphology of inclusions and time after infection at which they appeared on cell line, effect of chlamydial multiplication on the host cell cytoskeleton and the change in the number of cells infected in response to (DEAE-D) and cycloheximide.

Perez-Martinez and Storz (1985) revealed the limited usefulness of biotyping scheme because considerable experience was required and the process was very laborious.

Anderson (1986) had also utilized biotyping approach to differentiate *C. psittaci* isolates of divergent origin by foot pad inoculation into mice.

Abortion isolates were much more invasive than enteric isolates of *C. psittaci* in mice and sheep (Buzoni-Gatel and Rodolakis, 1983, Anderson and Baxter, 1986, Rodolakis and Souriau, 1989).

Rodolakis *et al.* (1989) observed that the mouse model could be used for graded differentiation between virulent and non virulent strains of *C. psittaci* isolated from ruminants.

2.3.2 Immunological typing techniques

Immunological typing techniques were employed to differentiate mammalian and avian strains of *C. psittaci.* The mammalian strains were further differentiated into that causing abortion, enteritis or polyarthritis.

Schachter *et al.* (1974) used neutralization test based on plaque assay to differentiate abortion strains from those associated with polyarthritis, conjunctivitis and encephalomyelitis.

Eb and Orfila (1982) observed the usefulness of micro immunofluroscence test to differentiate *C. psittaci* of ovine origin. They were separated into two antigenic groups.

Perez-Martinez and Storz (1985) immunotyped the different biotypes of *C. psittaci* of mammalian origin by indirect micro immunofluorescence test. They observed a close correlation between immunotypes and biotypes.

Eb *et al.* (1986) recognised eight immunotypes in *C. psittaci* based on micro immunofluorescence test.

Takahashi *et al.* (1988) classified the avian *C. psittaci* into three immunotypes based on indirect immunofluorescence test with monoclonal

antibodies. He identified that mammalian strains were distinct from the avian strains.

Puy *et al.* (1990) reported that serotyping using monoclonal antibodies could recognise genus, species, subspecies and type specificity of chlamydia, thus improving the diagnostic efficiency.

Anderson (1991) divided the avian isolates of *C. psittaci* into four serovars (turkey, psittacine, pigeon and duck) based on their reactivity to the monoclonal antibodies. On comparison of avian isolates with mammalian isolates, it was observed that they were distinctly different.

Souriau *et al.* (1993) serotyped the *C. psittaci* strains from ruminant into two serotypes. Serotype 1 was responsible for inducing abortion whereas the intestinal strains were serotype-2.

Vretou *et al.* (1996) classified *C. psittaci* abortion strains into four immunotypes based on microimmunofluorescence test.

Vanrompay *et al.* (1997) characterized *C. psittaci* strains using serovar specific monoclonal antibodies in a microimmunofluorescence test. They revealed four avian *C. psittaci* serovars.

2.3.3 Comparison of polypeptide profiles

Caldwell *et al.* (1981) electrophoresed the purified elementary body proteins on polyacrylamide gel and demonstrated proteins of molecular weight 105 kDa, 68 kDa, 57 kDa, 52 kDa and 45 kDa.

Vitu and Russo (1984) reported that the polypeptide profiles of *C. psittaci* isolates of ovine, bovine and caprine origin were almost similar.

Buzoni-Gatel *et al.* (1989) observed that the protein pattern of *C. psittaci* invasive and non-invasive strains showed some major differences at three regions. The invasive strains possessed 96 kDa, 90 kDa and 78 kDa polypeptides. The 90 kDa band could be regarded as a virulence marker for invasive strains.

Rodolakis *et al.* (1989) reported that the invasive and non-invasive strains differed in their protein profile. Invasive strains had an estimated molecular mass of 78 kDa and protein bands of molecular mass 96 and 90 kDa.

Baghian *et al.* (1990) reported that the MOMP of *C. psittaci* strains possessed genus-, species-, serovar-, specific epitopes and immune response to serovar specific epitopes of MOMP predominated when infectious elementary bodies were used for immunisation.

Cevenini *et al.* (1991) reported some difference in the major outer membrane protein (MOMP) of avian and abortion strains of *C. psittaci* by

immunoblot analysis, though high cross reactivity was detected between the polypeptides of the two strains. The MOMP of avian strain was recognized at molecular weight position of 40 kDa whereas in abortion strain it was at 38 kDa. A band of 97 kDa was detected in abortion strain but not in avian strain.

McClenaghan *et al.* (1991) reported that SDS-PAGE analysis of the purified elementary bodies of eight isolates of *C. psittaci* revealed four distinct profile types. All ovine abortion isolates had similar profiles.

Griffiths *et al.* (1992) compared placental and intestinal isolates of *C. psittaci* of ovine origin. The bands seen at 40 kDa, 49 kDa and 50 kDa in abortion isolates were not detected in intestinal isolates.

Markey *et al.* (1993) compared abortion and non-abortion isolates of *C. psittaci.* The profiles of the abortion isolates were similar to each other and readily distinguishable from non-abortion isolates.

Mani (2001) observed that protein profiling could be considered as a valuable tool in differentiating various strains of *C. psittaci.*

2.3.4 Genomic DNA analysis by restriction enzyme (RE) digestion

Studies on the nucleic acid of *C. psittaci* isolates presented clear differences in the size distribution of DNA fragments produced by restriction endonuclease digestion of the genome. The problems faced with RE profiling technique was that it required large scale culture and purification procedure

for the isolation of elementary body to yield enough DNA for analysis and again the profiles presented were complex and difficult to quantify.

Peterson and DeLa Maza (1983) characterised and compared the DNA from *C. psittaci* and *C. trachomatis* using nine restriction enzymes. Significant difference was noted between the DNA of both species but small or no difference was noticed between the strains.

McClenaghan *et al.* (1984) compared the DNA extracted from different isolates of *C. psittaci* by RE analysis and reported that the technique represented a valuable additional tool for the taxonomy and epidemiology of *C. psittaci.*

Fukushi and Hirai (1989) reported that the chlamydial genus could be classified into different groups by DNA fingerprinting, using restriction enzymes. They divided the *C. psittaci* into four groups – two avian, one feline and one ruminant group. The ovine abortion strain belonged to one of the avian group.

Anderson (1991) examined the genomic DNA of the four avian serovars of *C. psittaci* by RE analysis using three enzymes viz., *Eco RI, Bam HI* and *Pst I.* Turkey serovars differed from others by three to four bands with all enzymes. Duck and pigeon isolates showed only minor differences when compared with psittacine isolates.

Rodolakis and Souriau (1992) studied in detail the strains of *C. psittaci* isolated from ruminants by RE analysis of the DNA. They observed that the DNA fragments of invasive strains differed from that of non-invasive strains.

Anderson *et al.* (1996) analysed the genomic DNA of chlamydia isolated from various mammalian species by Southern blot hybridization and RE anlysis of DNA amplified by Polymerase Chain Reaction (PCR). The genomic RE profiles were found to be identical for all the isolates.

Pudjiatmoko *et al.* (1997a) amplified the DNA samples from *C. psittaci* isolates of different origin by Random Amplification of Polymorphic DNA (RAPD) and obtained eight distinct patterns of feline origin, five patterns of avian origin and one pattern of guinea pig origin.

2.3.5 Plasmid typing

Demonstration of plasmid in chlamydia led to the possibility of plasmid typing. Plasmid in both *C. trachomatis* and *C. psittaci* were first reported in 1980 by Lovett *et al.*

McClenaghan *et al.* (1984) reported the presence of 7.9kb plasmid in avian strains of *C. psittaci.*

Joseph *et al.* (1986) compared *C. psittaci* and *C. trachomatis* plasmids using RE analysis and southern blot analysis. The size of the plasmids were 6.2 kb and 7.3 kb respectively and the two plasmids were found to be distinct by RE analysis and partial homology was observed by southern blotting.

A broad survey to detect plasmids in avian and mammalian strains of *C. psittaci* was done by McClenaghan *et al.* (1988). They had purified plasmid DNA from avian strains of *C. psittaci* and estimated it to be 7.9 kb in size by REA.A 5.9 kb fragment of this plasmid was cloned and used to screen a range of chlamydial strains. They observed that while all the avian isolates investigated had plasmid, ovine abortion and ovine arthritis isolates lacked plasmid. But related sequences were detected in infective pneumonitis, guinea pig inclusion conjunctivitis and ovine conjunctivitis strains and in *C. trachomatis* serovar L2.

Timms *et al.* (1988) demonstrated plasmids with homologous DNA sequences in avian, ovine polyarthritis and sporadic encephalomyelitis strains of *C. psittaci,* but not in Koala kerato conjunctivitis, ovine abortion and feline conjunctivitis strains. Plasmid size determined was 6.2 to 7.2/7.3 kb.

Girjes *et al.* (1989) opined that plasmid DNA was not ubiquitous in *C. psittaci* and this casted some doubt on the importance of its role in the life cycle of the organism.

Hugal *et al.* (1989) compared the DNA sequence in three *C. psittaci* plasmids. A plasmid with size 7.4 kb was obtained from Koala strain and sporadic bovine encephalomyelitis strain. A 6.2 kb plasmid was noticed in avian *C. psittaci.*

Restriction mapping and southern blot hybridization revealed diversity among plasmids obtained from avian, feline equine and guinea pig isolates. Despite this diversity, they shared some sequence homology which was mapped to two separate regions in the plasmid molecule. One region showed high degree of homology between *C. psittaci* plasmids and also detectable homology with the *C. trachomatis* plasmid which might be representing a common replication coding region for plasmids of this genus (Lusher *et al.*, 1989).

Wills *et al.* (1990) characterised the plasmid DNA obtained from *C. psittaci* isolated from horse and a single 7 kb band was obtained.

Thomas *et al.* (1997) determined the complete nucleotide sequence for plasmids from *C.psittaci* avian strain and compared it with *C. pneumoniae* and *C. trachomatis*. More than 60 per cent sequence conservation was detected. The plasmid of avian *C. psittaci* was reported to be 7553 nucleotides in length and no biological function was identified for the plasmids.

2.3.6 Polymerase chain reaction (PCR) based techniques

PCR has emerged as one of the most widely used techniques in molecular biology and for good reason, it is a rapid, simple and reliable tool in the hands of microbiologist for disease diagnosis. As the PCR technique is based on production of relatively large number copies of DNA molecule from the target DNA, its significance as a diagnostic test is all the more high.

The PCR based characterization of chlamydia mainly depended on the amplification of the coding region of the MOMP gene followed by the RE analysis of the DNA.

Pickett *et al.* (1988) determined the complete nucleotide sequence coding the MOMP of abortion strain of *C. psittaci* and compared it with *C. trachomatis*. Thirty four per cent nucleotide homology and 65 per cent amino acid homology were revealed.

Herring *et al.* (1989) cloned and sequenced the MOMP gene from an ovine abortion strain. Subsequent RE analysis of the DNA showed that it had an avian type genomic profile and had sequential divergence from *C. trachomatis.*

Zhang *et al.* (1989) cloned and sequenced MOMP genes from two *C. psittaci* strains, guinea pig inclusion conjunctivitis and meningeopneumonitis strains. They observed that the similarity between the MOMP genes of the two strains were about 80.6 per cent. When *C. psittaci* was compared with *C. trachomatis* 68 per cent similarity was observed, thus indicating their evolutionary homology.

Holland *et al.* (1990) detected the MOMP gene sequences of three species of chlamydia using PCR and reported three corresponding genotypes as *C. trachomatis*, *C. psittaci* and *C. pneumoniae.*

Denamur *et al.* (1991) illustrated the usefulness of the MOMP gene restriction mapping in typing chlamydia. They compared the various isolates of *C. psittaci* by analysing *Alu I* restriction patterns of the MOMP gene after DNA amplification by PCR. He used a pair of nucleotide primers CTU (5'-ATGAAAAAACTCTTGAAATCGG-3') and CTL (5'-CAAGATTTTCTA GA (T/C) TTCAT (C/T) TT GTT-3') for PCR amplification.

Everett *et al.* (1991) cloned and sequenced the MOMP genes of *C. psittaci* psittacine strains and compared with other strains of *C. psittaci*. A 79 per cent identity was obtained with guinea pig inclusion conjunctivitis strain, 83 per cent with ovine abortion strain and 99 per cent with meningeo-pneumonitis strain. It was thus confirmed that psittacine and non-psittacine avian strains were closely related and distinct from guinea pig inclusion conjunctivitis and abortion strains of *C. psittaci*.

Frost *et al.* (1991) used PCR amplification of the coding region of the MOMP gene, followed by RE analysis of the DNA using *Alu I* and *Msp I* to show that the serovars of *C. trachomatis* could be distinguished.

Kaltenboeck *et al.* (1991) using two step PCR amplified the MOMP gene DNA sequences and digested with *Hae III*. They observed a close relationship between *C. psittaci* strains of avian and mammalian serovar I, while those of mammalian serovar seven and eight exhibited distinct restriction patterns. The other serovars could not be amplified by this technique.

Rasmussen and Timms (1991) described that PCR amplification of chlamydial DNA for conserved sequences of the MOMP gene enabled the detection of even very few elementary bodies in the clinical samples.

Storey *et al.* (1992) reported that an isolate of *C. psittaci* from a partridge had MOMP sequence related to the abortion strain rather than to the avian strain.

Kaltenboeck *et al.* (1993) amplified a large part of the MOMP gene coding region by multiplex PCR and classified all chlamydiae into four MOMP genotype groups as follows. (1) Trachoma group with *C. trachomatis* strain along with porcine isolate and mouse pneumonitis, (2) Pneumonia group with *C. pneumonia* and one Koala isolate, (3) psittacosis group with avian, ruminal abortion, feline pnumonitis and guinea pig strains and (4) the polyarthritis group with strains isolated from ruminants and pigs suffering mainly with symptoms of polyarthritis and enteritis.

Sayada *et al.* (1994) compared the various strains of *C. psittaci* MOMP gene after DNA amplification by PCR and observed genetic relatedness between various strains.

Griffiths *et al.* (1995) sequenced the MOMP gene of *C. psittaci* abortion strain from cattle and when compared with the type strain of ovine enzootic abortion, the gene sequence was identical, but different from that of *C. pecorum*. Thus it was confirmed as serotype 1.

Anderson *et al.* (1996) analysed the MOMP gene of *C. psittaci* isolated from various mammalian species. The results showed considerable heterogenity in the DNA sequence in the MOMP gene region of the *C. psittaci* isolated from ruminants and pigs. The porcine strain has more sequence homology to the *C. pecorum* and thus the isolate was included in this species.

Hewinson *et al.* (1997) reported the suitability of PCR assay for detection of *C. psittaci* DNA in clinical samples. The oligonucleotide primers selected were CPF (5'- GCAAGACACTCCTCAAGCC - 3') and CPR (5'– CCTTCCACATAGTGCCATC –3').

Martin and Gross (1997) compared the MOMP gene of *C. psittaci* isolates causing genital infection in Koala with isolates from other animal species using PCR amplification and RE analysis. There was immense variation in the gene sequence of *C. psittaci* isolates from different species of animals and also between isolates responsible for different diseases in the same animal species. Based on this the isolates could be classified into distinct groups.

Sykes *et al.* (1997) opined that PCR was less time consuming and less expensive than culture or genomic DNA extraction. The MOMP gene of *C. psittaci* feline strains from different parts of world were amplified and subsequent RE analysis indicated identical restriction patterns thus suggesting that the feline chlamydial strains causing conjunctivitis were similar throughout the world.

Longbottom *et al.* (1998) identified and isolated the four putative MOMP genes from *C. psittaci* and detected that these four genes were highly homologous, sharing 82 to 100 per cent similarity with each other.

Yoshida *et al.* (1998) amplified the MOMP gene of all the four species in the genus chlamydia using PCR. Subsequent RE analysis revealed four distinct patterns.

Bush and Everett (2001) used PCR primers CTU (5'- ATGAAAAAACTCTTGAAATCGG – 3') and TGLY (5'- GGCTACAGC TCTACCATTGA – 3') to amplify the major outer membrane protein (MOMP) gene of *C. psittiaci.* They observed that the invasive isolates of various origin shared restriction pattern, thus representing a strictly homologous group. This was not observed in the non-invasive strains. They had eight distinct restriction pattern suggesting the heterogenity in serotype-2.

2.3.7 Ribotyping

Weisburg *et al.* (1986) sequenced the 16S rRNA gene from *C. psittaci* and on comparing with other organisms showed that the organism was an eubacterium, peripherally related to plantomyces.

Fukushi and Hirai. (1993) analysed rRNA genes of the four chlamydia species by Restriction Fragment Length Polymorphism (RFLP) and observed that the RFLP were characteristic for each chlamydia species.

Herring (1993) amplified 16S rRNA since it was having less variable genes than MOMP and obtained distinct patterns for *C. trachomatis, C. psittaci, C. pneumonia* and *C. pecorum.*

Petterson *et al.* (1997) investigated the nucelotide sequences of 16S rRNA from strains of four species of chlamydia. Genetic homogenity were identified among the strains. The phylogenetic tree revealed two distinct lines of descent of the family *chlamydiaceae* which was built of two main clusters noted as *C. pneumonia* and *C. psittaci* clusters. The *C. trachomatis* was identified as a recently evolved species.

Pudjiatmoko *et al.* (1997b) compared the 16S rRNA sequences of the different strains of *C. psittaci, C. pecorum* and *C. trachomatis.* They identified eight genetic groups which formed two clusters. First cluster had *C. pecorum, C. pneumoniae* and *C. psittaci* with three genetic groups (one group with avian, human and ovine strains; two with feline strains and three with guinea pig strains). The second cluster of *C. trachomatis* also included three genetic groups.

Sheehy *et al.* (1997) determined the 16S nucleotide sequences of *C. psittaci* isolates of various origin. They observed interspecies sequence conservation. RE analysis of the nucleotide sequence clearly distinguished the various isolates.

Takahashi *et al.* (1997) determined the nucleotide sequence of 16S rRNA of *C. psittaci* avian and mammalian strains and *C. pecorum* strains. Sequence comparison revealed the interspecies conservation but diversity were also noticed suggesting at least four groups within the species of *C. psittaci.*

Everett and Anderson (1999) distinguished nine chlamydial species by PCR-RFLP test of 16S and 23S rRNA.

Materials and Methods

3. MATERIALS AND METHODS

Analytical grade chemicals procured from Sisco Research laboratory (SRL), Bombay were used, wherever the source is not mentioned. Glasswares of Borosil brand and Laxbro brand plastics were used in this study.

3.1 Revival of isolates

3.1.1 Chick embryo (CE) inoculation

3.1.1.1 Materials

(i) *Chlamydia psittaci* isolates

a. M-28 – An isolate from liver of an aborted caprine foetus.

b. M-430 – An isolate from lung of an aborted caprine foetus

c. M – 121 – An isolate from liver of an aborted bovine foetus

d. P-156 – The infected yolk sac (YS) material obtained from the Department of Microbiology, Veterinary College, Palampur, Himachal Pradesh as reference isolate.

All the local isolates were preserved as infected YS material in the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy.

(ii) Embryonated chicken eggs (CE)

Six to seven day-old embryonated eggs were procured from University Poultry Farm, Mannuthy. These eggs were obtained from hens fed on antibiotic free diet.

3.1.1.2 Method

Viable and healthy CE were selected by candling. The sample emulsion was prepared from the infected YS material and was brought to room temperature prior to inoculation. Inoculated 0.25 millilitres of the sample into the egg through YS route. Five embryos were used for each sample and incubated at 37°C.

The inoculated eggs were candled daily. The embryos that died within the first 48 h of inoculation was considered non specific and discarded. Those embryos which died on subsequent days were chilled by keeping in refrigerator overnight. The embryos found viable even after ten days post inoculation were killed by placing them in refrigerator overnight. Harvesting of inoculated CE was done on the next day.

