ISOLATION AND CHARACTERIZATION OF *Chlamydia psittaci* WITH EMPHASIS ON PROTEIN PROFILE

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

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MANNUTHY, THRISSUR - 680651
KERALA, INDIA

2001

DECLARATION

I hereby declare that the thesis entitled **"ISOLATION AND CHARACTERIZATION OF *Chlamydia psittaci* WITH EMPHASIS ON PROTEIN PROFILE"** is a bonafide record of research work done by me during the course of research and that this 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
20-02-2001

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CERTIFICATE

Certified that this thesis, entitled **"ISOLATION AND CHARACTERIZATION OF *Chlamydia psittaci* WITH EMPHASIS ON PROTEIN PROFILE"** is a record of research work done independently by **Mr. Binu K. Mani** 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 him.

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ACKNOWLEDGEMENTS

This research work was carried out under the able guidance and supervision of ***Dr. M. Mini****, Assistant Professor, Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy .It was indeed my great privilege to have been associated with her in this regard. When I began this project, I lacked patience and some of the finer qualities of a good research worker. However she helped in refining me from my original crude self. It is indeed hard to put down in words what ever she has done for me. I have respect and gratitude towards her.*

I am thankful to ***Dr. V. Jayaprakasan****, Associate Professor and Head, Dept. of Microbiology and also a member of advisory committee in giving me meticulous guidance during my work.*

This work could not have achieved present success but for the timely help rendered by ***Dr. G. Krishnan Nair****, Associate Professor, Dept. of Microbiology. I am extremely greatful to him for the same. My sincere thanks to him for all his efforts.*

Dr. M.R. Saseendranath*, Associate Professor and Head, Department of Epidemiology and Preventive Medicine, the other member of the advisory committee was extremely co-operative and I express my sincere gratitude to him.*

I am deeply indebted to ***Dr. S. Sulochana****, former Dean, College of Veterinary and Animal Sciences, Mannuthy for her valuable advice and unstinted help during the course of the work.*

I wish to express my profound sense of gratitude to ***Dr. K.T. Punnoose****, former Professor and Head, Dept. of Microbiology for his abiding interest and constructive criticism rendered through out the work.*

I am thankful to ***Dr. Koshy John****, Assistant Professor, Dept. of Microbiology, who was very considerative and took an active interest in my work.*

I acknowledge with gratitude the valuable help rendered by ***Dr. Lal Krishna****, ADG (AH), ICAR and* ***Dr. R.C. Katoch****, Professor and Head, Dept. of Microbiology, Veterinary College, Palampur, Himachal Pradesh.*

I wish to place my profound sense of gratitude ***Dr. P.P. Balakrishnan****, Professor and Head,* ***Dr. Athman****, Associate Professor and* ***Dr. Metilda Joseph****, Assistant*

Professor, Dept. of Animal Reproduction for their sincere co-operation and help extended to me during the work.

I am thankful to ***Dr. C.V. Sreeranjitkumar, Mrs. A.V. Vijayalakshmi*** *and* ***Mrs. T.K. Sreekalakumary,*** *Research Associates, in helping me through out this work.*

I thankfully acknowledge the timely help extended by ***Mr. Chandrasekharan,*** *Instrumentation Engineer, College of Veterinary and Animal Sciences, Mannuthy.*

The execution of this project would not have been possible but for ***Dr. Akilesh Ramachandran, Dr. Binu, T.V., Dr. Jaison George, Dr. Tressa Mary, Dr. Priya, P.M., Dr. Sreeja*** *and* ***Dr. Rajalakshmi*** *who extended a very sincere helping hand in connection with the collection of specimens during my project work. I am short of words to acknowledge this tremendous help.*

I would like to place on record all the help rendered by ***Mrs. Kumari, Miss. Lakshmi, Mrs. Lathika, Miss. Suja and Mrs. Chandramthi*** *during the course of execution of this project.*

Lastly but not the least, I profoundly appreciate my friends ***Dr. S. Anoop, Dr. P. Raveendran, Dr. Arun George, Dr. Nigil Mathew, Dr. A. Prasad, Dr. V. Dildeep, Dr. Vinu David, Dr. Shibu. K. Jacob, Dr. S. Harikumar, Dr. C.N. Dinesh, Dr. S. SooryaDas,*** *and* ***Dr. P. Mohan*** *for their remarkable co-operation and encouragement extended to me during this tenure. The cool shadow of our association has been very much rewarding.*

My sincere thanks to my beloved ***brother, mother*** *and* ***wife*** *for their abiding support during my project work.*

I acknowledge my sincere thanks to the ***Kerala Agricultural University,*** *Vellanikkara, Trissur for providing me the fellowship for the Post Graduate programme.*

Binu K. Mani

DEDICATED

TO MY

FATHER

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INTRODUCTION

1. INTRODUCTION

The success of livestock development programmes depend mainly on well organised animal health service which would ensure timely investigation of disease outbreaks, proper diagnosis and advocating suitable control measures. Though the economic losses due to infectious diseases have been brought down to a remarkable level, the importance of chlamydial disease is underlined by the frequently chronic and damaging nature of infection.

Chlamydiae have a wide distribution among warm and cold blooded animals. They are of medical interest because of the diverse diseases they cause in man and animals. Some of these diseases are of public health interest and others are of economic importance in farm animals. Chlamydiae are of microbiological interest because of their mode of interaction with eukaryotic host cells and their specialised life cycle with unique features of parasitism.

Chlamydia psittaci causes economically important diseases viz., pneumonitis, polyarthitis, enteritis,

encephalomyelitis, conjunctivitis and placentitis leading to abortion in domestic animals (Storz, 1971).

Research work carried out in India had proved the prevalence of chlamydial agents in the Northern States of India. In Kerala *Chlamydia psittaci* had been isolated from pneumonic lungs in goats, aborted foetus and semen samples from bovines.

Now a new classification has been introduced in the family Chlamydiaceae. This includes two genera Chlamydia and Chlamydophila. The organisms which cause abortion in ruminants have been characterised as *Chlamydophila abortus*.

Thirteen cases of abortion occurred in cows from November 1993 to March 1994 in the Livestock farm, Kerala Agricultural University, Mannuthy. Four of the aborted foetuses were subjected to detailed microbiological investigation. Three of them revealed chlamydia, both by direct microscopical examination and isolation in embryonated chicken eggs. Out of the three semen samples reported unfit for artificial insemination, one gave positive isolation for chlamydia (Sulochana, 1994).

There are reports of differentiation of mammalian isolates of *Chlamydia psittaci* by various methods. McClenaghan *et al.* (1991) compared *Chlamydia psittaci* isolates of diverse origin by analysis of protein from purified elementary bodies by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Markey *et al.* (1993) also used the study of proteins as one of the methods to compare the abortion and non-abortion causing isolates of *Chlamydia psittaci*.

The present study was designed to understand the prevalence and magnitude of chlamydia in incriminating abortions in livestock and also to characterise the chlamydial agents.

Previous research work and the clinical reports received in this laboratory showed ample evidences for the involvement of chlamydial agents in causing abortions in livestock of this state. The chlamydial isolateS from divergent origin are reported to be unique in morphological, biochemical, biological and serological characters. Later, workers have reported comparison of isolates based on genomic, protein and antigenic profile.

A knowledge about the protein profile will help to understand the variation if any, among different isolates. The studies in this line may help in developing a safe and potent vaccine suitable for this region.

Parameters included in this study are :

i. Isolation and identification of *Chlamydia psittaci* from livestock.

ii. Characterisation of the isolates with special emphasis on protein profile.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

A number of publications relevant to bovine and ovine chlamydial abortion have appeared in the last few years. With the notable exception of Australia and New Zealand, chlamydial abortion has been encountered in most sheep rearing areas of the world, particularly where flocks are closely herded.

2.1. Chlamydia and its taxonomic position

In the past, at least seven but, contradictory classifications were proposed by French, Russian and American taxonomists. Thus earlier literature contains references about genus Chlamydia as Rakeia, Miyagawanella, Bedsonia, Rickettsia, Ehrlichia or Chlamydozoon. Thus, the confusion of several names motivated many investigators to refer them as the agents of psittacosis- lymphogranuloma venereum-Trachoma group.

The scientific history leading to the recognition of chlamydia as a unique group of pathogenic bacteria is derived from the convergence of two independent paths of medical investigation. The first began in the pre-Christian era with the Egyptians describing it as

causing an ocular disease, which we know now as trachoma, followed three millennia later by the discovery of Chlamydozoa as named by Von Prowazek (1907).

The etiological agent of pneumonia in the owners of exotic psittacine birds was named as *Rickettsia psittaci* by Lillie (1930). The similarities between disease causing agents of this type of pneumonia, trachoma and many other related strains were recognised in 1945 (Jones *et al.*, 1945).

Because of the similarities in morphology, mode of reproduction and antigenically similar lipopolysaccharide moieties in the cell wall of the agents of trachoma and psittacosis, they have been assigned to one genus, Chlamydia for taxonomic purpose (Page, 1966 and 1974). Hence, the chlamydial isolates from any source were put into either of the two species *Chlamydia trachomatis* or *Chlamydia psittaci*.

Chlamydiae are classified as gram negative bacteria because they possess a number of properties that are highly characteristic to bacteria like division by binary fission, comparable cell wall, DNA

containing nucleoid with no nuclear membrane and the presence of ribosomes with antibiotic susceptibility, characteristic for prokaryotic ribosomes (Moulder, 1966).

Chlamydiae are classified as the single genus of the eubacterial order Chlamydiales and Family Chlamydiaceae (Page, 1966; Storz and Page, 1971).

The unique developmental cycle, the lack of muramic acid in the cell wall and absolute dependence of the host cell derived nutrients distinguish chlamydiae from other intracellular bacteria (Moulder *et al.* , 1984).

Based on DNA homology, Herring (1992) reported substantial intra-species homology with species other than *Chlamydia psittaci*. *Chlamydia psittaci* was extremely diverse in its host range and genome, with intra-species homology varying from 14 to 95 per cent.

The classification of the organism as *Chlamydia trachomatis* and *C. psittaci* was based mainly on the source, type of disease, iodine staining and sensitivity of sulphadiazine (Herring, 1993). The

research in this line led to the establishment of a third species *C. pneumoniae*.

Storz and Kaltenboeck (1993) reported the recognition of four species in the genus *Chlamydia*, namely *C. psittaci*, *C. pecorum*, *C. pneumoniae* and *C. trachomatis*. *C. trachomatis* was mainly a human pathogen, while *C. psittaci* and *C. pecorum* infected animals and *C. pneumoniae* was exclusively a human pathogen.

Eventhough *Chlamydia psittaci* stain best with Gimenez, Machiavello's, Castaneda and Giemsa stains, phenotypically it is indistinguishable from *Chlamydia pecorum* (Biberstein and Hirsh, 1999).

Everett *et al.* (1999) analysed the 16S and 23S rRNA in chlamydia and showed that Chlamydiaceae contained two monophyletic lineages and nine species level groups.

The above studies provided clear supporting evidence for nine different species in Chlamydiaceae and for the genetic differentiation of two new genera (Bush and Everett, 2000).

The order chlamydiales contains only one family Chlamydiaceae consisting of two genera, Chlamydia and Chlamydophila. The genus Chlamydia possesses three species- *C. trachomatis*- human pathogen; *C. suis* - isolates from swine and *C. muridarum* isolates from mice and hamsters. Six species were reported in the genus Chlamydophila viz., *Chlamydophila psittaci* - infecting birds; *Chlamydophila pneumoniae* - infecting human beings and koala; *Chlamydophila pecorum* - infecting cattle, sheep, goats, koalas and swine; *Chlamydophila felis* - endemic among house cats; *Chlamydophila caviae* recovered from guinea pigs and *Chlamydophila abortus* characterised as *Chlamydia psittaci* for many years. Genetic analysis indicated that *Chlamydophila abortus* had evolved from *Chlamydia psittaci* but was pathogenically different. *Chlamydophila abortus* colonized, the placenta and were primarily associated with cases of abortion and weak neonates (Everett, 2000).

2.2. Isolation of *Chlamydia psittaci*

Chlamydiae multiply in cells of the reticulo-endothelial system, in endothelial cells of the conjunctival, genital and intestinal tracts, in synoviocytes and in cells of the placenta and foetus.

This may result in any one or a combination of the following clinical manifestations like pneumonia, arthritis, polyserositis, diarrhoea, conjunctivitis and abortion/ still birth. Since abortion causes heavy economic loss in live stock industry, more emphasis has been given for isolation of the organism from cases of abortions. Literature supporting the isolation of *Chlamydia psittaci* from abortion will only be reviewed in this chapter.

Chlamydia psittaci was identified as the etiological agent of enzootic abortion in ewes and epizootic bovine abortion by Stamp *et al.* (1950) and Storz *et al.* (1960) respectively.

Ognyanov and Genchev (1970) could obtain nine isolates of *Chlamydia psittaci* from 24 samples of aborted foetuses and placentae.

Chlamydial isolation was made by Jain *et al.* (1976) in nine out of 14 samples of foetal tissues and placenta from aborted sheep.

Kurbanov *et al.* (1978) isolated *Chlamydia psittaci* from bovine abortion cases and they

reproduced the condition experimentally in heifers using their isolate.

Krishna and Mathur (1979) isolated *Chlamydia psittaci* from 40 per cent of the processed specimens from cases of abortions among sheep and goat in an organised farm in Himachal Pradesh.

Isolation of *Chlamydia psittaci* was done by Durand *et al.* (1980) in one herd out of the ten having history of abortions. The abortion rate was found to be four to ten percent in the various herds.

Wehner and Wehr (1980) from Germany isolated chlamydia from 33 of the 41 aborted foetuses referred to them for diagnosis.

Chlamydia psittaci could be isolated in 43.6 per cent of bovine abortion cases (Glavitis *et al,* 1982).

Sharma *et al.* (1983) isolated *Chlamydia psittaci* from cases of pneumonia and abortions in sheep. They identified the isolates based on the intra cytoplasmic nature of inclusions, presence of elementary bodies in

yolk sac smears, mouse inoculation and also by serology.

Purohit *et al.* (1986) achieved 6.66 per cent isolation from cases of abortion, still birth and premature birth in goats.

Khanna *et al.* (1987) reported isolation, identification and characterisation of *Chlamydia psittaci* from cases of sheep abortion. They could achieve two isolations from 36 specimens which were found to be negative for Brucella and mycoplasma.

Francis (1988) isolated two strains of *Chlamydia psittaci* from cases of bovine abortion, two from caprine pneumonic lungs and four from bovine semen samples. In this study a total of 71 clinical materials were screened.

Krishna (1990) reported an isolated case of abortion in a cow in Himachal Pradesh. *Chlamydia psittaci* was isolated from cotyledons by guinea pig inoculation through intra peritoneal route.

Wittenbrink (1991) processed 165 sheep foetuses obtained from abortion cases to isolate *Chlamydia psittaci*. He could get an isolation percentage of 36.4.

In cattle, chlamydial infection causes abortion in third trimester of pregnancy, while in ovines, towards the later stages of gestation (Blood *et al.*, 1994).

Of four abortions that occurred during November 1993 to March 1994 in Livestock farm, Kerala Agricultural University, Thrissur, three revealed Chlamydia, both by direct microscopic examinations and isolation in embryonated chicken eggs. One, out of the three semen samples which were found unfit for inseminating cows, gave Chlamydial isolate (Sulochana, 1994).

Griffiths *et al.* (1995) isolated chlamydial agent from the placental cotyledons of an aborted cow from a 100 -cow dairy herd in Cumbria.

Batta *et al.* (1996) carried out investigations on abortion outbreaks in nomadic sheep and goats of Himachal Pradesh. Out of the 26 flocks, 16 flocks

harboured *Chlamydia psittaci*. In two flocks there was mixed infection of *Chlamydia psittaci* and *Brucella melitensis*. *Chlamydia psittaci* could be isolated from six out of 26 samples processed from sheep, while 28 isolations of *Chlamydia psittaci* were possible from 71 vaginal samples from goats.

