ASSESSMENT OF DIFFERENT EXPERIMENTAL VACCINES AGAINST Chlamydophila abortus (Chlamydia psittaci) IN RABBITS

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DECLARATION

I hereby declare that the thesis entitled **"ASSESSMENT OF DIFFERENT EXPERIMENTAL VACCINES AGAINST *Chlamydophila abortus* (*Chlamydia psittaci*) IN RABBITS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis entitled **"ASSESSMENT OF DIFFERENT EXPERIMENTAL VACCINES AGAINST *Chlamydophila abortus* (*Chlamydia psittaci*) IN RABBITS"** is a record of research work done independently by **Dr. Sanjeetha L.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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SANJEETHA

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Introduction

1. INTRODUCTION

The success of livestock industry depends on the proper management of animals, their breeding and the early diagnosis and control of diseases. Many of the infectious diseases affecting the animals and causing heavy economic losses have been successfully kept under control by adopting proper prophylactic measures. But health hazards due to various infectious diseases still exist as a major impediment interfering with attainment of anticipated output of animal production. Chlamydiosis is one such important disease entity among livestock.

Chlamydia psittaci (Chlamydophila abortus), an obligate intracellular bacterium, is a heterogenic group with a number of antigenically different serotypes which do not confer any cross immunity.

Chlamydia psittaci is associated with a wide spectrum of diseases in avian and mammalian hosts, including man. Infection with chlamydiae result in pneumonia, abortion, rhinitis, conjunctivitis, arthritis and enteritis. In domestic animals, abortion is the common pathological effect caused by *C. psittaci.*

Chlamydial infections in domestic animals are increasingly being recognized in India. Association of *C. psittaci* in the causation of abortion in sheep and goats has been reported from different parts of the country (Krishna *et al.*, 1988 and Batta *et al.*, 1997).

Prevalence of chlamydial agents among the livestock of Kerala had been reported for the first time by Francis (1988). *Chlamydia psittaci* has been isolated from two cases of abortion in goats and one in bovines at the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy. These isolates are being maintained in the Department. Limited studies on the protein and nucleic acid profile of the isolates revealed variation amongst them.

Chlamydial infections can be treated effectively with antibiotics, but once the infection and pathology are established treatment may be less effective. A combination of flock management, screening and vaccination is needed to control such infections.

No commercial vaccine against chlamydiosis is available in India. Vaccines used in other countries do not contain all the serotypes. Infected yolk sac as such or elementary bodies from infected yolk sac or cell culture is used as seed material for the preparation of vaccines.

In the present study an attempt was made to prepare and assess the experimental vaccines against *Chlamydia*, incorporating the local isolate (M-430) obtained from a case of caprine abortion.

The parameters included in the study are,

- preparation and standardization of inactivated yolk sac and elementary body vaccines.
- immunization of rabbits
- assessment of immunity by challenge experiment
- monitoring of humoral immune response by passive haemagglutination and serum neutralization tests.

Review of Literature

2. REVIEW OF LITERATURE

Chlamydiae are a group of obligate intracellular gram negative bacteria that undergo a well defined life cycle in the cytoplasm of the host cell. This group of organisms are extremely wide spread in nature in wide spectrum of hosts.

2.1 TAXONOMIC STATUS

Halberstaedter and von Prowazek (1907) observed intracytoplasmic inclusions of *Chlamydia trachomatis* in the conjunctival scrapings from a human patient with trachoma. They thought the agent to be protozoa and conferred the name *Chlamydozoaceae.*

The classification and nomenclature of the chlamydiae were unsettled for long and these organisms have been referred to as *Miyagawanella*, *Bedsonia* and *psittacosis – lymphogranuloma – trachoma* agent.

Page (1966) proposed the generally accepted description of the genus *Chlamydia*.

Storz and Page (1971) grouped *Chlamydia* in a separate eubacterial order *Chlamydiales* as it differ from other gram negative bacteria by their morphology, developmental cycle and common group antigen. This order contains the family *Chlamydozoaceae.*

The family *Chlamydozoaceae* consists of a single genus C*hlamydia* with two species *C. psittaci* and *C. trachomatis* (Moulder *et al.*, 1984).

Within the genus *Chlamydia*, a diverse group of isolates comprise the species *C. psittaci.* DNA re-association studies showed divergence between the genomes of various types of *C. psittaci* to divide the group into atleast four separate species (Cox *et al.*, 1988).

Grayston *et al.* (1989) proposed a third species of C*hlamydia, C. pneumonia* which is a human pathogen.

The genus *Chlamydia* is currently divided into four species, *Chlamydia psittaci, C. trachomatis, C. pneumoniae* and the most recently classified *C. pecorum.* Both *C. pneumoniae* and *C. pecorum* were originally classified as strains of *C. psittaci* (Fukushi and Hirai, 1992).

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

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

2.2 ISOLATION OF CHLAMYDIA

Chlamydiae are energy dependent intracellular parasites and their isolation can be attempted using living systems like chicken embryo, small laboratory animals (mice and guinea pigs) and cell cultures/cell lines.

2.2.1 Isolation Using Chicken Embryo

Rake *et al* (1940) opined chicken embryos to be the most uniform and favourable medium for the cultivation of chlamydial agents.

Tang *et al.* (1957) reported chicken embryos as an important host for isolation and propagation of chlamydiae.

Page (1971) observed that growth rate of *C. psittaci* strains were increased, when infected chicken embryos were incubated at 39°C or 41°C than the usual incubation temperature of 37°C.

Sharma *et al.* (1982) reported that the chlamydial isolates from genital tract of repeat breeding cattle and buffaloe, were pathogenic to chicken embryos. The infected yolk sac was thin walled and deeply congested. Yolk was more liquid and had bright yellow colour. Embryos had stunted growth.

Francis (1988) observed that the embryos which died of chlamydial infection were hyperaemic and had cyanotic legs and toes.

Nanda *et al.* (1992) noticed 60-80 per cent mortality on third to eight days post inoculation (PI) of the embryos. The dead embryos showed haemorrhage and congestion, particularly on occipital region and legs.

Storz and Kaltenboeck (1993) reported that some chlamydial strains were adapted to multiply in the cells of chorio-allantoic cavity and release elementary bodies (EBs) into the allantoic fluid for easy harvest and purification.

Batta *et al.* (1997) isolated *C. psittaci* from a nasal swab of pig and 14 pneumonic lungs of goats, calves, sheep and buffaloe. Isolation was made in six to eight day-old chicken embryos. All the isolates revealed massive congestion of yolk sac membrane. All these isolates induced death of embryos within four to eight days after inoculation, except the goat pneumonic isolates that were more virulent and resulted in embryonic death from two to six days PI.

Ward and Ridgway (1998) observed that inoculation of seven to eight day-old embryos with chlamydial isolates led to their death from day four of incubation at 35°C.

Mani (2001) observed patchy haemorrhagic areas in the infected yolk sac. The consistency of yolk was thin. Dead and live embryos showed haemorrhagic patches on the skin.

Nair (2001) found that the chlamydial isolate from the liver of an aborted caprine foetus killed the inoculated embryos within five to seven days post inoculation. The yolk sac was thin walled with deeply injected blood vessels and embryo showed haemorragic patches on the skin all over the body.

2.2.2 Isolation in Laboratory Animals

Animal models are widely used for studying the pathogenesis and immunology of chlamydial infections. Some laboratory animals and birds are subject to natural infection. This must be kept in mind during investigations.

2.2.2.1 Isolation in Guinea Pigs

Cottral (1978) found guinea pigs as the laboratory animals of choice for the isolation of bovine and ovine chlamydiae. He also found that guinea pigs were more susceptible to the growth of chlamydiae than chicken embryos, when the numbers of chlamydia were small.

Senyk *et al.* (1981) studied cell mediated immune response and humoral response to chlamydial antigens in guinea pigs infected with the agent of guinea pig inclusion conjunctivitis.

Purohit *et al.* (1986) identified and characterised *Chlamydia psittaci* from abortions in goats, based on pathogenicity in chicken embryo and guinea pigs, staining reaction and complement fixation test.

Francis (1988) used guinea pigs aged four to six weeks for the primary isolation of chlamydiae from clinical specimens. Death of the animals was found to occur in 12 to 15 days after inoculation of the infective materials.

Asrani *et al.* (1997) studied the difference between isolates of *C. psittaci* from abortion in sheep and goat and pneumonia in goats, based on their pathogenic potential in guinea pigs. The abortion isolate induced slight enlargement of spleen, while there was significant splenic enlargement by goat pneumonia isolate.

Ward and Ridgway (1998) reported the possibility of natural infection of guinea pig with *C. psittaci* agent causing mild conjunctivitis. The chance of latent infection and presence of its antibody must be considered when these are used experimentally.

Mani (2001) studied differences among isolates of *Chlamydia psittaci*, from cases of abortion in cattle and goats, for their pathogenic potential in mice and guinea pigs. It was found that the organisms were less pathogenic to guinea pigs when compared to mice.

2.2.2.1 Isolation in Mice

Strains of *C. psittaci* from birds could be isolated and propagated in young laboratory mice. Strains causing lymplogranuloma venerum in human beings and severe enteritis in mammals could also be propagated in mice (Cottral, 1978).

On comparing an abortion isolate with an intestinal isolate Buzoni-Gatel and Rodolakis (1983) found that the abortion isolate was more virulent than the intestinal one, on the basis of the spleen weight to body weight ratio and re-isolation results.

Rodolakis (1983) estimated the virulence of two temperature sensitive *(ts)* mutant strains of *Chlamydia psittaci,* following intra peritoneal inoculation of pregnant mice, by the number of living offspring per litter and its survival which was monitored daily for eight days. The two *ts* mutant strains induced intrauterine mortality.

Anderson (1986) observed that among the five ovine chlamydial isolates, four isolates from abortion were more virulent to mice than an isolate from arthritis. Food pad inoculation of the abortion isolates produced significant splenic enlargement.

Francis (1988) observed that the inoculation of the infected yolk sac materials, passaged twice in chicken embryo, into mice aged three to four weeks produced 25 to 50 per cent mortality within 10 to 15 days.

Rodolakis *et al.* (1989) studied the virulence of chlamydial strains from disease process and intestinal strains, in mice. Non pregnant mice inoculated subcutaneously in the food pad with pathogenic strain showed an increase in splenic weight. Pregnant mice were inoculated intravenously to establish their ability to colonize the placenta and invade the foetus. The non-invasive strains colonized the placenta less than did the invasive strains.

Biolatti *et al.* (1991) observed that the mice infected orally with an isolate of *C. psittaci* showed clinical signs 46 h after infection. They had rough hair coat and stood in a corner of the cage without feeding. No differences could be noticed between the responses of mice infected with undiluted and diluted inoculum.

Rodolakis and Souriau (1992) differentiated the abortion strains of chlamydia from intestinal strains, based on their virulence in mice. Mouse invasive strains alone were recovered from the spleen following subcutaneous inoculation.

Mice could be infected by the intranasal, intraperitoneal, intracerebral routes and via the genital tract. Intravenous inoculation was not satisfactory as it resulted in rapid toxin mediated death or death within days from infection before the establishment of infection (Ward and Ridgway, 1998).

Mani (2001) studied the pathogenicity of *Chlamydia psittaci* isolates in mice and found them to be moderately virulent. Post mortem examination of dead mice revealed whitish stringy fibrinous exudate in peritoneum. Liver, lung and spleen were severely congested.

2.2.3 Isolation in Cell Culture/ Cell Line

Chlamydia can be grown in a wide range of animal derived cell lines/cell cultures. Different cell lines have been reported to be efficient, economical and quick tools for the isolation and identification of chlamydiae.

Piraino (1969) tested plaque forming ability of 15 chlamydial isolates (13 *C. psittaci* strains and two *C. trachomatis* strains) on chicken embryo fibroblast cells. Among these, twelve *C. psittaci* strain formed plaques. *Chlamydia trachomatis* and one *C. psittaci* strain did not form plaques. Plaque size was found to be related to virulence of chlamydia for laboratory animals.

Hobson *et al* (1977) re-isolated an egg adapted laboratory strain of enzootic abortion agent and propagated it serially in monolayer cultures of Mc Coy cells. The infectivity in tissue culture was enhanced by centrifugation and the titers were atleast 40 times higher than *in vivo*. Increase in the number and size of the chlamydial inclusions could be obtained on addition of cycloheximide.

Cottral (1978) reported that chlamydial strains recovered from birds were adapted to grow well in primary cultures of chicken embryo or in cell lines derived from mice and human beings. Strains of chlamydiae from domestic animals multiplied only to a limited extent in cell cultures.

Johnson *et al.* (1978) found that dark-ground microscopy of monolayers stained with methylene blue was quicker and allowed precise identification of chlamydial inclusions than the light microscopy of Giemsa stained preparations.