3.1.2 Harvesting of inoculated CE

3.1.2.1 Collection of YS

3.1.2.1.1 Materials

(i) Inoculated CE

(ii) Calcium – Magnesium Free Phosphate buffered saline (CMF-PBS) (0.15 M, pH 7.2)

Sodium chloride	-	8 g
Potassium chloride	-	0.2 g
Potassium dihydrogen phosphate anhydrous	-	0.02 g
Disodium hydrogen orthophosphate	-	1.15 g

Triple distilled water	-	1000 ml

Sterilized by autoclaving at 15 lbs pressure at 121°C for 15 min.

(iii) Sucrose-phosphate glutamate (SPG) buffer with antibiotics (pH 7.2)

Sucrose (0.218 M)	-	74.62 g
Potassium dihydrogen phosphate (0.0038 M)	-	0.52 g
Dipotassium hydrogen phosphate (0.0072 M)	-	1.64 g
Sodium glutamate (0.0049 M)	-	0.82 g
Triple distilled water	-	1000 ml

Autoclaved at 10 lbs pressure and 110°C for 20 min. This buffer was supplemented with the following antibiotics.

Streptomycin	-	500 µg/ml
Kanamycin	-	500 IU/ml
Nystatin	-	50 IU/ml
Vancomycin	-	100 µg/ml
Gentamicin	-	50 µg/ml

3.1.2.1.2 Method

The shell was disinfected with 70 per cent ethyl alcohol. It was opened by cutting and removing the shell over the air sac region using sterile scissors and forceps. The shell membrane and the chorioallantoic membrane in the area of the air sac were removed. With a pair of sterile forceps, the inner contents were emptied into a sterile petri dish. The YS and the embryo were washed in sterile PBS. Pieces of YS blotted dry on a blotting paper were used to prepare impression smears on clean grease free slides.

The YS so obtained was then suspended in SPG, with antibiotics, dispersed in sterile vials, labelled and kept at –20°C.

3.2 Staining of infected YS

3.2.1 Giemsa staining

3.2.1.1 Materials

(i) Impression smears from YS

(ii) Giemsa stain

Prepared by dissolving 0.6 gram of Giemsa stain powder in 50 ml of glycerol and kept at 55°C to 60°C till the stain powder was dissolved completely. Then added 50 ml of methanol and allowed to stand for a day.

3.2.1.2 Method

Impression smears prepared from yolk sac membrane were stained by the slow method of staining advocated by Cruickshank *et al.* (1975) with slight modifications.

The impression smears were fixed using methanol for five minutes. Then 0.5 millilitre of the stock solution of Giemsa stain was dissolved in 80 ml of distilled water. The smears were then left immersed in this dilute stain solution for 16 to 18 h. Finally they were washed, dried and then examined under oil immersion objective.

3.2.2 Modified Ziehl-Neelsen staining (MZN)

3.2.2.1 Materials

(i) Impression smears from YS

(ii) 1:10 dilution of Ziehl-Neelsen carbol fuchsin

(iii) Acetic acid (0.5 per cent)

(iv) Loeffler's alkaline methylene blue

3.2.2.2 Method

The smears were fixed by heat and stained with diluted Ziehl-Neelsen carbol fuchsin for 10 min, then washed in running tap water and decolourised with freshly prepared 0.5 per cent acetic acid for five seconds. They were then washed in running water, and counterstained with methylene blue for one minute, again washed in running water, dried and examined under oil immersion objective.

3.2.3 Gimenez staining

3.2.3.1 Materials

(i) Impression smears from YS

(ii) Primary stain

Stock solution

Solution I

Basic fuchsin - 10 g

95% ethanol - 100 ml

Solution II

Phenol crystals - 10 g

Triple distilled water - 250 ml

Solutions I and II were prepared separately and were mixed slowly and thoroughly. Then added 650 ml of triple distilled water to this mixture and was incubated at 37°C for 48 h and stored in amber coloured bottle.

(iii) Phosphate buffered saline (PBS) (0.01 M pH 7.5)

Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	-	0.15 g
Disodium hydrogen orthophosphate anhydrous	-	1.27 g
Sodium chloride	-	8.00 g
Triple distilled water	-	1000 ml

Working solution

Stock solution	-	1 part
PBS (0.01 M pH 7.5)	-	2 parts

(iv) Counter stain

Malachite green	-	800 mg
Triple distilled water	-	100 ml

3.2.3.2 Method

The smears were fixed over flame. The slides were flooded with filtered working solution for 15 min and washed with water. Counter stained with malachite green for three minutes. Washed the slides with water, dried and examined under oil immersion.

3.3 Grading of infected YS

3.3.1 Materials

Stained YS impression smears

3.3.2 Method

Grading of infected YS was performed based on the average number of chlamydial elementary bodies (EBs) present on stained YS smears.

YS (3+): those showing more than 100 EBs per microscopic field in the stained smear.

YS (2+): those showing about 50 to 100 EBs per microscopic field.

YS (1+): those showing about 10 to 50 EBs per microscopic field.

3.4 Propagation of chlamydial isolates in Mc Coy cell line

3.4.1 Maintenance of cell line

3.4.1.1 Materials

(i) Mc Coy cell line obtained from National Centre for Cell Sciences (NCSS), Pune

(ii) Eagles minimum essential medium (MEM)

Ready made medium purchased from Hi-media, Bombay was reconstituted as per manufacture's instruction and filtered using millipore filter (0.2 μm).

(iii) 7.5 per cent sodium bicarbonate in triple distilled water, sterilized by filtration.

(iv) 0.25 per cent trypsin (1:250), in CMF-PBS (0.15 M, pH 7.2) and sterilized by filtration.

(v) A stock solution of five per cent trypsin was prepared in CMF-PBS, sterilized by filtration through millipore filter (0.2 μm), distributed in small quantities and stored at –20°C. When needed the working

solution was prepared by diluting the stock solution with CMF-PBS to give a final concentration of 0.25 per cent.

(vi) CMF- PBS (0.15 M, pH 7.2) [3.1.2.1. (ii)]

(vii) Foetal calf serum (PAA Laboratories, Austria)

(viii) Cell culture growth medium: Eagles MEM containing 10 per cent foetal calf serum was prepared and pH was adjusted to 7.2 with 7.5 per cent sodium bicarbonate.

(ix) Cell culture maintenance medium: Differ from growth medium in having five per cent foetal calf serum.

3.4.1.2 Method

The maintenance medium was poured off from the bottle containing confluent monolayer. The monolayer was washed twice with MEM not containing serum. Two millilitres of 0.25 per cent trypsin solution was added to the bottle containing monolayer with gentle shaking and kept at 37°C for two minutes. When cells started dislodging from the monolayers, added double volume of maintenance medium and kept at room temperature for five minutes. The cells were detached from the surface by mechanical disruption using sterile pepette attached with bulb. A split ratio of 1:3 was employed for seeding into new tissue culture bottles and the cells were also seeded into test tubes containing coverslips. Enriched the tissue culture bottles and test tubes containing cover slips with growth medium at the rate of eight millilitre and two millilitre respectively. The bottles and tubes were incubated at 37°C. They were observed daily for the formation of monolayer. When monolayer

of cell was formed, they were used for further growth with chlamydial isolates.

3.4.2 Inoculation of cell line

3.4.2.1 Materials

(i) Mc Coy cell monolayer in tissue culture bottle and cover slips.

(ii) *Chlamydia psittaci* infected YS membrane from all isolates processed for inoculation.

(iii) SPG [3.1.2.1.1 (iii)]

(iv) Cycloheximide – 10 mg dissolved in 10 ml tripple distilled water, sterilized using millipore filter and stored at –20°C.

(v) Sterile glass beads

3.4.2.2 Method

Tissue culture bottles with monolayers were selected, the growth medium was poured off and, then washed with maintenance medium.

Twenty per cent suspension of heavily infected YS (3+) in SPG was prepared. Centrifuged at 3800 rpm for 15 min. The sediment and the fat layers were discarded and the middle layer was collected. It was diluted with maintenance medium at the rate of 1:100. This was used as inoculum. The monolayer was inoculated with three millilitres of inoculum and was incubated at 37°C for one hour to facilitate adsorption. The inoculum was then poured off. The monolayer was washed with maintenance medium and added maintenance medium containing cycloheximide at a concentration of

0.1 millilitre per 100 ml at the rate of eight millilitre per bottle. Control cover slips were prepared simultaneously in which instead of inoculum maintenance media was added.

All the tissue culture bottles were incubated at 37°C and were examined at 24 h intervals for a period of 96 h, under an inverted microscope for evidence of any cytopathic effect (CPE). When the monolayers were sufficiently infected and produced cytopathic changes, the cells were detached from the surface using sterile glass beads and collected aseptically.

For infecting cover slip cultures, 0.2 millilitres of inoculum and two millilitres of maintenance medium containing cycloheximide at a concentration as above were used. In control tubes maintenance medium alone was inoculated instead of the isolates. Infected coverslip cultures were collected at 24 h, 48 h, 72 h, 96 h interval and stained using May-Grunwald Giemsa stain as well as indirect immunoflorescence technique to appreciate the cytopathic effect and/or inclusion bodies. The control cover slips were also stained and studied.

3.4.3 Staining of coverslip cultures

3.4.3.1 May-Grunwald Giemsa staining

3.4.3.1.1 Materials

(i) Cover slip cultures

(ii) May – Grunwald stain

Prepared by dissolving 2.5 gram of May-Grunwald stain powder in 100 ml of absolute methanol and allowed to age for one month.

(iii) Giemsa stain

Prepared by dissolving one gram of stain powder in 66 ml of glycerol and kept at 60°C till the stain powder got dissolved completely. Then added 66 ml of absolute methanol and kept for a day.

(iv) Methanol

(v) Acetone

(vi) Xylene

(vii) DPX mountant

3.4.3.1.2 Method

The coverslip cultures were fixed overnight in methanol. They were stained for 10 min in May-Grunwald stain and for 20 min in 1 in 10 diluted Giemsa stain. The cover slips were rinsed rapidly in two changes of acetone and then in two parts of acetone and one part of xylene for five seconds. They were then placed in one part of acetone and two parts of xylene for one minute, cleared in two changes of xylene, two minutes each, dried and mounted with DPX on a clean grease free glass slide and examined for CPE and/or inclusion bodies under a microscope. The uninfected coverslips each were also fixed as described above and studied in detail.

3.4.3.2 Indirect immunofluorescence technique

3.4.3.2.1 Materials

(i) Coverslip cultures

(ii) Antichlamydial group specific monoclonal antibodies (Courtesy Dr. Buendia, Spain)

(iii) Rabbit antimouse IgG Fluorescin isothiocyanate conjugate (Bangalore, Genei)

(iv) PBS (0.15 M, pH 7.2) [3.1.2.1 (ii)]

(v) Glycerol saline

Glycerol - 50 ml

Physiological saline (0.85 per cent) - 50 ml

Mixed and sterilized by autoclaving at 15 lbs pressure for 15 min.

(vi) Evans blue - 0.5 per cent in CMF-PBS

3.4.3.2.2 Method

The glass coverslips with infected monolayer at intervals of 24 h, 48 h, 72 h and 96 h were fixed in ice cold acetone for five minutes. Antichlamydial group specific monoclonal antibodies developed in mice was diluted to 1:10 and added to each monolayer at the rate of 0.1 millilitre and incubated at 37°C for one hour. Monolayer was then thoroughly rinsed with PBS and triple distilled water thrice respectively. Allowed to react with 0.1 millilitre of fluorescin isothiocyanate conjugated antimouse antiglobulins produced in rabbit at a dilution of 1:30 in PBS and incubated at 37°C for one hour. Nine parts of the conjugated antiglobulin was mixed with one part of 0.5 per cent Evans blue before it was used to react with the monolayer culture. They were

again rinsed well in PBS and triple distilled water. The cover slips were mounted in glycerol saline to demonstrate chlamydial inclusion bodies. Uninfected coverslips were also processed as described above and studied in detail.

3.5 Characterization of chromosomal DNA of *C. psittaci*

3.5.1 Preparation of purified elementary bodies from infected Mc Coy cell line

3.5.1.1 Materials

(i) Mc Coy cell line infected with *C. psittaci* isolates

(ii) Urografin – 76 (Scherring AG, Germany)

(iii) Tris-potassium chloride (T-KCl) (pH 7.5)

Tris - 20 mM

KCl - 150 mM

Triple distilled water - 100 ml

Autoclaved at 121°C at 15 lbs pressure for 15 min and stored at 4°C.

3.5.1.2 Method

Chlamydia psittaci were harvested from Mc Coy cell line 96 h PI, when sufficient numbers of inclusions or CPE were seen. Cells were detached from culture bottles using sterile glass beads and the suspension was collected. Ten culture bottles were used per isolate and 10 ml of the cell suspension obtained per bottle was pooled and homogenized. The suspension obtained was centrifuged at 6000 rpm for five minutes at 4°C to remove the cell debris. The supernatant were layered on 30 per cent (v/v) urografin-76 diluted with

T-KCl and centrifuged at 25000 rpm for one hour at 4°C. The pellet was resuspended in one millilitre of T-KCl. It was layered on 30 to 60 per cent (v/v) urografin-76 gradients in T-KCl and centrifuged at 25000 rpm for two hours at 4°C. The diffuse band seen in between 40 and 50 per cent gradient was collected, diluted with three millilitre of T-KCl and centrifuged at 25000 rpm for one hour at 4°C. The pellet obtained was resuspended in 0.2 millilitre T-KCl and stored at –70°C. The purity of the suspension was checked by staining with Gimenez method.

3.5.2 Preparation of purified elementary bodies from infected yolk sac membrane

3.5.2.1 Materials

(i) YS (3+) infected with *C. psittaci* isolates

(ii) Hanks balanced salt solution (HBSS)

Readymade medium purchased from Hi-media, Bombay was reconstituted as per manufacturer's instruction.

(iii) Urografin-76 (Schering AG, Germany)

(iv) T-KCl (3.5.1.1 – iii)

(v) Silica gel

3.5.2.2 Method

Twelve yolksac membranes (3+) infected with *C. psittaci* isolate were washed in HBSS to remove the yolk completely. Disrupted thoroughly with sterile silica gel and T-KCl in mortar and pestle and centrifuged at 3800 rpm for 15 min to remove the coarse sediments. The supernatant collected was

centrifuged again at 6000 rpm for five minutes to remove the cell debris. The supernatant collected were layered on 30 per cent (v/v) urografin-76, diluted with T-KCl and centrifuged at 25000 rpm for one hour at 4°C. The pellet was resuspended in one millilitre of T-KCl. The remaining procedures done for purifying the elementary bodies obtained from Mc Coy cell line were followed here also. The purity of the final suspension was checked by staining with Gimenez method.

3.5.3 DNA extraction from purified EBS

3.5.3.1 Materials

(i) Purified EBS suspended in T-KCl

(ii) Tris-EDTA sucrose buffer (TES) (pH 7.5)

Tris - 20 mM

EDTA - 20 mM

Sucrose - 20 per cent

Triple distilled water – 100 ml

Autoclaved at 10 lbs pressure for 20 min. and stored at 4°C

(iii) Proteinase K (Genei Private Limited, Bangalore)

A stock solution was prepared by dissolving 10 mg of proteinase K in one millilitre of sterile triple distilled water and pre incubated at 37°C for one hour. The stock solution was then stored at –20°C.

(iv) Sodium dodecyl sulphate (SDS) – 10 per cent solution was prepared in triple distilled water and stored at room temperature.

(v) Phenol-chloroform-isoamyl alcohol (25:24:1 ratio) (Sigma-Aldrich, USA).

Distilled phenol equilibrated with Tris-EDTA (TE) buffer of pH 7.5 was mixed with chloroform isoamyl alcohol at the said ratio.

(vi) Chloroform isoamyl alcohol mixture – Chloroform and isoamyl alcohol mixture in the ratio of 24:1 (V/V) was prepared and stored at room temperature.

(vii) Tris EDTA (TE) buffer (pH 7.5)

Tris-Chloride - 10 mM

EDTA - 1 mM

Triple distilled water - 100 ml

Autoclaved at 15 lbs pressure for 15 min. and stored at 4°C.

(viii) Distilled ethanol

(ix) Sodium chloride (5 M) (pH 7.5)

Sodium chloride - 29.2 gram

Triple distilled water - 100 ml

Autoclaved at 15 lbs pressure for 15 min. and stored at 4°C.

3.5.3.2 Method

The chlamydial suspension was homogenized thoroughly in a glass homogenizer. Added 300 µl of TES buffer, gently mixed and then added proteinase K, at a final concentration of 200 µg per ml.

Mixed gently and incubated at 55°C for one hour, 20 µl of 10 per cent SDS was added to this mixture and incubated at 37°C for three hours. This solution was extracted with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged at 12000 rpm for 15 min. The upper aqueous phase collected was again extracted with chloroform isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 15 min. To the upper aqueous phase collected added 1/20 V of 5 M sodium chloride. Mixed slowly and added double volume ethanol. Slowly mixed for 10 min and kept at –20°C overnight. Centrifuged at 14000 rpm for 15 min. The pelleted DNA was washed with 70 per cent ethanol and resuspended in TE (pH 7.5).

3.5.4 Estimation of purity and concentration of DNA

3.5.4.1 Materials

(i) DNA samples from each isolate

(ii) TE buffer (pH 7.5) (3.5.3.1 – vii)

3.5.4.2 Method

The purity and concentration of DNA were assessed by spectrophotometry. Ten microlitre of DNA sample suspended in TE buffer was diluted with one millilitre of sterile triple distilled water. The spectrophotometer was set to zero with 10 microlitre of TE buffer diluted with one millilitre of sterile triple distilled water at 260 and 280 nm wavelength. The DNA samples from each of the isolate were taken in turn in the cuvette and optical density (OD) was measured at 260 and 280 nm. One OD at 260

nm was taken as 50 µg per ml of double stranded DNA. Ratio of OD 260/280 indicated the purity of the DNA samples. DNA samples having ratio of less than 1.8 were again subjected to DNA extraction procedure to obtain sufficiently pure DNA.

3.5.5 Restriction endonuclease (RE) digestion

3.5.5.1 Materials

(i) Purified DNA from *C. psittaci* isolates

(ii) Restriction enzymes (RE) (Genei Private Limited, Bangalore)

Eco RI	-	20u/µL
		10x assay buffer
Bam HI	-	10u / µL
		10x assay buffer
Hae III	-	10u/µL
		10x assay buffer
Lamda DNA (Genei)	-	250 µg/vial

Sequence specificities and source of restriction enzymes.

RE	Bacterial source	Sequence cleaved
Eco RI	*E. coli RY 13*	5' G↓AATTC 3' CTTAA↑G
Bam HI	*Bacillus amyloliquifaciens* H	5' G↓GATCC 3' CCTAG↑G
Hae III	*Haemophilus aegypticus*	5' GG↓CC 3' CC↑GG

3.5.5.2 Method

The purified DNA obtained from each isolate of *C. psittaci* was digested with the restriction enzymes *Eco RI, Hae III* and *Bam HI.* Ten microgram of the purified DNA from each isolate, 10x RE assay buffer of 2x concentration and RE of two units per microgram of DNA were taken in eppendorf tubes containing distilled water so that the total reaction volume in each tube was 20 µL. The digestion mixture was incubated at 37°C overnight in waterbath. The restriction enzyme activity was then inactivated by heating at 55°C for 15 min. The samples were prepared for agarose gel electrophoresis by mixing 20 µL of the RE digest with two microlitre of the sample dye.