Two hundred and forty isolates of *Chlamydia psittaci* were obtained from 15 species of animals and birds. The overall isolation from different morbid materials was found to be 17.05 per cent (Katoch, 1997).

2.3. Cultivation of *Chlamydia psittaci*

Since Chlamydiae are energy dependent intracellular parasites, their isolation can be attempted only by using living systems like chicken embryo, small laboratory animals and cell cultures.

2.3.1. Isolation using chicken embryo

The most favourable system for cultivation and isolation of chlamydial agents is yolk sac of developing chicken embryo and this property was first identified by Rake *et al.* (1940).

Developing chicken embryos, inoculated into the yolk sacs, represented an important host for isolation and propagation of chlamydiae in the past and they still play a useful role (Tang *et al.* 1957; Storz, 1971).

Eggs inoculated with *Chlamydia psittaci* strain should be incubated at 37 to 39°C at 60 per cent humidity. The rate of multiplication of *Chlamydia psittaci* strains is enhanced at higher temperature (Page, 1971).

The identification of chlamydiae during isolation is a difficult task unless the clinical specimens contain a heavy concentration of organism. Most of the clinical specimens might require two to three passages in chick embryo before chlamydia could be identified (Idtse, 1984).

Page (1984) reported that the time of death of embryos inoculated with the chlamydiae was related to the number of organisms, their virulence and the temperature of incubation. Depending on these factors embryos died three to fourteen days post inoculation (PI). He suggested that the multiplication of

Chlamydiae in the lining cells of the yolk sac produced vascular congestion and haemorrhages in the membrane.

The chicken embryos that died due to chlamydial infection might have hyperaemia, cyanotic legs and the toes and might be deep red in colour with patchy haemorrhages in the skin (Collier, 1984; Francis, 1988).

Sreeramulu *et al.* (1989) attempted isolation of *Chlamydia psittaci* by yolk sac inoculation in six to seven day-old embryonated chicken eggs. During initial passages, yolk sac membranes were congested and oedematous. Embryos were highly haemorrhagic with cherry red haemorrhages on the whole surface of the embryo. From the fifth passage onwards the yolk sac was thin and yolk was watery. Regular mortality of chicken embryos by 72 h PI was observed from the sixth passage onwards, reaching even 100 per cent.

Six to seven day-old embryonated chicken eggs were used for inoculating the processed clinical specimen for the purpose of isolation of *Chlamydia psittaci*. The identification was based on the presence

of elementary bodies in yolk sac membrane smears, regular mortality pattern of inoculated eggs and confirmed by indirect micro-immuno fluorescence test (Sharma *et al.*, 1996; Batta *et al.*, 1997).

2.3.2. Isolation using cell cultures

Isolation of chlamydia is most easily accomplished in cell cultures although the chick embryo yolk sac remains as a reliable medium for primary isolation. Most investigators have individual preferences as to which cell lines and which conditions produce the best result.

Piraino (1969) reported the use of chicken embryo fibroblast primary cell culture for isolation of *Chlamydia psittaci*.

Robinson and Anderson (1979) found that sheep thyroid cell cultures were most successful for supporting the growth of chlamydia.

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

Johnson *et al.* (1983) found Mc Coy cell line more sensitive for isolation of *Chlamydia psittaci* from cases of abortion. However Tessler (1984) found vero cells more suitable than Mc Coy cells.

Certain methods enhance the infectivity of chlamydiae for cultured cells. Ionising radiation was one of the several methods used to prepare non-dividing cells for chlamydial cultures (Gordon *et al.*, 1972).

Cells treated with hydrocortisone enhanced the susceptibility to chlamydia (Bushell and Hobson, 1978). Centrifugation of Chlamydial inoculum onto the cell monolayer resulted in 1200 fold increase in infectivity of *Chlamydia psittaci* on mouse L cell mono layers (Allan and Pearce, 1979).

Cycloheximide treatment enhanced infectivity of *Chlamydia psittaci* for mouse L cells (Spears and Storz, 1979).

Cells treated with diethyl amino ethyl- dextran (DEAE-D) (Spears and Storz, 1979), cytochalasin B (Evans and Taylor-Robinson, 1979), cyclic nucleotides

and prostaglandins (Ward and Salari, 1980) and colchicine (Dennis and Storz, 1982) became more susceptible for *Chlamydia psittaci* and *Chlamydia trachomatis*.

The success of chlamydial culture depended on the type and age of the host cells used and on the quality control and expertise of the lab culturing the organism (Vanrompay *et al.*, 1991).

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

2.3.2.1. Growth characteristics of *Chlamydia psittaci* in cell cultures

Spears and Stroz (1979) suggested that inclusion morphology in mouse L cell lines can be used to biotype *Chlamydia psittaci*. Chlamydial strains that caused abortion and genital tract infection were grouped in biotype I. These strains grew more slowly, round or oval inclusions usually appearing around 30 h

post inoculation. The compact inclusions maintained entire margin as they enlarged to fill the host cell cytoplasm which lacked changes in shape and they were almost similar to inclusions produced by faecal isolates.

Page (1981) reported that in infected cell cultures, cytopathogenicity was in the form of rounding, swelling and detachment of infected cells after two to six days of incubation. Cytochalasin B treated cells had numerous multinucleated giant cells.

Johnson (1984) described a faecal isolate that produced diffuse inclusions with viable progeny by 32 h, compared with the 42 h taken by the placental isolates.

Anderson and Baxter (1986) could observe *Chlamydia psittaci* inclusions as large, compact and dark staining bodies seen only after 48 h of inoculation. These isolates were of faecal or abortion origin. In the case of other isolates of arthritis or conjunctivitis origin, diffuse and light staining inclusions were seen even at 24 h PI.

In Mc Coy cell cultures, abortion isolates of *Chlamydia psittaci* typically developed compact deeply methylene blue stained inclusions which were just visible after 41 h and reached maturity around 72 h PI. However, *Chlamydia psittaci* isolated from sheep intestine produced diffuse, less deeply staining inclusion which developed more rapidly in culture and matured within 48 h (Griffiths *et al.*, 1992).

Storz and Kaltenboeck (1993) obtained the best results of cell line infection after 40 h post inoculation by staining using Giemsa and Gimenez staining methods and counting intracytoplasmic inclusions.

Markey *et al.* (1993) utilized inclusion morphology based on direct immunofluorescence as one of the methods to distinguish ovine abortion and non abortion strains of *Chlamydia psittaci*. In this study, abortion strains produced compact, helmet shaped inclusions with a well defined margin in Mc Coy cells. In the case of faecal isolates inclusions were irregular, had lobes with poorly defined margins, and had a granular appearance.

2.3.3. Isolation using guinea pigs and pathogenicity

Rivers and Berry (1931) introduced the use of guinea pigs as an experimental animal in chlamydial research.

Before selecting these animals for isolation attempts, they should be examined for the presence of natural chlamydial infection. Many guinea pig colonies had natural chlamydial infection which manifested as conjunctivitis (Murray, 1964).

Guinea pigs were the most suitable laboratory animals used for isolation of bovine and ovine chlamydial strains because these animals were more prone to pick up infection even though the numbers of chlamydiae were lesser (Cottral, 1978).

Guinea pigs infected intraperitoneally developed spleenomegaly, hepatomegaly and a stringy fibrinous exudate in the peritoneal cavity. Preceding death, the affected animals showed signs of lethargy, anorexia, hyperthermia and in co-ordination. Guinea pigs inoculated with virulent strains from birds might die of severe meningitis within a few days after intraperitoneal inoculation (Page, 1981). He also

emphasised that certain strains of *Chlamydia psittaci* which were capable of producing death or lesion in mice might not produce infection in guinea pigs and vice versa.

Francis (1988) reported the use of guinea pigs for isolation of *Chlamydia psittaci*. Out of the eight isolates recovered by chick embryo inoculation techniques, six produced an acute infection resulting in death of the animal after 12 to 15 days PI. The remaining two produced latent infection and the impression smears from visceral organs revealed extremely few organisms and chlamydia could be re-isolated from one such condition.

2.3.4. Isolation using mice and virulence

Most chlamydial agents could infect mice aged three weeks after intranasal instillation and this method was useful for the propagation and isolation of Chlamydial agents (Storz, 1971). The time taken for death varied very much with the virulence of the chlamydial strains. Death might occur in three to fourteen days depending on the virulence, or they might survive and remain latently infected. Strains from human and avian sources affected mice after

intracerebral inoculation leading to death within four to seven days.

Page (1981) reported that *Chlamydia psittaci* from birds could be isolated and propagated in young laboratory mice by intracerebral, intranasal or intraperitoneal route. Typical clinical signs and gross lesions developed within five to fifteen days, depending on the number and virulence of the organisms. Highly virulent strains produced severe systemic infection and necropsy revealed hyperaemic lungs, enlarged spleen and liver and sticky exudate in the peritoneal and thoracic cavities. There was fibrin film covering the liver and spleen.

Anderson and Baxter (1986) reported that abortion causing isolates of *Chlamydia psittaci* provoked significant spleenic enlargement in mouse inoculated in foot pad, but this method was not satisfactory for testing the virulence.

Three *Chlamydia psittaci* isolates were found to be moderately virulent for mice after intraperitoneal inoculation of yolk sac material. In the animals, which died of acute infection the lesions detected at

necropsy included a stringy yellowish fibrinous exudate on the peritoneal surface, severe congestion of the visceral organs and extensive pneumonia (Francis, 1988).

Rodolakis *et al.* (1989) evaluated the virulence of *Chlamydia psittaci* isolated from ruminants in mouse model which permitted graded differentiation between virulent and non-virulent strains.

Rodolakis and Souriau (1992) reported that, mouse invasive strain of *Chlamydia psittaci* from clinical cases was most often isolated in mice following subcutaneous inoculation. This did not occur with non-invasive strains, mostly of intestinal origin.

Ward and Ridgway (1998) stated that mouse could be infected by the intranasal, intraperitoneal and intracerebral routes and via the genital tract. Intravenous inoculation resulted in rapid death (in three to four hours) or death within days from infection.

2.4. Confirmation of Chlamydial isolates

2.4.1 Staining of elementary bodies

Demonstration of large numbers of small coccoid elementary bodies in stained smears of affected areas provide a basis for firm diagnosis. Suitable staining procedures included Giemsa, Macchiavello, Gimenez and Modified Ziehl Neelsen methods (Johnson, 1986; Francis, 1988; Nanda *et al.*, 1992 and Aitken, 1993).

Vanrompay *et al.* (1995) suggested the usefulness of other stains like acridine orange, castaneda and methylene blue.

2.4.2. Sulphadiazine sensitivity

To establish the differential identity of Chlamydiae at species level, test of sensitivity to sodium sulphadiazine was one most important criterion (Storz, 1971; Page, 1981; Francis, 1988; Sreeramulu *et al.*, 1989 and Herring, 1993).

2.4.3. Iodine staining

Iodine staining carbohydrate (glycogen) accumulated in inclusions of *C. trachomatis* but not in those of *Chlamydia psittaci* (Gordon and Quan, 1965; Becker, 1978; Spears and Storz, 1979; Ward, 1983;

Sreeramulu *et al.*, 1989; Herring, 1993; Storz and Kaltenboeck, 1993).

2.4.4 Use of serology for identification of *Chlamydia psittaci*

Serology is useful for identifying new isolates of chlamydia.

Fluorescence - conjugated antiserum preparation had been used to confirm the identity of *Chlamydia psittaci* by various investigators (Eugster *et al.*, 1970; Ardrey *et al.*, 1972; Stamm *et al.*, 1983).

Shatkin *et al.*(1976) used a modified immunoperoxidase method for the identification of *Chlamydia psittaci* in infected mouse L cells.

Tessler and Page (1977) developed a direct fluorescent antibody test on air sac exudates from *Chlamydia psittaci* infected birds.

All members of the genus Chlamydia shared a common heat stable complement fixing antigen (Allen, 1986). This antigenic fraction could be extracted from elementary bodies with ethyl ether, sodium

deoxycholate and sodium dodecyl sulphate. This was made use of for the conduct of complement fixation test (CFT) to elucidate chlamydia.

Malkinson *et al.* (1987) used the enzyme linked immunosorbent assay (ELISA) for identification of chlamydial antigen in broiler breeder stock, along with FAT.

Palmer *et al.* (1988) demonstrated *Chlamydia psittaci* antigen in smears and paraffin tissue sections using fluorescein isothiocyanate labelled monoclonal antibodies.

Kunz *et al.* (1991) compared the use of commercially available monoclonal antibodies of murine origin with vitelline immuno globulins (IgV) to detect the chlamydiae in formalin fixed tissue sections.

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

Katoch (1997) performed agar gel precipitation test to study seroprevalence of chlamydiosis in animals by preparing agar gel precipitating antigen from infected yolk sac. The sensitivity of this test was found to be 37.24 per cent.

A modified ELISA test to detect the chlamydial antigen in cloacal swabs to study the prevalence of *Chlamydia psittaci* in Italian turkey was performed by Fezia *et al.* (1999).

Yuejin *et al.* (1999) typed chlamydial strains, which were isolated in France from different species of birds, by indirect immunofluorescence test using a panel of nine monoclonal antibodies.

2.5. Characterisation of proteins of *Chlamydia psittaci*

The dominant antigen at the surface of the infectious chlamydial elementary body is the Major Outer Membrane Protein (MOMP), encoded by *ompl* gene. This antigen of approximately 40 kDa is the basis for serological classification of chlamydia.

Caldwell *et al.* (1981) when purified *Chlamydia trachomatis* from HeLa cell monolayer harvest using renografin gradients of 40, 44 and 52 per cent, elementary bodies' band was found to be located in between 44 and 52 per cent renografin interface. Thus purified elementary body proteins were electrophoresed on 12.5 per cent acrylamide slab gel. The results showed that a sharing of sub-unit molecular weight of 105 kDa, 68 kDa, 57 kDa, 52 kDa and 45 kDa was seen.

McClenaghan *et al.* (1984) purified elementary body from infected cell cultures by gradient centrifugation in renografin -76 gradients (30 to 60 per cent). This purified elementary bodies were used for polyacrylamide gel electrophoresis (PAGE).

Vitu and Russo (1984) reported that there was no substantial difference between the polypeptide profiles of *Chlamydia psittaci* isolates of ovine, caprine, bovine and canine origin. They could observe a minor quantitative change in the caprine strain at 88 kDa.

Buzoni- Gatel *et al.* (1989) compared the protein pattern between invasive and non-invasive ovine

strains of *Chlamydia psittaci*. Polypeptide pattern revealed three important differences between invasive and non-invasive strains, major differences being the presence of 96, 90 and 78 kDa polypeptide bands in invasive strains. They reported that the 90 kDa band was so distinct that it could be regarded as a virulence marker for invasive strain.

McClenaghan *et al.* (1991) studied the polypeptide profile obtained by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified elementary bodies from eight isolates of *Chlamydia psittaci*. Four profile types were evident. The three ovine abortion isolates showed very similar profiles although a minor qualitative difference was found in a band migrating at about 90 kDa in one of the isolates.

Griffiths *et al.* (1992) detailed the antigenic differentiation of placental and intestinal isolates of *Chlamydia psittaci* of ovine origin. At least forty components of each chlamydial isolate were detected by silver staining. Each isolate possessed densely staining major bands at 40 kDa, 49 kDa and 50 kDa in

all the abortion causing isolates which were not detected in the intestinal isolates.

Markey *et al.* (1993) compared abortion and non-abortion causing isolates of *Chlamydia psittaci* using various methodology including PAGE of elementary bodies. They reported more than 50 polypeptides with similar numbers and size distribution of bands on silver staining. The profiles of the four isolates from abortion cases were very similar to each other and readily distinguishable from those of the other isolates. Each of the abortion causing isolate profile exhibited a 23 to 24 kDa doublet, 57 to 60 kDa doublet and 97 to 100 kDa triplet.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Analytical grade chemicals procured from Sisco Research laboratory (SRL), Bombay were used, where ever the source is not mentioned. Glass wares used were of Borosil brand.