Spears and Storz (1979) grouped the 29 strains of *C. psittaci* from various sources into eight biotypes. This was based on the morphology of inclusions in

the mouse fibroblast cells, the effect of chlamydial multiplication on the host cell cytoskeleton and change in the number of cells infected in response to diethyl-amino ethyl-dextran and cycloheximide.

Page (1981) reported that both L-929 and Mc Coy cell line had rapidly growing cells that supported growth of most chlamydial strains.

Dennis and Storz (1982) studied the infectivity of two strains of *C. psittaci* of mammalian origin in mouse L-cells. Infectivity was enhanced by centrifugation of the inoculum onto cell monolayer. Infectivity was higher in cultures centrifuged at 37°C, than at room temperature.

Allan and Pearce (1983) assessed the effects of omission of individual amino acids from growth medium on the multiplication of *C. trachomatis* and *C. psittaci* strains in cycloheximide treated Mc Coy cells. All Chlamydiae required the addition of valine to medium and majority required leucine, phenylalanine and glutamine.

Anderson and Baxter (1986) studied the four ovine chlamydial isolates and one arthritis isolate in Baby Hamster kidney cells (BHK-21). Abortion isolates produced large compact and dark staining cytoplasmic inclusions which appeared after 48 h. The arthritis isolate formed inclusions at 24 h and were diffuse and light staining.

Wills *et al.* (1986) used a commercially available ELISA kit designed for the detection of *C. trachomatis* in human urogenital specimens for detection of *C.psittaci* in cat conjuctival swabs. They compared it with cell culture and found cell culture to be more sensitive than the ELISA.

Anderson (1987) used BHK-21 cells pretreated with 5-iodo-2 deoxy uridine for three days, for isolation of chlamydia and enhancement of infectivity of cells was noticed.

Rodolakis *et al.* (1989) compared the ability of chlamydial strains to induce persistent infections in Mc Coy cells. Mc Coy cells were persistently infected by both virulent and avirulent strains. Avirulent stains induced completely inapparent persistent infection.

Wills *et al.* (1990) isolated a strain of *C. psittaci* from a horse, in cycloheximide treated Mc Coy cell monolayers. This strain of *C. psittaci* grew relatively slow in cycloheximide treated Mc Coy cells and the yield of elementary bodies was relatively low.

Dhingra and Mahajan (1992) used neonatal buffalo calf serum to supplement foetal calf serum, in growth media for *C.psittaci.* Studies carried out revealed that neither of the sera gave satisfactory infectivity.

Griffiths *et al.* (1992) distinguished ovine abortion and intestinal isolate by inclusion development and morphology in cell culture. In Mc Coy cell line, ovine abortion isolate developed compact, deeply staining inclusions, whereas intestinal isolate produced diffuse, less deeply staining inclusions, when stained with methylene blue. The intestinal isolate developed more rapidly in culture, than the ovine isolate.

Philip and Clarkson (1992) found a rapid method for distinguishing abortion and enteric isolates, based on their growth in sheep fibroblast cell culture derived from the small intestine of a lamb. Abortion isolates produced large inclusions whereas enteric isolates produced sparse inclusions.

Amin and Wilsmore (1997) studied the effects of crude placental extract and erythritol on growth of *C. psittaci* in cell culture. No significant differences in the number of inclusions were noticed.

Nagal *et al.* (1997) infected the Mc Coy cell lines with chicken embryo adapted *C. psittaci* isolates from kid pneumonia, kid enteritis, sheep abortion and

goat abortion. Mc Coy cells could not support the replication of goat abortion isolate.

Eventhough chlamydiae could be grown in a wide range of animal derived cell lines, *Chlamydia pneumoniae* could not be cultivated easily in animal derived cell lines. For this, human fibroblast cells or Hep2 cells were used (Ward and Ridgway, 1998).

2.2.3.1 *Confirmation of Infection of Monolayer by Immunofluorescence*

Cottral (1978) reported that the complement fixation test and direct and indirect fluorescent antibody test could be used to detect chlamydial antigens and thus to identify the organism. The fluorescent antibody technique (FAT) was more reliable with infected cell cultures than with impression smears or tissues.

Farmer *et al.* (1982) employed immunofluorescence staining for detecting inclusion bodies in Mc Coy cell monolayers infected with *C. psittaci*, isolated from the eyes of domestic ducks with conjunctivitis and rhinitis.

Timms *et al.* (1988) compared DNA spot hybridization, cell culture and direct immunofluorescence staining for the detection of avian *C. psittaci* strains in cell culture dilutions and in routine samples. The direct immunofluorescence staining was found to be the test of choice for routine diagnosis.

Griffiths *et al.* (1992) studied the ewe placental and lamb intestinal isolates of *C. psittaci*, recovered from flocks affected with ovine enzootic abortion, by inclusion morphology, indirect immunofluorescence and immunoblot analysis. The intestinal and abortion isolates were distinguished in cell culture by indirect immunofluorescence.

Nagal *et al.* (1997) employed the indirect immunofluorescence with group specific monoclonal antibody for the detection of intracytoplasmic inclusions in the cell culture.

2.3 DETECTION OF ANTIBODY BY SEROLOGICAL TESTS

Diagnosis of chlamydial infections of domestic animals is a difficult task. Clinical signs associated with the different chlamydia induced diseases are not specific for an accurate diagnosis. The diagnosis of chlamydial infections in cattle, sheep and goat is based on serological tests. Presence of group specific antibodies in the sera of animals is an indication of the presence of chlamydial infection in the population.

Complement fixation test (CFT) is the most commonly used serological test. Alternative tests based on immunoflourescence (IF), Enzyme Linked Immunosorbent Assay (ELISA) are also used.

2.3.1 Complement Fixation Test (CFT)

Bassan and Ayalon (1971) observed that the complement fixation titres appeared and disappeared irregularly and the levels of the titres were also irregular, indicating that the complement fixation reaction was valuable in the diagnosis of epizootic bovine abortion as a herd test.

Storz (1971) reported CFT as a relatively reliable test to detect the group specific chlamydial antigen isolated from clinical specimen or to detect group specific antibodies in the serum.

Cottral (1978) found that the standard CFT could be used for detection of antibodies in sera from mammals. Indirect complement fixation test was recommended for sera from birds since avian sera did not fix guinea pig complement.

Purohit and Gupta (1983) employed CFT for screening serum samples from sheep and goat slaughtered for food purpose. A complement fixation titre of 1:8 and above was detected in the sera of 32 sheep and 27 goats.

Sharma and Baxi (1983) assessed chlamydial antibodies in serum samples from cattle and buffaloe at two farms in Punjab, by CFT. An incidence of 14.4 and 11.8 per cent was recorded in the cattle and buffalo sera respectively.

Tongaonkar *et al.* (1984) employed CFT for a serosurvey of chlamydial infection in milch animals of Gujarat.

Milon *et al.* (1985) conducted serological diagnosis of ovine chlamydiosis using three techniques, ELISA, IF and CFT and found CFT as the least sensitive test.

Perez – Martinez *et al.* (1986) used modified complement fixation test (MoCFT) to detect antibodies in sera from 11 cows experimentally infected with abortigenic strain of *C. psittaci*. The sensitivity of the test was notably improved by adding the bovine complement.

Huang *et al.* (1990) studied the lymphnode antibody response in sheep to experimental infection with an ovine abortion strain of *C. psittaci*, by the complement fixation and immunofluorescence technique. The tests showed a rise in titre that peak approximately two weeks after infection.

Nanda *et al.* (1992) employed Gimenez staining, CFT, fluorescent antibody technique, chlamydial isolation and histopathology, for diagnosis of chlamydial abortion. It was found that CFT coupled with histopathology was superior to Gimenez staining and FAT.

2.3.2 Enzyme Linked Immunosorbent Assay (ELISA)

Perez-Martinez *et al*. (1986) tested serum samples from 11 cows experimentally inoculated with different abortigenic strains of *C. psittaci*, by a modified complement fixation test (MoCFT), indirect inclusion fluorescence antibody (IIFA) test and ELISA. The IIFA test and ELISA detected larger number of seropositive cows than MoCFT and were easy to perform and interpret.

Souriau and Rodolakis (1986) developed an ELISA for the detection of *C. psittaci* in vaginal swabs of aborted ewes and goats. This was compared to direct isolation of the agent by plaque assay on Mc Coy cells. The ELISA technique permitted the detection of chlamydia even in the absence of special care in sampling and conservation of specimen.

Anderson *et al.* (1995) developed an indirect enzyme linked immunosorbent assay for detection of antibodies against abortion strains of *C. psittaci*.

Mohanty *et al.* (1996) employed an antigen capture enzyme immunoassay (EIA) for detection of chlamydia antigen. The test was evaluated against a direct immunofluorescence assay. The sensitivity and specificity of EIA was found to be 86.36 and 91.66 per cent respectively against the reference direct immunofluorescence assay.

2.3.3 Immunofluorescence (IF)

Anderson (1987) compared four ovine abortion isolates and an isolate from a case of lamb arthritis by indirect immunofluorescence, using antisera raised in chickens and mice. All the four abortion isolates gave homologous reaction with both antisera, but heterologous reaction with arthritis isolate.

Krishna *et al.* (1988) investigated and diagnosed an outbreak of chlamydial pneumonia in Gaddi goats in Himachal Pradesh, on the basis of clinicopathological, exfoliative cytological, pathological and immunofluorescent studies. Group specific chlamydial immunofluorescent antigen was detected by FAT.

Biolatti *et al.* (1991) detected chlamydial antigens in formalin fixed paraffin wax embedded samples by an immunofluorescent test based on monoclonal antibody.

Markey *et al.* (1993) compared ELISA with CFT and the indirect immunofluorescence test for the detection of antibodies to *C. psittaci* in sheep and found indirect immunofluorescence as the more discerning test.

Batta *et al.* (1996) used indirect immunofluorescence method, employing monoclonal antibodies against group specific antigen, for detection of *C. psittaci* in impression smears of lung tissue from sheep and goat. Efficacy of this test was compared with conventional isolation technique in chicken embryo and found the indirect immunofluorescence to be more specific.

2.3.4 Passive Haemagglutination (PHA) Test

For the assay of fluids containing small quantities of antibody, sensitive methods of antibody detection are required. One such test is the indirect or passive haemagglutination test.

Lewis *et al.* (1972) performed the PHA using tanned sheep erythrocytes sensitized with a deoxycholate extract of *C. psittaci*. They found that PHA was more sensitive than CFT.

Belden and Mc Kercher (1973) studied the feasibility of using PHA test for serodiagnosis of bovine chlamydial abortion. The test was found to be four times more specific than the CFT and it was found to be species specific also.

The lower limit of antibody detectable by the CFT is 0.01 to 1.0 µg/ml of test fluids, whereas the same for PHA is as low as 0.005 µg/ml (Carpenter, 1975).

Garvey *et al.* (1979) opined that the concentration of red blood cells to be sensitized with antigen in PHA test must be standardized to get reproducible results. When the concentration of cells was halved, antibody titre was doubled.

Yang *et al.* (1984) carried out the indirect haemagglutination test using sheep erythrocytes sensitized with ether extracted antigen. In cross reaction

experiments they could prove the specificity of the reaction. The titres obtained were eight to sixteen times higher than that of CFT.

Francis (1988) studied the seroprevalance of chlamydiosis in livestock of Kerala by PHA and obtained prevalence rate of 22.8 and 19.4 per cent in bovine and caprine respectively.

2.3.4 Serum Neutralization Test (SNT)

Neutralization test estimate the ability of antibody to neutralize the biological activity of antigen when they are mixed *in vitro*. This test is highly specific and sensitive.

Mc Ewen and Foggie (1956) estimated antibody in the sera of vaccinated sheep by SNT and CFT. It was found that complement fixing and neutralizing antibodies persisted for three years and probably for longer period.

Cottral (1978) opined that neutralization test could be done in chicken embryos, cell cultures or mice which are protected by the inhibiting effect of the specific antiserum. Success of the test depends upon the use of high titered antisera.

Caldwell and Perry (1982) found that rabbit immunoglobulin antibodies raised against the major outer membrane protein of the *C. trachomatis lymphogranuloma venereum* strain 434 neutralized the infectivity of chlamydia for human epithelial (HeLa 229) cells.

Su and Caldwell (1991) reported that monovalent Fab antibodies to serovar and subspecies specific epitopes of the major outer membrane protein of *C.trachomatis* neutralized infectivity for hamster kidney cells by preventing chlamydial attachment.

Su *et al.* (1991) studied the neutralizing activities of murine immunoglobulin G_3 monoclonal antibody specific for the major outer membrane

protein of *C. trachomatis* and its monovalent Fab fragments, using Syrian hamster kidney (HaK) cells and HeLa 229 cells. Intact IgG_3 was neutralizing for HaK but not for HeLa cells. Monovalent Fab antibody fragment neutralized chlamydial infectivity for both cells.