3.5.6 Agarose gel electrophoresis

3.5.6.1 Materials

(i) Agarose (Sigma)

Agarose - 0.8 g

Tris-borate EDTA buffer – 100 ml

Agarose was dissolved by heating and then cooled to 60°C.

(iii) Tris-Borate – EDTA buffer (TBE) stock solution (5x) (pH 8.0)

Tris base - 54 g

Boric acid - 27.5 g

0.5 M EDTA - 20 ml

Triple distilled water - 980 ml

The stock solution was diluted to 1x before use.

(iv) Gel loading buffer

Bromphenol blue	-	0.25 per cent
Xylene cyanol	-	0.25 per cent
Glycerol	-	30 per cent

(v) Ethidium bromide stock solution

Ethidium bromide	-	10 mg
Triple distilled water	-	1 ml

The solution was mixed using a magnetic stirrer and the solution was then dispensed in amber coloured bottles and stored at room temperature.

Ethidium bromide was added at a concentration of 0.5 μg/ml to the agarose at 60°C.

3.5.6.2 Method

The clean and dry platform edges were sealed with adhesive tape and the comb was kept in position before pouring the agarose solution into the glass plate for setting. A gap of one millimetre was provided between the glass plate and the comb tip so as to form a seal of agarose within the well formed beneath the comb tip. Agarose solution was left at room temperature for one hour to set into a gel. The tape and the comb were then removed, the platform with the gel placed within the tank filled with TBE buffer (1x), so that the agarose gel was completely submerged within the buffer. The DNA digest of each isolate mixed with the sample buffer were loaded into each slot

of the prepared gel. Lambda DNA digested with *Hind III* was taken as the molecular weight standard.

Electrophoresis was carried out at 50 V till the loading dye reached three fourth of the anode end of the gel. The gel was viewed and photographed in Alpha imager (Alpha Innotech Corporation, USA).

3.6 Plasmid profile of *C. psittaci* isolates

3.6.1 Extraction of plasmid DNA

3.6.1.1 Materials

(i) Purified EBS of *C. psittaci* isolates

(ii) Tris EDTA glucose (TEG) buffer (pH 8.0)

Tris-Cl (pH 8.0)	-	25 mM
EDTA (pH 8.0)	-	10 mM
Glucose	-	50 mM
Triple distilled water	-	100 ml

Autoclaved at 10 lbs pressure for 20 min and stored at 4°C.

(iii) Sodium dodecyl sulphate (SDS) – 10 per cent (3.5.3.1-iv)

(iv) Sodium hydroxide (NaOH) (IN) solution

Sodium hydroxide	-	4g
Triple distilled water	-	100 ml

(v) SDS-NaOH solution

SDS	-	1.0 g
Sodium hydroxide	-	0.2 N
Triple distilled water	-	100 ml

The solution was prepared fresh from the stock solution of 10 per cent SDS and IN NaOH.

(vi) Sodium acetate solution (3 M) (pH 4.8)

Sodium acetate - 40.81 g

Triple distilled water - 100 ml

pH was adjusted to 4.8 with glacial acetic acid

Autoclaved at 15 lbs pressure for 15 min and stored at 4°C.

(vii) Distilled ethanol

(viii) TE buffer pH 7.5 (3.5.3.1 – vii)

3.6.1.2 Method

The chlamydial suspension was homogenized in a glass homogenizer. Added 100 µL of ice cold TEG buffer, mixed thoroughly and kept in ice for 15 min. To this suspension added 200 µl of freshly prepared SDS-NaOH solution and the contents were mixed gently till the solution became translucent. Incubated in ice for 15 min.

Sodium acetate (3M) was added at the rate of 150 µL and mixed gently and incubated in ice for 10 min. The solution was then centrifuged at 12000 rpm for 15 min at 4°C. The supernatent was collected into a fresh eppendorff tube and to this added double the volume of ice cold ethanol. Mixed gently but thoroughly and kept at –20°C over night. The DNA was pelleted by centrifuging at 14000 rpm for 15 min. The pellet was washed with 70 per cent ethanol and resuspended in 50 µL of TE buffer.

3.6.2 Extraction of plasmid from *E. coli* V 517

3.6.2.1 Materials

(i) *E. coli* V 517 (Microbial Type Collection Centre) (MTCC Chandigarh)

(ii) Luria Bertani (LB) Medium (Hi-media)

(iii) TEG buffer pH 8.0 – (3.6.1.1 ii)

(iv) Lysozyme (Merck)

Five milligram per ml was prepared as fresh solution in TEG buffer (pH 8.0)

(v) SDS – 10 per cent (3.5.3.1-iv)

(vi) NaOH (IN) – (3.6.1.1 – iv)

(vii) SDS-NaOH solution (3.6.1.1. – v)

(viii) Sodium acetate (3M) (3.6.1.1. – vi)

(ix) Distilled ethanol

(x) TE buffer pH 7.5 (3.5.3.1 – vii)

3.6.2.2 Method

E. coli V 517 procured from MTCC, Chandigarh was inoculated into 10 ml of LB medium and incubated at 37°C for 18 h with intermittent shaking. The cells were harvested by centrifugation at 6000 rpm for 20 min at 4°C and then subjected to plasmid extraction as described for *C. psittaci,* except that the cell pellet was dissolved in 100 µL of TEG buffer containing lysozyme at a final concentration of five milligram per ml.

3.6.3 Agarose gel electrophoresis of plasmid DNA (Done as per 3.5.6)

E. coli V 517 was taken as the molecular weight standard having eight plasmids.

Results

4. RESULTS

4.1 Revival of isolates

4.1.1 Propagation in chick embryo

The four isolates (M-28, M-430, M-121 and P-156) which were preserved in the Department of Microbiology as infected yolk sac (YS) were revived by YS route of inoculation at the rate of 0.25 millilitre per egg into six to seven day-old embryonated chicken eggs.

The isolate M-28 killed the inoculated embryos within five to seven days PI where as M-430 killed the embryos between eight to ten days PI. The other two isolates had not caused mortality within ten days, but the embryos appeared stunted. The typical changes noticed on the embryo were haemorrhagic patches on the skin all over the body. The legs were hyperemic. The YS was thin walled with deeply injected blood vessels. The Yolk was found to be more liquid than normal (Fig.1). The isolates M-28 and M-430 presented more pronounced lesions on the embryo and YS than P-156 and M-121. The lesions observed in chick embryo when inoculated with different isolates of *C. psittaci* are presented in Table (1).

4.1.2 Staining of infected YS

The impression smears prepared from the YS membranes revealed elementary bodies of Chlamydia by different staining reactions.

The impression smear prepared from YS membrane infected by the isolates when stained by the slow method of Giemsa staining revealed reddish purple extracellular elementary bodies in dark blue background.

Modified Ziehl-Neelsen staining presented deep red EB in blue background but differentiation of the EB appeared to be difficult.

The impression smear from the infected YS membrane revealed pink stained extracellular EB in bluish green background when stained by Gimenez method. This staining method was found to be the most suitable one for differentiation and identification of the EB (Fig. 2).

4.2 Grading of infected YS

Grading of the infected YS was performed based on the average number of Chlamydial EB present on stained YS smears. The smears prepared from the YS of embryo that died five to seven days PI had the maximum number of organisms per microscopic field. The concentration of EB decreased as the age of the embryo increased. The impression smears which were prepared from areas of marked congestion, exhibited varying numbers of EB per microscopic field. Based on the number of EB present per microscopic field of the stained smear, grading of the YS into 3+, 2+ and 1+ was done and they were preserved separately.

Fig.1 Chick embryo eight days PI.

C – Control

I – Infected showing stunting and injected YS vessels

Fig.2 YS impression smear - Gimenez staining (1000X) revealing chlamydial EBs

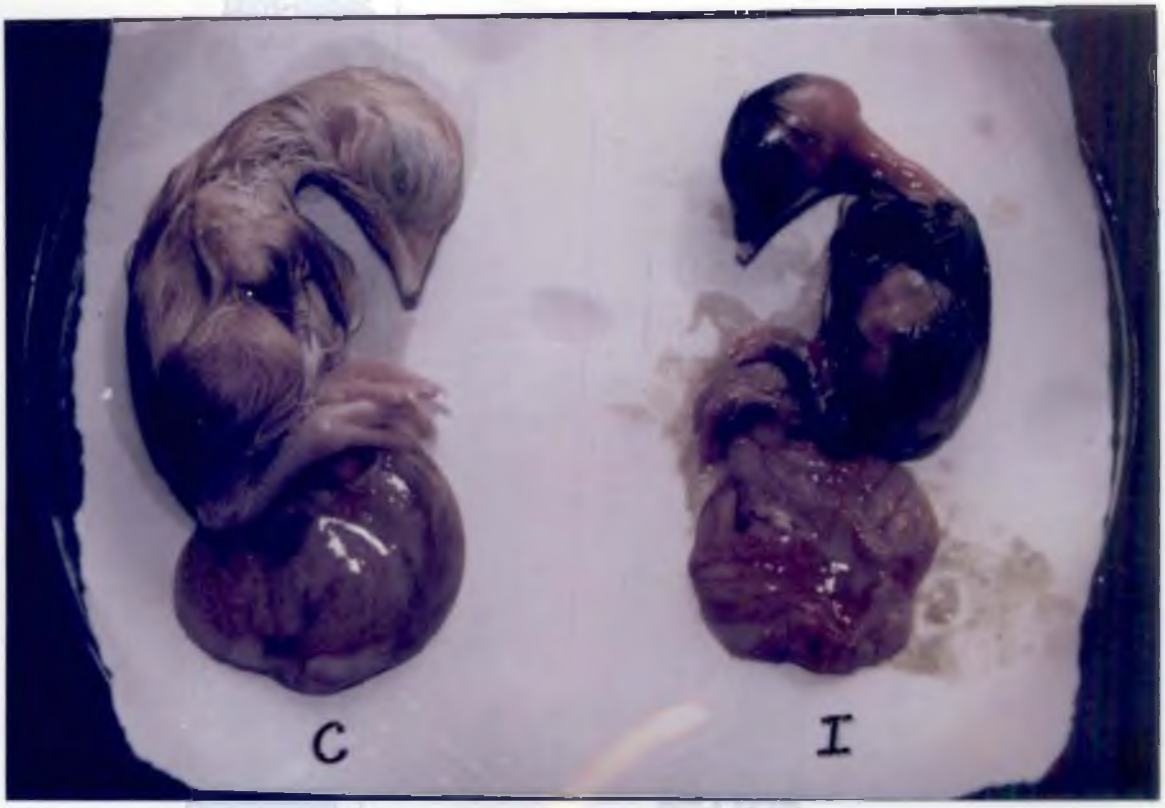


Fig.1

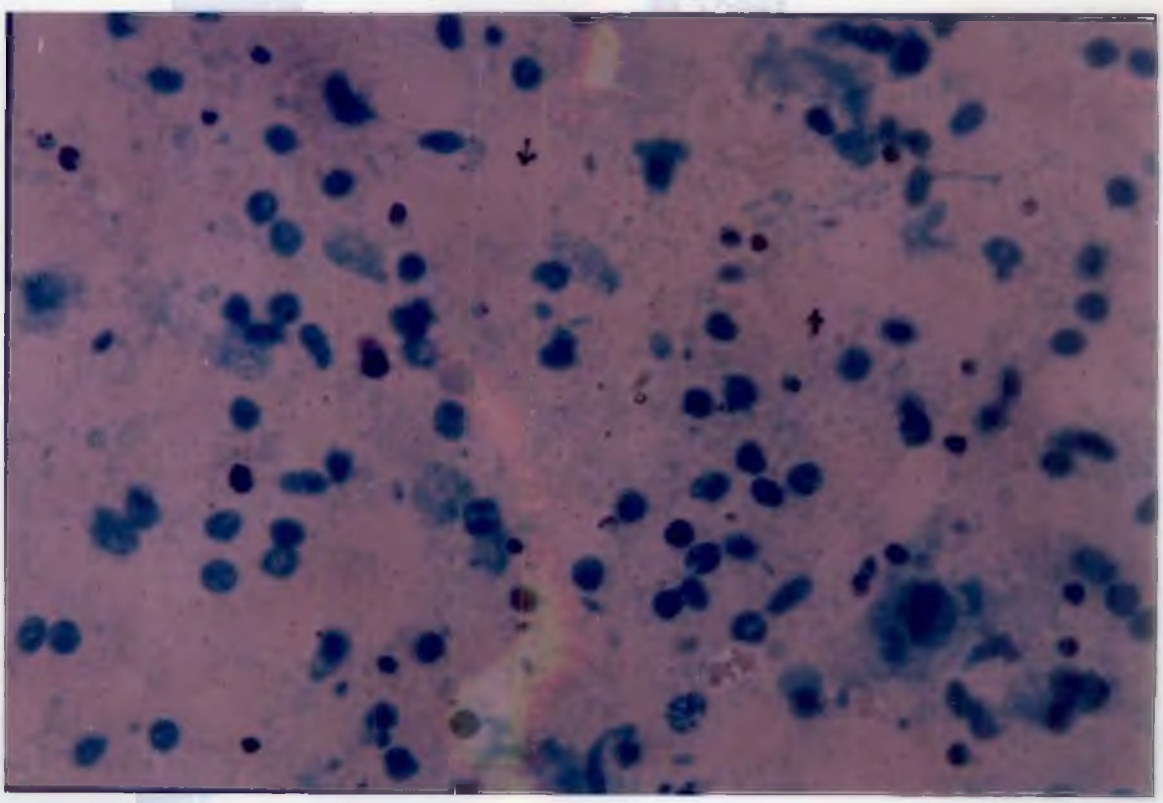


Fig.2

Table 1. Changes in developing CE after inoculation with isolates of *C. psittaci*

Isolates	Lesions observed
M-28	Death of embryo in five to seven days PI. The YS blood vessels deeply injected. Yolk more fluidy than normal. Stunting of embryo more pronounced. Haemorrhagic patches all over the embryo.
M-430	Death of embryo in eight to ten days. Dwarfing of embryo, unabsorbed yolk and injected yolk sac blood vessels noticed.
M-121	Death of the embryo not usual within 10 days but yolk sac haemorrhagic with unabsorbed yolk and stunting of embryo noticed.
P-156	Death of the embryo not noticed within 10 days. Moderate congestion of blood vessels supplying yolk sac. Yolk with a tendency for inspissation. Stunting of embryo noticed. Haemorrhagic patchy areas on the skin not exhibited.

4.3 Propagation of Chlamydial isolates in Mc Coy cell line

Mc Coy cell line obtained from NCCS, Pune was subcultured into tissue culture bottles and coverslips. Eagles MEM with 10 per cent foetal calf serum was used as growth medium. The seeded plates/tubes were incubated at 37° C and observed at intervals of 24 h for growth characteristics. Confluent monolayer (Fig. 3) developed 24 h after subculturing. All the four isolates were used for propagation in Mc Coy cell line. The monolayer with inoculum was incubated with maintenance medium containing cycloheximide (1µg/ml) at 37°C. The rounding and swelling of cells observed at 24 h interval indicated the multiplication of *C. psittaci* in the inoculated cells.

To study the CPE, the coverslip cultures inoculated with the isolates were stained and examined at 24 h intervals. The control culture tubes in which maintenance medium was inoculated instead of the isolates, showed no changes during the observation period and remained as confluent monolayers (Fig. 5, 7).

4.3.1 Staining of coverslip cultures

The coverslip cultures fixed in methanol overnight were stained with May – Grunwald Giemsa stain. The stained monolayers exhibited specific cytopathic changes such as rounding and swelling of the fibroblastic cells. The coverslip cultures inoculated with all the four isolates presented rounding and swelling of cells by 24 h PI. Clumping of cells with syncytia formation was additionally presented by the isolates M-28 and M-430 (Fig.4).

Mc Coy cell line 24 h PI (May-Grunwald Giemsa Stain – 400X)

Fig.3 Control

Fig.4 M-430 infected showing rounding and swelling of cells

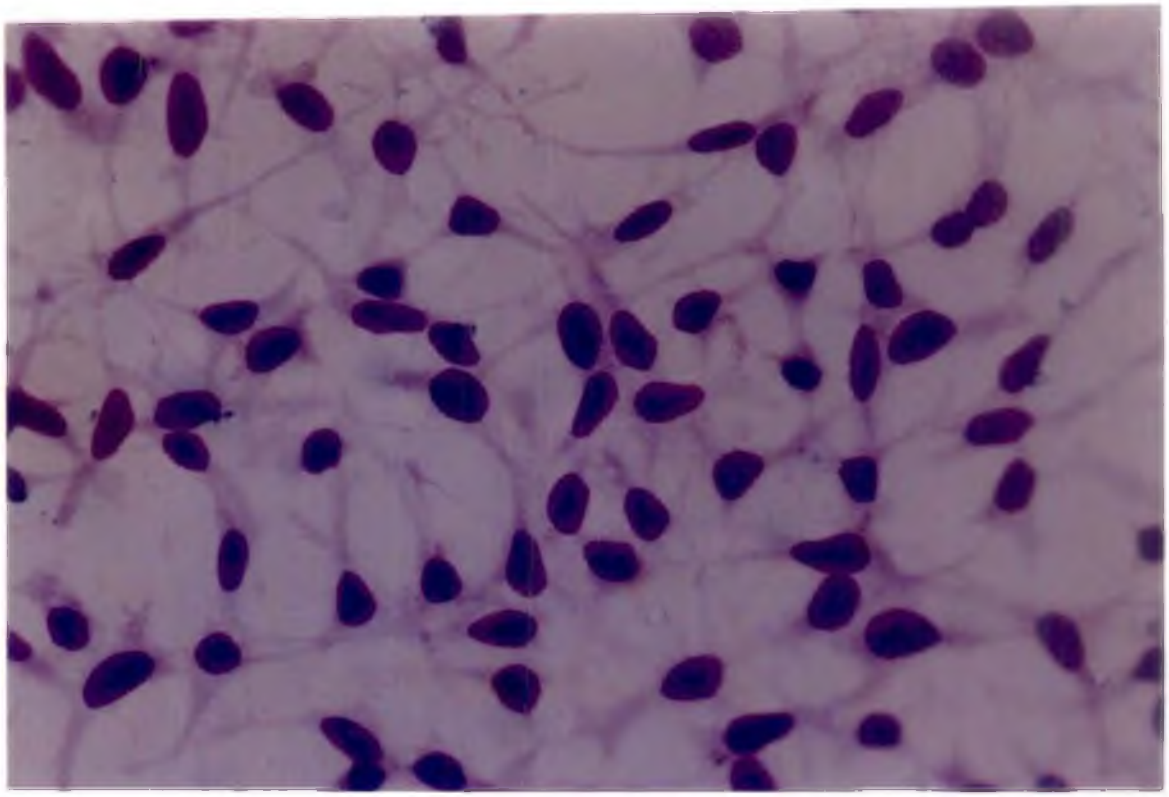


Fig. 3

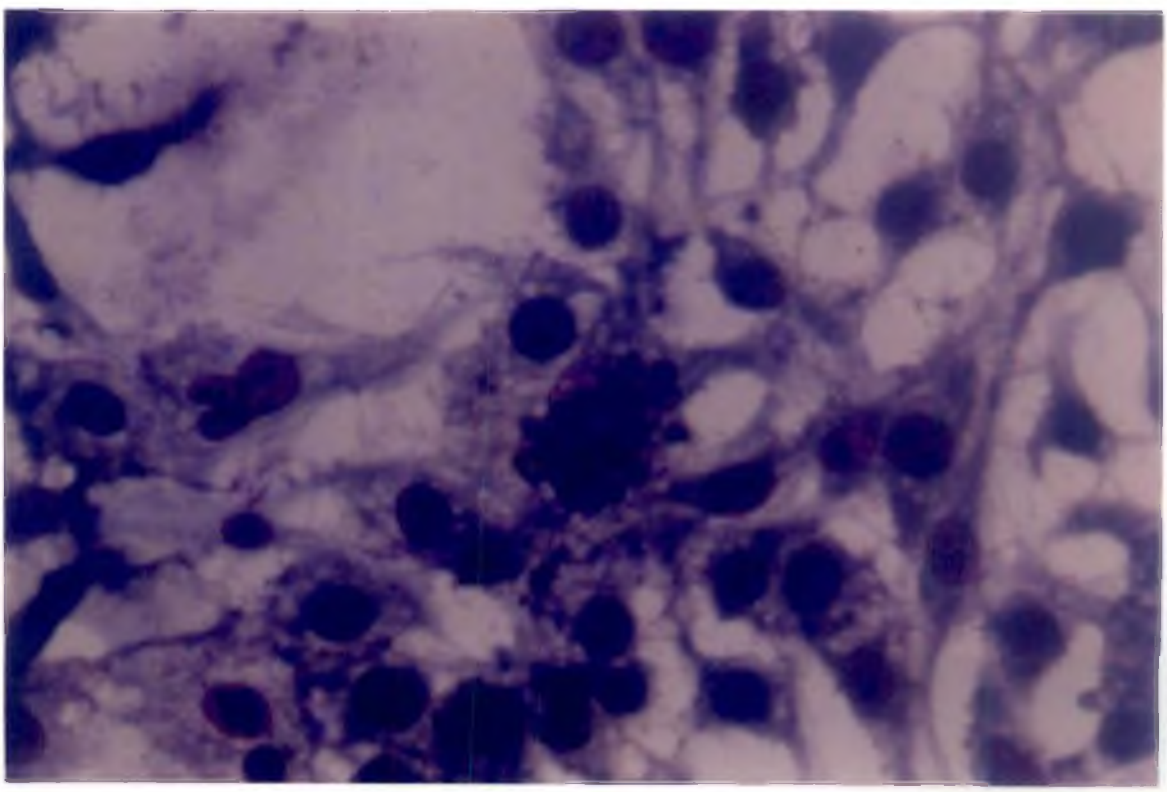


Fig.4

The CPE was found progressively increase as time passed.