3.1. Isolation of *Chlamydia psittaci*

3.1.1. Specimen collection

3.1.1.1. Materials

Specimens for the isolation were collected from reported cases of abortion in livestock brought to the Veterinary Hospitals in and around Thrissur and from Kerala Agricultural University Farms. They included the liver, spleen, lungs and stomach contents of aborted foetuses/ still born young ones, cervical mucus and uterine discharge (within 20 days of abortion) and placenta. Similarly fresh semen samples showing poor motility and sperm abnormality which were found unfit for insemination in the Artificial Insemination Centre, Veterinary College Hospital, Mannuthy, were also collected.

3.1.1.2. Method

Specimens were collected observing aseptic techniques. While collecting the visceral organs, special care was taken to include both the areas of active lesion and normal tissue. Pipette method and cervical swab method were used to collect uterine discharges and cervical samples respectively.

3.1.2. Transportation of clinical specimens

3.1.2.1. Materials

Sucrose Phosphate Glutamate (SPG) buffer with antibiotics (pH- 7.2)

Sucrose (0.218 M)	-	74.62 g
Potassium dihydrogen phosphate (0.0038M)	-	0.52 g
Dipotassium hydrogen phosphate (0.0072M)	-	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

3.1.2.2. Method

The clinical materials were preserved in chlamydial transport medium (SPG) and brought to the laboratory immediately. If it was not possible to transport the specimens to the laboratory within a period of one hour, they were transported over ice in thermocole box.

3.1.3. Staining of smears from clinical specimens

3.1.3.1. Giemsa staining

3.1.3.1.1. Materials

i. Impression smears from clinical material

ii. Giemsa's stain: Prepared by dissolving one gram of stain powder in 66 ml of glycerol and keeping at 55°C overnight. Then 66 ml of absolute alcohol was added. Dilute Giemsa's stain was prepared by diluting the above prepared stain 20 times in distilled water.

3.1.3.1.2. Method

Impression smears prepared from clinical materials were stained by the slow method of staining advocated by Cruickshank *et al.* (1975). The stained smears were washed, dried and then examined under oil immersion objective of the microscope.

3.1.3.2. Modified Ziehl Neelsen Staining

3.1.3.2.1. Materials

i. Smears from clinical specimens

ii. Dilute carbol fuchsin

iii. Acetic acid (0.5 per cent)

iv. Loeffler's alkaline methylene blue

3.1.3.2.2. Method

The smears were fixed by heat over flame and stained with 1:10 dilution of carbol fuchsin for 10 min and then washed in running tap water. Decolourisation was done with freshly prepared 0.5 per cent acetic acid for two to five seconds. The smears were immediately washed again in running water and were counter stained with Loeffler's alkaline methylene blue for one to two minutes. They were washed again in running tap water, dried and examined under oil immersion objective.

3.1.3.3. Gimenez staining

3.1.3.3.1. Materials

i. Smears

ii. Phosphate buffered saline (PBS)-0.01M pH- 7.5

Sodium dihydrogen phosphate (NaH_2PO_4. $2H_2O$) -0.15g

Disodium hydrogen ortho phosphate -anhydrous (Na_2HPO_4)-1.27 g

Sodium chloride (NaCl) -8.00 g

Triple distilled water -1000 ml

iii. Primary stain

Stock solution:

Solution I Basic fuchsin -10 g

Ethanol (95 per cent) -100 ml

Solution II Phenol crystals -10g

Triple distilled water -250 ml

Solutions I and II were prepared separately. Before use these solutions were mixed slowly and thoroughly. Then added 650 ml triple distilled water, mixed and stored in amber coloured bottle. The solution was incubated at 37°C for 48h.

Working solution:

Stock solution	-	1 part
Phosphate buffered saline (PBS) [0.01 M, 7.5 pH]	-	2 parts

iv. Counter stain

Malachite green	-	800 mg
Triple distilled water	-	100 ml

3.1.3.3.2. Method

The smears were fixed by heat over flame. Flooded the slides with filtered working solution of primary stain for 10 to 15 min and washed with water. Counter stained with malachite green for two minutes. Washed the slide with water, dried and examined under oil immersion objective.

3.1.4. Storage of specimen for isolation of *Chlamydia psittaci*

3.1.4.1. Materials

i. Clinical specimens

ii. SPG

3.1.4.2. Method

Tissue specimens (3.1.1.1) brought to the laboratory were washed two to three times in SPG

buffer containing antibiotics. Large pieces of tissue were cut into smaller ones and kept in sterile vials with SPG supplemented with antibiotics and stored at 4°C. When the tissues could not be processed within 24h, they were preserved at −20°C in SPG.

Uterine discharges and other fluid specimen transported to the laboratory in SPG buffer were either immediately processed or stored at −20°C.

3.1.5. Processing of samples

3.1.5.1. Materials

i. Tissue pieces in SPG

ii. Fluid specimens

iii. Sterile silica gel

3.1.5.2. Method

Tissue pieces were again cut into smaller bits. The materials were homogenised with sterile silica gel in a sterile mortar and pestle in SPG containing antibiotics to obtain 20 per cent (w/v) suspension. The tissue homogenate was transferred to properly labelled test tubes and kept at 4°C for 20 to 30 min to facilitate settling of coarse particles and silica gel. The supernatant was then centrifuged in three

steps at 600 x g, 1100 x g and 1700 x g for 30 min each. In the first two steps, the supernatant collected were stored for six to seven hours at 4°C. The final supernatant after storing at 4°C for overnight was tested for sterility by inoculation onto blood agar plates. After overnight incubation of the inoculated plates at 37°C, they were examined for bacterial contamination. The emulsion established to be free of bacterial contamination was used for inoculation into embryonated chicken eggs.

The cervical and uterine discharges collected by swab and pipette method respectively were diluted with equal amount of SPG containing antibiotics, incubated at 37°C for 30 min and centrifuged at 1000 x g for 15 min. The supernatant was collected. After ruling out bacterial contamination, it was used for inoculation in chick embryo (CE).

3.1.6. Chick embryo inoculation

3.1.6.1. Materials

i. Processed samples

ii. Six to seven day-old embryonated eggs.

Hatching eggs from hens fed on antibiotic free ration were procured from University Poultry Farm,

Mannuthy and were pre-incubated for six to seven days in a humid atmosphere at 37°C.

3.1.6.2. Method

The embryonated eggs were candled to check the viability. Viable and healthy eggs were selected for inoculation. The sample emulsion was kept outside the refrigerator to bring it to the room temperature. For embryo inoculation 0.25 ml of the emulsion was used by the yolk sac (YS) route (Storz, 1971). Four embryos were used for each sample and incubated at 37°C.

The inoculated eggs were candled daily. Death of embryos within the first 48h was considered non-specific and were discarded. Embryos died on subsequent days were placed in a refrigerator overnight. The inoculated embryos found viable even after 12 days were killed by placing them in refrigerator overnight. Harvesting was done on the next day.

3.1.7. Harvesting of inoculated embryonated eggs

3.1.7.1. Materials

i. Inoculated CE after incubation

ii. Phosphate buffered saline(PBS)(0.15M, pH 7.2)

Sodium chloride	-	8 g
Potassium chloride	-	0.20 g
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	-	0.02 g
Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)	-	1.15 g
Triple distilled water	-	1000ml

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

3.1.7.2. Method

The shell over air cell region was disinfected with 70 per cent ethyl alcohol. Then that area was cut and removed with sterile scissors and forceps. Subsequently the shell membrane and the chorio-allantoic membrane in the region of air cell were also removed with a pair of sterile forceps, the inner contents were emptied into a sterile Petridish. The YS and embryo were washed in sterile PBS (pH- 7.2). Small pieces of YS blotted dry on a blotting paper were used to prepare impression smear on clean grease free slides.

Hemorrhagic lesions on YS and over embryo were considered as typical for chlamydial infection.

The YS so obtained was then suspended in SPG with antibiotics, stored in sterile vials, labelled and kept at -20°C until further use.

The YS smear were stained by Giemsa, Modified Ziehl Neelsen and Gimenez stains for detecting chlamydial elementray body (EB) or inclusion. Even if the YS smear failed to reveal typical chlamydial bodies, the YS materials were subjected to three serial blind passages before they were discarded as negative. For repeated passaging, the YS were processed as mentioned earlier in the method of processing of tissue specimens.

Based on the average number of chlamydial elementary bodies present on stained YS smears, a grading of infected YS was performed. 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.

The infected YS material (*Chlamydia psittaci* isolate) obtained from the Department of Microbiology, Veterinary College, Palampur, Himachal Pradesh was

also passaged three times in CE and stored at -20°C for further studies.

The infected YS material obtained from the Department of Microbiology, Veterinary College, Palampur was considered as reference isolate.

3.2. Identification of isolates

The following tests were done to confirm the identity of the suspected *Chlamydial* isolates, along with reference isolate.

3.2.1. Sensitivity for sodium sulphadiazine

3.2.1.1. Materials

i. Isolates

ii. Seven-day-old embryonated eggs

iii. Sodium sulphadiazine (Hi-Media, Pvt. Ltd, Bombay)

iv. PBS (0.15 M, pH 7.2)

3.2.1.2. Method

Yolk sac (3+) obtained by inoculating the suspected and reference isolates was made into a 20 per cent suspension in PBS (pH-7.2) treated with one milligram of sodium sulphadiazine. The suspension was

kept at 4°C for 24h. The supernatant was used as inoculum. The test was done using two sets of CE, one of which was inoculated with the isolate treated with sulphadiazine and the other without treatment with sulphadiazine (control).

3.2.2. Pathogenicity in mice

3.2.2.1. Materials

i. *Chlamydia psittaci* isolates: reference and two local isolates

ii. BALB/C mice aged three to four weeks obtained from Small Animal Breeding Station, Mannuthy were utilized for the study.

3.2.2.2. Method

Six mice were inoculated intra peritoneally with 0.2 ml each of the isolate (20 per cent of YS (3+) suspension). This was prepared out of the YS of CE after third passage in SPG. Along with them two mice were kept each inoculated intraperitoneally with 0.2 ml SPG.

The animals were fed *ad lib* and clean drinking water was provided.

The inoculated animals were observed daily for the development of clinical signs or death. The dead animals were necropsied and examined for any gross lesions. Representative samples from organs viz., lungs, liver, spleen, and peritoneal exudate were collected. Impression smears of affected areas of these organs and exudate were prepared for staining by Giemsa, modified Ziehl Neelsen and Gimenez methods. The animals which remained apparently healthy after three weeks following inoculation were sacrificed. The pathological changes were noted and lungs, liver, spleen and peritoneal exudate were collected for demonstration of chlamydiae by staining impression smears and also for re-isolation.

3.2.3. Pathogenicity in guinea pigs

3.2.3.1. Materials

i. Isolates

ii. Guinea pigs (NIH- coloured) - healthy sero negative guinea pigs aged around four to five weeks and weighing one kilogram each, were procured from Small Animal Breeding Station, Mannuthy.

3.2.3.2. Method

Two guinea pigs each were inoculated intraperitoneally with three milliliters of YS (3+) material suspension of each of the isolate in PBS (pH- 7.2). Along with them, a guinea pig inoculated with three millilitre of PBS alone, was kept as control. The animals were housed separately and fed with commercially available ration. Feed and water were given *ad lib*. The guinea pigs were observed daily for the development of clinical signs or death. Once death of the animal had occurred, postmortem (PM) examination was performed immediately to note the gross pathological changes and to collect tissue specimens and exudates. The impression smears of peritoneum and visceral organs were prepared and stained to demonstrate chlamydial bodies. The tissues were also collected for processing to re-isolate the agent.

The animals that remained apparently healthy even after three weeks following inoculation were sacrificed. Postmortem examination was carried out and gross lesions on the organs viz., lung, liver and spleen were noted. Impression smears were prepared

from organs revealing lesions and looked for chlamydial bodies after staining. These organs and peritoneal exudate were also collected for re-isolation of the agent.

3.3. Confirmation of identity of the isolate by Agar Gel Precipitation Test (AGPT)

3.3.1. Antigen preparation

3.3.1.1. Materials

i. Standard *Chlamydia psittaci* isolate, local isolates and uninfected YS

ii. SPG

iii. Trypsin (Difco) 1:250

iv. Phosphate buffered saline (PBS) (0.2 M, pH 8.2)

Dipotassium hydrogen orthophosphate anhydrous (K_2HPO_4)	-	1.16 g
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	-	0.91 g
Sodium chloride	-	8.5 g
Distilled water	-	1000ml

v. PBS (pH 7.2)(3.1.7.1.)

vi. Celite (Hi-Media, Bombay)

vii. One per cent sodium deoxycholate solution in Normal Saline

Sodium deoxycholate	-	1 g
Sodium chloride	-	0.85 g
Triple distilled water	-	100 ml

3.3.1.2. Method

Step I. Heavily infected YS (3+) membranes (100 numbers) from each of the isolates were added to 300 ml of SPG and homogenised with sterile silica gel (3.1.5.1).

Step II. One per cent trypsin solution in PBS (0.2M, pH- 8.2) was added at a ratio of 1.7 : 1 (trypsin solution : gram of YS membrane). It was then incubated at 37°C for 60 min with constant stirring.

Step III. Centrifuged the above suspension at 12000 x g for 60 min at 4°C. Middle layer and fat layer seen above were discarded. Sediment was resuspended in 50 ml PBS (pH- 7.2). Added 20 g celite to it. Centrifuged at 500 x g for five minutes at 4°C. Sediment and fat were discarded. Five gram of celite was added to the supernatant and centrifuged at 500 x g for five minutes at

4°C.Again sediment and fat were discarded and the supernatant was centrifuged at 12000 x g for 60 min at 4°C. The supernatant thus obtained was discarded.

Step IV. The sediment thus obtained in the previous step was resuspended in 10 ml of one per cent sodium deoxycholate solution in saline. Incubated at 45°C for four hours. Centrifuged at 12000 x g for 30 min at 4°C. The supernatant was collected and labelled as AGPT antigen.

Similarly YS antigen was prepared from uninfected YS membrane (25 numbers) following the above procedure.

3.3.2. Preparation of antiserum

3.3.2.1. Materials

i. Antigen prepared from the reference isolate.

ii. Freund's complete adjuvant (Sigma)

iii. Rabbits (New Zealand white). Two rabbits of six months of age, each weighing approximately 1.5 kg obtained from Small Animal Breeding Station, Mannuthy were utilised for antiserum preparation. The animals were fed with

commercially available feed. Water was given *ad lib.*

3.3.2.2. Method

Antiserum was prepared in rabbits by subcutaneous inoculation with one millilitre of antigen mixed with equal quantity of Freund's complete adjuvant. A second dose was given 10 days later without adjuvant. Fifteen days following the second injection, the animals were bled, serum was separated and inactivated at 56°C for 30 min. This was adsorbed with normal YS emulsion. Equal quantities of antiserum and uninfected YS emulsion were mixed and incubated at room temperature for four hours with repeated shaking, followed by incubation at 4°C for 12 h. After which, it was centrifuged at 1100 x g for 15 min to remove the precipitate containing cross reacting antibody-antigen complexes.

3.3.3. Test proper

3.3.3.1. Materials

i. Antigens

ii. Antiserum from rabbit

iii. Normal saline

iv. Agarose (Sigma)

v. Microscopic slide pre-coated with 0.5 per cent agarose in distilled water.

3.3.3.2. Method

Agarose (0.7 per cent) was prepared in Normal saline. Three milliliters of melted agarose was poured on to pre-coated slide. After the agarose was set, five wells of four-millimeter diameter each were cut in a pattern in such a way that the central well formed was equidistant from the surrounding four wells and the wells were about four millimeters apart. The central well was charged with antiserum against the antigen of reference isolate. Peripheral wells were charged with the antigen prepared from reference and local isolates and non-infected YS antigen. Slide was incubated in a humid chamber at 37°C and the results were read after 48h.

3.3.4. Staining of the gel

3.3.4.1. Materials

i. Agarose gel

ii. Normal saline

iii. Triple distilled water

iv. Filter paper

v. Amidoblack stain :

Amidoblack 10B (SRL, Bombay)	-	0.1 g
Sodium chloride	-	0.9 g
Distilled water	-	100 ml

vi. Decolouriser I.