Peeling and Brunham (1991) used monoclonal antibodies to the major outer-membrane protein of *C. trachomatis* to neutralize its infectivity in HeLa 229 cells. The neutralization of *C. trachomatis* infectivity proceeded as a first order reaction. The rate of neutralization was linear with respect to antibody concentration and reaction temperature.

Byrne *et al.* (1993) evaluated an *in vitro* antibody-mediated chlamydial neutralization, assay for its utility as a method to assess functional correlates of antibody responses to *C. trachomatis*.

2.4 VACCINE TRIAL

Mc Ewen and Foggie (1956) reported that a single or double injection of an adjuvanated vaccine, prepared from formalinised infected ovine foetal membrane tissues precipitated by alum and emulsified in mineral oil, stimulated the appearance of complement fixing and virus neutralizing antibodies in the sera of sheep.

Frank *et al.* (1968) tested the efficacy of a commercial enzootic abortion vaccine against a strain of the *psittacosis-lymphogranuloma – venereum trachoma* organism which caused natural disease in Idaho sheep. Incidence of abortion and weak lambs which died soon after birth was less among vaccinated ewes. Vaccine induced substantial protection against infection as well as clinical disease.

Meinershagen *et al.* (1971) reported significant reduction of abortion in ewes vaccinated with bacterins containing *Vibrio foetus* and *Chlamydia* species,

after challenge inoculation with virulent chlamydial agent or live cultures of *Vibrio foetus*.

Sorodoc *et al.* (1979) used a vaccine against chlamydial ovine abortion, to more than 5,00,000 animals in a large breeding unit. The vaccine induced a considerable seroconversion and reduced the damage due to chlamydiosis by three to five times. Vaccination of primiparous ewes followed by two to three annual revaccination conferred resistance to the vaccinated animals.

Waldhalm *et al.* (1982) compared the efficacy of a bacterin prepared from *C. psittaci* grown in mouse L-cells, to a similar bacterin prepared from chicken embryos. Both bacterins significantly reduced incidence of abortion and weak lambs, compared to non vaccinated control ewes. The L-cell bacterin elicited a greater antibody response than the chicken embryo bacterin.

Popov and Martinov (1983) prepared a formalin inactivated concentrated depot – vaccine against chlamydial abortion of sheep. Efficacy of the vaccine was tested in guinea pigs. Guinea pigs immunized singly responded with complement fixing antibodies on the 30^{th} day, with titres ranging from 1:32 to 1:64. Second immunization on the 30^{th} day led to the production of antibodies with titres ranging from 1:256 to 1:512. This vaccine was characterized by low cost and safety.

Martinov and Popov (1985) immunized sheep against chlamydial abortion with a purified, concentrated and adsorbed vaccine. No abortion, still births and birth of under developed lambs were seen.

Souriau *et al.* (1988) tested different combinations of live brucella, chlamydia and salmonella vaccines using potency control models in mice. The efficacies of the salmonella and brucella vaccines were not affected by combinations, but efficacy of chlamydia vaccine was decreased when used in combination with brucella vaccine.

Anderson *et al.* (1990) reported that a vaccine prepared from purified, inactivated elementary bodies of *C. psittaci* protected sheep against abortion, after subcutaneous challenge with live chlamydiae. Immunoblot analysis of serum samples revealed a consistently dominant antibody response against the chlamydial major outer membrane protein, in all vaccinated sheep.

Wilsmore *et al.* (1990) studied the protection afforded by an experimental, killed, adjuvanated vaccine derived from the A-22 strain of *C. psittaci*. The vaccine was used undiluted (Group A), at a dilution of 10^{-3} (Group B) and at a dilution of 10^{-6} (Group C). A fourth group (Group D) was kept as control. The experimental vaccines protected the ewes in groups A and B against challenge with *C. psittaci*.

Gajdosova *et al.* (1994) evaluated the immunogenicity of vaccine against enzootic abortion of ewes in sheep and white mice. The vaccine contained purified and formalin inactivated corpuscles of *C. psittaci.* The antibody response was measured by complement fixation reaction and ELISA. Immunization of mice with one dose of vaccine significantly reduced multiplication of *C. psittaci* in the lungs of mice.

Jones *et al.* (1995) prepared vaccines containing inactivated semi purified antigen of abortifacient, *C. psittaci* from tissue culture grown harvest of two strains. These were evaluated for efficacy against experimentally reproduced enzootic abortion of ewes.

Chalmers *et al.* (1997) prepared a lyophilised chlamydial vaccine from the temperature sensitive strain of ovine *C. psittaci.* Ewes inoculated with the low titre of the live vaccine four weeks before artificial insemination were challenged on day seventy of gestation. Chlamydial abortion rate was significantly lowered in vaccinated group than in unvaccinated group. The vaccine also reduced the number of infected ewes and severity of the infection.

Caro *et al.* (2001) studied immune mechanisms of chlamydial infections using a mouse model, to evaluate the protection conferred by four inactivated vaccines, and one attenuated vaccine. The results obtained were compared with an experimental inactivated vaccine. The four inactivated vaccines promoted a residual protection in flocks if the *C. abortus* burden was not very high. A good degree of protection was conferred by the experimental vaccine.

Materials and Methods

3. MATERIALS AND METHODS

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

3.1 REVIVAL OF THE ISOLATES

3.1.1 Chicken Embryo Inoculation

3.1.1.1 Materials

(i) Two isolates of *C. psittaci* were used in the study. One was from the lung of an aborted caprine foetus (M-430) and the other from liver of an aborted caprine foetus (M-28). They were maintained in the Department of Microbiology as infected yolk sac membranes at -70ºC.

(ii) Embryonated chicken eggs (CE)

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

3.1.1.2 Method

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

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

3.1.2 Harvesting of Inoculated CE

3.1.2.1 Collection of YS

3.1.2.1.1 Materials

(i) Inoculated CE

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

Sodium chloride	-	8 g
Potassium chloride	-	0.2 g
Potassium dihydrogen phosphate anhydrous	-	0.02 g
Disodium hydrogen orthophosphate	-	1.15 g
Triple distilled water	-	1000 ml

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

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

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

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

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

3.1.2.1.2 Method

The egg shell was disinfected with 70 per cent ethyl alcohol. It was opened by cutting and removing the shell over the air sac region using sterile scissors and forceps. The shell membrane and the chorioallantoic membrane in the area of the air sac were removed. With a pair of sterile forceps, the inner contents were emptied into a sterile Petri- dish. The YS and the embryo were washed in sterile PBS (pH 7.2). Small pieces of YS blotted dry on a blotting paper were used to prepare impression smears on clean grease free slides.

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

3.2 STAINING OF INFECTED YS

3.2.1 Giemsa Staining

3.2.1.1 Materials

(i) Impression smears from YS

(ii) Giemsa stain

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

3.2.1.2 Method

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

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

3.2.2 Modified Ziehl-Neelsen Staining (MZN)

3.2.2.1 Materials

(i) Impression smears from YS

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

(iii) Acetic acid (0.5 per cent)

(iv) Loeffler's alkaline methylene blue

3.2.2.2 Method

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

3.2.3 Gimenez Staining

3.2.3.1 Materials

(i) Impression smears from YS

(ii) Primary stain

Stock solution

Solution I

Basic fuchsin - 10 g

95% ethanol - 100 ml

Solution II

Phenol crystals - 10 g

Triple distilled water - 250 ml

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

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

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

Working solution

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

(iv) Counter stain

Malachite green	-	800 mg
Triple distilled water	-	100 ml

3.2.3.2 Method

The smears prepared on clean grease free glass slides were fixed over flame. The slides were flooded with filtered working solution of basic fuchsin for 15 min and washed with water. Counter stained with malachite green for three minutes. Washed the slides with water, dried and examined under oil immersion.

3.3 GRADING OF INFECTED YS

3.3.1 Materials

Stained YS impression smears

3.3.2 Method

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

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

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

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

3.4 PROPAGATION OF CHLAMYDIAL ISOLATES IN Mc COY CELL LINE

3.4.1 Maintenance of Cell Line

3.4.1.1 Materials

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

(ii) Eagles minimum essential medium (MEM)

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

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

(iv) CMF- PBS (0.15 M, pH 7.2)

(v) A stock solution of five per cent trypsin was prepared in CMF-PBS, sterilized by filtration through millipore filter (0.2 µm), distributed in small aliquotes and stored at –20°C. When needed the working solution was prepared by diluting the stock solution with CMF-PBS to give a final concentration of 0.25 per cent.

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

(vii) Neonatal calf serum (collected aseptically from colostrum deprived male calves maintained in University Livestock farm, Mannuthy).

(viii) Cell culture growth medium: Eagles MEM containing 12 per cent foetal calf serum was prepared and pH was adjusted to 7.2 with 7.5 per cent sodium bicarbonate.

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

3.4.1.2 Method

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

3.4.2 Inoculation of Cell Line

3.4.2.1 Materials

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

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

(iii) SPG [3.1.2.1.1. (iii)]

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

(v) Sterile glass beads

3.4.2.2 Method

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

Twenty per cent suspension of heavily infected YS (3+) in SPG was prepared. Centrifuged at 2600 x g for 15 min. The sediment and fat layers were discarded and the middle layer was collected. It was diluted with maintenance medium at the rate of 1:100. This was used as inoculum. The monolayer was inoculated with three milliliters of inoculum. Incubated at 37°C for one hour to facilitate adsorption. The inoculum was then poured off. The monolayer was washed with maintenance medium and added maintenance medium containing cycloheximide at a concentration of 0.1 milliliter per 100 ml at the rate of eight milliliter per bottle. Control culture bottles were prepared simultaneously in which instead of inoculum maintenance media was added.

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

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

3.4.3 Staining of Cover Slip Cultures

3.4.3.1 May-Grunwald Giemsa Staining

3.4.3.1.1 Materials

(i) Cover slip cultures

(ii) May – Grunwald stain

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

(iii) Giemsa stain

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

(iv) Methanol

(v) Acetone

(vi) Xylene

(vii) DPX mountant

3.4.3.1.2 Method

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

3.4.3.2 Indirect Immunofluorescence Technique

3.4.3.2.1 Materials

(i) Cover slip cultures

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

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

(iv) PBS (0.15 M, pH 7.2)[3.1.2.1.1(ii)]

(v) Glycerol saline

Glycerol	-	50 ml
Physiological saline (0.85 per cent)	-	50 ml

Mixed and sterilized by autoclaving at 15 lbs pressure and 115°C for 15 min.

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

3.4.3.2.2 Method

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

saline to demonstrate chlamydial inclusion bodies. Uninfected cover slips were also processed as described above and studied in detail.

3.5 PREPARATION OF VACCINES

Chalamydial isolate (M-430) was chosen for preparation of vaccine. The heavily infected yolk sacs as such, and partially purified elementary bodies were used for the preparation of vaccine.

3.5.1 Yolk Sac (YS) Vaccine

3.5.1.1 Materials

(i) Heavily infected yolk sac membranes

(ii) Ten-broeack tissue grinder

(iii) Sterile silica gel

(iv) PBS (0.15M pH 7.6)

(v) Formaldehyde 40 per cent

3.5.1.2 Method

Hundred heavily infected yolk sac membranes were selected and they were chopped and ground completely in a Ten-broeack tissue grinder with sterile silica gel. The ground membrane was suspended in phosphate buffered saline, pH 7.6, to a final concentration of 20 per cent. The suspension was centrifuged at a low speed to remove the crude tissue debris and silica gel if any. The supernatant fluid was again centrifuged at 10,000 x g for one hour at 4°C. The pellet was resuspended in small volume of buffered saline and a thin smear was prepared from the suspension and stained by Gimenez technique to ensure the high yield of chlamydia. The heavily suspended chlamydial organisms formed the seed material for the preparation of vaccine.

The seed material was treated with formalin to a final concentration of 0.4 per cent and stored for one week at 37ºC.

3.5.2 Elementary Body Vaccine (EB vaccine)

3.5.2.1 Preparation of Purified Elementary Bodies from Infected Mc Coy Cell Line

3.5.2.1.1 Materials

(i) Mc Coy cell line infected with *C. psittaci* isolate M-430

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

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

Tris - 20 mM

KCl - 150 mM

Triple distilled water - 100 ml

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

3.5.2.1.2 Method

Chlamydia psittaci were harvested from Mc Coy cell line 96 h post inoculation (PI), when sufficient numbers of inclusions or CPE were seen. Cells were detached from culture bottles using sterile glass beads and the suspension was collected. Ten culture bottles were used and 10 ml of the cell suspension obtained per bottle was pooled and homogenized. The suspension obtained was centrifuged at 3500 x g for five minutes at 4°C to remove the cell debris. The supernatant were layered on 30 per cent (v/v) urografin-76 diluted with T-KCl and centrifuged at 65000 x g for one hour at 4°C. The pellet was resuspended in one milliliter of T-KCl. It was layered on 30 to 60 per cent (v/v) urografin-76 gradients in T-KCl and centrifuged at 65000 x g for two hours at 4°C. The diffuse band seen in between 40 and 50 per cent gradient was collected, diluted with three milliliter of T-KCl and centrifuged at 65000 x g for one hour at 4°C. The pellet obtained was resuspended in 0.2 milliliter T-KCl and stored at –70°C. This formed the seed material for the preparation of vaccine. The purity of the suspension was checked by staining with Gimenez method.