By 48 h, rounding, swelling and syncytia formation of cells were prominent with M-28 and M-430 (Fig.6). Similar changes were observed to a lesser degree in coverslip cultures infected with P-156 and M-121. In these cultures the clumping of cells were comparatively less when compared to the changes produced by M-28 and M-430.

Complete rounding of most of the attached cells infected with all four isolates was observed by 72 h PI (Fig.8). By this time inclusion bodies (IB) were noticed in cells inoculated with M-28 and M-430. The IB were seen as round, dense, pink stained intracytoplasmic structures. They were not discretely present in cells inoculated with P-156 and M-121. By 72 h PI the cells inoculated with M-28 and M-430 started getting detached from the glass surface.

By 96 h PI, intracytoplasmic inclusions were appreciable in coverslip cultures inoculated with all the four isolates (Fig.9). Extracellular EB were also observed in cells that were infected with M-28 and M-430 (Fig.10). By this time the monolayers infected by all the four isolates were almost detached.

The CPE in Mc Coy cell line after inoculation with different Chlamydial isolates at 24 h intervals are presented in Table 2.

Mc Coy cell line 48 h PI (May-Grunwald Giemsa - 400X)

Fig.5 Control

Fig.6 M-28 infected showing syncytia formation

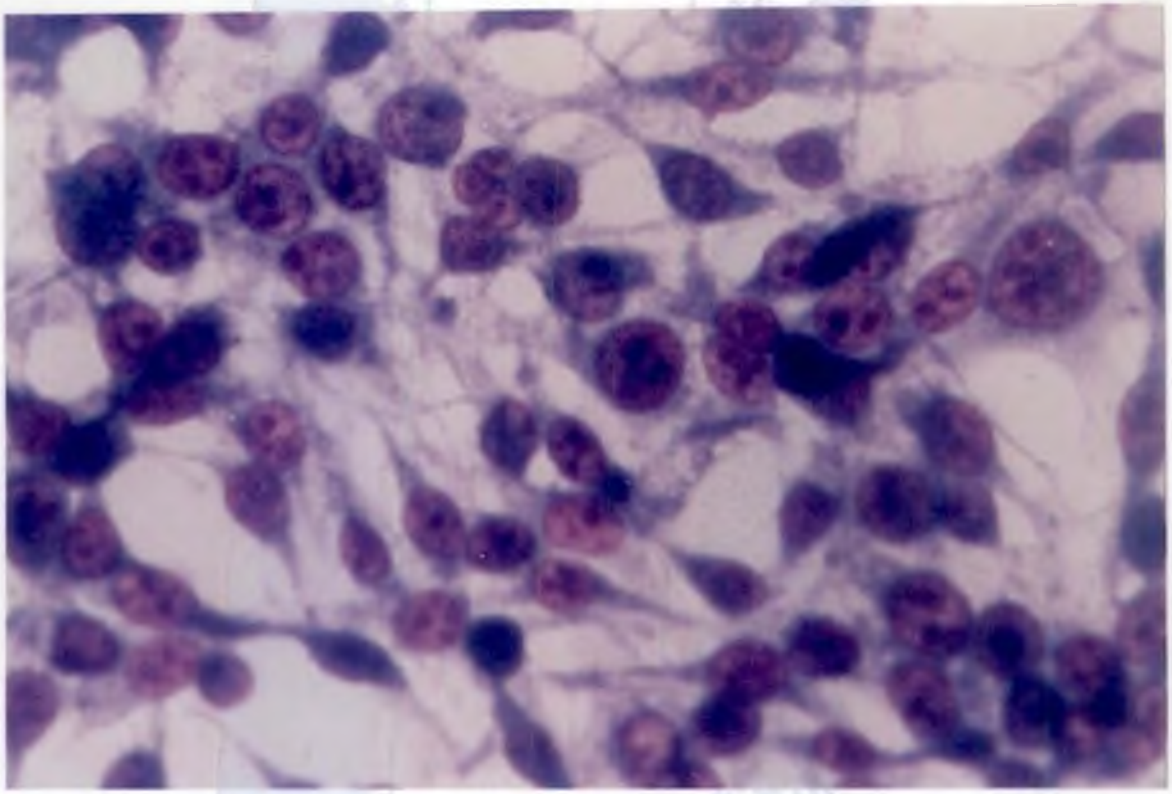


Fig.5

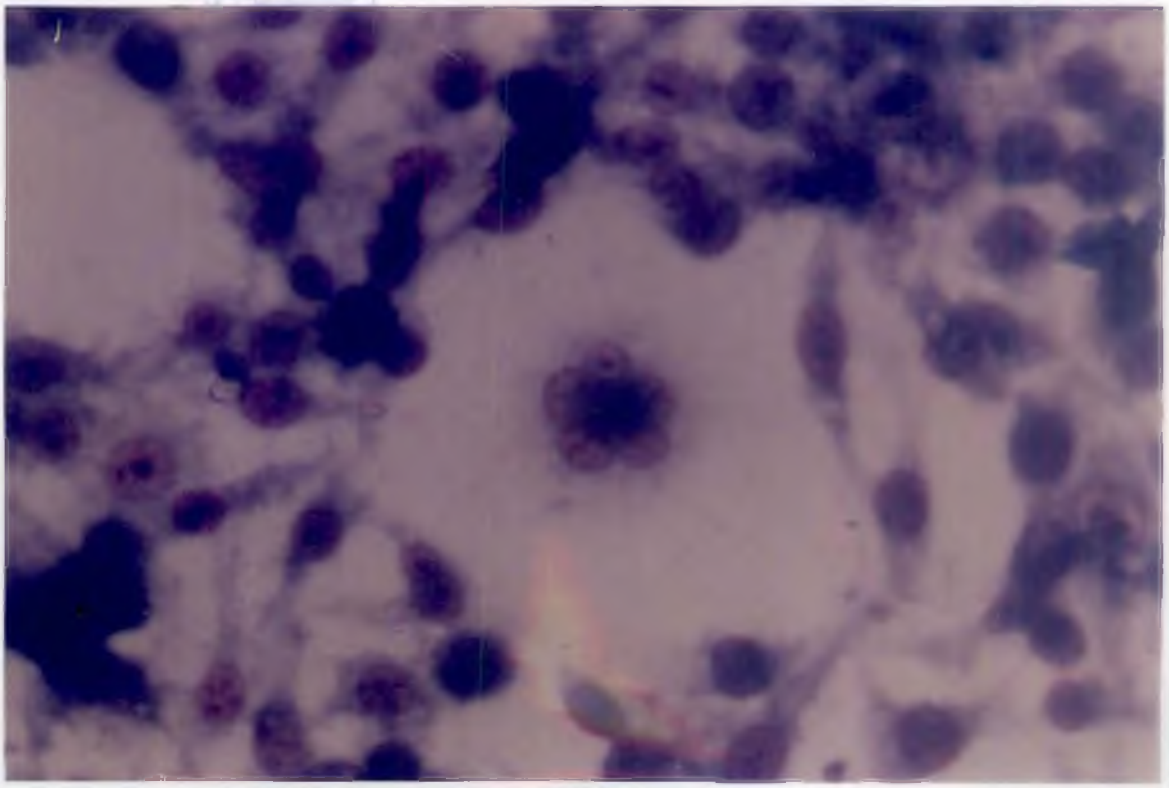


Fig.6

Mc Coy cell line 72 h PI (May-Grunwald Giemsa - 400X)

Fig.7 Control

Fig.8 M-430 infected showing detachment of cells

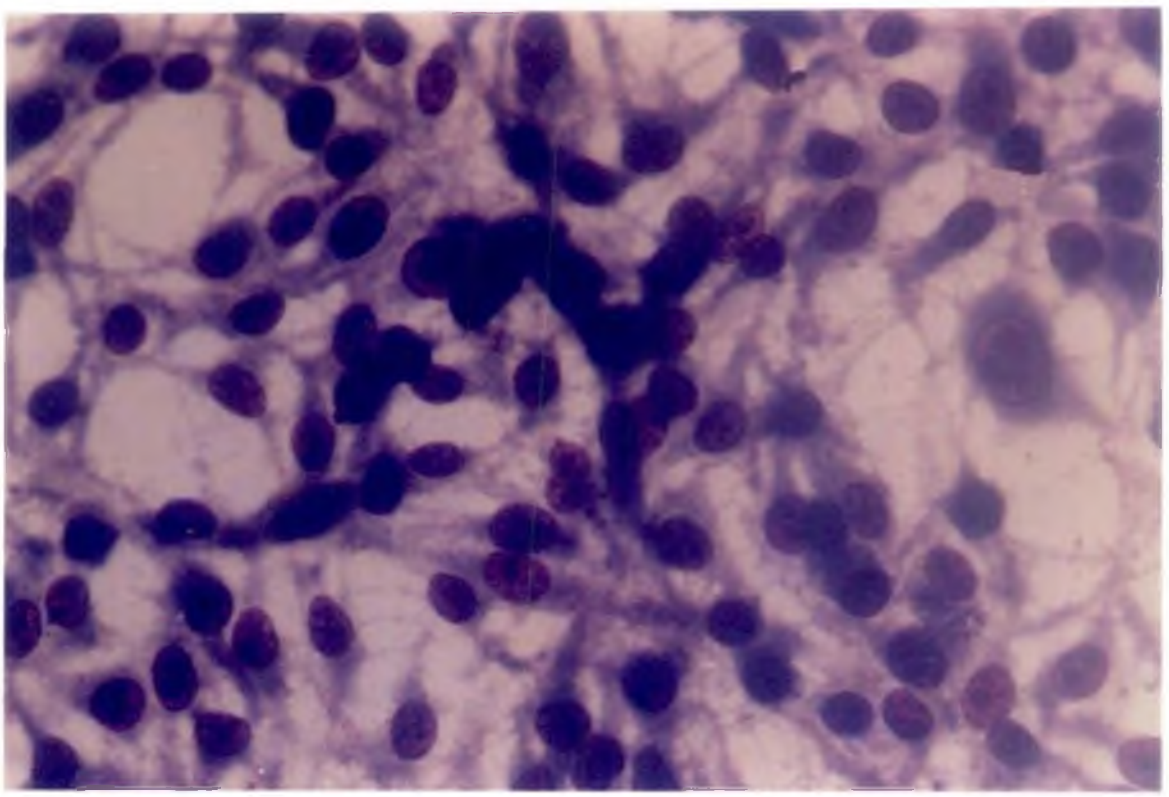


Fig.7

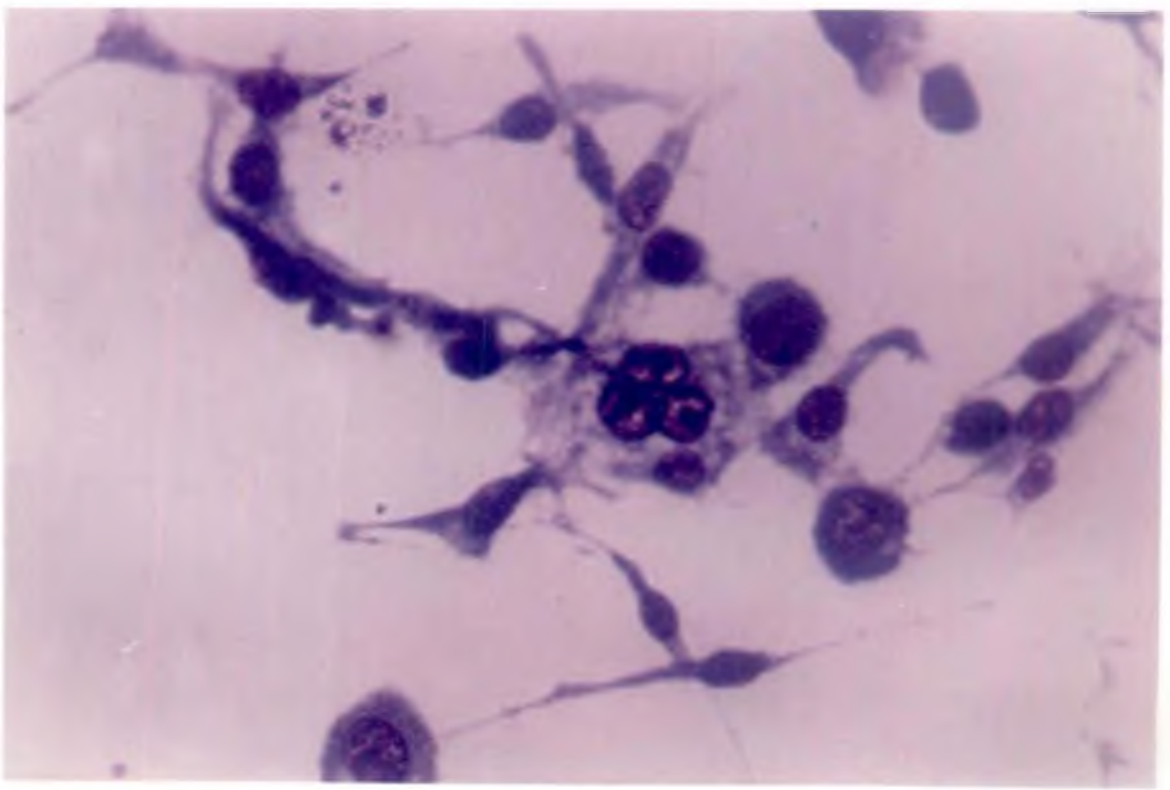


Fig.8

Mc Coy cell line 96 h PI (May-Grunwald Giemsa - 1000X)

Fig.9 M-430 infected showing Chlamydial inclusions

Fig.10 M-28 infected showing extracellular chlamydial EBs

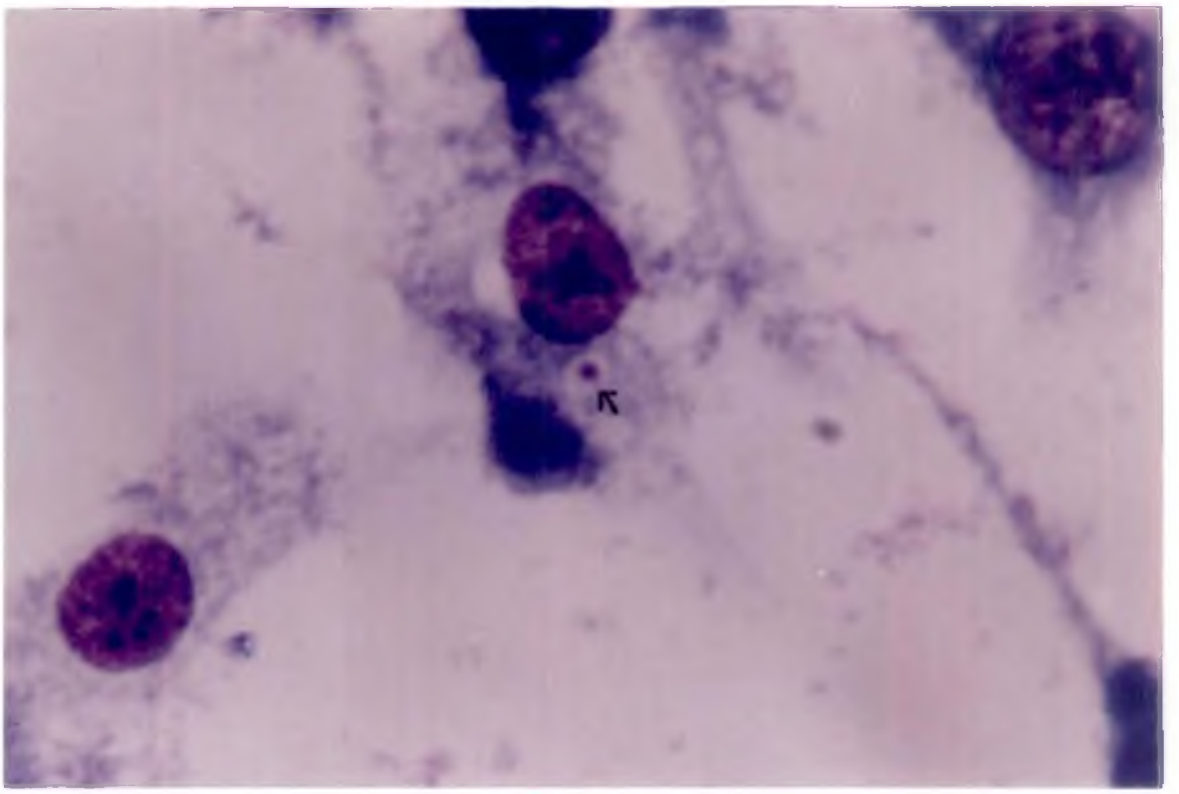


Fig.9

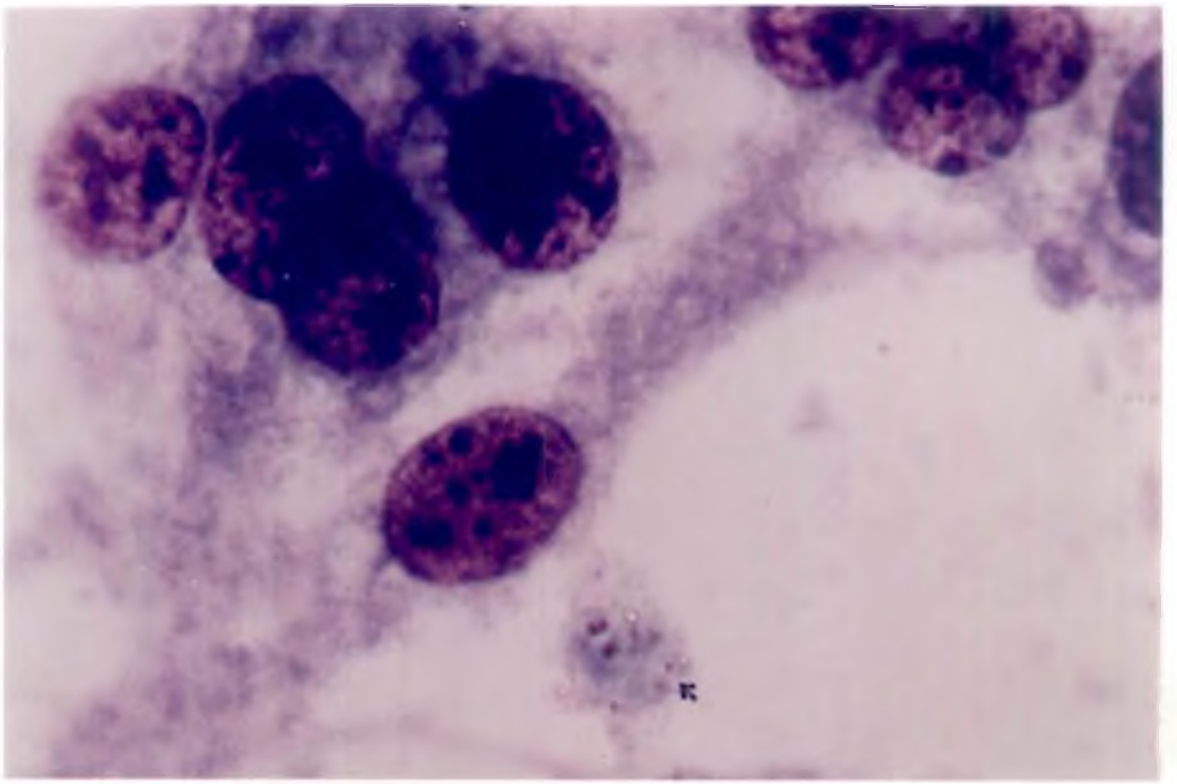


Fig.10

Table 2. CPE in Mc Coy cell line after inoculation with isolates of *C. psittaci*