Methanol	-	120 ml
Acetic acid	-	30 ml
Distilled water	-	30 ml

vii. Decolouriser II

Absolute alcohol	-	140 ml
Acetic acid	-	20 ml
Distilled water	-	40 ml

3.3.4.2. Method

After incubation in moist chamber at 37°C for 48 h, the slide with gel was placed in normal saline for 48h (two changes in normal saline) and finally four hours in triple distilled water. Then a moist filter paper was placed above in such a way that no air bubble was trapped in between and dried at room temperature. The side was stained with Amidoblack stain for 15 min, decolourised with decolourising solution I and II for 20 min each and examined.

3.4. Protein profile analysis

3.4.1. Maintenance of cell line

3.4.1.1. Materials

i. Mc Coy cell line obtained form National Centre for Cell Sciences (NCCS), Pune.

ii. Eagle's Minimum essential medium (MEM) without non-essential amino acids. Ready-made medium purchased from Hi-Media, Bombay was reconstituted as per manufacturer'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. Foetal bovine serum (PAA Laboratories, Austria)

v. 0.25 per cent trypsin (1:250) (Difco), in Calcium Magnesium Free (CMF) PBS and sterilised by filtration -

Trypsin (1:250)	-	0.5 g
Sodium chloride	-	1.6 g
Potassium chloride	-	0.04 g
Disodium hydrogen phosphate (Na_2HPO_4- $7H_2O$)	-	0.42 g
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	-	0.04 g

Triple distilled water - 200 ml

vi. Cell culture growth medium : Eagle's Minimum essential medium (MEM) without non-essential amino acids containing 10 per cent foetal bovine serum was prepared and pH was adjusted to 7.2 with 7.5 per cent sodium bicarbonate.

vii. Cell culture maintenance medium : same as growth medium except that the foetal bovine serum concentration was reduced to five per cent

3.4.1.2. Method

Mc Coy cell line obtained from NCCS, Pune was incubated at 37°C for 24h to complete the monolayer. Then to the monolayer was added two milliliters of 0.25 per cent trypsin solution with gentle shaking, kept for two minutes at room temperature and decanted. The monolayer was kept at room temperature for five minutes with maintenance medium. The cells were detached from the surface by mechanical disruption using a sterile pipette attached with bulb. A split ratio of 1:3 to 1:4 was employed by seeding into tissue culture bottles. The cells were also seeded into test tubes containing cover slips. The tissue

culture bottles and tubes were enriched with growth medium at the rate of 10 ml and 2 ml respectively.

The bottles and tubes were incubated at 37°C and observed daily for formation of monolayer in the bottle surface and cover slip. When a suitable monolayer of cells was obtained, they were used for further infection with isolates.

3.4.2. Propagation of Chlamydial isolates in Mc Coy cell line (infection of the cell line).

3.4.2.1. Materials

i. Monolayer bottles and coverslip cultures of Mc Coy cells
ii. Reference and local isolates
iii. SPG
iii. Maintenance medium
iv. Sterile glass beads

3.4.2.2. Method

Twenty per cent suspension of heavily infected YS in SPG was centrifuged at 1000 x g for 15 min. The sediment and the fat layers were discarded and the middle layer was used as inoculum. The monolayer in the bottle was inoculated with three milliliters of

inoculum and was allowed to adsorb for one hour at 37°C. Then the inoculum was poured off and washed the monolayer with maintenance medium. The maintenance medium was added at the rate of 10 ml per bottle. Control bottles were prepared simultaneously in which instead of inoculum, three millilitres of maintenance medium was added. All the bottles were incubated at 37°C and were examined at 24h intervals, under an inverted microscope, for a period of 72 h PI. The cells were detached from the surface by the use of sterile glass beads, when the monolayer was sufficiently infected and produced cytopathic changes 72 h post inoculation (PI).

For infecting cover slip culture, 0.2 milliliters of inoculum and two millilitres of maintenance medium were used. In control tubes instead of isolates, 0.2 ml of SPG was inoculated. Rest of the procedures were the same as for the bottle culture. At 24 h intervals infected coverslip cultures were stained using May-Grunwald Giemsa stain to appreciate the cytopathic effect produced by isolates in the monolayers.

The unifected coverslip cultures were also stained and studied in detail.

3.4.3. Staining of cover slip cultures

3.4.3.1. Materials

i. Coverslip culture

ii. May- Grunwald Giemsa stain:- prepared by dissolving 2.5 g of May- Grunwald stain powder in 100 ml of absolute methanol and allowing to age for one month. Giemsa stain prepared as in 3.1.3.1.1 (ii) was diluted 10 times in distilled water.

iii. Methanol

iv. Xylene

v. Acetone

3.4.3.2. Method

The cover slip cultures were fixed overnight in methanol. They were stained for 10 min in May-Grunwald stain and for 20 min, in dilute Giemsa stain. The cover slips were then rinsed rapidly in two changes of acetone and again in two parts of acetone and one part xylene for five seconds. They were then placed in one part acetone and two parts of xylene for one minute and cleared in two changes of xylene, two minutes

each, dried and mounted with DPX on a clean glass slide and examined for cytopathic effect under oil immersion objective of the microscope.

3.4.4. Preparation of purified elementary bodies (EB)

3.4.4.1. Materials

i. Cell culture bottles inoculated with *Chlamydia psittaci* isolates

ii. Urografin -76 (Schering AG, Germany)

iii. Tris-potassium chloride (T-KCl) (pH-7.5) containing 20 mM Tris and 150 mM KCl.

3.4.4.2. Method

Chlamydial cell culture harvests of 100 ml were disrupted with Teflon coated magnetic pellet and centrifuged at 1400 x g for five minutes at 4°C to remove cell debris. The supernatant was layered onto 30 per cent (v/v) urografin-76 (diluted with T-KCl). After centrifugation at 50000 x g for 45 min the pellet was re-suspended in one millilitre of T-KCl. It was layered on to 30 to 60 per cent (v/v) urografin-76 gradients in T-KCl and centrifuged at 50000 x g for two hours. The band seen at the middle of the 40 and 50 per cent gradient was collected, diluted with three millilitres of T-KCl and centrifuged at 50000 x g for 45 min. The pellet was resuspended in 0.5 ml of T-KCl.

This was taken as purified elementary bodies (EB). Fifty millilitres of uninfected Mc Coy cell harvest was disrupted with the Teflon coated magnetic pellet. This was sonicated at 40 per cent duty cycle for five minutes at 4°C in Branson sonifier 450 using microtip. This was used as cell control.

3.4.5. Estimation of protein content (Biuret method)

3.4.5.1. Materials

i. Purified elementary body prepared from the isolates

ii. Protein estimation kit (Boehringer Mannheim, Bombay)

3.4.5.2. Method

The purified elementary body was prepared using the reagents for protein estimation in the kit. The absorbance of each processed material was read on a Photometer 5010 at 546 nm. The absorbance of each sample was used for estimating the total protein content by the formula Total protein = 373 x 546 x Absorbance.

3.4.6.Sodium Dodecyl Sulphate -Polyacrylamide Gel Electophoresis.

Sodium dodecyl sulphate - poly acrylamide gel electrophoresis (SDS-PAGE) was used for characterisation of protein fractions of different *Chlamydia psittaci* isolates, following the procedures described by Laemmli (1970).

3.4.6.1. Materials

i. Solution A

Acrylamide	-	30.0g
N,N methylene bis acrylamide	-	0.8 g
Distilled water	-	100 ml

The solution was filtered through Whatman No.1 filter paper and stored at 4°C in amber coloured bottles.

ii. Solution B (pH- 8.8)

Tris base	-	12.1 g
Distilled water	-	50 ml

pH was adjusted to 8.8 with 1N hydrochloric acid and made up to 100 ml with double glass distilled water, filtered and stored at 4°C.

iii. Solution C (pH- 6.8)

Tris base	-	6.06 g
Distilled water	-	80 ml

pH was adjusted to 6.8 with 1N HCl. Final volume was made up to 100 ml, filtered and stored at 4°C.

iv. Sodium dodecyl sulphate (SDS) 10 per cent

SDS	-	10 g
Distilled water	-	100 ml

Filtered through Whatman No.1. filter paper and stored at room temperature.

v. Ammonium persulphate (5 per cent)

Ammonium persulphate	-	50 mg
Distilled water	-	1 ml

Prepared freshly before use.

vi. Electrode Buffer (Tris glycine) (pH- 8.3)

Tris base	-	3.03 g
Glycine	-	14.4 g
SDS	-	1.0 g
Distilled water	-	800 ml

pH was adjusted to 8.3 with 1N HCl and final volume was made up to one litre with double glass

distilled water, filtered and stored at room temperature.

vii. Resolving gel (10 per cent)

Solution A	-	6.7 ml
Solution B	-	5.5 ml
SDS	-	2.0 ml
Distilled water	-	5.2 ml
TEMED	-	10 µl
Ammonium persulphate	-	100 µl
Total volume	-	20.00 ml

viii. Stacking gel (5 per cent)

Solution A	-	1.65 ml
Solution C	-	2.5 ml
SDS	-	1.0 ml
Distilled water	-	4.75 ml
TEMED	-	5 µl
Ammonium persulphate	-	50 µl
Total volume	-	10.00 ml

ix. Sample buffer

Solution C	-	8.5 ml
SDS (10 per cent)	-	0.2 ml

Glycerol	-	1.0 ml
2- mercaptoethanol	-	0.5 ml
Pinch of bromophenol blue		

x. Staining Solution

Coomassie Brilliant Blue R-250-		200 mg
Methanol	-	50 ml
Acetic acid	-	10 ml
Distilled water	-	40 ml

Filtered through Whatman No.1 filter paper and stored at room temperature.

xi. Destaining Solution I.

Methanol	-	50 ml
Acetic acid	-	10 ml
Distilled water	-	40 ml

xii. Destaining solution II.

Methanol	-	25 ml
Acetic acid	-	50 ml
Distilled water	-	425 ml

xiii. Purified elementary bodies of isolates and Mc Coy cell protein (control)

3.4.6.2. Method

3.4.6.2.1. Sample preparation

Sample containing about 10 µg of purified elementary body was mixed with equal volume of sample buffer, heated in a water bath at 90°C for one minute, cooled and stored at 4°C. The cell protein (control) was also prepared as above.

3.4.6.2.2. Electrophoresis

The discontinuous system of polyacrylamide gel electrophoresis was employed.

The gels were prepared in between 16 x 10 cm glass plates supplied with the vertical gel electrophoresis apparatus (Amersham Pharmacia biotech, Hong Kong). One millimeter thick spacers were used in between the glass plates. Ten per cent resolving gel was prepared and poured in between the glass plates. Over this, three millilitre of distilled water was added to get a level surface and allowed to polymerize for 30 min. When the polymerization was completed, the water was poured off and five per cent stacking gel was added and allowed to polymerize for 30 min, after inserting the comb. The comb was removed after

complete polymerization of the gel and the wells were loaded with 20µl quantities each of the above prepared samples. The standard protein marker (3 kDa to 97.4 kDa) from Genei, Bangalore and the processed cell disrupt were loaded in the same way.

Electrophoresis was initially done at 100V till the dye reached the interface of stacking and resolving gel and then the voltage was changed to 150V and was continued till the dye reached the bottom of the gel.

3.4.6.2.3. Staining of the gel

The gel was removed and stained overnight with Coomassie Brilliant blue staining solution and then destained with decolouriser I for three hours, followed by decolouriser II for one hour each with three changes to complete destaining.

3.4.6.2.4. Photography of the gel and estimation of molecular weight

After destaining, the gel was placed in the Alpha imager gel documentation system (Alpha-Innotech, USA) and photographed. The molecular weights of the

different elementary body proteins were directly obtained by comparing the distance migrated by the elementary body proteins with that of the standard marker proteins, whose molecular weights were already known. Colour photograph of the gel was also taken.

RESULTS

4. RESULTS

4.1 Isolation of *Chlamydia psittaci*

4.1.1. Staining of smears from clinical specimens

For isolation of *Chlamydia psittaci*, 46 clinical cases from livestock were subjected to detailed examination. The clinical cases included animals from livestock farms and cases presented to veterinary hospitals in and around Thrissur. Out of these, 29 were bovine cases, including eight aborted foetuses, three semen samples, six uterine discharges, eight cervical swabs and four placentae, 13 were caprine cases, which included 11 aborted foetuses and two uterine discharges and four were porcine, comprising two presented cases of abortion and two still births (Table 1).Bovine abortions were of three to eight months stage, caprine were of three to four months and porcine were of three months stage.

Clinical specimens were used for preparing smears to detect chlamydial bodies. In case of abortion and still birth, smears were prepared as impression smears of cotyledons and cut surfaces of internal organs of the foetus such as liver, lungs and spleen. Direct

Table 1. The details of clinical specimens screened for chlamydial EBs by different methods of staining

Species of animal	Nature of sample	Number screened	Number positive	Percent positive	Percent positive for each species
Bovine	Uterine discharge	6	-	-	3.4
	Cervical swab	8	-	-	
	Semen	3	-	-	
	Aborted foetus (liver, lungs, spleen, stomach content)	8	1	12.5	
	Placenta	4	-	-	
Total		**29**	**1**	-	
Caprine	Uterine discharge	2	-	-	7.7
	Cervical swab	-	-	-	
	Aborted foetus (liver, lungs, spleen, stomach content)	11	1	9.1	
	Placenta	-	-	-	
Total		**13**	**1**	-	
Swine	Still birth (liver, lungs, spleen, stomach content)	2	-	-	-
	Aborted foetus (liver, lungs, spleen, stomach content)	2	-	-	
	Placenta	-	-	-	
Total		**4**	-	-	

smears were prepared from stomach contents of foetus, uterine discharge, cervical swab and semen.

The elementary bodies(EBs) of chlamydia were detected as purple coloured oval or coccoid bodies in dark blue background by Giemsa staining, reddish EBs in blue back ground by modified Ziehl Neelsen staining and red or eosinophilic purple colour in a bluish green background by Gimenez staining.

Smears made from two cases of abortion were positive for EBs of chlamydia by all the three staining techniques. One was from bovine abortion (M-121) and the other from caprine abortion (M-430). The cow had aborted at third trimester of gestation and the goat aborted at second half of gestation.

Impression smears of lungs and liver of M-121 revealed EBs in all the three staining methods, while elementary bodies could not be detected in the smears of spleen and stomach content. But when the impression smears of cotyledons were stained, a high degree of bacterial contamination was observed along with the EBs.

The impression smears from congested lungs of M-430 presented EBs, but that of stomach content, liver and spleen did not reveal any chlamydial bodies on staining.

The different methods of staining to detect chlamydial EBs could give a positive result in 3.4 percent of bovine cases and 7.7 percent of caprine cases. All the clinical samples collected from the swine were found to be negative for EB by staining (Table 1).

The smears prepared from any of the uterine discharge, cervical swab or semen samples did not reveal the presence of elementary body.

One hundred and fifteen processed clinical material (lungs, liver, spleen and stomach content of aborted foetus were processed separately as detailed in Table.2) were used for checking bacterial contamination.

Contaminated samples as ascertained by inoculation in blood agar plates were discarded. Thirty six samples were obtained free of bacterial

Table 2. Statistics showing clinical materials processed

Species	Uterine discharge	Cervical swab	Semen	Liver	Lungs	Spleen	Stomach content	Placenta	Total
Bovine	6	8	3	8	8	8	8	4	115
Caprine	2	-	-	11	11	11	11	-	
Swine	-	-	-	4	4	4	4	-	

contamination. They comprised of 11 liver, 14 lungs, and two spleen samples, six stomach contents and three uterine discharges. These samples were used for CE inoculation.

4.1.2 Chick embryo inoculation

The processed samples after ruling out bacterial contamination were inoculated separately into embryonated chicken eggs through YS route at the rate of 0.25ml per egg. Each sample was inoculated in four embryos and were kept for incubation at 37°C for 12 days. Out of 36 samples, 34 were not able to kill the embryos or produce any lesion in the embryo or YS, even after three blind passages. Impression smears prepared from these YS did not present EBs by the various staining methods.

Two samples ie; liver of M-121 and lung of M-430, revealed the presence of EBs by all the three staining methods in the YS impression smears in the first passage itself.