3.5.2.2 Inactivation of the Purified Elementary Body

3.5.2.2.1 Materials

(i) Formalin (40 per cent)

(ii) Purified EB

3.5.2.2.2 Method

The purified elementary body was treated with formalin to a final concentration of 0.4 per cent and was stored for one week at 37°C and then tested for sterility.

3.5.3 Sterility and Toxicity Test

3.5.3.1 Sterility Test

A quantity of 0.2 ml each of the two vaccines were separately inoculated on to blood agar (BA), Trypticase Soya agar (TSA) and on to Sabouraud's Dextrose agar (SDA) plates. BA and TSA plates were incubated under aerobic and increased CO_2 tension at 37°C. Sabouraud's Dextrose agar plates were incubated at room temperature and 37°C. The plates were observed for 7 days for any growth or contamination.

3.5.3..2 Toxicity Test

Six albino white mice of three weeks of age were injected intra peritoneally with 0.5 ml each of the vaccine and were observed for 7 days for the development of any untoward reaction.

3.6 VACCINATION TRIAL

3.6.1 Materials

(i) Eighteen rabbits purchased from small animal breeding station, KAU, Mannuthy.

(ii) Inactivated yolk-sac vaccine

(iii) Inactivated elementary body vaccine

3.6.2 Method

Six rabbits were immunized with YS and EB vaccines, two doses of 3ml each, subcutaneously at 14 days interval. The three non vaccinated rabbits formed the control group. Blood samples were collected for serology at '0' and 7, 14, 21, 28, 42, 56 and 70 days post vaccination. Serum samples collected at intervals were analysed by passive haemagglutination (PHA) test and serum neutralization test (SNT). Challenge infection of three vaccinated and the control rabbits with homogenous preparation of *C. psittaci* from yolk sac membranes heavily infected with M-28 isolate was done on 28^{th} day post vaccination and rest on 70^{th} day post vaccination. The rabbits were given three milliliter of the suspension intraperitoneally. Challenged rabbits were sacrificed on seventh day post challenge. Impression smears were taken from lung, liver, lymphnode and spleen for monitoring the infection and clearance of elementary body of *C. psittaci* in tissues.

3.7 MONITORING OF ANTIBODY RESPONSE

3.7.1 Passive Haemagglutination (PHA) Test

The antibody titre in the serum collected from the vaccinated and control group of rabbits were analysed by performing PHA .

3.7.1.1 Collection of Serum

3.7.1.1.1 Materials

(i) Vaccinated and control rabbits

(ii) Sodium azide

3.7.1.1.2 Method

The serum samples were collected from both vaccinated and control groups of rabbits on day 0, 7, 14, 21, 28, 42, 56 and 70 days post vaccination. The serum samples were preserved at –20°C after the addition of sodium azide to the concentration of 0.1 per cent.

3.7.1.2 Preparation of 50 per cent Sheep Red Blood Cells (SRBC)

3.7.1.2.1 Materials

(i) Alsever's solution

Dextrose	10.25 g
Trisodium citrate	4 g
Sodium chloride	2.10 g
Citric acid	0.275 g
Triple distilled water	500 ml

Autoclaved at 10 lbs and 110°C for 20 min

(ii) PBS (0.15 M pH 7.2) [3.1.2.1.1(ii)]

3.7.1.2.2 Method

Sheep blood was collected in Alsever's solution from the University Sheep and Goat Farm, Mannuthy, and stored at 4°C for 24 hours. The SRBC in Alsever's solution was washed thrice in sterile PBS (pH 7.2) and finally a 50 per cent suspension was prepared in sterile PBS.

3.7.1.3 Gluteraldehyde Stabilization of SRBC

3.7.1.3.1 Materials

Neutral gluteraldehyde (one per cent)

Normal saline solution (0.85 per cent)

Phosphate buffer (pH 8) as recommended by Cruickshank *et al*.,1975 SRBC (50 per cent)

3.7.1.3.2 Method

The gluteraldehyde SRBC mixture was prepared as described below:

Neutral gluteraldehyde	1.5 ml
Normal saline solution	5.0 ml

Phosphate buffer	1.0 ml
Sheep RBC	1.0 ml

This mixture was stored at 4°C for 24 hours and then washed with PBS (pH 7.2) five times. Finally a 10 per cent even suspension was made in PBS (pH 7.2) and stored at 4°C.

3.7.1.4 Preparation of Antigen for the Passive Haemagglutination Test

3.7.1.4.1 Materials

(i) Infected yolk sac membrane

(ii) Sterile PBS (0.15 M pH 7.2) [3.1.2.1.1 (ii)]

3.7.1.4.2 Method

Chlamydia pittaci of caprine origin (M-430) was propagated in the yolk sac of developing chicken embryo. Yolk sacs revealing high concentration of the chlamydial agent were pooled together after washing thoroughly in sterile PBS (pH 7.2). A 40 per cent w/v emulsion of the YS was prepared in sterile PBS (pH 7.2). The emulsion was cleared of its debris and was centrifuged at 1100 x g for half an hour in a refrigerated centrifuge.

The supernatant so obtained was centrifuged twice at 1700 x g for half an hour. The supernatant was collected and centrifuged again at 3500 x g for half an hour. The final supernatant obtained was decanted and fresh sterile PBS was added to resuspend the pelleted mass to its original volume. This suspension was centrifuged once again at 3500 x g for half an hour. After discarding the supernatant, the pellet was carefully resuspended in PBS (pH 7.2) to obtain a moderately turbid uniform suspension. The concentration of the organism in the suspension was verified by examining a stained smear under oil immersion. A relatively uniform distribution of the EBs, free from extraneous tissue debris was considered for the preparation of antigen.

3.7.1.5 Sonication of the Antigen

3.7.1.5.1 Materials

(i) Suspension of *C.psittaci*

(iii) Sonifier (Branson 450)

3.7.1.5.2 Method

The suspension of the semi-purified *C. psittaci* was held on an ice bath and disrupted by sonication. Sonication was carried out at 150 volts with a 15 second burst followed by an interruption of 15 seconds between each burst for cooling.

The antigen was sonicated for four minutes and this was used to sensitize the SRBC.

3.7.1.6 Sensitization of Stabilized as well as Fresh SRBC

3.7.1.6.1 Materials

(i) PBS (pH 7.2) (0.15 M)) [3.1.2.1.1(ii)]
(ii) PBS (pH 6.4) as recommended by Cruickshank *et al.*, 1975.
(iii) Tannic acid
(iv) BSA
(v) Sodium azide

3.7.1.6.2 Method

Ten per cent suspension of SRBC was washed thrice in PBS (pH 7.2) and then resuspended to 2.5 per cent suspension in PBS (pH 7.2). Equal volumes of 2.5 per cent SRBC and 1:20,000 tannic acid in PBS (pH 7.2) were mixed and incubated at 37°C for 10 minutes. The cells were washed twice in PBS (pH 7.2) and the tanned SRBC were then resuspended in PBS (pH 6.4) to a concentration of 2.5 per cent. Three milliliter aliquots of this suspension was drop wise mixed with 0.25 ml, 0.5 ml and 0.75 ml of the sonicated antigen. These mixtures were then incubated at 37°C for one hour. Following the sensitization, the cells were centrifuged and washed twice with sterile PBS (pH 7.2). The cells were

resuspended in PBS containing one per cent BSA to provide a one per cent suspension. Finally 0.1 per cent sodium azide was added and it was stored at 4°C until used.

3.7.1.7 Titration of Antigen

A checkerboard titration was carried out to determine the optimum concentration of the sonicated antigen required for use in the passive haemagglutination test. Hyperimmune sera raised in rabbits against *C. psittaci* of caprine origin (M-430) was used for the titration. The hyperimmune serum was inactivated at 56˚C and mixed with an equal volume of one per cent SRBC in PBS (pH 7.2) to remove the heterophile antibodies. This serum of 1:2 dilution was serially diluted with PBS-BSA to provide two fold dilutions ranging from 1:4 to 1:1024. Fifty microlitre of one per cent sensitized SRBC carrying different concentrations of chlamydial antigen were added individually to each serum dilution. The mixture was gently agitated and incubated at room temperature for one and a half hours. The lowest concentration of the antigen that gave a maximum haemagglutination titre with the highest serum dilution was taken as the optimum concentration of antigen required for the sensitization of SRBC.

3.7.1.8 Bulk Sensitization of SRBC with the Sonicated Antigen

3.7.1.8.1 Materials

Phosphate buffer (pH 6.4)) [3.7.1.8.1]

Tannic acid

Sodium azide

BSA

3.7.1.8.2 Method

A 2.5 per cent suspension of tanned SRBC was prepared in PBS (pH 6.4). To 3 ml of 2.5 per cent tanned SRBC, 0.5 ml of sonicated antigen of optimum concentration as assessed previously was mixed. SRBC antigen mixture was incubated for one hour at 37°C. The sensitized cells were washed twice in PBS

(pH 7.2) and then resuspended in PBS-BSA to obtain one per cent suspension. Finally 0.1 per cent sodium azide was added and it was stored at 4°C till further use.

3.7.1.9 Protocol for the Passive Haemagglutination Test (PHA test)

3.7.1.9.1Materials

(i) Sera samples collected from rabbits (Vaccinated and control)

(ii) Sensitized SRBC

(iii) U bottom micro titre plate

(iv) PBS-BSA

3.7.1.9.2 Method

All test sera samples were heat inactivated in water bath at 56°C for half an hour. The sera samples were then mixed with an equal volume of one per cent unsensitized SRBC in PBS (pH 7.2) and incubated at room temperature for one hour. The unsensitized SRBC was sedimented by centrifugation and supernatant was diluted to 1:2 and harvested. Two fold serial dilutions of this serum (50 µl) were prepared in PBS-BSA to provide a dilution ranging from 1:4 to 1:512 in U bottom microtitre plate. Fifty microlitre of the sensitized SRBC was added to all the dilutions. Gently agitated the plates and incubation was carried out at room temperature for one and a half hours. The reciprocal of the highest dilution of the serum in which complete haemagglutination occurred was taken as the end titre of the serum.

3.7.2 Serum Neutralization Test (SNT)

3.7.2.1 Materials

(i) 75 cm^2 tissue culture flasks

(ii) 96 well flat – bottom plates

(iii) 96 well round – bottom plates

(iv) SPG [3.1.2.1.1 (iii)]

(v) CMF – PBS [3.4.1.1. (iv)]

(vi) HBSS – (reconstituted as per manufacturers instruction)

(vii) Cell culture growth medium [3.4.1.1. (viii)]

(viii) Test serum (control and vaccinated group)

(ix) Stock elementary body preparation)[3.5.2.1]

(x) PBS [3.1.2.1.1. (ii)]

(xi) Cycloheximide - [3.4.2.1. (iv)]

(xii) Methanol - [3.4.3.1.1. (v)]

3.7.2.2 Method

Mc Coy cells were grown to confluence in 75 cm^2 tissue culture flasks. The confluent monolayers were washed, trypsinized and diluted to 10^6/ml in medium and 100 μl of cells were added to each will of a 96 well flat – bottom microtitre plate. Cells then were incubated for 24 h at 37°C in five per cent CO_2 in air.

The serial two fold dilutions of the test sera were made in SPG in 96 well round – bottom cell culture dishes. The final volume in each well was 90 μl.

The stock EB preparation was diluted in SPG to contain 2 x 10^4 inclusion forming units per ml (ifu/ml) and 90 μl of this added to serum containing wells. Diluted EB (90 μl) was added to the SPG containing well to ensure that the non neutralizing antibody control contained no neutralizing activity. The EB-antibody mixture were incubated for 30 min at 37°C with continuous shaking and then inoculated on to the previously prepared Mc Coy cell in 96 well flat – bottom microtiter plates. To do this, monolayers were first washed in 100 μl of Hank's balanced salt solution (HBSS) and inoculated with 50 μl of the antibody – EB mixtures in triplicate for each dilution of each antibody. Plates were incubated for 2 h at 37°C, then inoculum was removed, plates were washed with 100 μl of HBSS, 200 μl of MEM containing 1 μg/mg cycloheximide was added

and the plates were incubated for 48 h at 37°C in an atmosphere of five per cent CO_2 in air and examined simultaneously for development of cytopathic effect (CPE).

The medium was removed and the monolayers were washed with 200 µl of PBS. Plates were then fixed with 100 µl of absolute methanol for 20 min and washed with 200 µl of PBS.

Plates were examined at 24 h interval until the antigen control wells showed CPE. Serum dilution showing CPE or no CPE were noted down.