Isolate	CPE			
	24 h	48 h	72 h	96 h
M-28 M-430	Rounding and swelling of cells with syncytia formation	Syncytia formation pronounced	Inclusion bodies visible as pink intracyto-plasmic round bodies; cells started detaching from glass surface	Inclusion bodies very clear. Extra cellular elementary bodies also visible. Cell detachment from glass surface almost complete
M-121 P-156	Rounding and swelling of cells	Clumping of cells with syncytia formation started	Syncytia formation more prominent	Intracytoplasmic inclusion bodies visible and cells started detaching from glass surface

4.3.2 Indirect immunofluorescence technique

The infected coverslip cultures were subjected to immunofluorescence reaction at 24 h interval until 96 h. Specific apple green fluorescence was observed when the infected cells were treated first with mouse monoclonal *C. psittaci* antibody and then with antimouse antiglobulin conjugated with fluorescin isothiocyanate, revealing intracytoplasmic chlamydial inclusions (Fig.12). The fluorescence was detected from 72 h PI with M-28 and M-430 isolates and maximum fluorescence was detectable by 96 h PI. In the cells infected with P-156 and M-121, fluorescence was noticed only by 96 h PI. In control coverslip cultures no specific fluorescence was noticed (Fig.11).

4.4 Characterization of chromosomal DNA

4.4.1 Preparation of purified EB

Purified EB were obtained from both cell culture and YS membrane harvest. From both the sources sufficient concentration of elementary bodies could be obtained, though *Chlamydiae* grown in chick embryo YS membrane involved lengthier purification procedures.

4.4.2 DNA extraction from purified EB

The method employed for extraction of DNA from EB was successful in obtaining purified DNA.

FAT stained Mc Coy cell line (400X)

Fig.11 Control

Fig.12 M-28 infected revealing apple green fluorescence

Fig.11

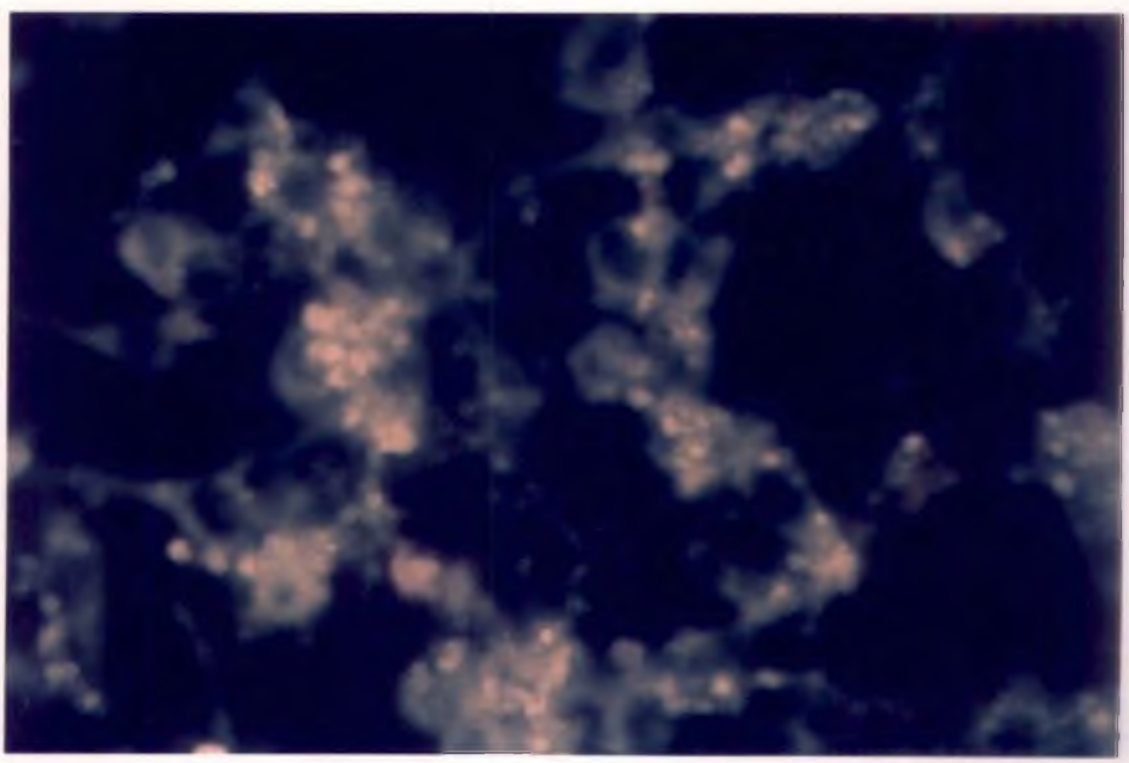


Fig.12

The DNA extracted from all the four isolates of *C. psittaci* was suspended in TE buffer. The concentration and purity of the extracted DNA of the four isolates were assessed by spectrophotometry. The optical density (OD) at 260 nm and at 280 nm for the different isolates are shown in Table 3. The concentration of DNA in each sample was calculated by the formula OD 260 x 50 (a constant for double stranded DNA) x 100 (dilution factor). The purity of the DNA was measured by calculating the ratio of OD 260/280 which indicated absence of extraneous material and hence the purity of the sample.

4.4.3 Restriction endonuclease analysis (REA) of genomic DNA

Pure DNA samples having the OD ratio 1.8 or more were subjected to restriction enzyme digestion. The DNA extracted from all the four isolates were digested separately by *Eco RI, Bam HI* and *Hae III* enzymes. The DNA could be digested by all the REs employed in the study when used in two units per microgram of DNA. When the enzyme digested mixture was electrophoresed in 0.8 per cent agarose gels containing ethidium bromide 0.5 µg/ml, it presented discrete bands which could be appreciated by UV illumination. The *Hind III* digested Lambda DNA electrophoresed along with the DNA digested mixture when used for comparison, the molecular size of different restriction fragments generated by different enzymes could be calculated based on the comparative distance migrated by them in the agarose gel.

Table 3. Concentration and purity of DNA from *C. psittaci* isolates

	M-28	M-430	M-121	P-156
OD 260	0.342	0.226	0.309	0.295
OD 280	0.188	0.122	0.168	0.164
Concentration (µg/ml)	1710	1130	1545	1475
Purity	1.82	1.85	1.84	1.80

When the electrophoresed gel was viewed in Alpha Imager for reading the results, fewer number of discrete bands could only be appreciated. But when the same gel was autoqueried for calculating the molecular size of the bands, more bands could be discerned with different molecular weights. These additional bands were detected by the autoanalyses in the diffused zones of the gel (Fig.13).

4.4.3.1 REA using *Eco RI*

On digestion with *Eco RI* enzyme the M-28, M-430, M-121 and P-156 isolates yielded eight, nine, ten and nine fragments respectively and the molecular sizes are listed in Table 4 and diagrammatically represented in Fig.15.

The molecular sizes of the restriction fragments of the isolates ranged from 11.1 kbp to 0.2 kbp with respect to M-28, from 12.3 to 0.2 kbp for M-430 from 17.3 to 0.2 kbp for M-121and from 10.6 to 0.2 kbp for P-156.

All the four isolates had five bands in common among them at the 0.2 kbp, 0.3 kbp, 0.7 kbp, 2.0 kbp and 3.3 kbp region. In addition to these bands, isolate P-156 shared a band withM-121 at 8.3 kbp region and with M-430 at 10.6 kbp region.

The M-28 isolate shared one band with M-430 at 6.8 kbp region.

Unique fragments were shown at 11.1 kbp and 10.1 kbp region by M-28, 12.3 kbp and 1.3 kbp region by M-430, 17.3 kbp, 11.7 kbp, 6.2 kbp and at 0.8 kbp by M-121 and at 5.7 kbp and 0.6 kbp region by P-156.

4.4.3.2 REA using *Hae III*

The DNA of the isolates M-28, M-430, M-121 and P-156 on digestion with *Hae III* enzyme yielded eight, eight, seven and nine fragments respectively. The molecular sizes are listed in Table 5 and diagrammatically represented in Fig.16.

The digested DNA presented fragments ranging in molecular sizes from 7.5 kbp to 0.2 kbp (M-28), 8.3 kbp to 0.2 kbp (M-430), 9.1 kbp to 0.2 kbp (M-121) and 11.7 kbp to 0.2 kbp (P-156) respectively.

Common bands were noticed at 0.2 kbp 0.3 kbp and 2.8 kbp for all the four isolates. P-156 shared bands with M-430 at 0.8 kbp and with M-28 at 0.4 kbp. M-121 isolate shared bands with M-430 at 4.6 kbp and with M-28 at 0.7 kbp and 2.1 kbp. Unique bands for P-156 isolates were observed at 11.7 kbp, 7.9 kbp, 3.1 kbp and 2.0 kbp region. For M-121 they are noticed at 9.1 kbp, region alone whereas for M-430 at 8.3, 2.2 and 0.9 kbp and for M-28 at 7.5 and 4.2 kbp respectiely.

4.4.3.3 REA using *Bam HI*

On digestion with *Bam HI* the DNA of the isolates M-28, M-430, M-121 and P-156 yielded eight, eight, seven and eight fragments respectively. The

molecular sizes are represented in Table 6 and diagrammatically represented in Fig.17.

The molecular sizes of the restriction fragments ranged from 10.6 kbp to 0.2 kbp for M-28; 12.3 kbp to 0.2 kbp for M-430; 10.1 kbp to 0.2 kbp for M-121 and 9.1 kbp to 0.2 kbp for P-156 isolates respectively.

Sharing of bands between all the four isolates were observed at 0.2 kbp and 0.7 kbp only. P-156 shared common bands with M-28 and M-430 at 0.3 kbp and with M-121 at 9.1 kbp and 4 kbp.

Unique bands were noticed at five region viz. 10.6 kbp, 8.7 kbp, 5.6 kbp, 3.9 kbp and 0.9 kbp molecular size region for M-28 and at 12.3 kbp, 9.6 kbp, 5.9 kbp, 4.2 kbp and 1 kbp for M-430. For M-121 unique bands were observed at 10.1 kbp, 5.3 kbp and 0.8 kbp molecular size region and for P-156 at 7.9 kbp and 4.9 kbp and 0.6 kbp region respectively.

4.5 Plasmid profile of *C. psittaci* isolates

The procedure for extraction of plasmid DNA from all the four siolates of *C. psittaci* employed in the present study could not procure any plasmid DNA, while with the above procedure the *E. coli* V517 yielded plasmid DNA. This was evidenced when the extracts from *C. psittaci* isolates and *E. coli* V517 where subjected to agarose gel electrophoresis. The *E. coli* V517 generated eight plasmid bands of molecular sizes ranging from 48.1 kb to 1.9 kb (Fig.14).

Fig.13 Restriction enzyme analysis of chromosomal DNA

Lane 1, 14 - λ DNA *Hind III* digest

Lane 2-5 - *EcoRI* digested genomic DNA. 2 (M-28); 3 (M-430); 4 (M-121) and 5 (P-156)

Lane 6-9 - *Hae III* digested genomic DNA. 6 (M-28); 7 (M-430); 8 (M-121) and 9 (P-156)

Lane 10-13 - *Bam HI* digested genomic DNA. 10 (M-28); 11 (M-430); 12 (M-121) and 13 (P-156)

Fig.14 Plasmid profile of *C. psittaci* isolates

Lane 1 - *E. coli* V517

Lane 2 (M-28); 3 (M-430); 4 (M-121) and 5 (P-156)

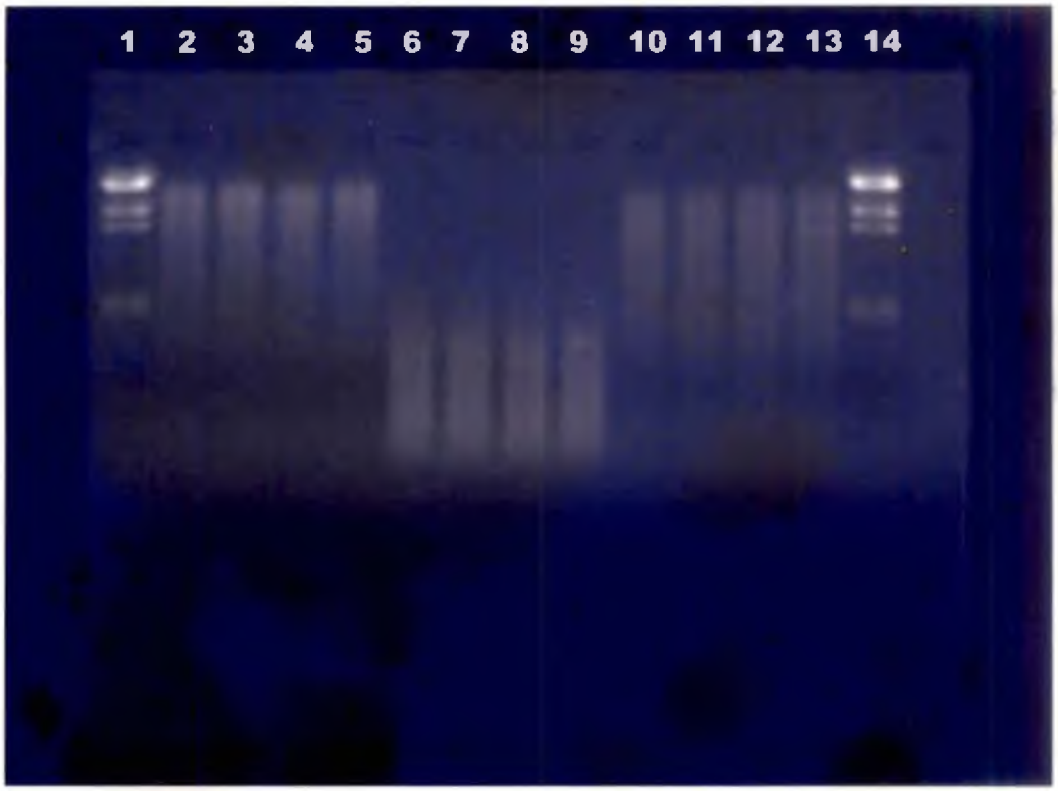

1 2 3 4 5 6 7 8 9 10 11 12 13 14
(kbp)
23·1
9·4
6·6
4·4
2·3
2·0
0·6

Fig.13

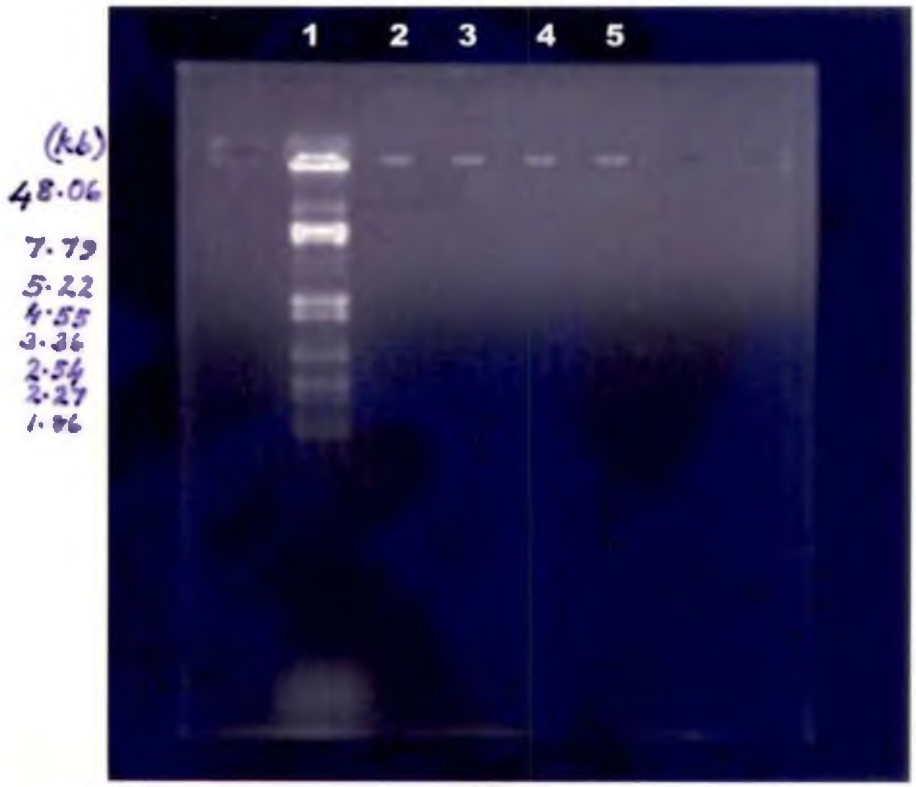
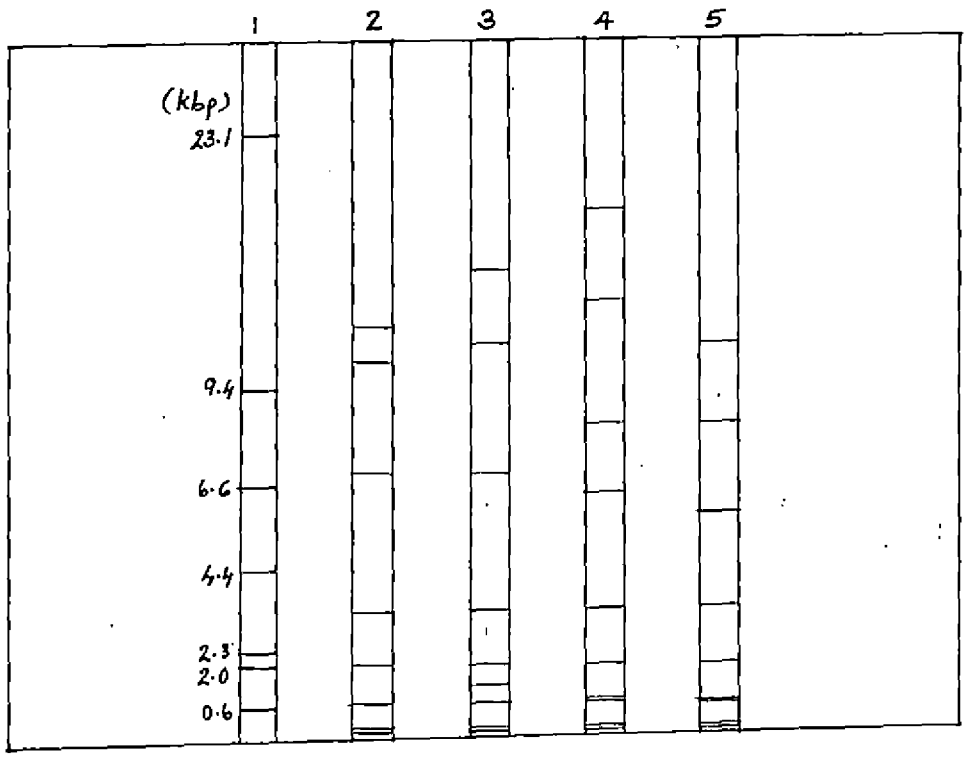