On passaging in CE using M-121, out of four embryos in first passage, only one died on seventh day PI. On second passage of each YS harvest in four

embryos, only three died on seventh, eighth and tenth day PI. On third passage, out of 64 embryos, eight embryos died, of which six were on eighth day and two on tenth day. The YS harvested from all the three passages revealed patchy haemorrhagic areas. Dead and even live embryos showed haemorrhagic patches on the skin. The consistency of the yolk became thin by third passage. The chlamydial isolation was confirmed by staining the impression smears of YS, which revealed EBs.

Out of four embryos ,on first passage, M-430 was able to kill two embryos at eighth day PI. On second passage, out of 16 embryos, ten died. Seven of them died on seventh day and three of them on tenth day. On third passage, out of 64 embryos, thirty died, of which 18 embryos died on seventh day, ten died on ninth day and two on tenth day. The YS harvested from 90 per cent of the inoculated eggs revealed patchy haemorrhages. Dead and even live embryos revealed haemorrhagic patches on the skin. The consistency of the yolk become thinner from second passage onwards. Elementary bodies on stained smears of YS by all the three methods confirmed chlamydial isolation.

The four embryos inoculated with SPG, served as control and did not show any mortality. Lesions were absent in the embryo and YS. Stained smears of YS did not show chlamydial bodies.

The isolate obtained from foetal liver of bovine abortion was designated as M-121 and that obtained from lungs of caprine foetus was named as M-430. An isolation percentage of 4.3 was obtained.

To study the pattern of the embryo mortality and lesions produced in the embryo, 250 CE were inoculated per isolate including the reference isolate (designated as P-156). Five passages were done at the rate of 50 embryos per passage. Number of CE, which produced haemorrhagic lesions in the embryo and YS are shown in the Table 3. On inoculating isolates P-156, M-121 and M-430, haemorrhagic YS of dead embryo was found to be at the rate of 78.1 per cent, 77.8 per cent and 75 per cent respectively. From fourth passage onwards the mortality rate of M-430 was reduced.

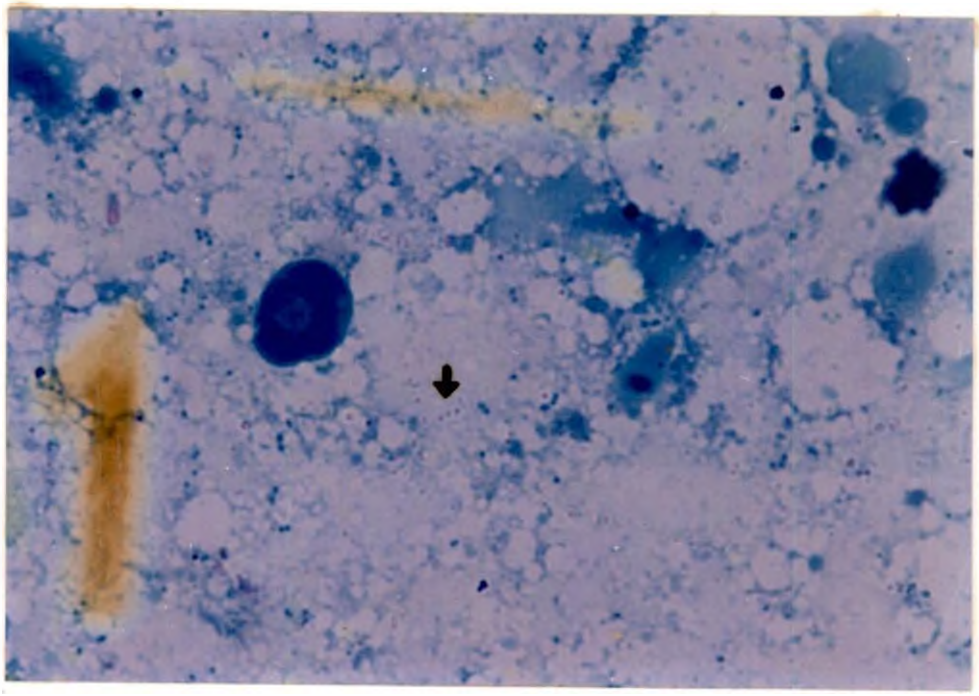
Impression smears of the YS (Fig. 1,2), inoculated with P-156, M-121 and M-430 revealed

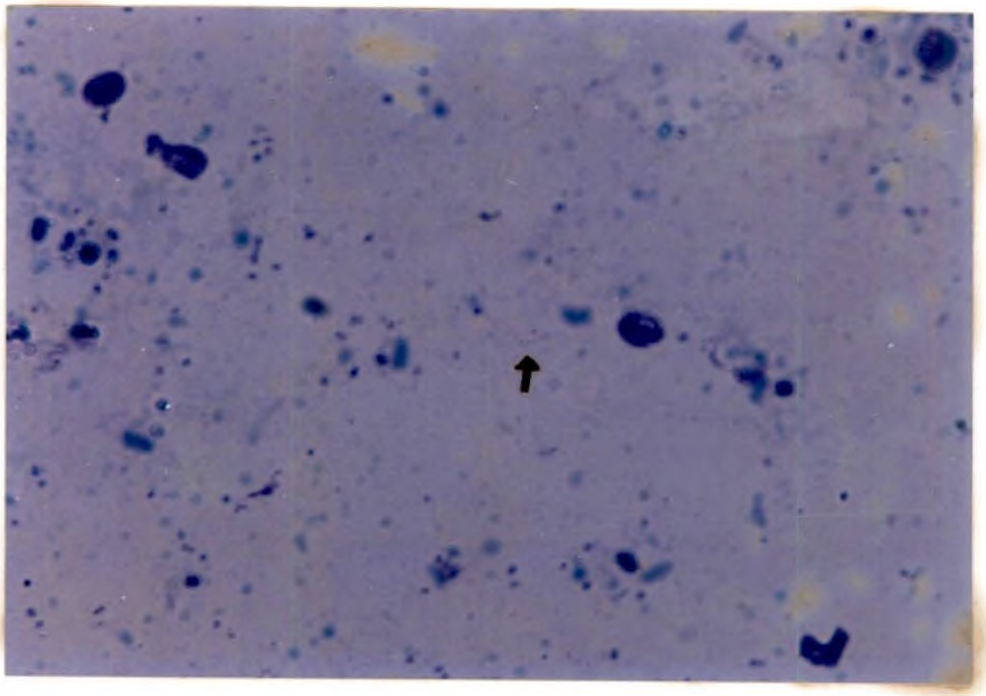
Table 3. Changes in chicken embryos after inoculation with isolates of Chlamydia

Strain	Number of eggs inoculated	Number of Non specific deaths (in 48h)	Number of CE which remained after non-specific death	Embryos which died between 3rd to 12th day PI	Dead embryos with haemorr-hagic YS	Per cent of dead embryos with haemorrhagic YS	Embryos alive after 12 days PI	Live embryos with haemorrhagic YS	Per cent of live embryos with haemorrhagic YS
P-156	250	21	229	32	25	78.1	197	120	60.9
M-121	250	34	216	27	21	77.8	189	104	55.0
M-430	250	37	213	104	78	75.0	109	71	65.1

Fig. 1. Yolk sac impression smear of P-156 showing elementary bodies stained by Gimenez (x 1000)

Fig. 2. Yolk sac impression smear of M-121 showing elementary bodies stained by Giemsa (x 1000)





chlamydial bodies at the rate of 82.1 per cent, 90.3 per cent and 79.3 per cent respectively (Table 4).

4.2. Identification of isolates

4.2.1 Sensitivity for sodium sulphadiazine

A set of 10 CE was used for each of the samples, P-156, M-121 and M-430. Five CE of each set were inoculated with isolates after sulphadiazine treatment whereas the remaining five were inoculated with sample suspension free of sulphadiazine.

Two CE, which received M-121 without sulphadiazine treatment died on eighth day. No mortality was observed in sulphadiazine treated sample inoculated eggs (Table 5). Three CE inoculated with sulphadiazine treated sample of P-156 were found dead, one each on eighth, tenth and eleventh day PI. Three CE inoculated with sulphadiazine treated sample of M-430 died on ninth day PI. The YS of all CE inoculated with the three samples, both those with and without sulphadiazine treatment, elicited haemorrhagic lesions and congestion. All the YS smears irrespective of the treatment and isolate also showed chlamydial bodies in different methods of staining viz., Giemsa, Modified Ziehl Neelsen and Gimenez.

Table 4. Yolk sac showing Chlamydial bodies on staining

Strain	No. of dead embryos (third to twelfth day post inoculation)	Dead embryos showing chlamydial bodies on staining the yolk sac impression smear	Percent of dead embryos showing chlamydial bodies on staining the yolk sac impression smear	No. of Live embryos (after 12 days P.I.)	Live embryos showing chlamydial bodies on staining the yolk sac impression smear	Per cent of live embryos showing chlamydial bodies on staining the yolk sac impression smear	Per cent of embryo showing chlamydial bodies on staining the yolk sac impression smear (excluding non-specific death)
P-156	32	28	87.5	197	160	81.2	82.1
M-121	27	25	92.6	189	170	89.9	90.3
M-430	104	84	80.8	109	85	78.0	79.3

Table 5. Sensitivity to sulphadiazine of different isolates of Chlamydia species

Material	Treatment	No. of CE inoculated	No. of dead embryos	No. of live embryos	Chlamydial bodies on Gimenez staining	Chlamydial bodies on Giemsa staining	Chlamydial bodies on modified Ziehl Neelsen staining
YS of P-156	With Sodium sulphadiazine	5	3	2	+	+	+
	Without Sodium sulphadiazine	5	-	5	+	+	+
YS of M-121	With Sodium sulphadiazine	5	-	5	+	+	+
	Without Sodium sulphadiazine	5	2	3	+	+	+
YS of M-430	With Sodium sulphadiazine	5	3	2	+	+	+
	Without Sodium sulphadiazine	5	-	5	+	+	+

4.2.2. Pathogenicity in mice

The results of pathogenicity studies of three chlamydial isolates in mice are given in Table 6.

Six mice were used for each sample. The YS (3+) from third passage of the isolates in CE were used for inoculation in mice. Five of the M-121 inoculated mice showed anorexia, dullness and ruffled hairs by seven to eight days. By nine to ten days their extremities became cold and their movement was found to be sluggish. Of these five, one died on 14^{th} day and other four survived. The other mouse showed anorexia and dullness by tenth day, but survived within four to five days, after showing clinical symptoms. The mortality rate in mice was 16.7 per cent for M-121

On post-mortem examination of the mouse dead after receiving M-121 showed whitish stringy fibrinous exudate in the peritoneal cavity. Severe hepatic congestion with patchy necrosis of liver was noticed. But congestion found in lungs and spleen were of milder degree. Impression smears of the affected areas of these organs revealed chlamydial EBs on staining with Giemsa, Modified Ziehl Neelsen and Gimenez

Table 6. Mortality pattern and morbid anatomy of mice (three to four weeks of age) inoculated with Chlamydial isolates

Strain	Material used	Number of mice used	Number of mice died	Per cent of mortality	Period of death (days)	Postmortem finding	
						Dead	Sacrificed
M-121	Yolk sac of third passage	6	1 (14^{th} day post inoculation)	16.7	14	Whitish stringy fibrinous exudate in peritoneum. Congested liver spleen and lungs	Whitish peritoneal exudate, moderate peritonitis.
M-430	Yolk sac of third passage	6	4 (2 each on 12^{th} and 14^{th} day post inoculation)	66.7	12-15	Whitish stringy fibrinous exudate on peritoneal surface. Congested liver spleen and lungs	Whitish peritoneal exudate, moderate peritonitis.
P-156	Yolk sac of third passage	6	2 (12^{th} and 15^{th} day post inoculation)	33.3	12-15	Whitish stringy fibrinous exudate on peritoneal surface. Congested liver spleen and lungs	Whitish peritoneal exudate, moderate peritonitis.

methods. Smears of liver and lungs presented comparatively more number of EB than in spleen.

The five mice inoculated with M-121 isolate, which survived were sacrificed after three weeks PI. On post-mortem examination whitish stringy peritoneal exudate, and adhesion of internal organs and mild degree of peritonitis were noticed. On staining, impression smears of internal organs (liver, lung and spleen) and smear of exudate showed few numbers of chlamydial agents.

All the mice inoculated with M-430 showed anorexia, dullness and ruffled hairs by eight to nine days. By tenth day four of them exhibited cold extremities. They went down in condition by eleventh day and two of them died on 12^{th} day and the other two died on 14^{th} day. The other two mice showed anorexia and dullness by tenth day. they survived within four to five days after showing clinical symptoms.

Four mice which received sample M-430 and died within 12 to 14 days, were necropsied. Severe pulmonary congestion and pneumonic lesions were noticed in them. Liver and spleen showed mild degree

of congestion. Impression smears of affected areas of lung presented numerous EBs (Fig. 3), while liver and spleen were with lesser number of EBs.

The two mice that survived after receiving M-430 were sacrificed after three weeks PI. Whitish stringy peritoneal exudate, adhesion of internal organs and moderate degree of peritonitis were noticed on postmortem. Stained impression smears of liver, lungs, spleen and smear of peritoneal exudate revealed few numbers of EBs.

Those six mice inoculated with P-156 showed anorexia and dullness by seven to eight days PI. Out of these, two mice exhibited cold extremity, sluggishness in movement on tenth day. One of these died on twelfth day and the other on 15^{th} day PI. The remaining four mice even though showed clinical manifestation as mentioned above, survived.

Post-mortem examination of two mice, dead after receiving P-156, presented whitish stringy fibrinous exudate in the peritoneal cavity and severe hepatic and pulmonary congestion with patchy necrosis in liver and lungs were noticed. But the congestion of spleen

was of milder degree. Impression smears of the liver, lungs and spleen and smears of peritoneal exudate revealed chlamydial EBs by all the three methods of staining.

The four mice that survived after inoculation with P-156, were sacrificed after three weeks PI. On necropsy, whitish peritoneal exudate, mild degree of adhesion of liver to the peritoneum and moderate degree of peritonitis, were observed. On staining, impression smears of liver, lungs and spleen and smears of exudate, moderate number of chlamydial EBs were revealed.

The pooled tissue samples and peritoneal exudate obtained from each mouse were used for re-isolation of chlamydia, employing four CE per isolate. One embryo each inoculated with pooled samples of P-156, and M-121 and two which received M-430 pooled samples, died within ten days. The impression smears of YS of harvested eggs (both live and dead) showed high concentration of EBs for all the isolates.

The pooled samples of liver, lungs and spleen collected from survived but sacrificed mice did not

kill the CE within 12 days for all the isolates. But chlamydial EBs were detected in the YS impression smears confirming the re-isolation .

Control mice inoculated with SPG alone did not show any clinical manifestation even after three weeks PI and were sacrificed. On necropsy they did not present gross lesion in any of the visceral organs, and were negative for EB after staining.

4.2.3. Pathogenicity in Guinea pigs

Results of pathogenicity study of chlamydial isolates in guinea pig are presented in Table 7.

Each sample was inoculated in two guinea pigs. The YS (3+) from third passage of the isolates were used for inoculating them. Mortality rate of M-430 was found to be 50 per cent, while P-156 and M-121 (bovine isolates) did not kill the guinea pig at all.

The guinea pigs, which received M-121 even though showed a moderate degree of clinical manifestations viz., anorexia and dullness by eighth day, recovered to normal health in four to five days.

Table 7. Results of pathogenicity of chlamydial strains in Guinea pigs (four to five weeks of age)

Material inoculated	Number of guinea pigs inoculated	Number of guinea pigs died	Mortality per cent	Period of death (days)	Postmortem finding	
					dead	Sacrificed
M-121	2	-	-	-	-	Stringy peritoneal exudate, mild peritonitis
M-430	2	1	50	10	Yellowish stringy fibrinous exudate, Congested lung and spleen, necrotic liver	Sticky exudate, congested liver and lungs
P-156	2	-	-	-	-	Mild peritonitis, congested liver, lungs and spleen

Both the guinea pigs that received M-121 were sacrificed after three weeks PI. Stringy exudate in the peritoneal cavity and mild degree of peritonitis were noticed on post-mortem. Moderate congestion of liver and lungs were seen. Stained smears of affected areas of liver, lungs and spleen and peritoneal exudate presented EBs.