The neutralization titre was calculated by Reed and Muench method (1938).

Results

4. RESULTS

4.1. REVIVAL OF THE ISOLATES

4.1.1 Chicken Embryo Inoculation

The two isolates (M-430 and M–28) maintained in the Department of Microbiology, were revived by inoculation of six to eight day-old chicken embryo through yolk sac route.

Both the isolates had not caused death of the embryos even after 10 to15 days of incubation at 37°C. Characteristic lesions like stunted embryo and haemorrhagic patches on the skin all over the body were noticed. The yolk sac was thin walled and with injected blood vessels. The yolk was more liquid in nature than normal and had a bright yellow colour.

4.1.2 Staining of Infected Yolk Sac Smear

The impression smears prepared from the yolk sac membrane of the inoculated embryos revealed elementary bodies, on staining by different methods like Giemsa, Modified Ziehl-Neelsen and Gimenez.

Slow method of Giemsa staining revealed reddish purple extracellular elementary bodies in a dark blue background.

Modified Ziehl Neelsen staining presented deep red EBs in a blue background.

Gimenez staining of the impression smears from the infected yolk sac membrane revealed pink stained extracellular EBs in bluish green background. This staining method was found to be the most suitable one for identification and easy differentiation of EBs.

4.2 GRADING OF INFECTED YOLK SAC

Grading of the infected YS was done based on the average number of EBs present on stained YS smears. The smears prepared from the yolk sac of M-430 isolate showed more number of EBs per microscopic field than M-28.

Based on the number of EBs present per microscopic field, the infected yolk sac was graded as 3+, 2+ and 1+ and were preserved separately.

4.3 PROPAGATION OF CHLAMYDIAL ISOLATE IN Mc COY CELL LINE

Mc Coy cell line obtained from NCCS, Pune was subcultured into tissue culture bottles and coverslips in test tubes. The seeded bottles and tubes were incubated at 37°C and observed at 24h intervals for visualizing the growth characteristics. Confluent monolayer was formed 24h after seeding. M-430 isolate was used for propagation in Mc Coy cell line. The monolayer with inoculum was incubated with maintenance medium containing cycloheximide (1 µg/ml) at 37°C. The multiplication of *C. psittaci* in the cells was indicated by examining changes in the cells at 24 h interval.

To study the CPE, the coverslip cultures inoculated with the isolate and the control were stained and examined at 24 h intervals.

4.3.1 Staining of Coverslip Cultures

The coverslip cultures fixed in methanol overnight were stained with May-Grunwald Giemsa stain. The stained monolayers exhibited specific cytopathic changes such as rounding and swelling of the fibroblastic cells by 24 h PI.

The characteristic changes in the cells increased as time passed. By 48 h, rounding, swelling and syncytia formation of cells were prominent. Complete rounding of most of the attached cells was observed by 72 h PI and the cells

started getting detached from the surface of the coverslip. By 96 h the monolayers were almost detached.

No cytopathic changes were noticed in the cells in the control tubes.

The control cells and the changes in the cell line after inoculation with M-430 at different intervals are shown in Plate 1 and 2.

The changes in Mc Coy cell line after inoculation with M-430 isolate at 24 h intervals are presented in Table. 1

4.3.2 Confirmation of Infection by Immunofluorescence

Infected and negative coverslip cultures were subjected to immunofluorescence reaction at 24 h intervals until 96 h. The infected cells were first treated with mouse monoclonal *C. psittaci* antibody and then with antimouse antiglobulin conjugated with fluorescein isothiocyanate. Specific apple green fluorescence was detected 48 h PI and the maximum fluorescence at 96 h PI. [Plate 3. (B)].

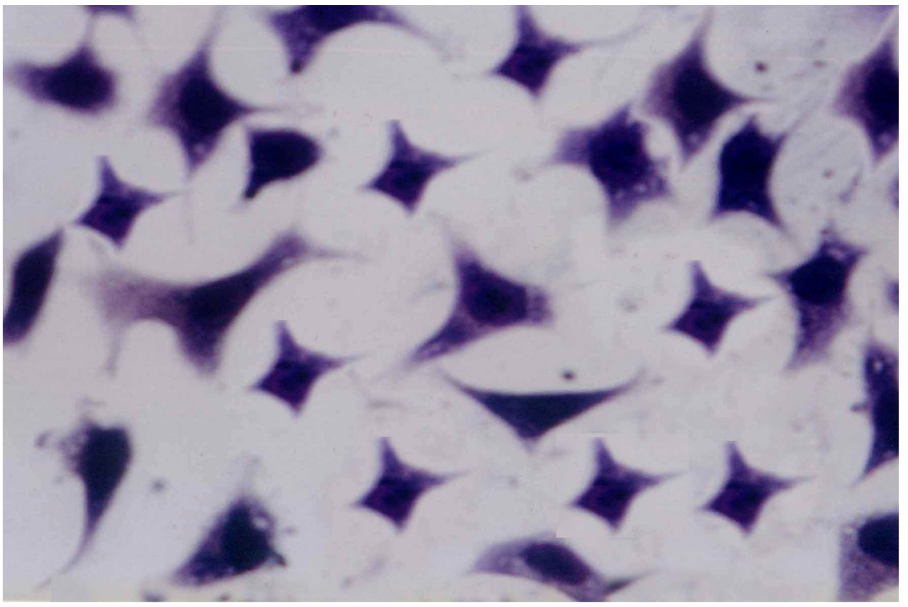
No specific fluorescence was noticed in negative coverslip [Plate 3. (A)].

4.4 PREPARATION OF VACCINE

The inactivated yolk sac and elementary body vaccines were used for vaccinating the rabbits.

4.4.1 Yolk Sac Vaccine

A homogenous suspension of chlamydial isolate M-430 was prepared from one hundred heavily infected yolk sac membranes. This suspension was treated with formalin to a final concentration of 0.4 per cent and stored at 37°C for one week.



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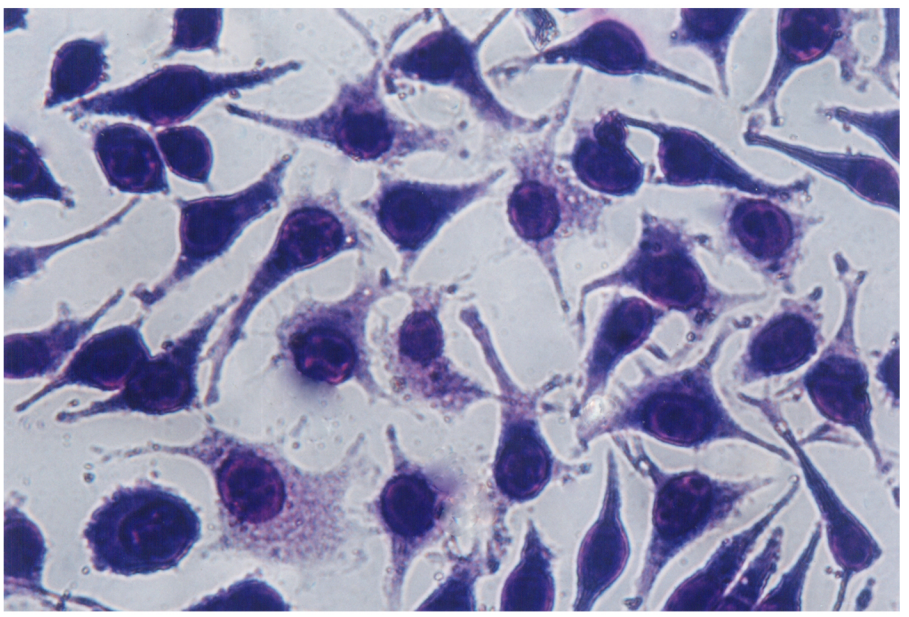
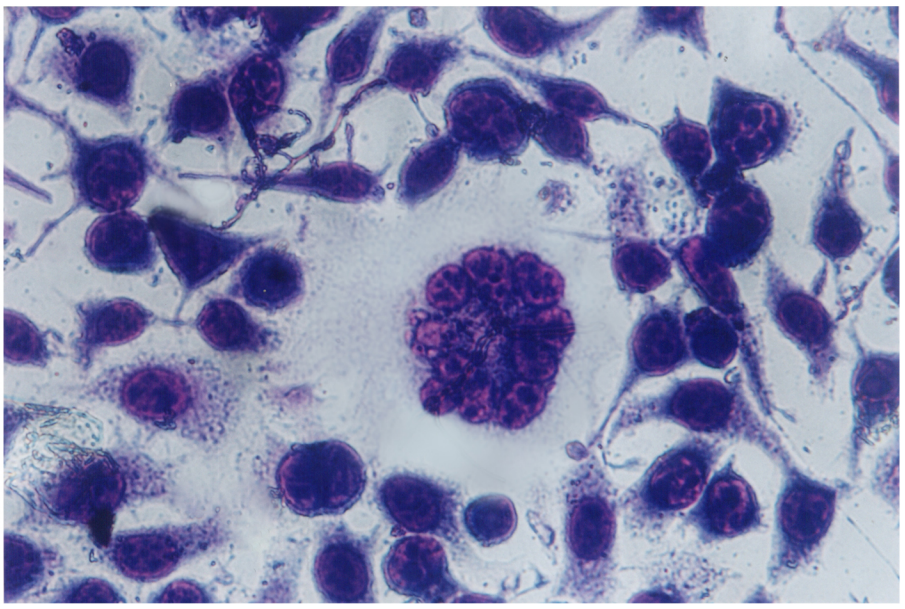


B

Plate 1. Mc Coy cell line stained by May-Grunwald Giemsa (400X):

(A) Control (B) Rounding and swelling of cells-24h PI



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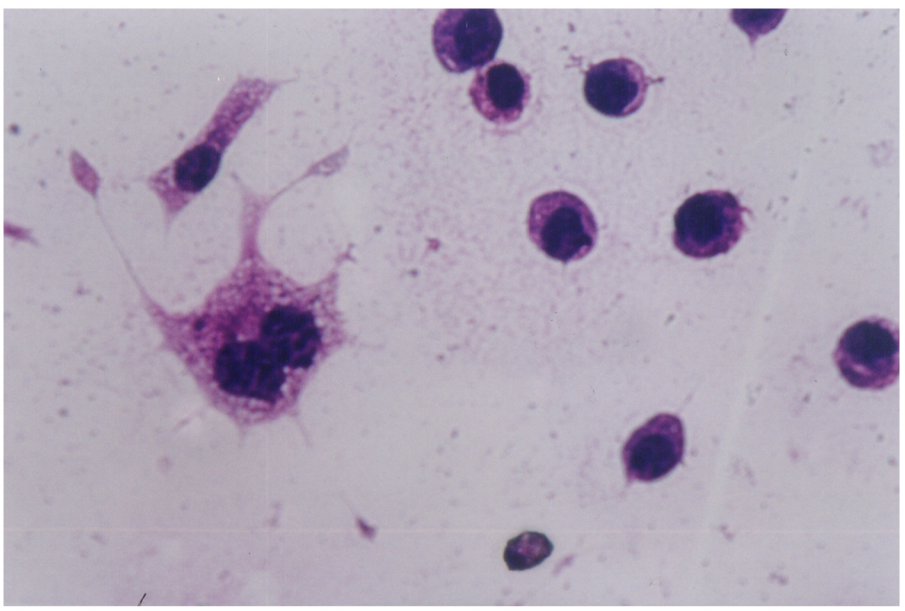
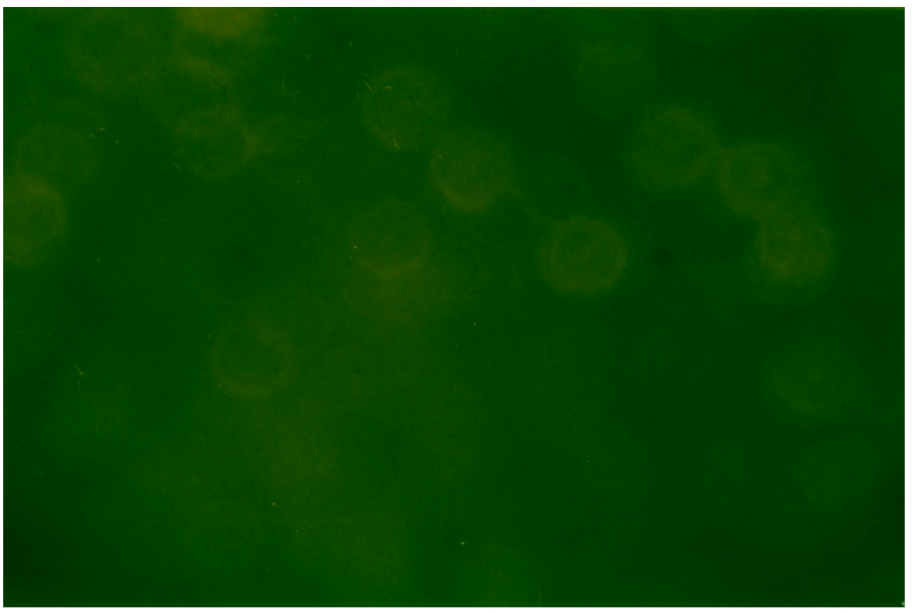