1 2 3 4 5
(kb)
48·06
7·79
5·22
4·55
3·36
2·54
2·27
1·96

Fig.14

Fig.15 Diagrammatic representation of restriction pattern of *C. psittaci* isolates on digestion with *Eco RI*

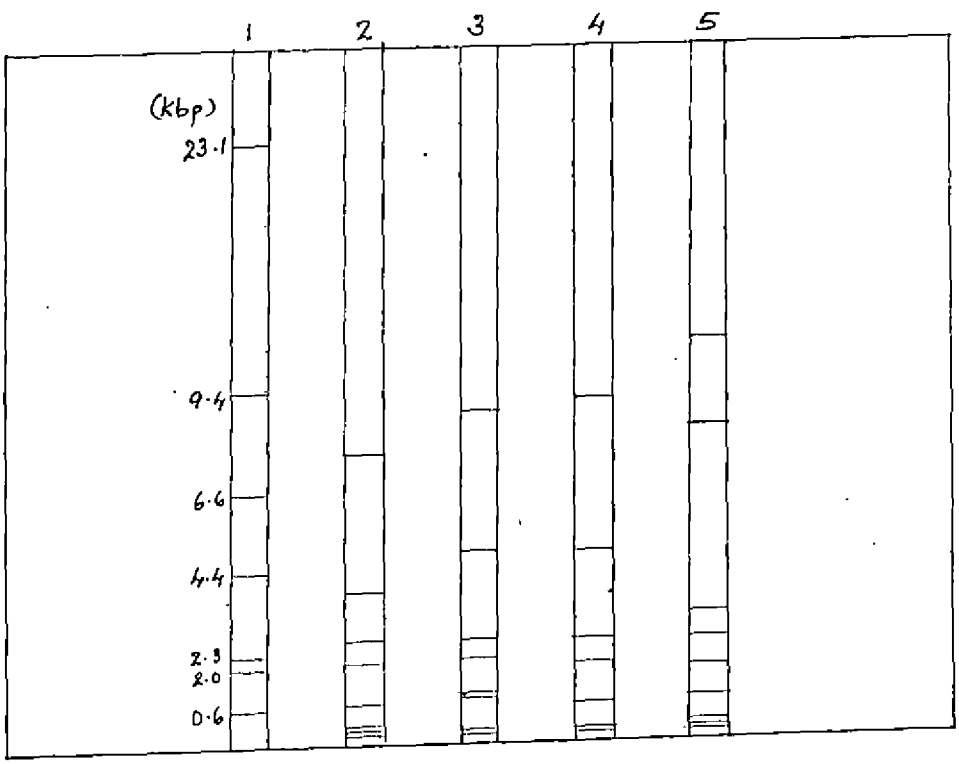


Lane 1 - λ DNA *Hind III* digest
Lane 2 - M-28
Lane 3 - M-430
Lane 4 - M-121
Lane 5 - P-156

Table 4. REA using *EcoRI*

DNA fragment	Molecular sizes (kbp) of the restriction fragments of the isolates			
	M-28	M-430	M-121	P-156
I	--	--	17.3	--
II	--	12.3	--	--
III	--	--	11.7	--
IV	11.1	--	--	--
V	--	10.6	--	10.6
VI	10.1	--	--	--
VII	--	--	8.3	8.3
VIII	6.8	6.8	--	--
IX	--	--	6.2	--
X	--	--	--	5.7
XI	3.3	3.3	3.3	3.3
XII	2.0	2.0	2.0	2.0
XIII	--	1.3	--	--
XIV	--	--	0.8	--
XV	0.7	0.7	0.7	0.7
XVI	--	--	--	0.6
XVII	0.3	0.3	0.3	0.3
XVIII	0.2	0.2	0.2	0.2
Total number of fragments	8	9	10	9

Fig.16 Diagrammatic representation of restriction pattern of *C. psittaci* isolates on digestion with *Hae III*

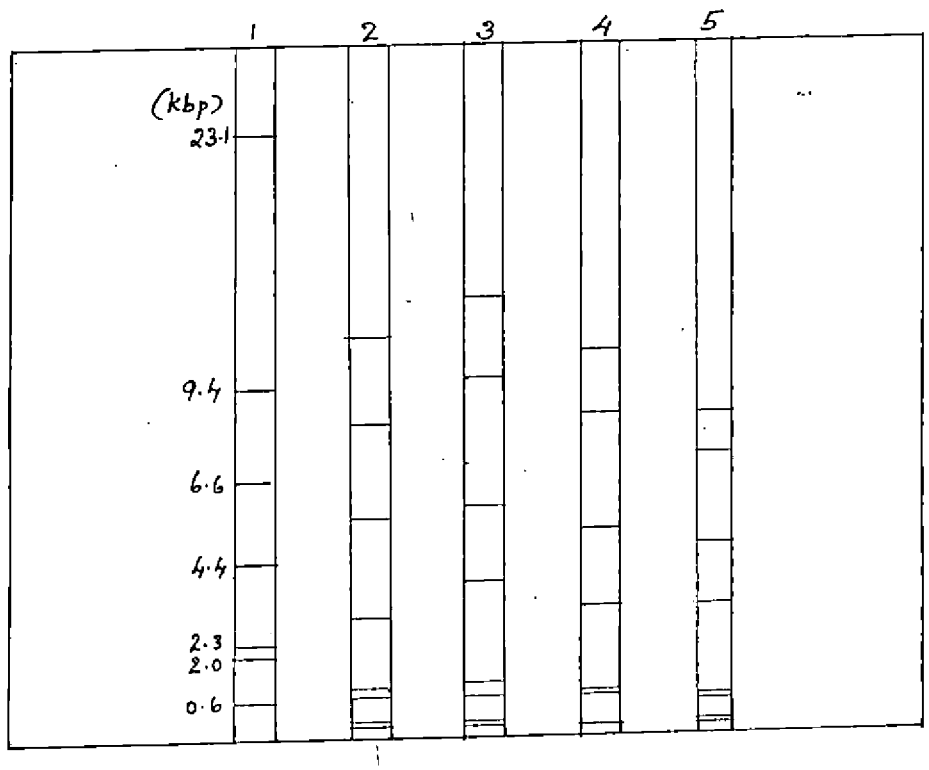


Lane 1	-	λ DNA *Hind III* digest
Lane 2	-	M-28
Lane 3	-	M-430
Lane 4	-	M-121
Lane 5	-	P-156

Table 5. REA using *Hae III*

DNA fragment	Molecular sizes (kbp) of the restriction fragments of the isolates			
	M-28	M-430	M-121	P-156
I	--	--	--	11.7
II	--	--	9.1	--
III	--	8.3	--	--
IV	--	--	--	7.9
V	7.5	--	--	--
VI	--	4.6	4.6	--
VII	4.2	--	--	--
VIII	--	--	--	3.1
IX	2.8	2.8	2.8	2.8
X	--	2.2	--	--
XI	2.1	--	2.1	--
XII	--	--	--	2.0
XIII	--	0.9	--	--
XIV	--	0.8	--	0.8
XV	0.7	--	0.7	--
XVI	0.4	--	--	0.4
XVII	0.3	0.3	0.3	0.3
XVIII	0.2	0.2	0.2	0.2
Total number of fragments	8	8	7	9

Fig.17 Diagrammatic representation of restriction pattern of *C. psittaci* isolates on digestion with *Bam HI*



Lane 1	-	λ DNA *Hind III* digest
Lane 2	-	M-28
Lane 3	-	M-430
Lane 4	-	M-121
Lane 5	-	P-156

Table 6. REA using *Bam HI*

DNA fragment	Molecular sizes (kbp) of the restriction fragments of the isolates			
	M-28	M-430	M-121	P-156
I	--	12.3	--	--
II	10.6	--	--	--
III	--	--	10.1	--
IV	--	9.6	--	--
V	--	-	9.1	9.1
VI	8.7	--	--	--
VII	--	--	--	7.9
VIII	--	5.9	--	--
IX	5.6	--	--	--
X	--	--	5.3	--
XI	--	--	--	4.9
XII	--	4.2	--	--
XIII	--	--	4	4
XIV	3.9	--	--	--
XV	--	1.0	--	--
XVI	0.9	--	--	--
XVII	--	--	0.8	--
XVIII	0.7	0.7	0.7	0.7
XIX	--	--	--	0.6
XX	0.3	0.3	--	0.3
XXI	0.2	0.2	0.2	0.2
Total number of fragments	8	8	7	8

Discussion

5. DISCUSSION

Chlamydia psittaci is an obligate intracellular bacterium which causes a wide variety of clinical syndromes in animals and birds. Among the pathogenic manifestations, abortion in bovines and ovines in late pregnancy is known to cause heavy economic loss in livestock industry and is recognized as a world wide problem. The discrimination of the isolates of *C. psittaci* from divergent origin at the molecular level is essential as variations are shown by different strains in biological and serological characterization. The present study reports the results of genetic characterization of local isolates of *C. psittaci* in comparison to that of a reference strain obtained from Himachal Pradesh.

5.1 Revival of isolates

All the four isolates used in this study could be propagated in six to eight day old embryonated chicken eggs by yolk sac route of inoculation and produced characteristic lesions in YS and embryo.

The lesions seen in the embryo were almost similar for all the isolates. When compared with uninoculated YS, the infected YS were thin walled, severely congested and the yolk tended to be more liquid in nature. The typical changes observed in the embryo included hyperemic legs and toes and patchy haemorrhages on the skin. Though the lesions on the YS and embryo were similar with different isolates, the isolate M-28 had more pronounced lesions.

This isolate, M-28, was the one isolated last and it had been passaged considerably less number of times when compared to other *C. psittaci* isolates employed in this study.

Collier (1984) and Batta *et al.* (1997) have also reported a similar death pattern of the CE as observed in this study.

The lesions on the YS and embryo observed in the present study are in conformation with the observations of Page (1984), Francis (1988) and Sreeramalu *et al.* (1989).

5.1.1 Staining of the infected YS

Impression smears prepared from the YS membrane were stained and examined to detect Chlamydial EB. Giemsa, modified Ziehl-Neelsen and Gimenez staining technique were employed. All the staining techniques could demonstrate successfully chlamydial elementary bodies. Among the three different staining techniques, Gimenez staining method was found to give more reliable and consistent results. Storz (1971) and Hanna *et al.* (1974) recommended the use of Giemsa staining whereas Krishna (1984) and Francis (1988) advocated MZN staining technique for direct demonstration of chlamydial bodies in infected materials. However, Katoch (1997) had reported the Gimenez staining method to be the most suitable one for the demonstration of *C. psittaci* in clinical materials/infected tissue.

5.2 Propagation in Mc Coy cell line

Schachter and Dawson (1978) suggested that cell culture methods superseded chick embryo in chlamydial isolation as the latter procedure was cumbersome and messy. But, for high yield of antigens both the procedures went hand in hand.

Page (1981) reported that Mc Coy cell lines were having rapidly growing cells that supported growth of most chlamydial strains and so were ideal for propagation of *C. psittaci* from cases of abortion.

In the present study, Mc Coy cell line was used for propagation of *C. psittaci* and characteristic growth of organism could be obtained.

When the isolates were passaged in Mc Coy cells, marked CPE were visible from 24 h onwards. Rounding and swelling of cells were produced by all the isolates. Formation of syncytia also started by 24 h in the monolayer infected with M-28 and M-430. By 48 h, syncytia formation were more pronounced. By 72 h, intra cytoplasmic inclusion bodies started appearing and were prominent by 96 h and cells were seen to get detached from adjacent ones. Even extra cellular bodies were visible by this time. Complete dislodgement of cells from the glass surface occurred at the end of 96 h.

On comparison, the degree and onset of cytopathic changes were faster in cell lines infected with the caprine isolates viz., M-28 and M-430 as against the bovine isolates P-156 and M-121.

Page (1981) reported similar cytopathic changes in cell cultures infected with *C. psittaci* in two to six days of incubation. The time required for the inclusion of CPE as observed in the present study was almost similar to the observations made by Page (1981).

Spears and Storz (1979) demonstrated intracytoplasmic inclusions in cells 30 h PI similar to that observed in the present study.

Anderson and Baxter (1986) and Griffiths *et al.* (1992) also observed large compact inclusions in infected cell line 48 h PI for isolates causing abortion which reached maturity by around 72 h.

Katoch (1997) and Nagal *et al.* (1997) described intracytoplasmic granular inclusions in Mc Coy cell line 72 h PI. Thus, the observations made in the present study corroborate with reports by earlier workers.

5.2.1 Enhancement of infectivity of cell lines

Various metabolic inhibitors have been reported to be employed in cell lines to enhance chlamydial growth and suppress the host cell replication. These included treatment of cells with Cytochalasin, Mitomycin C, cycloheximide, DEAE-D etc.

Spears and Storz (1979) enhanced infectivity of cell line to *C. psittaci* by treatment with cycloheximide. A three fold increase of inclusions were noticed.

In the present study, the treatment of Mc Coy cell line with cycloheximide has resulted in increase of infectivity of all the isolates and was found to contribute to the development of discrete inclusions.

Dennis and Storz (1982) observed that in vitro infectivity of *C. psittaci* of bovine origin had been enhanced by centrifugation. Physically the infectivity could be enhanced by the use of centrifugation of cells at the rate of 3000 g or more. This increased the infectivity by a combination of pressure and directional force, resulting in cell surface changes and reduced the electrostatic repulsive barriers of cells.

Katoch (1997) used a combination of centrifugation and cycloheximide treatment to increase the infectivity of cells.

5.2.2 Staining of coverslip cultures

In the present study, May-Grunwald Giemsa staining and indirect immunofluorescence staining techniques were used to confirm the presence of *C. psittaci* in cell culture.

Using May-Grunwald Giemsa staining, compact deeply pink staining intracellular inclusions were noticed 72 h PI which became prominent by 96 h. Elementary bodies which were lightly pink were also noticed by 96 h. These bodies were extracellular in nature. Similar observations have been made by Spears and Storz (1979) and Anderson (1986).

Thomas *et al.* (1997), Stamm *et al.* (1983), Griffiths *et al.* (1992), Vanrampay *et al.* (1994) and Nagal *et al.* (1997) reported that the Fluorescent Antibody Technique (FAT) was a superior technique for detection of chlamydial inclusions in cell cultures.

In the present study, our attempt to demonstrate chlamydia in cell lines employing monoclonal antibodies revealed apple green flourescing inclusions 96 h PI in sufficient number to be of diagnostic level. These observations were in confirmation with those of Vanrampay *et al.* (1994) and Nagal *et al.* (1997).

5.3 Characterization of chromosomal DNA

In this study, purified EB of *C. psittaci* were obtained from Mc Coy cell and YS membrane. Sufficient concentration of EB was obtained by both the methods, eventhough chlamydiae grown in chicken embryo yolk sacs required a lengthier purification procedure as reported by McClenaghan *et al.* (1984).

The homogenized EB in TES buffer were treated with proteinase K, followed by SDS and then extracted with phenol-chloroform isoamyl alcohol mixture. Nucleic acid was precipitated from the aqueous phase by addition of 5 M NaCl and double volume ethanol. The concentration and purity of DNA extracted from all the four isolates were assessed by spectrophotometry. The purity of DNA from isolates was more than 1.80. The concentration of DNA varied from 1130 to 1710 μg/ml for different isolates.

The procedure of McClenaghan *et al.* (1984) varied from our procedure in the first step only. In their study they had treated homogenized EB in TES buffer containing N-lauroyl sarcosine and proteinase K. But in the present experiment we have used proteinase K in the first step followed by SDS. We were able to get enough concentration of sufficiently pure DNA.

5.3.1 REA of genomic DNA

Restriction enzyme analysis is one of the typing methods that has been used with increasing frequency to identify and differentiate the strains and to test their homogenity without any ambiguity.

Peterson and DeLa Maza (1983) characterized the DNA from *C. psittaci* and *C. trachomatis* using a panel of nine restriction enzymes and observed small or no variation between the strains of one species, though marked difference was noticed between the DNA of different species.

Anderson (1991) reported the use of three restriction enzymes *Eco RI*, *Bam HI* and *Pst I* to characterise the avian serovar of *C. psittaci.*

Isolates causing abortion in sheep had been surveyed by RE analysis (McClenaghan *et al.*, 1984; Herring *et al.*, 1989) but no convincing variation within UK isolates had been revealed.

In this study four isolates of *C. psittaci* were compared by REA with *Eco RI, Hae III* and *Bam HI.*

5.3.1.1 *Eco RI* profile

Digestion of DNA of isolates with *Eco RI* resulted in eight, nine, ten, and nine fragments for M-28, M-430, M-121 and P-156 respectively. The banding pattern were complex together with poorly resolved fragments. The DNA of M-28 had presented fragments of their own at 11.1 and 10.1 kbp region and M-430 at 12.3 and 1.3 kbp region. The M-121 had unique segments at 17.3, 11.7, 6.2 and 0.8 kbp. P-156 had unique fragments at molecular size of 5.7 kbp and 0.6 kbp. The other fragments of caprine isolates were similar but different from bovine isolates, especially at higher molecular size range.

McClenaghan *et al.* (1984) obtained a low number of discrete fragments on *Eco RI* digestion in higher molecular weight range of 10 to 20 kbp whereas the resolution of smaller fragments were poor. They opined that too many fragments cut by the enzymes presented difficulty in their analysis and identification, appearing as poorly resolved bands on electrophoresis.

5.3.1.2 *Hae III* profile

Hae III digestion of the DNA of isolates presented eight fragments for M-28 and M-430 and seven fragments for M-121. Nine cuts were generated by this enzyme in the DNA of P-156. The enzyme produced similar bands for all the isolates at 0.2, 0.3 and 2.8 kbp range. At higher molecular range there was marked variation among the isolates.

Rodolakis and Souriau (1992) used this enzyme for comparison of the various *C. psittaci* isolates. Minor difference between the fragments were observed by them. Better resolution of fragments was noticed at lower molecular range region than at higher levels.

Restriction enzymes with four base recognition sequences produced fragments too small for effective resolution in low concentration agarose gels (McClenaghan *et al.*, 1984). Similar results also were observed in the present study.