One guinea pig, which was inoculated with M-430 died at tenth day PI. By seventh day both guinea pigs inoculated with M-430 were found anorectic and lethargic. On eighth day one of it ran down in condition and exhibited convulsions. Death occurred on tenth day.

The other guinea pig inoculated with M-430 showed milder degree of clinical manifestation like anorexia and dullness by seventh to eighth day and this persisted for two days. It recovered to normal health by twelfth day.

On post-mortem examination of dead guinea pig, given M-430, pulmonary congestion of severe degree was noticed. Hepatic necrosis with enlargement of liver

was also observed. The peritoneal cavity contained moderate amount of yellowish stringy fibrinous exudate. Peritonitis and inflammation of spleen were the other lesions. Impression smears of all affected organs (liver, lungs, and spleen) and smears of peritoneal exudate revealed chlamydial EBs (Fig. 4).

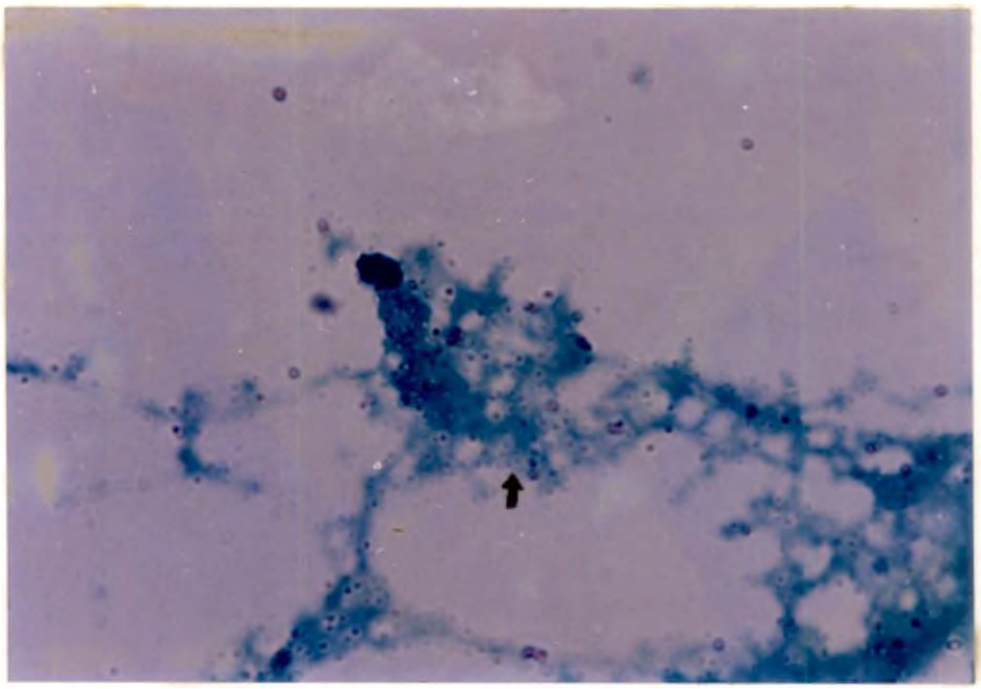
The guinea pig, which survived after receiving M-430, was sacrificed on completion of 21 days PI. Post-mortem examination showed small quantity of sticky exudate in the peritoneal cavity. Mild congestion in lungs, liver and visceral peritoneal layer were noticed along with necrotic areas in liver. Stained smears of affected areas in lungs and liver and peritoneal exudate showed EBs. No change was noticed in spleen and their impression smears did not reveal any chlamydial bodies.

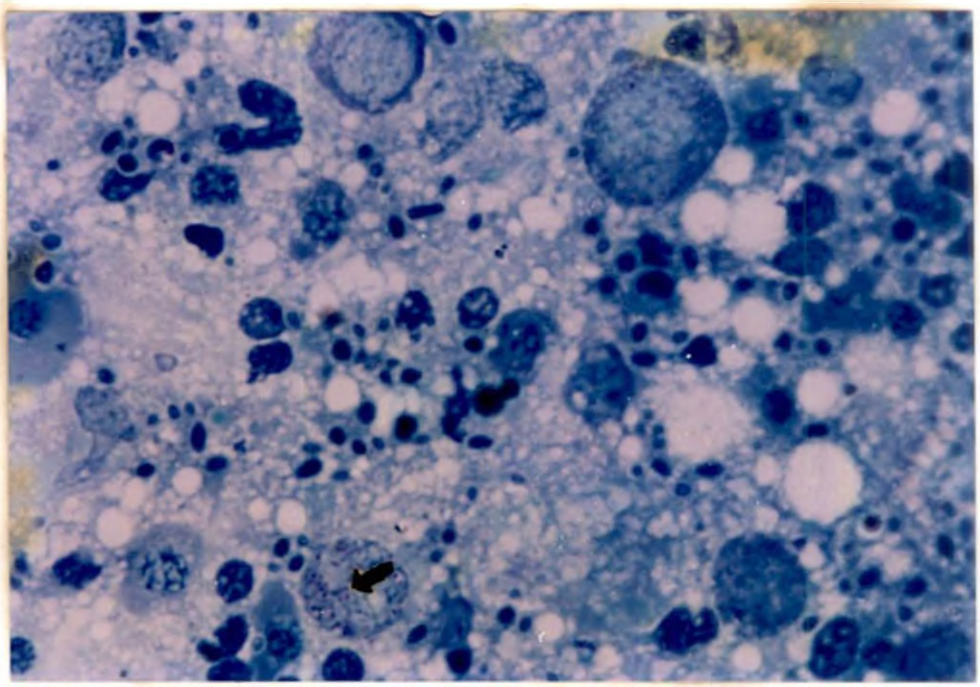
Those two guinea pigs inoculated with P-156 became weak and anorectic by seven to eight days. Their movements became sluggish by ten days, but they survived after four more days.

P-156 inoculated guinea pigs, which survived, were sacrificed after three weeks PI. Adhesion of

Fig. 3. Impression smear from lungs of mouse inoculated with M-430 showing elementary bodies (Gimenez x 1000)

Fig. 4. Impression smear from lungs of guinea pig inoculated with M-430 showing elementary bodies (Modified Ziehl Neelsen x 1000)





internal organs in the abdominal cavity and mild degree of peritonitis were observed. Moderate congestion was noticed in lungs, liver and spleen. Smears of affected areas of lungs, liver and spleen and peritoneal exudate revealed EBs on staining.

Lungs, liver and peritoneal exudate collected from both dead and sacrificed guinea pigs, were used to reisolate the organism. These samples from each animal were pooled separately and inoculated into four CE per isolate. One embryo inoculated with pooled organ samples of P-156 inoculated guinea pig and two embryos each, which received M-121 and M-430 inoculated organ samples, died within ten days. Stained impression smears of YS showed numerous EBs for all the isolates.

Control guinea pig, which was inoculated with PBS was without any signs of illness throughout the observation period of three weeks. The animal was sacrificed and subjected to post-mortem examination. No gross lesion was present in any of the visceral organs and smears were negative for EBs after staining.

4.3. Confirmation of identity of the isolates by Agar gel precipitation test (AGPT)

The identity of P-156 (reference isolate) was confirmed as *Chlamydia psittaci*, at the Department of Microbiology, Veterinary college, Palampur, using monoclonal antibody. By inoculating YS antigen of P-156, antiserum was raised in rabbit. A sufficient level of antibody titre was obtained in the anti serum collected after 15 days following the booster dose, as ascertained by performing Agar gel precipitation test against P-156 antigen.

On diffusing the prepared antigen of all the three isolates and YS antigen against the adsorbed antiserum, a clear and thick and another faint precipitin lines were formed against all the isolate antigens. The thick precipitin lines of different isolates proved the line of identity of the antigens, thus confirming that the isolates were all *Chlamydia psittaci*. No precipitin line was present in between the central well and the well loaded with YS antigen.

4.4. Protein profile analysis

4.4.1. Maintenance of cell line

Eagle`s Minimum essential medium, without non-essential amino acids and containing 10 percent foetal bovine serum, at pH7.2 was able to support the formation of confluent monolayer by 24h of incubation of the Mc Coy cell line obtained from NCCS, Pune at 37°C. It was subcultured into tissue culture bottles and cover slips.

The newly subcultured bottles and cover slip cultures with the above used growth medium were incubated at 37°C for formation of monolayer. They were continuously examined under inverted microscope to observe the growth characteristics at an interval of 24h. By 72 h after subculturing, almost confluent monolayers were obtained (Fig. 5). For maintaining the confluent monolayer maintenance medium was used.

4.4.2. Propagation of chlamydial isolates in Mc Coy cell line

All the three isolates were used for propagation in Mc Coy cell line. After a period of one hour of incubation at 37°C for adsorbtion of the organism to the cell surface, the monolayer bottles were incubated

with maintenance medium.The cytopathic effect from 24 h onwards showed that the organisms multiplied in the cells.

Cover slip cultures inoculated with these isolates to study the cytopathic effect also showed similar changes as that in monolayer bottles.

4.4.3. Staining of cover slip cultures

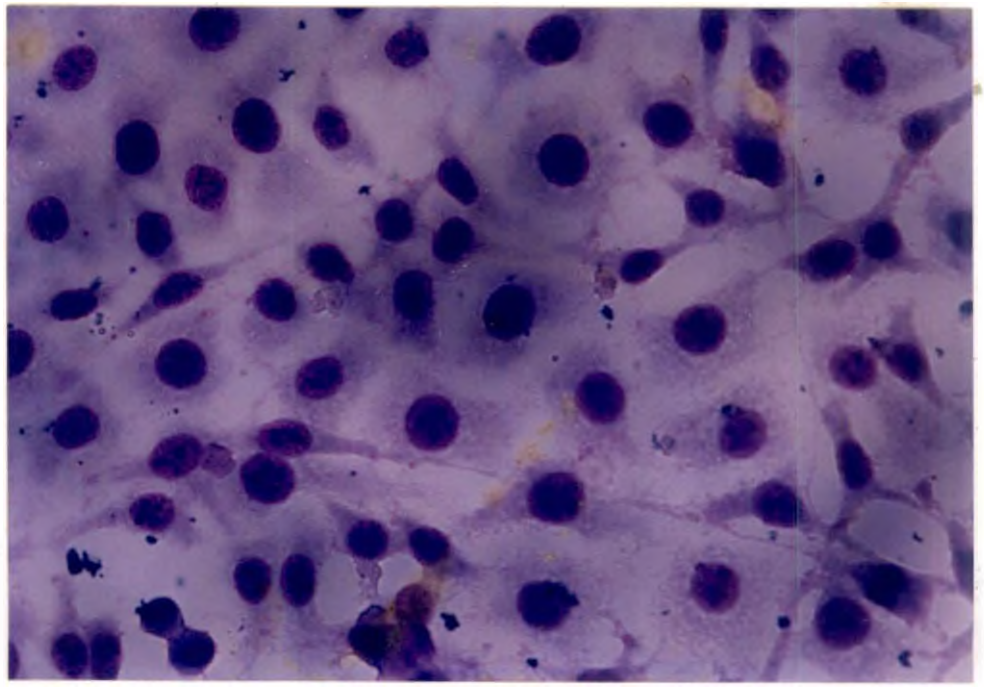
In the stained cover slip cultures inoculated with P-156, rounding and swelling of fibroblast cells started in first 24h. No detachment of cell was noticed at that time. Few cells were found clumped together forming syncytia (Fig. 6).

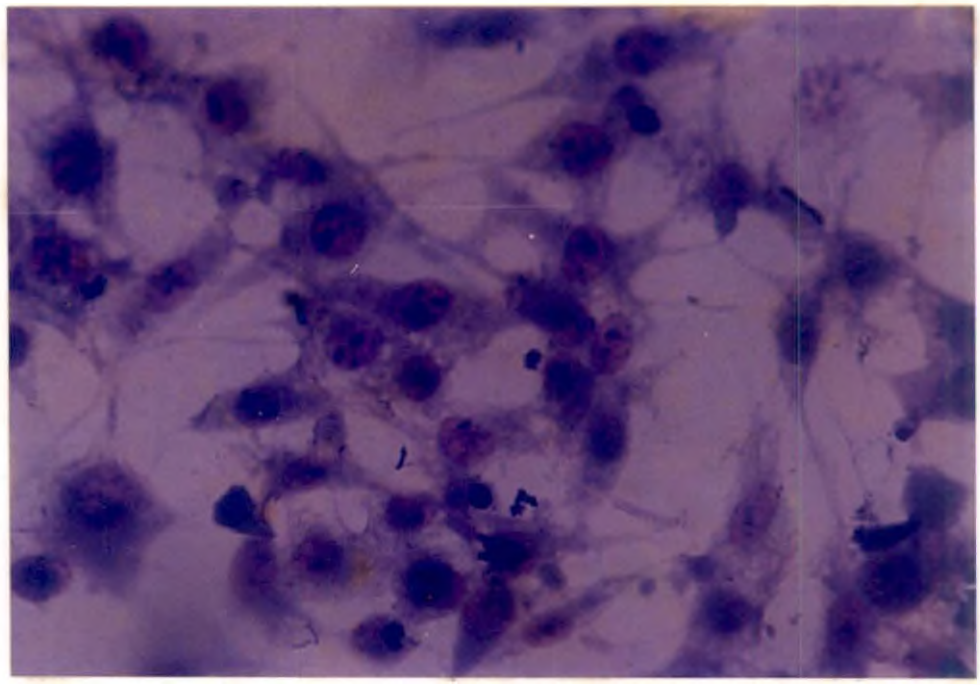
In M-121 and M-430 inoculated cultures, similar type of cytopathic effect as found in that inoculated with P-156, was observed by staining the cover slips at 24 h PI.(Fig. 7).

By 48 h, rounding and swelling of cells were found more prominent and the cells had started detaching from adjacent cells and also from the glass surface in all the three isolate infected monolayers. Clumping of cells was very characteristic in cell

Fig. 5. Uninfected Mc Coy cell line after 72 h of incubation (May-Grunwald Giemsa x 1000)

Fig. 6. P-156 infected Mc Coy cell line after 24 hours post inoculation (May-Grunwald Giemsa x 1000)





lines infected with P-156 and M-430. (Fig.8). Similar cytopathic changes were observed in M-121 infected cover slip monolayer after staining, with the exception that, clumping of cells were less compared to that produced by the other two isolates.

At 72h PI, most of the attached cells infected with all the three isolates were found rounded. Majority of cells got detached from the inner surface.

The control tubes in which, instead of isolates, 0.2 ml of SPG was inoculated, showed no changes during the observation period and remained as confluent monolayer.

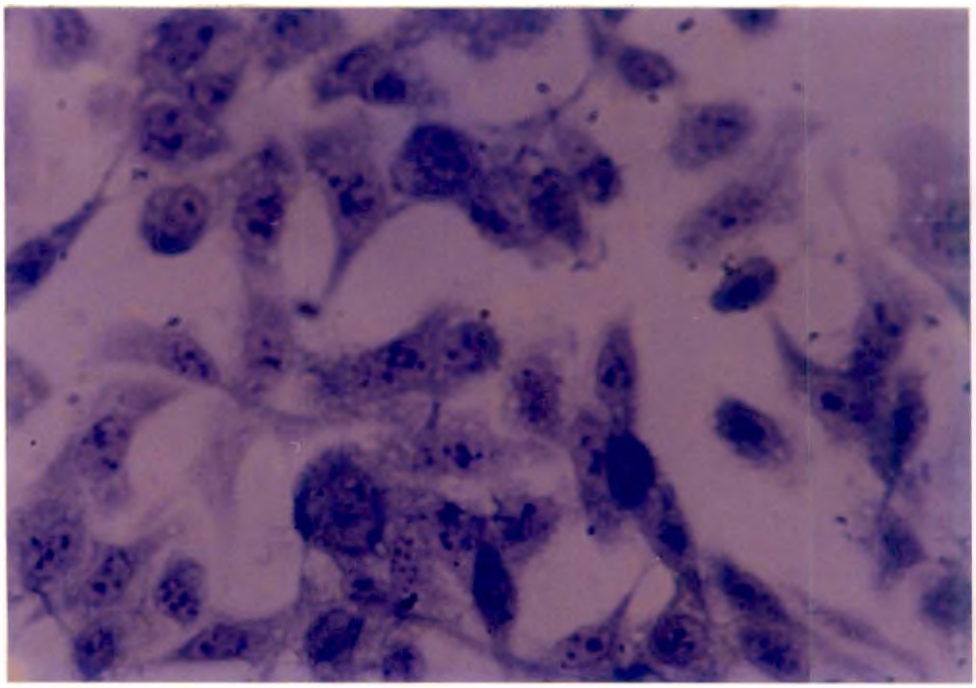
Infected and uninfected cells in bottle cultures were harvested after 72h with the help of sterile glass beads and using fresh maintenance medium.