B

Plate 2. M-430 infected Mc Coy cell line May-Grunwald Giemsa stain (400X):

(A) Syncytia formation- 48h PI (B) Detachment of cells -72h PI



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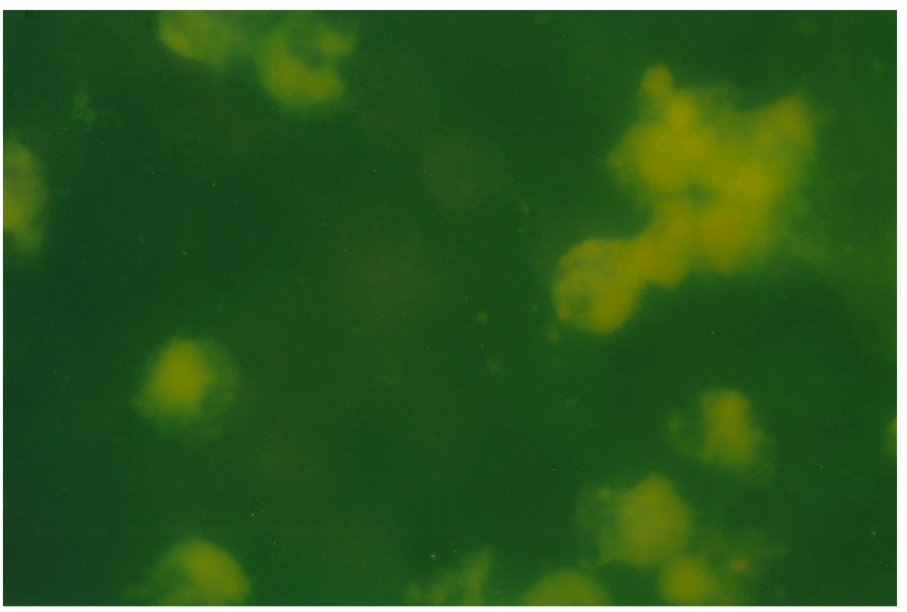


B

Plate 3. Immunofluorescence staining of Mc Coy cell line (400X):

(A) Control (B) Infected cells revealing apple green fluorescence

4.4.2 Elementary Body Vaccine

Purified elementary bodies were prepared from infected Mc Coy cell line, treated with 0.4 per cent formalin and stored for one week at 37°C.

Both the vaccines were tested for sterility and toxicity before used for animal inoculation.

4.4.3 Sterility Test

The sterility of the vaccines was tested individually on BA, TSA and SDA plates.

The plates were inoculated with 0.2 ml of the prepared vaccine.

The BA and TSA plates were incubated both at aerobic condition and in increased CO_2 tension at 37°C for seven days.

The SDA plates were incubated at 37°C and at room temperature for up to seven days for detecting fungal contamination.

The inoculated media were observed daily for any growth in the media. There was no growth in any of the above media, indicating that the vaccines prepared were sterile and without any contamination.

4.4.4 Toxicity Test

The toxicity of the vaccines was assessed by inoculating 0.5 ml each of the vaccines intraperitoneally into six albino mice of three weeks of age. These inoculated mice were observed for a period of seven days for any untoward reaction at the site or clinical manifestations.

Vaccines did not produce any local and systemic reaction in any of the inoculated mice.

4.5 ASSESSMENT OF IMMUNITY

Three month old seronegative rabbits were used for the assessment of immunity. The rabbits were grouped into two, with nine rabbits in each group. Of the nine, six animals formed the vaccine group and rest three formed the control. Each group received two doses of three milliliter of each vaccine subcutaneously at 14 days interval. Three vaccinated and the control rabbits were challenged with three milliliter of the homogenous suspension of M-28 isolate intraperitoneally on 28th day post vaccination.

Immunity was assessed by monitoring of clearance of EB of *C. psittaci* in tissues (lung, liver, lymphnode and spleen) of challenged animals and by measuring humoral immune response by PHA and SNT.

First dose of vaccines was given at three months of age and the second dose 14 days after the first. The three vaccinated and control rabbits were challenged intraperitoneally with three milliliter of heavily infected yolk sac (3+) suspension of M-28 chlamydial isolate on 28th day post vaccination. Rest of the rabbits were challenged on 70th day post vaccination.

4.5.1. Monitoring of Clearance of Elementary Bodies in Tissues

4.5.1.1 Group1

The rabbits in group I were used to assess the immunity provided by inactivated yolk sac vaccine. Of the nine rabbits, six formed vaccine group and three formed the control.

The rabbits were vaccinated with three milliliter of the yolk sac vaccine subcutaneously. Each of the control animals received three milliliter of sterile PBS. Second dose of vaccine was given after 14 days.

Three of the vaccinated and the control rabbits were challenge infected. All the animals were observed for the development of any clinical symptoms or

death. Clinical manifestation like dullness and anorexia were observed in control rabbits.

The challenged rabbits were sacrificed on 7th day post challenge.

Necropsy of control animals that were challenged presented lesions suggestive of chlamydiosis. Pulmonary congestion of severe degree was noticed. Hepatic necrosis with enlargement of liver and inflammation of spleen were noticed.

Impression smears were prepared from lung, liver, lymphnodes and spleen and stained by Gimenez method. Stained smears revealed EBs in the 3+ or 2+ grade. Concentration of EBs were more in spleen than in other organs. The EB concentration was more in mesenteric lymphnode than other lymphnodes.

The vaccinated animals that were challenged did not show appreciable gross lesions in any of the visceral organs.

The elementary bodies in the Gimenez stained impression smears from lung, liver, lymphnodes and spleen were in the grade 2+, 1+, 1+ and 2+ respectively. The EBs were less in number when compared to control group.

4.5.1.2 Group II

Efficacy of elementary body vaccine was assessed in this group. Vaccination and challenge experiment were conducted as in the case of yolk sac vaccine.

Impression smears taken from the lung, liver, lymphnodes and spleen of control animals when stained by Gimenez method revealed elementary bodies to 3+ or 2+ grade.

In the smears from organs of the vaccinated group EBs were to the grade of 1+ or even less than that.

EB clearance from the organs was faster with EB vaccine than with yolk sac vaccine.

Number of EBs per microscopic field was less when compared with yolk sac vaccine.

The remaining animals in vaccinated group were challenged on 70th day post vaccination. These were observed for seven days and then sacrificed. No gross lesions could be noticed.

Complete clearance of EBs from the organs was noticed on staining of impression smears.

Impression smears from lung and spleen revealing EBs were shown in Plate 4.

Results of challenge experiment is shown in Table 2.

4.5.2 Monitoring of the Humoral Immune Response

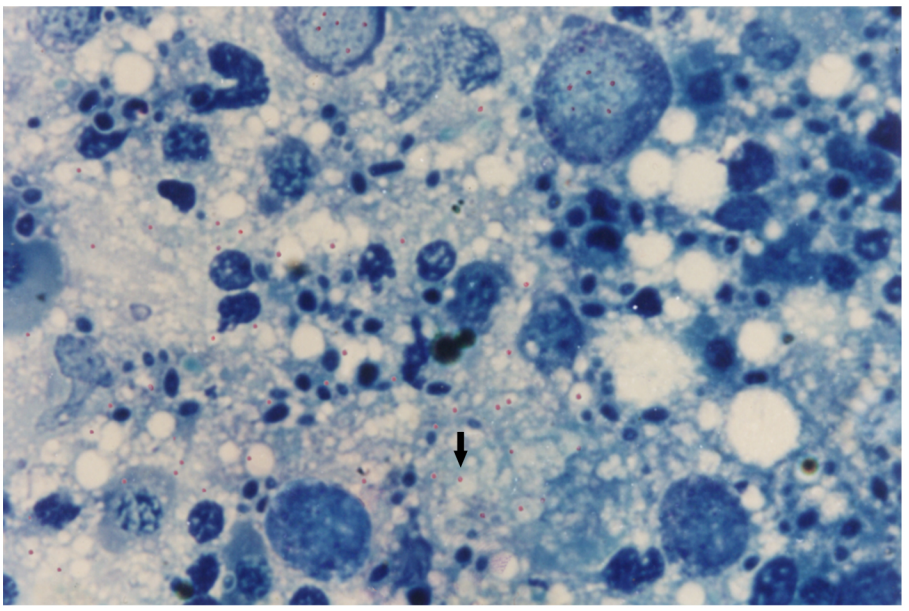
Test sera samples collected from control and vaccinated rabbits at 0, 7, 14, 21, 28, 42, 56 and 70 days post vaccination were subjected to PHA and SNT, to assess the humoral immune response.

4.5.2.1 Passive Haemagglutination (PHA) Test

The gluteraldehyde fixed sheep erythrocytes sensitized with sonicated antigen were used for assessing the immune status of rabbits. The sensitized SRBC were specifically agglutinated by the rabbit antichlamydial serum. Controls of sensitized SRBC with diluent buffer (PBS-BSA) showed a definite negative reaction (Plate 5).

Group I

The average titres of the six rabbits vaccinated with yolk sac vaccine were 0, 6, 24, 53, 64 on days 0, 7, 14, 21 and 28 respectively. Since three of the



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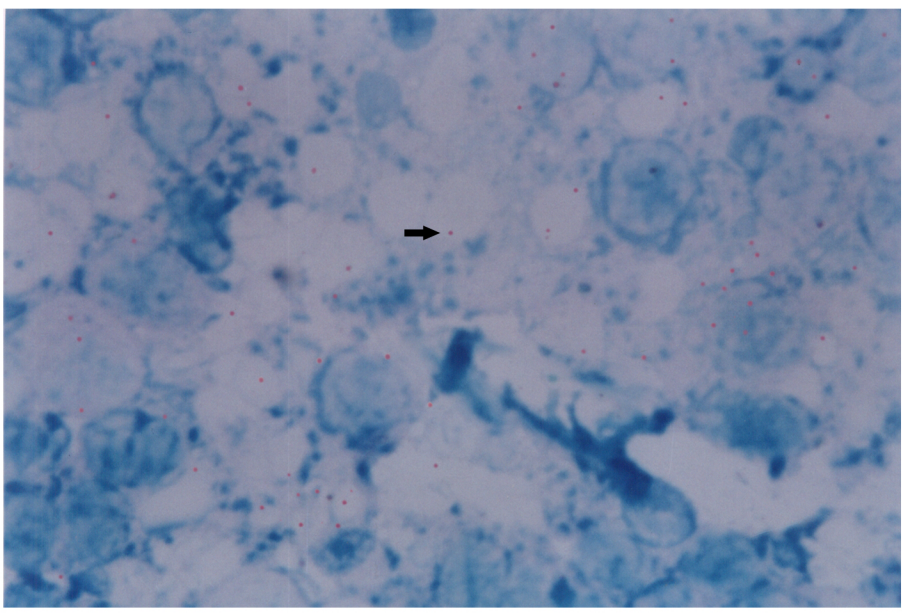
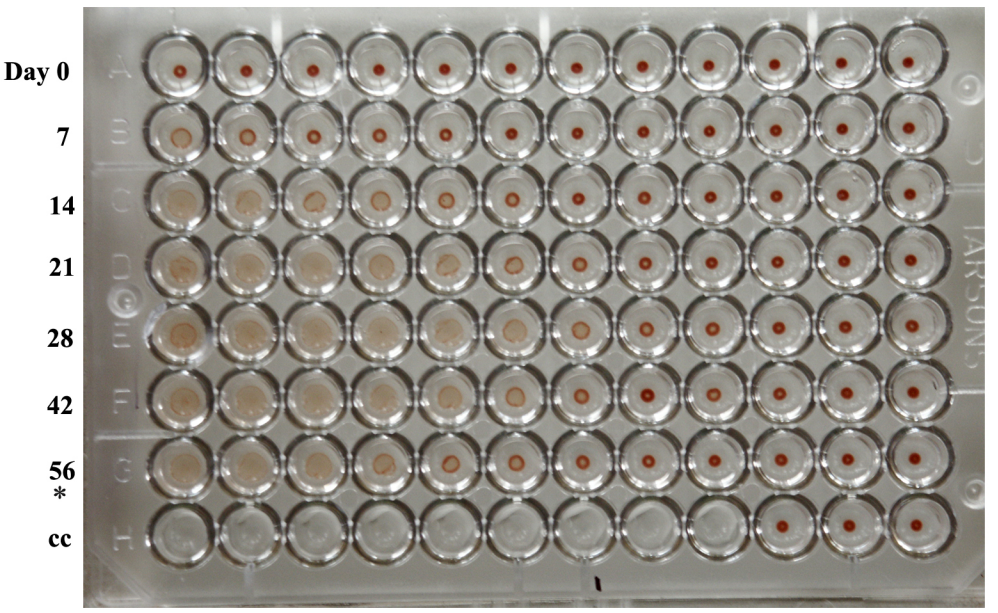