5.3.1.3 *Bam HI* profile

This enzyme digested the DNA of isolates M-28, M-430, M-121 and P-156 into eight, eight, seven and eight fragments respectively. All the isolates had bands at molecular size of 0.2 and 0.7 kbp in common. M-28, M-430 and P-156 had additionally common bands at 0.3 kbp whereas M-121 and P-156 had common bands at 9.1 and 4 kbp regions. Unique fragments were also observed for different isolates.

Rodolakis and Souriau (1992) obtained almost similar RE digestion fragments with *Bam HI* and hence recommended its use in the discrimination of strains of *C. psittaci*. The restriction pattern evidenced in this study also confirm with the observation of the above workers.

5.4 Plasmid profile of *C. psittaci*

Lovett *et al.* (1980) reported the presence of plasmids in *C. psittaci.* Later on, McClenaghan *et al.* (1988) carried out a broad survey to detect plasmids in avian and mammalian strains of *C. psittaci.* They observed plasmids in avian isolates, whereas, certain mammalian isolates were free of plasmids.

In this study, efforts were made to separate and characterize plasmids from *C. psittaci.* Repeated attempts employing the techniques which had proved fruitful for isolation of plasmids from gram negative bacteria including chlamydia had failed to detect the presence of plasmids in all isolates. Everett (2000) also reported the absence of an extra chromosomal plasmid in all strains of *Chlamydophila abortus.*

Conclusion

Characterization of chromosomal DNA of *C. psittaci* by REA revealed a near homogenity among the isolates. The lack of plasmids in all isolates also indicate the homogenity of their origin and probably the genetic relationship. Thus, the restriction enzyme digestion analysis with other genetic tools can better resolve the similarity/dissimilarity among the isolates.

The electrophoresis of digested DNA on PAGE is accredited as an approved technique for higher resolution of individual fragments. This can

further delineate the homogenity/heterogenity in *C. psittaci*. This has been the approach by McClenaghan *et al.* (1984).

There are two problems with the RE profiling technique. First, it requires the large scale culture and purification of EB to yield DNA for analysis. Second, although small differences can be easily distinguished by eye, the profiles are complex and difficult to quantify.

These problems can be partially overcome by incorporating the "Southern blotting" technique in which the fragment pattern is transferred to a nitrocellulose membrane and analysed by hybridization.

Summary

6. SUMMARY

Abortion due to *Chlamydia psittaci* has caused significant economic loss in livestock production industry throughout the world. Reports on the prevalence of the disease in this part of the country had indicated the need for an indepth study.

The present study was undertaken to unearth differences, if any, among the various isolates of *C. psittaci* at molecular level. The pathological alterations in embryonated eggs, cytopathic changes in cell line, plasmid profiling and restriction enzyme analysis of genomic DNA were used for comparison of the isolates.

Four isolates of *C. psittaci* (M-28, M-430, M-121 and P-156) preserved in the Department of Microbiology were revived by passaging into six to seven day old developing chick embryo through YS route. The embryos that died due to specific infection had patchy haemorrhage all over the body and hyperemic legs. The yolk sac was thin walled with deeply injected blood vessels and contained thin and watery yolk. Death of the embryo occurred five to seven days PI for M-28 whereas death occurred in eight to ten days PI for M-430. The other two isolates had not caused mortality within ten days, but the embryos were stunted in growth. The impression smears from infected YS revealed EB by different staining techniques.

Marked CPE was exhibited by M-28 and M-430 in inoculated Mc Coy cell lines. In stained preparations rounding and clumping of cells, syncytia formation and intracytoplasmic inclusion bodies were noticed. For the isolates P-156 and M-121 the CPE developed later.

Fluorescent antibody staining technique was used in coverslip cultures to confirm the identity of the isolates. Specific apple green fluorescing inclusions were observed which were very prominent by 96 h PI.

The purified EB of each isolate was prepared from infected cell culture harvest and directly from YS by urografin density gradient centrifugation. These were used for DNA extraction. The purity and concentration of the extracted DNA were determined by spectrophotometry. The DNA concentration of M-28, M-430, M-121 and P-156 were 1710 µg/ml, 1130 µg/ml, 1545 µg/ml and 1475 µg/ml respectively.

Extracted DNA of the four isolates were digested by *Eco RI, Hae III* and *Bam HI.* Electrophoresis was done in 0.8 per cent agarose gel containing ethidium bromide (0.5 µg/ml) to study the restriction profile of each strain. The DNA fragments were observed in Alpha imager and photographed. The molecular sizes of the restriction fragments were estimated by comparison of the distance migrated by them with that of standard molecular weight marker. *Hind III* digested Lambda DNA was used as molecular weight marker in this study.

All the four isolates of *C. psittaci* DNA were cleaved into fragments ranging from eight to ten with slight differences in fragment size on digestion with *Eco RI*. The isolates differed mainly in size of the heavy fragments. Most of the light fragments were similar in size.

On digestion with *Hae III* the number of fragments ranged from seven to nine. The caprine isolates were having eight fragments each, but fragment size varied between the isolates. In case of bovine isolates the fragment size and number differed.

Digestion of DNA of the isolates with *Bam HI* yielded eight fragments for all the isolates except M-121 wherein seven fragments were only noticed. But variation was noticed in fragment size among the isolates.

All the three REs produced some difference in restriction profile of the DNA of isolates of *C. psittaci* and hence these enzymes are found to be useful in distinguishing the isolates used in this investigation.

The isolates were screened for the presence of plasmids. Repeated attempts revealed no plasmids at all in any of the isolates.

Hence, the present study revealed that genotyping and plasmid profiling could be used as an adjunct with other typing methods to differentiate the strains of *C. psittaci* in epidemiological studies.

References

REFERENCES

Amin, J.D. and Wilsmore, A.J. (1994). Detection of *Chlamydia psittaci (ovis)* antigen in tissue sections and Mc Coy cells using streptavidin-biotin and the Imagen staining method. *Br. Vet. J.* **150**(6): 555-560.

Amin, J.D.. and Wilsmore, A.J. (1997). The effects of crude placental extract and Erythritol on growth of *Chamydia psittaci* (*ovis*) in Mc Coy cells. *Vet. Res. Commun.* **21**: 431-435.

Anderson, A.A. (1991). Serotyping of *Chlamydia psittaci* isolates using serovar-specific monoclonal antibodies with the micro-immunofluorescence test. *J. Clin. Microbiol.* **29**(4): 707-711.

Anderson, I.E. (1986). Comparison of the virulence in mice of some ovine isolates of *Chlamydia psittaci. Vet. Microbiol.* **12**: 213-220.

Anderson, I.E. (1987). Comparison of some ovine *Chlamydia psittaci* isolates by indirect immunofluorescence. *Vet. Microbiol.* **13**: 69-74.

Anderson, I.E. and Baxter, T.A. (1986). *Chlamydia psittaci*: Inclusion morphology in cell culture and virulence in mice of ovine isolates. *Vet. Rec.* **119**: 453-454.

Anderson, I.E., Baxter, S.I.F., Dunbar, S., Rae, A.G., Philips, H.L., Clarkson, M.J. and Herring, A.J. (1996). Analyses of the genomes of Chlamydial isolates from ruminants and pigs support the adoption of a new sp. *Chlamydia pecorom. Int. J. Syst. Bacteriol.* **46**(1): 245-251.

Baghian, A., Shaffer, L. and Storz, J. (1990). Antibody response to epitopes of chlamydial major outermembrane protein on infectious elementary bodies and of the reduced polyacrylamide gel electrophoresis separated form. *Infect. Immun.* **58**(5): 1379-1383.

Batta, M.K., Katoch, R.C., Asrani, R.K., Sharma, M., Joshi, V.B. and Nagal, K.B. (1997). Prevalence of chlamydial pneumonia among livestock in Himachal Pradesh. *Indian Vet. J.* **74**: 824-826.

Biolatti, B., Dagnall, G.J.R., Bollo, E., Cornaglia, E., Wilsmore, A.J. and Donn, A. (1991). The use of fluorescein conjugated monoclonal antibodies, cell culture and transmission electron microscopy to detect *Chlamydia psittaci* and associated lesions in experimentally infected mice. *J. Comp. Pathol.* **105**: 17-26.

Bollo, E., Biolatti, B., Donn, A., Turilli, C. and Wilsmore, A.J. (1992). Preparation and use of a monoclonal antibody to detect *Chlamydia psittaci* antigen in paraffin-embedded tissue sections. *Res. Vet. Sci.* **53**(3): 393-396.

Bush, R.M. and Everett, K.D.E. (2001). Molecular evolution of the Chlamydiaceae. *Int. J. Syst. Evol. Microbiol.* **51**: 203-220.

Buzoni-Gatel, D. and Rodolakis, A. (1983). A mouse model to compare virulence of abortive and intestinal ovine strains of *Chlamydia psittaci*: influence of the route of inoculation. *Ann. Microbiol.* (Inst. Pasteur) 134 A: 91-99. Cited by Anderson, I.E. (1986). pp: 213-220

Buzoni-Gatel, D., Layachi, K., Dubray, G. and Rodolakis, A. (1989). Comparison of protein patterns between invasive and non-invasive ovine strains of *Chlamydia psittaci*. *Res. Vet. Sci.* **46**: 40-42.

Caldwell, H.D., Kromhout, J. and Schachter, J. (1981). Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect. Immun.* **31**(3): 1161-1176.

Campbell, L.A., Kuo, C.C. and Grayston, J.T. (1987). Characterization of the new *Chlamydia* agent, TWAR, as a unique organism by Restriction Endonuclease Analysis and DNA-DNA hybridization. *J. Clin. Microbiol.* **25**(10): 1911-1916.

*Cevenini, R., Donate, M., Brochi, E., Simone, F.D.E. and Placa, M.LA. (1991). Partial characterization of an 89 kDa highly immunoreactive protein from *Chlamydia psittaci* A/22 causing ovine abortion. *FEMS Microbiol. Let.* **81**(1): 111-115.

*Coles, A.C. (1930). Microorganisms in psittacosis. *Lancet* **1**: 1011-1012. Cited by Vanrompay *et al.* (1994). PP: 1470-1474

Collier, L.H. (1984). Chlamydia In "Topley and Wilson's Principles of Bacteriology, Virology and Immunology". Vol.2 (M.G. Parker Ed.). Edward Arnold Publishers Ltd., 7th ed. pp. 510-523.

Cottral, G.E.. (1978). "Manual of Standard Methods for Veterinary Microbiology". Comstock Publishing Associates, Ithaca, London. pp: 485-492.

Cox, R.L., Kuo, C., Grayston, T. and Campbell, L.A. (1988). Deoxy ribonucleic acid relatedness of *Chlamydia* sp. strain TWAR to *Chlamydia trachomatis* and *Chlamydia psittaci*. Int. J. Syst. *Bacteriol.* **38**(3): 265-268.

Cruickshank, R., Duguid, J.P., Marmion, B.P. and Swain, R.H.A. (1975). "Medical Microbilogy". Churchil, Livingstone, Edinburgh, 12th ed. pp. 516-517.

Denamur, E., Sayada, C., Souriau, A., Orfila, J., Rodolakis, A. and Elison, J. (1991). Restriction pattern of the major outer-membrane protein gene provides evidence for a homogenous invasive group among ruminant isolates of *Chlamydia psittaci*. *J. Gen. Microbiol.* **137**: 2525-2530.

Dennis, M.W. and Storz, J. (1982). Infectivity of *Chlamydia psittaci* of bovine and ovine origins for cultured cells. *Am. J. Vet. Res.* **43**(11): 1897-1902.

Duguid, J.P., Marmion, B.P. and Swain, R.H.A. (1978). "Mackie and Mc Cartney Medical Microbiology". The English Language Book Society and Churchil Livingstone, Edinburgh, 13th ed. pp. 515-518.

Eb, F. and Orfila, J. (1982). Serotyping of *Chlamydia psittaci* by micro immunofluorescence test: isolates of ovine origin. *Infect. Immun.* **37**: 1289-1291.

*Eb, F., Orfila, J., Milon, A. and Geral, M.F. (1986). Interet epidemiologique du typage por immunofluorescence de *Chlamydia psittaci*. *Annales de l Istitut Pasteur de Microbiologie* 137B: 77-93.

Escanlente-Ochoa, C., Ducatelle, K. and Haesebrouck, F. (2000). Dynamics of the development of *Chlamydia psittaci* inclusions in epithelial and fibroblast host cells. *J. Vet. Med. Series B.* **47**(5): 343-349.

Evans, R.T. and Taylor-Robinson, D. (1979). Comparison of various Mc Coy cell treatment procedures used for detection of *Chlamydia trachomatis*. *J. Clin. Microbiol.* **10**: 198-201.

Everett, K.D.E. (2000). Chlamydia and Chlamydiales more than meets the eye. *Vet. Microbiol.* **75**: 109-126.

Everett, K.D.E. and Anderson, A.A. (1999). Identification of nine species of the Chlamydiaceae using PCR-RFLP. *Int. J. Syst. Bacteriol.* **49**(2): 803-813.

Everett, K.D.E., Andersen, A.A., Plaunt, M. and Hatch, T.P. (1991). Cloning and sequence analysis of the MOMP gene of *Chlamydia psittaci* 6BC. *Infect. Immun.* **59**(8): 2853-1855.

Francis, R. (1988). Prevalence of chlamydial agents in livestock in Kerala. M.V.Sc. thesis, Kerala Agricultural University. pp: 68-142

Frost, E.H., Deslandes, S., Veilleux, S. (1991). Typing Chlamydia trachomatis by detection of restriction fragment length polymorphism in the gene encoding the major outer membrane protein. *J. Infect. Dis.* **163**: 1103-1107.

Fukushi, H. and Hirai, K. (1989). Genetic diversity of ovian and mammalian *Chlamydia psittaci* strains and relation to host origin. *J. Bacteriol.* **171**(5): 2850-2855.

Fukushi, H. and Hirai, K. (1992). Proposal of *Chlamydia pecorum* sp. nov for Chlamydia strains derived from ruminants. *Int. J. Syst. Bacteriol.* **42**: 306-308.

Fukushi, H. and Hirai, K. (1993). Restriction fragment length polymorphism of RNA as genetic marker to differentiate Chlamydia spp. *Int. J. Syst. Bacteriol.* **43**(3): 613-617.

Gatel, B.D., Layachi, K., Dubray, G. and Rodolakis, A. (1989). Comparison of protein patterns between invasive and non invasive ovine strains of *Chlamydia psittaci*. *Res. Vet. Sci.* **46**: 40-42.

Girjes, A.A., Weighler, B.J., Hugall, A.F., Carrick, F.N. and Lavin, M.F. (1989). Detection of *Chlamydia psittaci* in free ranging Koalas (*Phascolaretos cinereus*): DNA hybridization and Immuno-Slot Blot analyses. *Vet. Microbiol.* **21**: 21-30.

Gorden, F.B. and Quan, A.L. (1965). Occurrence of glycogen in inclusions of the psittocosis lymphogranuloma venereum trachoma agents. *J. Infect. Dis.* **115**: 186-196.

Grayston, J.T., Kuo, C.C., Campbell, L.A. and Wang, S.P. (1989*). Chlamydia pneumoniae* sp. nov. for Chlamydia sp. strain TWAR. *Int. J. Syst. Bacteriol.* 39: 88-90.

Griffiths, P.C., Philips, H.L., Dawson, M., Clarkson, M.J. (1992). Antigenic and morphological differentiation of placental and intestinal isolates of *Chlamydia psittaci* of ovine origin. *Vet. Microbiol.* **30**(2-3): 165-177.

Griffiths, P.C., Plater, J.M., Martin, T.C. Hughes, S.L., Hughes, K.J., Hewinson, R.G. and Dawson, M. (1995). Epizootic bovine abortion in a dairy herd: Characterization of a *Chlamydia psittaci* isolate and antibody response. *Br. Vet. J.* **151**:683.

*Halberstaedter and Von Prowazek, S. (1907). Uber Zelleinschlusse Parasitarer natur beim Trachom. *Arb. Gesundheitsamt Berlin.* **26**: 44-47. Cited by Storz, J. (1971). pp: 146-246

Hanna, L., Schachter, J. and Jawetz, E. (1974). Chlamydiae. In "Manual of Clinical Microbiology". Eds. Lennette, E.H., Spaulding, E.H. and Traunt, J.P. American Society for Microbiology, Washington, 2nd ed. pp. 795-803.

*Hellerstorm, S. and Wassen, E. (1930). Meningo-enzephalitische veranderungen bei Affennach intracerebraler Imptung mit Lymphogranuloma inguinale. Proc. Septieme Congres International de Dermatologie et de Syphiliegraphie, Copenhagen pp: 1147-1151. Cited by Vanrompay *et al.* (1994). pp: 1470-1474

Herring, A.J. (1993). Typing of *Chlamydia psittaci* – A review of methods and recent findings. *Br. Vet. J.* **149**: 455-475.

Herring, A.J., Tan, T.W., Baxter, S., Inglis, N.F. and Dunbar, S. (1989). Sequence analysis of the major outer membrane protein gene of an ovine abortion strain of *Chlamydia psittaci. FEMS Microbiol. Let.* **65**(1-2): 153-158.

Hewinson, R.G., Griffiths, P.C., Bevan, B.J., Kirwan, S.E.S, Field, M.E., Woodward, M.J. and Dawson, M. (1997). Detection of *Chlamydia psittaci* DNA in avian clinical samples by PCR. *Vet. Microbiol.* **54**: 155-166.

Holland, S.M., Gaydos, C.A. and Quinn, T.C. (1990). Detection and differentiation of *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneuomoniae* by DNA amplification. *J. Infect. Dis.* **162**(4): 984-987.

Hugal, A., Timms, P., Girjes, A.A. and Lavin, M.F. (1989). Conserved DNA sequences in Chlamydial plasmids. *Plasmid* **22**: 91-98.

Idtse, F.S. (1984). Chlamydia and Chlamydial disease of cattle: a review of literature. *Vet. Med.* **79**(4): 543-546.

*Johnson, F.W.A. (1984). Abortion-continuing flock problem: enteric infection in sheep associated with enzyootic abortion (*Chlamydia psittaci*). *Irish Vet. News* (December): 10-15. Cited by Anderson and Baxter (1986). pp: 453-454

Johnson, F.W.A., Clarkson, M.J. and Spencer, W.N. (1983). Direct isolation of the agent of enzootic abortion of ewes (*Chlamydia psittaci*) in cell cultures. *Vet. Rec.* **113**: 413-414.

Joseph, T., Nano, F.E., Garon, C.F. and Caldwell, H.D. (1986). Molecular characterization of *Chlamydia trachomatis* and *Chlamydia psittaci*; plasmids. *Infect. Immun.* **51**: 699-703.

Kaltenboeck, B. and Storz, J. (1992). Biological properties and genetic analysis of the omp A locus in chlamydiae isolated from swine. *Am. J. Vet. Res.* **53**(9): 1482-1487.

Kaltenboeck, B., Kousoulas, K.G. and Storz, J. (1991). Detection and strain differentiation of *Chlamydia psittaci* mediated by a two-step PCR. *J. Clin. Microbiol.* **29**(9): 1969-1975.

Kaltenboeck, B., Kousoulas K.G and Storz, J. (1992). Two-step polymerase chain reactions and restriction endonuclease analyses detect and differentiate omp A DNA of *Chlamydia* spp. *J. Clin. Microbiol.* **30**(5) 1098-1104.

Kaltenboeck, B., Kousoulas, K.G. and Storz, J. (1993). Structures of and allelic diversity and relationships among the MOMP (omp A) genes of the four chlamydial species. *J. Bacteriol.* **175**(2): 487-502.