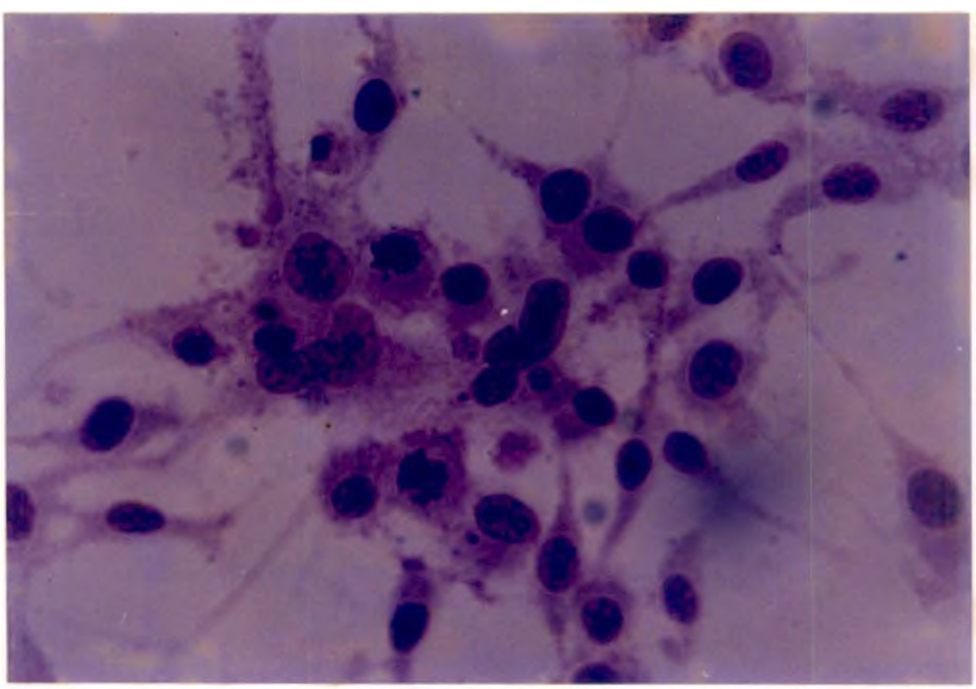
4.4.4. Preparation of purified elementary bodies

Cell culture harvests of about 100 ml of each isolate were disrupted with Teflon coated magnetic pellet and the cell debris were removed by centrifugation. Further pelleting and gradient centrifugation resulted in diffuse band at the

Fig. 7. M-121 infected Mc Coy cell line after 24 hours post inoculation (May-Grunwald Giemsa x 1000)

Fig. 8. P-156 infected Mc Coy cell line after 48 hours post inoculation (May-Grunwald Giemsa x 1000)





interface of 40 per cent and 50 per cent urografin gradients. The pellet was collected using a Pasteur pipette, suspended in T-KCl and centrifuged to pellet it. The resulting pellet was resuspended in 0.5 ml of T-KCl.

A drop of the above suspension was smeared on a slide, stained by Gimenez and examined for chlamydial bodies. There was sufficient concentration of EBs in the samples prepared from all the three isolates and these formed the purified EBs.

4.4.5. Estimation of protein content of elementary body

The protein concentration of elementary body determined by Biuret method showed that, P-156, M-121 and M-430 harvests contained 1099.75 mg/ 100 ml, 651.71 mg/ 100 ml and 549.88 mg/ 100 ml respectively.

4.4.6. Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

The standard protein marker, protein from the isolates and that made from Mc Coy cells were used for PAGE . On lane 1, Standard protein marker, and on lane 5, Mc Coy cell protein (control) were loaded. Lanes

2- 4 were loaded with the proteins from the respective isolates P-156,M-121 and M-430.

After electrophoresis the polypeptide bands were clearly visible on staining with coomassie brilliant blue.

The standard protein marker yielded eight bands with molecular weights ranging from 3 kilo Dalton (kDa) to 97.4 kDa. The molecular weights of the test samples were calculated, in comparison with that of standards using Alpha Imager Gel Documentation System. The results are shown in Table 8. A colour photograph of the gel was also taken (Fig. 9). The bands are graphically represented (Fig.10).

Control showed six bands, of which the band at 36 kDa was present in all the three isolates and so was not taken in to account. Thus a total of 12 bands were observed in P-156 and M-121 samples and 10 bands were seen in M-430 sample of their own.

Of the 12 bands observed for P-156 and M-121, most of them were common. Additional bands of molecular weight 148 kDa and 135 kDa were present in

Table 8. SDS-PAGE analysis of proteins of *Chlamydia psittaci*

Protein fractions	Molecular weights of the proteins of the isolates (kDa)		
	P-156	M-121	M-430
I	-	-	155
II	-	152	-
III	148	-	-
IV	-	137	-
V	135	-	-
VI	41	41	41
VII	32	32	-
VIII	30	30	30
IX	23	23	23
X	21	21	21
XI	-	-	19
XII	18	18	-
XIII	15	15	-
XIV	-	-	12.2
XV	9	9	9
XVI	7	7	-
XVII	-	-	6.4
XVIII	5	5	5
Total number of bands in each isolate	12	12	10

Fig. 9. SDS-PAGE of proteins from the isolates
Lane 1- Standard protein marker
Lane 2- P-156
Lane 3- M-121
Lane 4- M-430
Lane 5- Mc Coy cell protein (control)

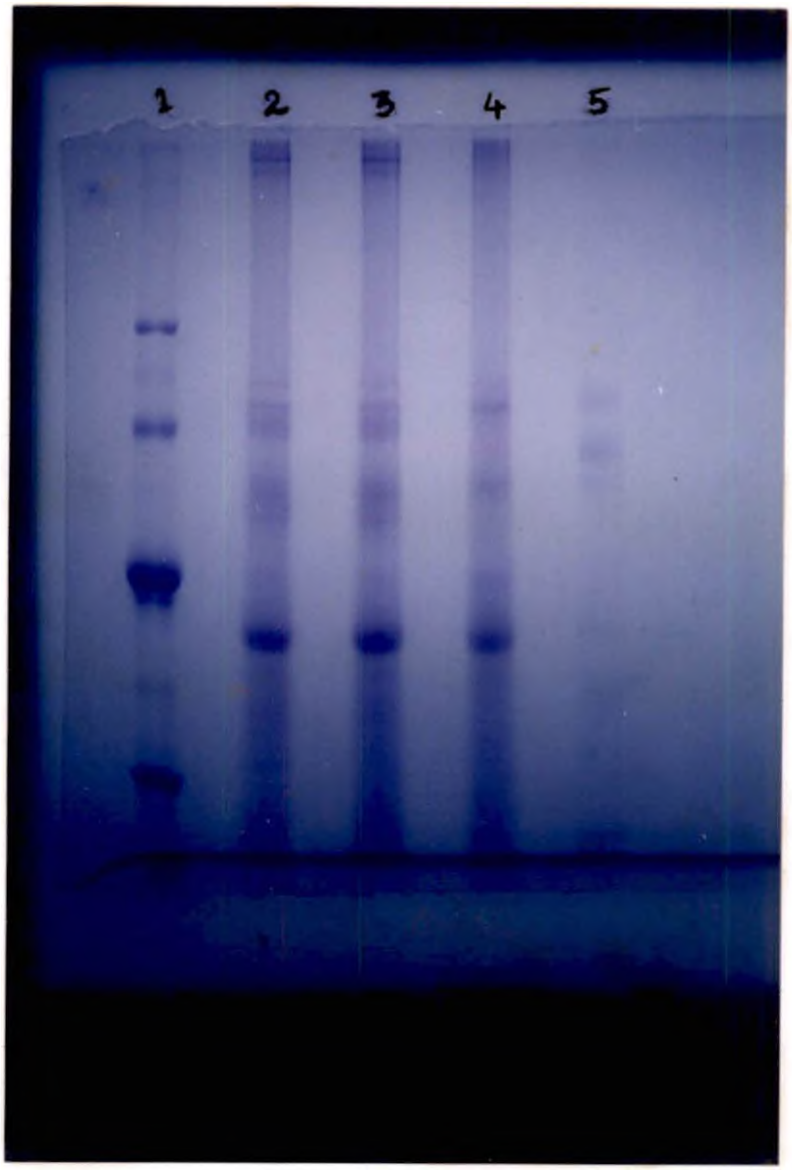
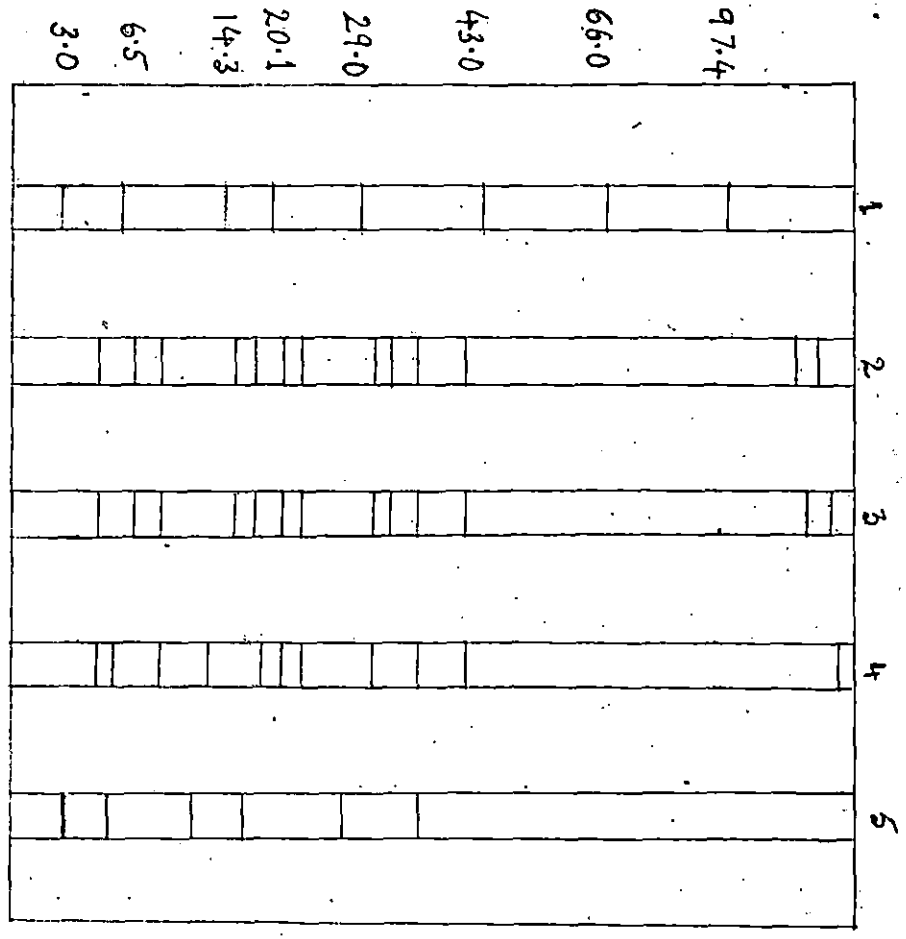


Fig. 10. Graphical representation of SDS-PAGE
Lane 1- Standard protein marker
Lane 2- P-156
Lane 3- M-121
Lane 4- M-430
Lane 5- Mc Coy cell protein (control)

MOLECULAR WEIGHT IN kDa



P-156, while 152 kDa and 137 kDa bands were unique for M-121. But M-430 (caprine sample) contained only 10 bands and was lacking bands at ranges of 152 kDa, 148 kDa , 137 kDa, 135 kDa, 32 kDa, 18 kDa 15 kDa and 7 kDa. Bands of 155 kDa, 19 kDa, 12.2 kDa and 6.4 kDa were present only in M-430.

DISCUSSION

5. DISCUSSION

Epizootic abortion in bovines and enzootic abortion of ewes in late pregnancy as a consequence of placental necrosis is among the most dramatic and economically devastating manifestations of pathogenicity of *Chlamydia psittaci*.

5.1. Isolation of *Chlamydia psittaci*

5.1.1. Staining of smears from clinical specimen

A total of 46 clinical cases were selected for isolation of chlamydia. The clinical materials were screened for EBs of chlamydia by various staining methods viz., Giemsa, modified Ziehl Neelsen and Gimenez. Out of these, two samples indicated the presence of chlamydiae by revealing EBs by these staining methods. One was from lungs and liver of bovine aborted foetus and the other from lungs of aborted foetus of caprine origin. Krishna (1990) and Aitken (1993) also found the EBs of chlamydia on staining the smears of clinical specimens using the above mentioned methods.

5.1.2 Chick embryo inoculation

The clinical materials processed for isolating chlamydia were tested for bacterial growth by culturing on blood agar. Thirty six specimens were found to be un-contaminated and they were used for CE inoculation by YS route. On CE inoculation of these samples and further incubation at 37°C for 12 days,death of the embryo in seven to ten days, gross lesions in the embryos and YS were observed only for two samples. It was found that these samples were those two, which showed positive results in primary screening by different methods of staining (M-121 and M-430). Thus an isolation percentage of 4.3 was obtained in the study. This comprised of 3.4 per cent from bovine cases, 7.7 per cent from caprine cases and zero per cent from swine samples.

Purohit *et al.* (1986) had reported an isolation percentage of 6.7, while Jain *et al.* (1976) could make isolation of 64.3 percentage from cases of abortion.

Majority of isolations made by Storz (1971), were from placentae and cotyledons. He opined that the isolation of chlamydia from bovine aborted foetuses might be difficult due to the pronounced cellular

immune response of the foetal calves to the infectious agent. Fresh placentae were the most suitable samples for attempting chlamydial isolation. In the present study placental tissue was not used for isolation because of the high degree of contamination. This may be the probable reason for reduced percent of isolation in this study.

M-121 (bovine) abortion occurred during third trimester of gestation, while M-430 (caprine) abortion occurred at the second half of gestation. Krishna (1990) reported bovine abortion due to chlamydial etiology occurred during 7^{th} month. Aitken (1993) and Blood *et al.* (1994) observed enzootic abortion in ewes towards the later stages of gestation due to placental necrosis.

The stages of abortion in the present study was in conformity with the results of the above mentioned investigators.

The source of bovine isolate was foetal liver while that of caprine was foetal lungs, both from cases of abortion. Most cotyledens obtained were contaminated and so were unsuitable to use for

isolation. Krishna and Mathur (1979), Sharma *et al.* (1983) and Krishna (1990) isolated chlamydial organisms from foetal organs and placental cotyledens. Isolation from internal organs of aborted foetus was in conformity with previous studies mentioned above.

Both bovine isolates (P-156 and M-121) less frequently produced death of the embryo in CE inoculation in first two to three passages. But caprine isolate (M-430) frequently produced death of the embryo, usually during seven to ten days PI.

The mortality pattern in CE was in accordance with the findings of Sreeramulu *et al.* (1989), who reported the mortality of CE during three to ten days PI.

The identification of chlamydiae during isolation is a difficult task unless the clinical specimens contain a heavy concentration of the organism. Most of the clinical specimens might require two to three passages in CE before chlamydia can be identified (Idtse, 1984). But in this study chlamydial bodies could be easily identified in YS smears of the two cases, which were found positive, in the primary

passage itself. This indicated that both these clinical materials had high concentration of organisms. No samples which were negative in the primary passage turned out positive in the subsequent passages.