B

Plate 4. Chlamydial EBs in impression smear stained by Gimenez (1000X):

(A) Lung impression smear (B) Spleen impression smear



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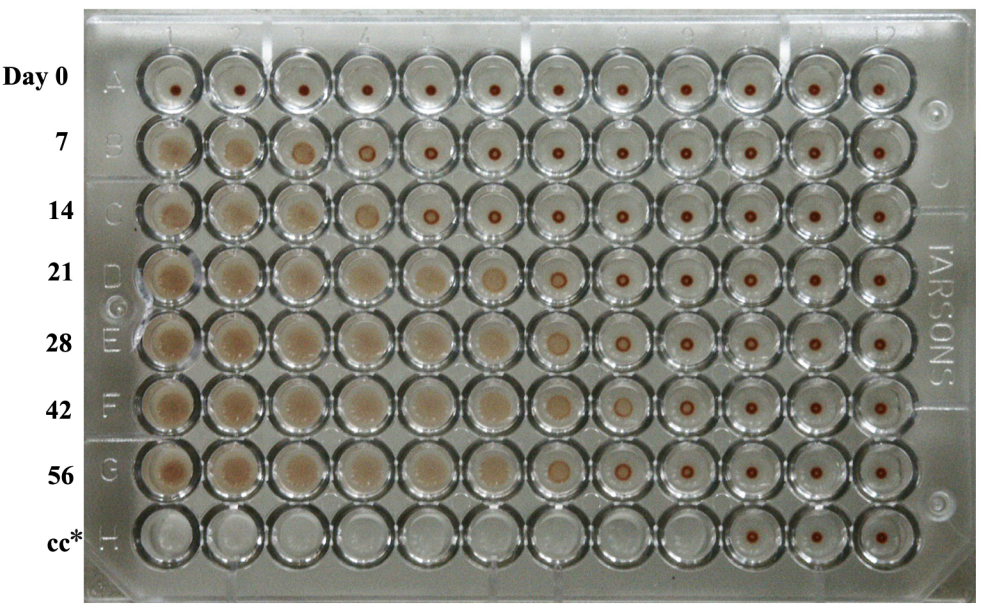


B

Plate 5. Passive haemagglutination test:

(A) Yolk sac vaccine (B) Elementary body vaccine

* cell control

immunized rabbits were sacrificed on 35th day post vaccination, the average titres of only three remaining rabbits were taken into account and the titres were 64, 37 and 26 on days 42, 56 and 70.

Group II

The PHA titres of the rabbits immunized with inactivated elementary body vaccine were calculated as for YS vaccine and these were 0, 12, 24, 59, 107, 107, 107 and 64 respectively on days 0, 7, 14, 21, 28, 42, 56 and 70.

The PHA titres of group I and II are given in Tables 3 and 4.

The average PHA titre of yolk sac and elementary body vaccines is given in Table 5.

4.5.2.2 ***Serum neutralization test (SNT)***

The serial two fold dilutions of test sera were mixed with equal quantities of EB suspension and the mixture was incubated at 37°C for 30 min. This mixture was added to previously prepared Mc Coy cell monolayer in 96 well flat bottom microtitre plate. Plate was incubated at 37°C and examined at 24 h interval until the antigen control wells showed CPE.

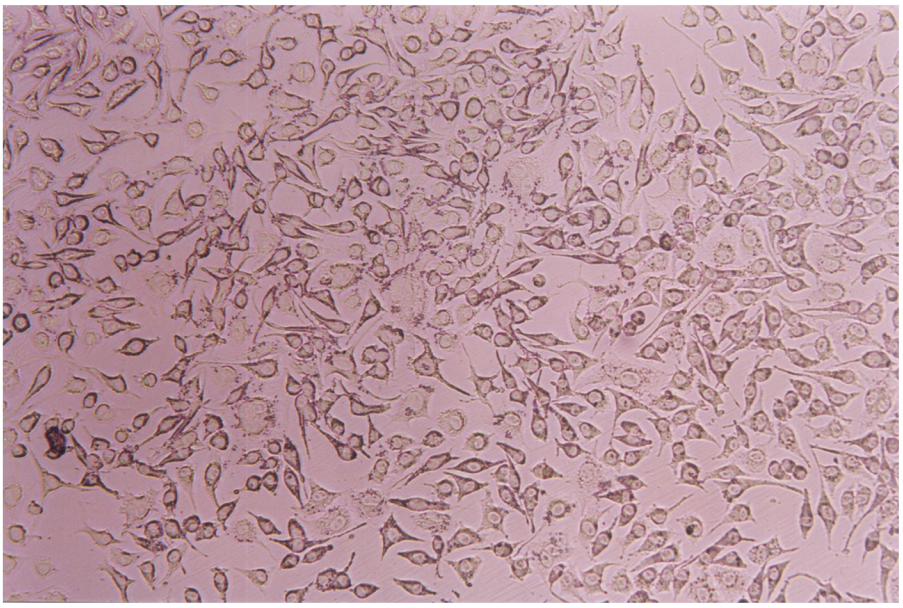
Rounding and detachment of cells from the surface was noticed 72 h PI (Plate 6).

The dilution of sera showing CPE or no CPE were noted down and the neutralization titre was calculated by Reed and Muench method (1938).

The neutralization titre obtained for the first group were 10, 11, 22, 22, 22, 14 and 14 on days 7, 14, 21, 28, 42, 56 and 70 respectively.

Neutralization titre for group – II were 14, 22, 32, 89, 89, 89 and 60 on days 7, 14, 21, 28, 42, 56 and 70 respectively.

Results of SNT are presented in Table 6.



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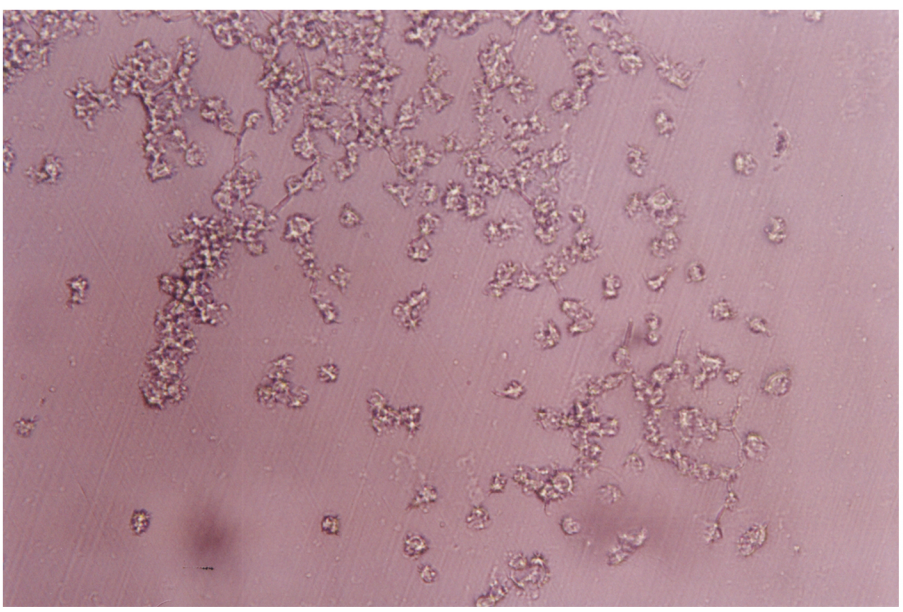

B

Plate 6. Serum neutralization test:

(A) Cell control

(B) Rounding and detachment of cells in lower dilutions of serum

Table 1. CPE in Mc Coy cell line after inoculation with M-430 isolate

Time after inoculation	Type of change
24 h	Rounding and swelling of cells with syncytia formation.
48 h	Syncytia formation pronounced.
72 h	Cells started detaching from the glass surface
96h	Cell detachment from glass surface almost complete.

Table 2. Results of challenge experiment

Tissue	Concentration of EBs in challenge experiment			
	Yolk sac vaccine		EB vaccine	
	Control	Vaccinated	Control	Vaccinated
Lung	3+	2+	3+	1+
Liver	2+	1+	2+	-
Lymphnode	1+	1+	2 +	-
Spleen	3+	2 +	3+	1+

Table 3. PHA titre obtained in Group I

Animals	PHA titre							
	0^{th}day	7^{th}day *pv	14^{th}day *pv	21^{th}day *pv	28^{th}day *pv	42^{th}day *pv	56^{th}day *pv	70^{th}day *pv
1	0	4	16	64	64	64	16	16
2	0	8	16	32	64	64	64	32
3	0	8	32	64	64	64	32	32
4**	0	4	32	64	64	-	-	-
5**	0	8	32	64	64	-	-	-
6**	0	4	16	32	64	-	-	-
Control**	0	0	0	0	0	-	-	-

* - post vaccination
**Rabbits sacrificed on 35^{th} day post vaccination

Table 4. PHA titre obtained in Group II

Animals	PHA titre on different days post vaccination							
	0^{th} day	7^{th} day	14^{th} day	21^{st} day	28^{th} day	42^{nd} day	56^{th} day	70^{th} day
1	0	16	32	64	128	128	128	64
2	0	8	32	64	128	64	64	64
3	0	16	32	64	128	128	128	64
4*	0	8	16	32	64	-	-	-
5*	0	8	16	64	64	-	-	-
6*	0	16	16	64	128	-	-	-
Control*	0	0	0	0	0	-	-	-

*Rabbits sacrificed on 35^{th} day post vaccination

Table 5. Average PHA titre of YS and EB vaccine

Days of post vaccination	PHA titre	
	YS vaccine	EB vaccine
0	0	0
7	6	12
14	24	24
21	53	59
28	64	107
42	64	107
56	37	107
70	26	64

Table 6. Neutralization titre of YS and EB vaccines

Days post vaccination	Neutralization titre of	
	YS vaccine	EB vaccine
0	-	-
7	10	14
14	11	22
21	22	32
28	22	89
42	22	89
56	14	89
70	14	60

Discussion

5. DISCUSSION

Chlamydophila abortus (Chlamydia psittaci) is a gram negative obligate intracellular bacterium and the etiological agent of chlamydial abortion, an economically important disease in many countries. There are reports of occurrence of chlamydial abortion among the livestock of Kerala. This study deals with the design of experimental vaccines, viz., inactivated yolk sac and elementary body vaccines, incorporating the local chlamydial isolate and immune studies in rabbit models.

5.1 REVIVAL OF THE ISOLATES

5.1.1 Chicken Embryo Inoculation

The chlamydial isolates, M-430 and M-28 maintained in the Department of Microbiology, were revived by inoculating to six to eight day-old chicken embryos through yolk sac route.

Rake *et al.* (1940) and Tang *et al.* (1957) recommended the use of embryonated chicken embryos for the isolation and propagation of chlamydiae.

Nanda *et al.* (1992) and Batta *et al.* (1997) noticed the death of the embryos within three to eight days post inoculation.

In the present study both isolates had not caused death of the chicken embryos, even after 10 days of incubation at 37°C. Both isolates produced characteristic lesions on embryo and yolk sac. Embryos were stunted and haemorrhagic patches were seen on the skin all over the body. Yolk sac was thin walled and with injected blood vessels. The yolk was more liquid in nature than normal and had a bright yellow colour.

The lesions on the YS and embryo observed in the present study are in accordance with the observations of Sharma *et al.* (1982), Francis (1988) and Nair (2001).

5.1.2 Staining of the Infected YS

Impression smears prepared from the YS membrane were stained and examined to detect chlamydial EBs. Giemsa, MZN and Gimenez staining techniques were employed.

Storz (1971) recommended the use of Giemsa staining whereas Francis (1988) and Krishna (1988) advocated MZN staining technique for direct demonstration of chlamydial bodies in infected materials.

All the staining techniques could demonstrate chlamydial EBs in the smears. In the present study, Gimenez staining method was found to give more reliable and consistent results.

Batta *et al.* (1996) and Nair (2001) had reported the Gimenez staining method to be the most suitable one for the demonstration of *C. psittaci* in infected tissue.

5.2 PROPAGATION IN Mc COY CELL LINE

Nagal *et al.* (1997) studied the suitability of Mc Coy cell line to support the growth of chicken embryo adapted *C. psittaci* isolates and found it suitable for propagation and maintenance of *C. psittaci* isolates in the laboratory.

In the present study, Mc Coy cell line was used for propagation of M-430 isolate for vaccine preparation.

Characteristic changes in the cells were noticed suggesting the growth of chlamydiae.

When the isolate was passaged in Mc Coy cells, marked CPE were visible from 24 h onwards and showed typical changes within 72 h.