Katoch, R.C. (1997). Epidemiology and immunodiagnosis of *Chlamydia pasittaci* infection in sheep and goats. Technical report submitted to Director, Far Eastern Regional Research Office, American Embassy and Indian Council of Agricultural Research, Krishi Bhavan, New Delhi. pp. 28-39

Krishna, L. (1984). Chicken embryo inoculation for the isolation of chlamydia. In Summer Institute on Neonatal Disease of Livestock. Vol.II. Indian Veterinary Research Institute, Izatnagar. pp. 304-308.

Krishna, L. and Rajya, B.S. (1985). Isolation of Chlamydia from cases of perinatal mortality in lambs and kids. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.* 6: 36-39.

Lee, C.K. and Moulder, J.W. (1981). Persistent infection of mouse fibroblast (Mc Coy cells) with a trachoma strain of *Chlamydia trachomatis*. *Infect. Immun.* **32**(2): 822-829.

*Lillie, R.D. (1930). Psittacosis-rickettsia like inclusions in man and in experimental animals. *Public Health Reports* **45**: 773-778. Cited by Page (1981). pp; 2210-2220

Longbottom, D., Russel, M., Donbar, S.M., Jones, G.E. and Herring, A.J. (1998). Molecular cloning and characterization of genes coding for the highly immunogenic cluster of 90 kelodalton envelope proteins from the *Chlamydia psittaci* subtype that causes abortion in sheep. *Infect. Immun.* **66**(4): 1317-1324.

Lovett, M., Kuo, C.C., Holmes, K. and Falkow, S. (1980). Plasmids of the genus Chlamydia. In "Current Chemothrapy and Infectious Diseases. (J.D. Nelson and C, Grassi, eds.. Washington, D.C. American Society for Microbiology. pp: 1250-1252

Lusher, M., Storey, C.C. and Richmond, S.J. (1989). Plasmid diversity within the genus Chlamydia. *J. Gen. Microbiol.* **135**: 1145-1151.

Mani, B.K. (2001). Isolation and characterization of *Chlamydia psittaci* with emphasis on protein profile. M.V.Sc. thesis, Kerala Agricultural University. pp: 33 – 104.

Markey, B.K., McNutty, M.S., Tood, D., Mackie, D.P. (1993). Comparison of ovine abortion and non abortion isolates of *Chlamydia psittaci* using inclusion morphology, Poly Acrylamide Gel Electrophoresis, Restriction Endonuclease Analysis and reactivity with monoclonal antibodies. *Vet. Microbiol.* **35**(1,2): 141-159.

Martin, J.L. and Gross, G.F. (1997). Comparison of the ompI gene of *Chlamydia psittaci* between isolates in Victorian Koalas and other animal species. *Aust. Vet. J.* **75**(8): 579-582.

McClenaghan, M., Herring, A.J. and Aitken, I.D. (1984). Comparison of *Chlamydia psittaci* isolates by DNA restriction endonuclease analysis. *Infect. Immun.* **45**(2): 384-389.

McClenaghan, M., HoneyCombe, J.R., Bevan, B.J., Herring, A.J. (1988). Distribution of plasmid sequences in avian and mammalian strains of *Chlamydia psittaci. J. Gen. Microbiol.* **134**: 559-565.

McClenaghan, M., Inglis, N.F. and Herring, A.J. (1991). Comparison of isolates of *Chlamydia psittaci* of ovine, avian and feline origin by analysis of polypeptide profiles from purified elementary bodies. *Vet. Microbiol.* **26**: 269-278.

*Meissler, M. and Krauss, K. (1986). Zurtechnik der Isolierung Und Zuchtung Von Chlamydieu in der Zellkultur Fortschr. *Vet. Med.* **30**: 224-230. Cited by Vanrompay *et al.* (1994). pp: 1470-1474

*Meyer, K.F. (1960). Characteristics of a Psittacosis viral agent isolated from a turkey. *Proc. Soc. Exp. Biol. Med.* **83**: 99. Cited by Vanrompay *et al.* (1994). pp: 1470-1474

*Moulder, J.W.. (1966). The relation of the psittacosis group (chlamydia) to bacteria and viruses. *Annu. Rev. Microbiol.* **20**: 107-130. Cited by Vanrompay *et al.* (1994). pp: 1470-1474

Murray, E.S. (1964). Guinea pig inclusion conjunctivitis virus. I. Isolation and identification as a member of the psittacosis – lymphogranuloma – trachoma group. *J. Infect. Dis.* **114**: 1-12.

Nagal, K.B., Katoch, R.C., Joshi, V.B., Batta, M.K. and Sharma, M. (1997). Infectivity of *Chlamydia psittaci* in Mc Coy cells. *Indian J. Anim. Sci.* 67(5): 402-408.

Page, L.A. (1966). Interspecies transfer of psittacosis-lymphogranuloma venerum trachoma agents: pathogenicity of two avian and two mammalian strains for eight species of birds and mammals. *Am. J. Vet. Res.* 27(1): 397-407.

*Page, L.A. (1971). Influence of temperature on the multiplication of Chlamydiae in chicken embryos. Excerpta Medica International Congress Series 223: 40-51. Cited by Page, L.A. (1981). pp : 2210-2220

Page, L.A. (1981). Obligatory intracellular bacteria. The genus chlamydia. In: "The prokaryotes", Vol. II (M.P. Starr, H. Stolp, H.G. Truper, A. Balows and H.G. Schlegel, Eds.). Springler-verlag, New York. pp. 2210-2220.

Page, L.A. (1984). Chlamydiosis: In "Isolation and identification of avian pathgoens". (M.S. Hofstad, H.J. Barnes, B.W. Calnek and W.M. Reid, Eds.) Arnold Printing Corporation, Ithaca, New York, 8th ed. pp. 118-131.

Perez-Martinez, A.J. and Storz, J.(1985). Antigenic diversity of *Chlamydia psittaci* of mammalian origin determined by micro immunofluorescence. *Infect. Immun.* **50**(3): 905-910.

Peterson, E.M. and DeLa Maza, L.M. (1983). Characterization of Chlamydial DNA by restriction endonuclease cleavage. *Infect. Immun.* **41**(2): 604-608.

Petterson, B., Anderson, A., Leither, T., Olsvik, O., Uhlen, M., Storey, C. and Black, C.M. (1997). Evolutionary relationship among members of the genus Chlamydia based on 16S ribosomal DNA analysis. *J. Bacteriol.* **179**(13): 4195-4205.

Philips, H.L.. and Clarkson, M.J. (1992). Culture of sheep Chlamydia in a sheep fibroblast cell culture. *Res. Vet. Sci.* **53**(2): 267-268.

Pickett, M.A., Everson, J.S. and Clarke, I.N. (1988). *Chlamydia psittaci* ewe abortion agent complete nucleotide sequence of the major outer membrane protein gene. *FEMS. Microbiol. Let.* **55**(2): 229-234.

Piraino, F. (1969). Plaque formation of chicken fibroblast cells by chlamydia isolated from avian and mammalian sources. *J. Bacteriol.* **98**: 475-480.

Pudjiatmoko, H., Fukushi, H., Ochiai, Y., Yamaguchi, T. and Hirai, K. (1997a). Phylogenetic analysis of the genus Chlamydiae based on 16S rRNA gene sequences. *Int. J. Syst. Bacteriol.* **47**(2): 425-431.

Pudjiatmoko, H., Fukushi, H., Ochiai, Y., Yamasuchi. T. and Hirai, K. (1997b). Diversity of feline *Chlamydia psittaci* revealed by Random Amplification of Polymorphic DNA. *Vet. Microbiol.* **54**(1): 73-83.

Puy, H., Fuentes, V., Eb, F. and Orfila, J. (1990). Immunological specificity of monoclonal antibodies to *Chlamydia psittaci* ovine abortion strain. *Immunol. Let.* **23**(3): 217-222.

*Rake, G., McKee, C.M. and Shaffer, M.F. (1940). Agent of lymphogranuloma venereum in the york sac of the developing chick embryo. *Proc. Soc. Exp. Biol. Med.* **43**: 332-335. Cited by Storz (1971). pp: 146-246

Rasmussen, S. and Timms, P. (1991). Detection of *Chlamydia psittaci* using DNA probes and the PCR. *FEMS Microbiol. Let.* 77(2-3): 169-173.

Rodolakis, A. and Souriau, A. (1989). Variations in the virulence of strains of *Chlamydia psittaci* for pregnant ewes. *Vet. Rec.* **125**(4): 87-90.

Rodolakis, A. and Souriau, A. (1992). Restriction endonuclease analysis of DNA from ruminant *Chlamydia psittaci* and its relation to mouse virulence. *Vet. Microbiol.* **31**: 263-271.

Rodolakis, A., Bernard, F., Souriau, A., Layachi, K. and Buzoni-Gatel (1989). Relationship between virulence of *Chlamydia psittaci* strains and establishment of persistent infections of Mc Coy Cells. *Vet. Microbiol.* **19**: 65-73.

Sayada, C., Anderson, A., Rodriguez, P., Eb, F., Milon, A., Elion, J. and Denamus, E. (1994). Homogenity of the major outer membrane protein gene of feline *Chlamydia psittaci. Res. Vet. Sci.* **56**(1): 116-118.

Schachter, J., Banks, J., Sugg, N., Sung, N., Storz, J. and Meyer, K.F. (1974). Serotyping of Chlamydia: Isolates of bovine origin. *Infect. Immun.* **11**: 904-907.

*Schachter, J., Dawson, C.R. (1978). Human Chlamydial infections, PSG Publishing Company, Littleton, M.A. Cited by Ward, M.E. and Ridgway, G. (1998). pp: 1331-1346

*Shatkin, A.A., Berkina, S.R., Pankrstova, V.N., Popov, V.L. and Zakharova, N.A. (1976). Indication of halprowia (Chlamydia) antigens by the direct immunoperoxidase method. *Zh. Mikrobiol. Epidemiol. Immunobiol.* (September) 9: 74-78.

Sheehy, N., Markey, B. and Quinn, P.J. (1997). Analysis of partial 16S rRNA nucleotide sequences of *Chlamydia pecorum* and *Chlamydia psittaci. FEMS Immun. Med. Microbiol.* **17**(4): 201-205.

Souriau, A., Le Rouzic, E., Bernard, F. and Rodolakis, A. (1993). Differentiation of abortion inducing and intestinal strains of *Chlamydia psittaci* isolated from ruminants by microimmuno-fluorescence test. *Vet. Rec.* **132**(9): 217-219.

Spears, P. and Storz, J. (1979). Biotyping of *Chlamydia psittaci* based on inclusion morphology and response to Diethyl Aminoethyl-Dextran and Cycloheximide. *Infect. Immun.* **24**(1): 224-232.

Sreeramalu, P., Krishnaswamy, S. and Roa, P.R. (1989). Isolation characterization and pathogenicity of *Chlamydia psittaci* from pneumonia in lambs. *Indian Vet. J.* **66**: 485-488.

Stamm, W.E., Tam, M., Koester, M. and Cles, L. (1983). Detection of *Chlamydia trachomatis* inclusions in Mc Coy cell cultures with fluorescein conjugated monoclonal antibodies. *J. Clin. Microbiol.* **17**: 666.

Storey, C., Lusher, M. and Richmond, S.J. (1992). Use of comparative MOMP gene sequence data for subdivision of *Chlamydia psittaci* species. In: Proceedings of the European Society for Chlamydia Research. eds. P.A. Mardh *et al.* Uppsala University Centre for STD Research, Sweden. Cited by Herring, A.J. (1993). pp: 455-475

Storz, J. (1971). Chlamydia induced diseases. In "Chlamydia and Chlamydia induced diseases". C.C. Thomas, Springfield, Illinois: pp. 146-246.

Storz, J. and Kaltenboeck, B. (1993). The chlamydiales. In "Rickettsial and chlamydial diseases of domestic animals". (Z. Woldeniwet and M. Ristic, Eds.) Pergamon Press, Oxford, First edn. pp. 27-64.

Storz, J. and Page, L.A. (1971). Taxonomy of the chlamydiae: reasons for classifying organisms of the genus Chlamydia, family Chlamydiaceae in a separate order, Chlamydiales Ord. Nov. *Int. J. Syst. Bacteriol.* **21**: 332-334.

Sulochana, S. (1994). Kerala Agricultural University. Personal Communication.

Sykes, J.E., Studdert, V.P., Anderson, G. and Brownery, G.F. (1997). Comparison of *Chlamydia psittaci* from cats with upper respiratory tract disease by PCR analysis of the omp A gene. *Vet. Rec.* **140**: 310-313.

Takahashi, T., Masuda, M., Tsuruno, T., Mori, T., Takashima, I., Hiramune, T. and Kikuchi, N. (1997). Phylogenetic analysis of *Chlamydia psittaci* straining from birds based on 16S rRNA gene sequence. *J. Clin. Microbiol.* **35**(11): 2908-14.

Takahashi, T., Takashima, I., Hashimoto, N. (1988). Immunotyping of *Chlamydia psittaci* by indirect immunofluorescence antibody test with monoclonal antibodies. *Microbiol. Immunol.* **32**(3): 251-263.

Tang, F.F., Chang, H.L., Huang, Y.T. and Wang, K. (1957). Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. *Clin. Med. J.* **75**: 429-447. Cited by Storz and Kaltenboeck (1993). pp : 27-64

Thomas, N.S., Lusher, M., Storey, C.C. and Clarke, I.N. (1997). Plasmid diversity in Chlamydia. *Microbiology* **143**: 1847-1854.

Timms, P., Eaves, F.W., Rodwell, B.J. and Lavin, M.F. (1988). Comparison of DNA spot hybridization cell culture and direct immunofluorescence staining for the diagnosis of avian Chlamydia. *Vet. Microbiol.* **18**: 15-25.

Vanrompay, D., Butaye, P., Sayada, C., Ducatelle, R. and Haesebrouck, F. (1997). Characterization of avian *Chlamydia psittaci* strains using omp I restriction mapping and serovar specific mono clonal antibodies. *Res. Immunol.* **148**(4): 327-333.

Vanrompay, D., Charlier, G., Ducatelle, R. and Haesebrouck, F. (1996). Ultrastructural changes in avian *Chlamydia psittaci* serovar A-, B-, C-, D- infected buffalo green monkey cells. *Infect Immun.* **64**(4): 1265-1271.

Vanrompay, D., Ducatelle, R. and Haesebrouck, F. (1992). Diagnosis of avian chlamydiosis: Specificity of the modified Gimenez staining on smears and comparison of the sensitivity of isolation in eggs and three different cell cultures. *J. Vet. Med. B.* **39**: 105-112.

Vanrompay, D., VanNerom, A., Ducatelle, R. and Haesebrouck, F. (1994). Evaluation of five immunoassays for detection of *Chlamydia psittaci* in cloacal and conjunctival specimens from turkeys. *J. Clin. Microbiol.* **32**: 1470-1474.

*Vitu, C. and Russo, P. (1984). Comparison de differentes Souches de *Chlamydia psittaci* par electrophorese en gel de polyacrylamide. *Les Colloques de l'INRA* number 28. Cited by McClenaghan *et al.* (1991).
PP : 269-278

Vretou, E., Loutrari, H., Mariani, L., Costelidou, K., Eliades, P., Conidou, G., Karamanou, S., Mangana, O., Siurkou, V. and Papadopoulos, O. (1996). Diversity among abortion strains of *Chlamydia psittaci* demonstrated by inclusion morphology, polypeptide profiles and monoclonal antibodies. *Vet. Microbiol.* **51**(3,4): 275-289.

Ward, M.E. and Ridgway, G. (1998). Chlamydia In "Topley and Wilson's Microbiology and Microbial Infections". Vol. 2 (L.H. Collier, A. Balwos and M. Sussman Eds.). Arnold Publishers, London, 9th Edition. Pp. 1331-1346.

*Ward, M.E. and Salari, H.S. (1980). Modulation of *Chlamydia trachomatis* infection of cyclic nucleotide and prostaglandins. *FEMS Microbiol. Let.* 7: 141-143. Cited by Dennis and Storz (1982). pp: 1897-1902

Weisburg, W.G., Hatch, T.P. and Woese, C.R. (1986). Eubacterial origin of Chlamydia. *J. Bacteriol.* **167**(2): 570-574.

Wills, J.M., Watson, G., Lusher, M., Nair, T.S. and Wood, D. (1990). Characterization of *Chlamydia psittaci* isolated from a horse. *Vet. Microbiol.* **24**: 11-19.

Yoshida, H., Kishi, Y., Shya, S., Hajiwara, J. (1998). Differentiation of Chlamydia species by combined use of polymerase chain reaction and restriction endonuclease analysis. *Microbiol. Immunol.* **42**(5): 411-414.

Zhang, Y.X., Morrison, S.G., Caldwell, H.D. and Baehr, W. (1989). Cloning and sequence analysis of the major outer membrane protein genes of two *Chlamydia psittaci* strains. *Infect. Immun.* **57**(5): 1621-1625.

* Originals not consulted

CHARACTERIZATION OF GENOMIC AND PLASMID DNA OF *Chlamydia psittaci* ISOLATES

By

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ABSTRACT OF A THESIS

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ABSTRACT

Four isolates of *Chlamydia psittaci* (M-28, M-430, M-121, P-156) obtained from ruminant abortion were subjected to restriction enzyme analysis and plasmid profiling to assess molecular level differences/homogenity among the isolates. The changes produced in developing chick embryo and the cytopathic effect in Mc Coy cell line by the isolates were also investigated in this study.

The *C. psittaci* isolates preserved in the Department of Microbiology were revived by passaging in embryonated chicken eggs through yolk sac route. Six to seven day-old developing chick embryo were used for propagation of the isolates. The isolates, M-28 and M-430 were found to produce death of the embryos within 10 days. The isolates M-121 and P-156 were not able to cause death within 10 days. The embryosappeared stunted with patchy haemorrhages all over the body and hyperemic legs. The yolk sac was thin walled, with deeply injected blood vessels and the yolk was thin and watery. M-28 and M-430 produced more severe lesions of the inoculated embryoscompared with the other isolates.

The isolates were grown in Mc Coy cell line. All isolates revealed marked CPE, characteristic of *C. psittaci*, with rounding and clumping of cells to form syncytia at 48 h PI and formation of intra cytoplasmic inclusion bodies

by 96 h PI. The isolates M-121 and P-156 took more time for the production of CPE compared with that of M-28 and M-430. The isolates were confirmed as *C. psittaci* by fluorescence antibody technique which revealed, apple green fluorescing chlamydial inclusions in the infected cell line by 96 h PI.

The CE as well as cell line propagated isolates were used as a source of elementary bodies for DNA extraction. The purified EBs were prepared by urografin density gradient centrifugation. After obtaining sufficient amount of EBs, it was subjected to DNA extraction. The isolates M-28, M-430, M-121 and P-156 had DNA concentrations of 1710 µg/ml, 1130 µg//ml, 1545 µg/ml and 1475 µg/ml respectively.

Restriction enzyme digestion of all the isolates were done separately with *Eco RI, Hae III* and *Bam HI.*

Eco RI cleaved DNA of the isolates M-430 and P-156 into nine fragments, M-28 into eight and M-121 into ten fragments respectively. The molecular size of bands for all isolates had variation in heavier fragments. Common bands were observed at five regions for all isolates.

Hae III cleaved the caprine isolates into eight fragments and the bovine isolate M-121 into seven and P-156 into nine fragments. Variation could be observed on the molecular size of the fragments.

Bam HI digested all isolates except M-121 into eight fragments. For M-121, seven fragments were obtained. Common bands were noticed at 0.2 and 0.7 kbp region.

All enzymes were found to be useful in the differentiation of *C. psittaci* isolates as these REs produced variation as well as similarity in the restriction fragment sizes.

Plasmid profiling revealed the absence of plasmids in all the four isolates screened.

Thus, a combination of phenotypic and genotypic methods could be used as epidemiological tools in the differentiation of *C. psittaci* strains of mammalian origin.