5.2. Identification of isolates

5.2.1 Sensitivity for sodium sulphadiazine

There was not much difference in the sulphadiazine treated and non-treated groups regarding mortality rate in all the three isolates. All the YS smears which were stained (both sulphadiazine treated and not treated) revealed chlamydial bodies. There were no difference between the sulphadiazine treated and non-treated YS smears indicating that the isolates were insensitive to sodium sulphadiazine. The resistance of *Chlamydia psittaci* organism to sodium sulphadiazine was also reported by Storz (1971) and Page (1981).

5.2.2. Pathogenicity in mice

In the present study mice were not made use for the primary isolation of chlamydia from clinical specimens. They were used to study the pathogenicity of three different *Chlamydia psittaci* isolates, two

from bovine and one from caprine origin by intraperitoneal inoculation.

Younger age groups (three to four weeks) of animals were used in this study. Francis (1988) reported younger age group of animals are preferred for better infectivity of chlamydia.

In the present study depending upon the severity of infection, mortality in mice ranged from 16.7 to 66.7 per cent for different isolates. Mortality of mice occurred during 12 to 15 days PI. Intraperitoneal inoculation of mice with virulent strains produced death in three to fourteen days depending on the virulence (Storz, 1971). This indicated that isolates in the present study were of moderate virulence in mice.

By one week the inoculated mice showed anorexia, dullness and ruffled hairs and later manifested sluggish movement. On post-mortem examination pulmonary and hepatic congestion and pneumonia were noticed with focal necrotic lesions on liver and mild congestion of spleen. Stringy fibrinous exudate was seen in peritoneal cavity of the inoculated mice.

From the present study it could be inferred that the two bovine strains of *Chlamydia psittaci* were of moderate virulence while that of caprine strain was of greater virulence in mice, compared to the other two. However, Storz (1971) had observed that chlamydial isolates from ruminants were of low pathogenicity for mice.

5.2.3. Pathogenicity in Guinea pigs

Pathogenicity study of the three isolates was conducted in guinea pigs. Out of two guinea pigs inoculated with caprine isolate (which was more virulent in mice), one animal died of chlamydial infection. The other two isolates M-121 and P-156 did not kill any guinea pig at all.

Page (1981) observed that *Chlamydia psittaci* organisms were less pathogenic to guinea pigs compared to mice. These results were in accordance with the observations made in present study.

At the end of one week PI, each guinea pig inoculated with P-156, M-121 and M-430 showed anorexia and dullness. Clinical manifestations shown by those

guinea pigs inoculated with M-430 were more severe compared to others. One of them died in two weeks. On post-mortem examination of dead guinea pig and live guinea pigs, which were sacrificed, congestion of internal organs and necrotic changes in liver of varying degree were noticed. Dead guinea pig showed stringy fibrinous exudate in the peritoneal cavity, but others which were sacrificed showed only sticky exudate. Chlamydial infection was confirmed in all the guinea pigs by staining impression smears of their internal organs.

Page (1981) showed that within few days of intraperitoneal inoculation in guinea pigs, they manifested lethargy, anorexia, and inco-ordination. Stringy fibrinous exudate in peritoneal cavity also was found. He emphasised that certain strains of *Chlamydia psittaci* were capable of producing death or lesions in guinea pigs while some were not. The clinical manifestations and lesions observed in this study were mostly in accordance with the results obtained by previous investigators, with the exception that, only caprine isolate was able to kill the guinea pig.

Impression smears of infected guinea pig liver, lungs and spleen revealed chlamydial bodies. Re-isolation of *Chlamydia psittaci* isolates from the liver, lungs and peritoneal exudate of the dead and sacrificed guinea pig was achieved in this study. Elementary bodies in tissue impression smears were also demonstrated by staining. Francis (1988) was able to re-isolate the organism from the affected organs of dead guinea pigs which was in conformity with the observations made in this study. Krishna (1990) also showed chlamydial bodies on staining impression smears of organs of infected guinea pig. He also was successful in re-isolating the organism from organs of infected guinea pig.

From the present study, it came to light that the two bovine strains were not virulent enough to produce mortality in guinea pigs, while the caprine strain was found to be moderately virulent and was able to produce mortality in one of the inoculated guinea pigs. This caprine isolate was pathogenic to mice also. Page (1981) reported that some strains of *Chlamydia psittaci* multiplied and caused death or lesions in mice, but not in guinea pigs and vice versa.

5.3. Propagation of chlamydial isolates in Mc Coy cell line

Page (1981) reported that Mc Coy cell lines were having rapidly growing cells that supported growth of most chlamydial strains and so was ideal for propagation of *Chlamydia psittaci*. Johnson *et al.* (1983) also found that these cell lines could be used for isolation of *Chlamydia psittaci* from cases of abortion.

On inoculation in Mc Coy cell line cytopathic changes were visible from 24h onwards and showed typical cytopathic effect within 72 h.

No additives were introduced into the medium for enhancing the infectivity of chlamydiae. But various workers have introduced additives in the medium to enhance the infectivity of chlamydiae viz., cycloheximide, diethyl amino ethyl -dextran (Spears and Storz, 1974), placental extract and erythritol (Amin and Wilsmore, 1997). These treatments resulted in easy infection of the cell monolayer and for the development of cytopathic effect and inclusions in the infected cells.

Even without any pre-treatment, the samples P-156, M-121 and M-430 were able to infect sufficiently the Mc Coy cell line in this study.

Cytopathic effects such as rounding and swelling of fibroblast cells started in first 24 h for all the three samples and by 48 h and 72 h, rounding, swelling, clumping and detachment became more prominent for all isolates. Clumping of cells was more prominent for P-156 and M-430 but was less for M-121 in 48 h. Detachment of cells from adjacent cells and from glass surface started at 48h PI and was very prominent by 72 h in the case of all the three isolates.

Spears and Storz (1979) found that abortion causing chlamydial isolates slowly developed round and oval inclusion bodies which appeared 30 h PI. Page (1981) reported rounding, swelling and detachment of infected cells after two to six days of incubation.

Development of cytopathic effects and inclusions in infected cell culture was not always certain, since some of the chlamydial species did not produce these effects in all cell lines of cultured cells (Page,

1981). This might be the reason for the difficulty in identifying inclusions in infected monolayer in the present study.

These cytopathic effects were found to be similar to the results obtained by Page (1981).

5.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The polypeptide constituents of *Chlamydia trachomatis* EBs have been described by several authors, while published reports regarding polypeptide pattern of *Chlamydia psittaci* are rare. However Vitu and Russo (1984) reported no substantial difference in polypeptide pattern between isolates obtained from different species of animals.

Only 12 polypeptide bands were prominent in both the bovine isolates while 10 bands were present for the caprine isolate.

Griffiths *et al.* (1992) detailed about forty components of polypeptide bands on PAGE analysis of placental and intestinal isolates, with prominent bands at 40 kDa, 49 kDa and 50 kDa regions.

Markey *et al.* (1993) reported more than 50 polypeptides with similar profiles among abortion isolates.

The difference in observation of present study and results of previous investigation may be due to strain variation of the isolates. The staining method was using Coomassie blue as against silver staining by Griffiths *et al.* (1992) and Markey *et al.* (1993). Silver stain is more sensitive towards polypeptide bands. This can be a reason for the reduction in bands during staining in the present study.

Eventhough P-156 and M-121 gave the same number of bands (12), the bands at the molecular weights 148 kDa and 135 kDa were unique for P-156, while the bands at 152 kDa and 137 kDa were seen only in M-121. For the caprine isolate (M-430), the polypeptide bands of molecular weights 155 kDa, 19 kDa, 12.2 kDa and 6.4 kDa were unique. Six bands of molecular weights 41 kDa, 30 kDa, 23 kDa, 21 kDa, 9kDa and 5kDa were common for bovine and caprine isolates while four bands viz., 32 kDa, 18 kDa, 15 kDa and 7 kDa were lacking in caprine isolate.

Buzoni-Gatel *et al.* (1989) correlated invasiveness in sheep with the presence of 78 kDa, 90 kDa and 96 kDa polypeptide bands which were distinct for them. But these three polypeptide bands were absent in all the isolates used in the present study, eventhough these isolates were capable of producing abortion in their respective host.

Caprine isolate differed from the other two bovine isolates in the number of polypeptide bands, which may be due to the strain variation and the difference in host affected.

Polypeptide bands of 21 kDa to 23 kDa for all the three isolates were in conformation with those found in SDS-PAGE pattern by Markey *et al.* (1993) and the band at 41 kDa was in agreement with those in pattern detailed by Griffiths *et al.* (1992).

Even though protein profiling can be used for differentiating the strains, in the present study only two local isolates were obtained. So standardisation for strain classification cannot be done based on polypeptide pattern by SDS-PAGE.

SUMMARY

6. SUMMARY

Chlamydia psittaci, an important etiological agent associated with abortion, has caused significant economic loss in livestock production industry. In Kerala, there are reports on the prevalence of the organism as one of the causative agents of livestock abortion. The present study was undertaken to ascertain the extent to which *Chlamydia psittaci* is involved in abortions in livestock of Kerala. The parameters of the study comprised of isolation and identification of *Chlamydia psittaci* from cases of abortion in livestock; sensitivity of the agent to sodium sulphadiazine; the pathogenicity in mice and guinea pigs and the protein profile analysis.

A total of 46 clinical cases including animals from livestock farms and cases presented to Veterinary hospitals in and around Thrissur, were subjected to detailed examination. The clinical materials comprised of aborted foetuses, semen samples, uterine discharges, cervical swabs and placentae in case of bovines. In caprine they comprised of aborted foetuses and uterine discharges only, while from porcine

species, the samples were from cases of abortion and still birth.

Smears of the above clinical materials comprised of impression smears from cotyledons, liver, lungs and spleen and smears of stomach content of aborted foetus. They were stained using Giemsa, Modified Ziehl Neelsen and Gimenez stains. Elementary bodies could be revealed only in two cases, both of which were from aborted foetuses. One was from bovine foetal lung and liver, aborted at third trimester of gestation and the other from caprine foetal lung at second half of gestation. The bovine sample was designated as M-121 and the caprine as M-430.

For isolation of the organism, CE inoculation was adopted, by YS route. On inoculation of 36 suspected clinical samples, after ruling out bacterial contamination, only M-121 and M-430 revealed EBs in YS impression smears, by the first passage itself.

Death of the embryos occurred during seven to ten days PI for both M-121 and M-430 in all the three passages. Ninety per cent of these inoculated eggs revealed patchy haemorrhages on skin of the

embryos and YS of both dead and live embryos. An isolation percentage of 4.3 was obtained.

The embryo mortality pattern of the reference isolate (P-156), isolates M-121 and M-430 revealed a high mortality rate for M-430 in first three passages, compared to the other two samples. Over 75 per cent of dead CE revealed haemorrhagic YS.

With regard to sensitivity to sodium sulphadiazine, all the three isolates were resistant.

Mortality rates of 33.3 per cent, 16.7 per cent and 66.7 per cent were observed for P-156, M-121 and M-430 isolates in mice. Death occurred within a period of 12 to 15 days PI. Post-mortem examination of the dead mice revealed whitish stringy fibrinous exudate in peritoneal cavity and congestion of liver, spleen and lungs. Re-isolation of the agent was possible from dead and sacrificed mice using liver, lungs and spleen.

Only one guinea pig inoculated with M-430 died of chlamydial infection. Death occurred on 10^{th} day PI. Yellowish stringy fibrinous exudate in peritoneal

cavity and congestion of lungs and spleen were noticed in dead guinea pig on post-mortem examination. Re-isolation of the agent was possible from liver, lungs and peritoneal exudate of dead and sacrificed guinea pigs.

Identity of the local isolates (M-121 and M-430) were confirmed as *Chlamydia psittaci* by AGPT in comparison with reference isolate (P-156).

Coverslip cultures with P-156, M-121 and M-430, when stained with May-Grunwald Giemsa revealed rounding and swelling of fibroblast cells and syncytia formation at 24 h PI. By 48 h PI, the rounding and swelling of cells became prominent, with their retraction from adjacent cells and detachment from glass surface. Clumping of cells were prominent in cell lines infected with P-156 and M-430. By 72 h PI, most of the cells, infected with all the three isolates, got detached from glass surface.

Purified EBs for each isolate was prepared from infected cell culture harvest by urografin gradient centrifugation of the disrupted harvest. Elementary bodies were seen as a diffuse band at the interface of

40 and 50 per cent urografin gradients in T-KCl. This was re-suspended in 0.5 ml of T-KCl and the protein content estimated.

Estimation of protein content by biuret method for P-156, M-121 and M-430 harvests, revealed a protein concentration of 1099.75 mg/ 100 ml, 651.71 mg/ 100 ml and 549.88 mg/ 100 ml respectively.

On SDS-PAGE 12 bands were obtained for P-156 and M-121 while only 10 bands were observed for M-430. Most of the polypeptide bands of bovine isolates were common. Molecular weights of 148 kDa and 135 kDa were present only in P-156 and 152 kDa and 137 kDa were unique for M-121. Polypeptide bands of 152 kDa, 148 kDa, 137 kDa, 135 kDa, 32 kDa, 18 kDa, 15 kDa and 7 kDa were lacking in M-430, compared to bovine isolates. Bands of 155 kDa, 19 kDa, 12.2 kDa and 6.4 kDa were unique for M-430.

From the study the following conclusions were made.

1. *Chlamydia psittaci* could be isolated from 4.3 per cent of clinical cases associated with abortions,

indicating the importance of this organism as one of the etiological agents of livestock abortion.

2. The local as well as the reference isolates of *Chlamydia psittaci* were found to be resistant to sodium sulphadiazine

3. All the three isolates were of moderate virulence for both mice and guinea pigs.

4. On PAGE analysis, there was not much difference in the polypeptide pattern between the bovine isolates, though they differed much from the caprine isolate.

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*- Originals not consulted.

ISOLATION AND CHARACTERIZATION OF *Chlamydia psittaci* WITH EMPHASIS ON PROTEIN PROFILE

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ABSTRACT OF THE THESIS

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ABSTRACT

For isolation of *Chlamydia psittaci* 46 clinical cases associated with abortions in livestock from livestock farms and Veterinary hospitals in and around Thrissur were screened. Impression smear staining of the clinical materials using Giemsa, Modified Ziehl Neelsen and Gimenez revealed two positive cases. Both were from aborted foetuses, one from bovine (M-121) and the other from caprine (M-430).

Chick embryo inoculation by YS route was used for isolation of the organism. The organisms could be isolated only from the two cases positive by preliminary screening, as confirmed by staining of YS impression smears.

The mortality rate in CE was more in case of M-430 compared to the other two isolates (M-121 and reference isolate, P-156). All the three isolates produced patchy haemorrhagic lesions in YS and skin of embryos. An overall isolation percentage of 4.3 was obtained in this study.

All the three isolates were resistant to sodium sulphadiazine.

Pathogenicity studies indicated that, all the three isolates were of moderate virulence for mice and guinea pigs.

Based on AGPT, the local isolates were confirmed as *Chlamydia psittaci*.

All the three isolates caused rounding and swelling of fibroblast cells and syncytia formation at 24h PI in Mc Coy cell line. By 48 to 72 h PI, these effects became more prominent with detachment of cells from adjacent cells and from the glass surface.

Purified EBs were prepared from infected Mc Coy cell line by Urografin gradient centrifugation. After confirming the presence of sufficient amount of protein by Biuret method, they were used for SDS-PAGE. A total of 12 polypeptide bands were obtained for both the bovine isolates while the caprine isolate gave 10 bands. Molecular weights of 148 kDa and 135 kDa were present only in P-156 and 152 kDa and 137 kDa were unique for M-121. Bands at 152 kDa, 148 kDa, 137 kDa, 135 kDa, 32 kDa, 18 kDa, 15 kDa and 7 kDa were lacking in M-430. This isolate was having unique bands of 155 kDa, 19 kDa, 12.2 kDa and 6.4 kDa.