Rounding and swelling of the fibroblast cells were noticed 24 h PI. Formation of syncytia also started by 24 h PI and more pronounced by 48 h PI. By 72 h PI cells started detaching from the surface and detachment of cells were completed by 96 h PI.

Spears and Storz (1979), Andersen and Baxter (1986) and Griffiths *et al.* (1992) observed large intracytoplasmic inclusions in the infected cell lines within 48 h PI for the isolates causing abortion.

In the present study, chlamydial inclusions could not be detected in the infected monolayers.

Development of cytopathic effects and inclusions in infected cell cultures were not always seen since some of the chlamydial species did not produce these effects in all cell line or cell cultures (Page, 1981). This might be the reason for the difficulty in identifying inclusions in infected monolayers in the present study.

These cytopathic effects were found to be similar to the results observed by Mani (2001).

5.2.1 Staining of Coverslip Cultures

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

Using May-Grunwald Giemsa staining technique, coverslips were stained at 24 h intervals to observe the characteristic cytopathic changes. No intracellular inclusions were noticed in stained coverslip cultures.

Spears and Storz (1979) and Anderson (1986) noticed compact, deeply staining intracellular inclusions in 72h PI, which became prominent by 96 h PI.

Griffiths *et al.* (1992) and Nagal *et al.* (1997) reported fluorescent antibody technique (FAT) to be a superior technique for detection of chlamydial inclusions in cell culture.

In the present study, chlamydiae in cell line were demonstrated employing monoclonal antibodies against *C. psittaci*. Specific apple green fluorescence was noticed 72 h PI and that became maximum by 96 h PI. These observations were in confirmation with that of Nagal *et al.* (1997).

5.3 VACCINE TRIAL

The two experimental vaccines used in the study viz., inactivated yolk sac and elementary body vaccines, were prepared from the M-430 isolate of chlamydia.

The isolate was revived by inoculating to six to eight day-old chicken embryo through yolk sac route and heavily infected yolk sac membrane (2+ or 3+) was used for vaccine preparation.

The same isolate was propagated in Mc Coy cell line for high yield of EBs for the preparation of EB vaccine.

Mc Ewen and Foggie (1956) developed an effective vaccine against ovine enzootic abortion, containing a single chlamydial isolate grown in yolk sac membrane of embryonated chicken eggs. Homogenized suspension of egg grown organisms was inactivated with formalin. This vaccine successfully controlled the disease to a point where it was no longer considered a problem.

Waldhalm *et al.* (1982) prepared bacterins from mouse L cells and chicken embryos infected with *C. psittaci*. Both vaccines were inactivated with formalin to a final concentration of 0.4 per cent.

Anderson *et al.* (1990) used an experimental vaccine prepared from purified inactivated elementary bodies of *C. psittaci*.

The prepared vaccines were inactivated with formalin to a final concentration of 0.4 per cent as recommended by Waldhalm *et al.* (1982) and tested for their sterility and toxicity.

Sterility was checked by inoculating vaccines individually on to BA plates and TSA plates and inoculated media were incubated under aerobic condition and at increased CO_2 tension at 37°C. SDA plates seeded with vaccines were incubated at room temperature and 37°C. The vaccines were found to be free from contamination.

Toxicity studies were carried out by inoculating each of these vaccines into mice and all of them were found to be without any toxic effects, either local or systemic.

5.4 ASSESSMENT OF IMMUNITY

Caro *et al.* (2001) employed a mouse model to evaluate the protection conferred by four inactivated chlamydial vaccines and one attenuated vaccine.

In the present study rabbits were used as experimental animals. The immunizing efficacy of the two vaccines were studied by active immunization of the rabbits and challenge experiment.

The rabbits were immunized by giving two doses of the respective vaccines. The first dose was given at three months of age and the second dose was given 14 days after the first. The control animals in each group received three millilitre of sterile PBS.

Three immunized rabbits and control rabbits were challenged with M-28 isolate on 28^{th} day post vaccination and remaining three rabbits on 70th day post vaccination.

Serum samples were collected on days 0, 7, 14, 21, 28, 42, 56 and 70 post vaccination to assess the antibody levels.

Various workers used different serological tests for detecting antibody response following vaccination.

Mc Ewen and Foggie (1956) detected the antibody titre in the serum of ewes vaccinated with three vaccines by complement fixation test and serum neutralization test.

Waldhalm *et al.* (1982) employed ELISA for assay of antibody response.

Complement fixation test was used for evaluation of antibody response by Anderson *et al.* (1990) and Chalmers *et al.* (1997).

In the present study antibody levels in the sera were assessed by PHA and SNT.

In the present study, none of the rabbits died nor showed clinical signs of illness during the period of observation.

The challenged rabbits were sacrificed on 7^{th} day post-infection. Necropsy of the control rabbits presented lesions suggestive of chlamydiosis. Severe pulmonary congestion was noticed. Hepatic necrosis with enlargement of liver and inflammation of spleen were observed.

Purohit *et al.* (1986) found lesions in the liver, lung and spleen of an aborted goat foetus. Severe congestion and necrosis of lung and liver were noticed. Histopathological lesions suggestive of chlamydial infection were present in these organs.

Similar type of lesions were observed by Francis (1988), Asrani *et al.* (1997) and Mani (2001) in the organs of guinea pig and mice inoculated with chlamydial isolate for the pathogenicity studies.

The impression smears from lung, liver, lymphnode and spleen of control animals on staining by Gimenez method revealed EBs in the grade 3+, 2+, 1+ and 3+ respectively. Concentration of EBs was more in spleen than in other tissues.

The results obtained were in correlation with those of Anderson (1986). He detected more number of EBs in the spleen of the mice, following foot pad inoculation of ovine isolate of *Chlamydia psittaci*, than any other organs.

In the immunized rabbits no characteristic macroscopic lesions were noticed. The concentration of EBs in the lung, liver, lymphnode and spleen were very less than the control rabbits.

The concentration of EBs in the lung and spleen of the rabbits immunized with EB vaccine was reduced from 3+ to 1+. And there was complete clearance of EBs from liver and mesenteric lymphnode.

No EBs could be detected in the impression smears prepared from the rabbits which were challenged on 70^{th} day post vaccination.

The results of the challenge experiment indicated the degree of protection conferred by the vaccines. A better protection was noticed with EB vaccine in comparison with yolk sac vaccine.

5.4.1 Monitoring of Humoral Immune Response

5.4.1.1 Passive Haemagglutination Test

Passive haemagglutination test is one of the most sensitive method for detecting antibodies against proteins. As little as 0.02-0.04 μg of antibody could produce detectable agglutination (Carpenter, 1975).

In this study, along with challenge experiment, the antibody levels of the vaccinated rabbits were also monitored, using passive haemagglutination test for which sheep red blood cells (SRBC) fixed and stabilized by one per cent

gluteraldehyde (GA-SRBC) were employed. The GA-SRBC was found to be stable at 4°C without any deterioration in quality during the period of the study. Sonicated antigen adsorbed on to GA-SRBC was used to assess antibody titre in the serum. The serum samples were collected at regular intervals of 0, 7, 14, 21, 28, 42, 56 and 70 days from all the vaccinated rabbits and titrated with homologous antigen adsorbed on to GA-SRBC.

In the present study, antibody titre varied with the type of vaccine and with different stages at which serum samples were collected to assess the antibody level.

In the case of inactivated yolk sac vaccine, the titre varied from 0 to 64. The highest antibody titre of 64 was observed on 28^{th} day, two weeks after the second dose of vaccine and there was a decline in the antibody level from 56^{th} day post vaccination.

The results obtained for EB vaccine were comparable with yolk sac vaccine where in the highest titre obtained was 107 on day 28 post vaccination and then there was a decline in the antibody level to 64.

Yang *et al.* (1984) proved the specificity of haemagglutination test using sheep erythrocytes sensitized with *C. trachomatis* and *C. psittaci*. The PHA titre were eight to sixteen and 64-128 times higher than that of direct and indirect CFT.

Belden and Mc Kercher (1973) and Francis (1988) used PHA for detection of chlamydial antibodies in the sero-prevalence studies.

5.4.1.2 Serum Neutralization Test

In vitro serum neutralization test minimize the need for expensive and time consuming *in vivo* protection studies. Serum neutralization test provides an appropriate *in vitro* correlate for the study of the protective activity of the

vaccines. This test could be readily standardized and reproduced (Byrne *et al.*, 1993).

In the present study, serum neutralization test was also included for assay of humoral immune response along with PHA.

The neutralization titres obtained in the serum samples were 10, 11, 22, 22, 22, 14 and 14 on days 7, 14, 21, 28, 42, 56 and 70 for yolk sac vaccine and 14, 22, 32, 89, 89, 89 and 60 for EB vaccine.

Peak titres were noticed by two weeks (28^{th}day post vaccination) following the booster dose and then gradually decreased.

An identical pattern of antibody response was observed for EB vaccine, but with higher titre values.

Antibody response observed in the present study was in accordance with the findings of Mc Ewen and Foggie (1956). They employed serum neutralization test to assess the antibodies in the sera of sheep vaccinated with adjuvanted chlamydial vaccine and they observed increase in titre gradually reaching a peak and then declining.

The study indicated that both the yolksac vaccine and the elementary body vaccines were efficacious in rabbits with the latter eliciting a better immune response.

Further studies by conducting elaborate field trials on target species is necessary for advocating the use of the vaccine in the field.

Summary

6. SUMMARY

Chlamydia psittaci causes a wide variety of clinical syndromes in animals and birds. Among the pathogenic manifestations, abortion in bovines and ovines in late pregnancy cause heavy economic loss in livestock industry and is recognized as a world wide problem. There are reports of occurrence of chlamydial abortion among livestock of Kerala. In this study an attempt was made to prepare and assess the experimental vaccines against chlamydia, incorporating the local isolate.

The chlamydial isolates, M-430 and M-28, maintained in the Department of Microbiology, were revived by inoculating to six to eight day-old chicken embryos through yolk sac route. M-430 isolate was used for vaccine preparation and M-28 for challenge experiment.

No death of the embryos was noticed even after 10 days of incubation at 37°C. Both isolates produced characteristic lesions in the embryos and yolk sac membrane.

The yolk sac membranes that revealed elementary bodies to the grade of 2+ or 3+ were used for the preparation of yolk sac vaccine.

The M-430 isolate was also propagated in Mc Coy cell line for the high yield of elementary bodies for preparation of elementary body vaccine.

The infected cell lines showed cytopathic changes 24 h PI. Rounding and swelling of the cells noticed 24 h PI and cells started detaching from surface by 72 h PI and was almost complete by 96 h PI.

Confirmation of infection of cell line was done by immunofluorescence reaction of the coverslip cultures. Specific apple green fluorescence could be obtained in the infected cultures.

Infected monolayers were separated and further processed for preparation of purified EBs.

Thin smear prepared from the processed suspension revealed sufficient concentration of EBs on staining by Gimenez method.

Homogenous suspensions of the yolk sac and EBs were made in PBS and were inactivated with formalin to a final concentration of 0.4 per cent.

Sterility of the vaccines was tested by inoculating to culture plates and toxicity was tested by mice inoculation.

The sterile and safe preparations were used for vaccination trial.

Two groups of the rabbits were immunized with three millilitre of each of the vaccines at an interval of 14 days. Three rabbits in the vaccine group and the control rabbits were challenged with M-28 isolate on 28^{th} day past vaccination and observed for any clinical manifestations. Rest of the rabbits were challenged on 70^{th} day post vaccination.

Sera samples were collected at days 0, 7, 14, 21, 28, 42, 56 and 70 for antibody assay by PHA and SNT.

Immunity of vaccines were assessed by three parameters viz., clearance of EBs from tissues (lung, liver, lymphnode and spleen), antibody titration by passive haemagglutination and serum neutralization test.

Rabbits were sacrificed on 7^{th} day post challenge. Impression smears prepared from tissues of vaccinated animals on staining by Gimenez method revealed less number of EBs, compared with control group.

Complete clearance of EBs was noticed in immunized rabbits challenged on 70^{th} day post vaccination.

Clearance of EBs was faster and more with EB vaccine than yolk sac vaccine.

Serum samples from rabbits vaccinated with inactivated yolk sac vaccine showed PHA titres of 0, 6, 24, 53, 64, 64, 37 and 26 respectively at days 0, 7, 14, 21, 28, 42, 56 and 70.

The PHA titres observed in the serum samples of rabbits vaccinated with inactivated EB vaccine at days 0, 7, 14, 21, 28, 42, 56 and 70 were 0, 12, 24, 59, 107, 107, 107 and 64 respectively.

The peak neutralization titre of Group I and II were 22 and 89 respectively.

The passive haemagglutination and neutralization titres of yolk sac vaccine were lower than that of EB vaccine.

Based on the results obtained from tissue clearance of EBs (challenge experiment), PHA and SNT, a better immune response was noticed with EB vaccine than yolk sac vaccine.

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ASSESSMENT OF DIFFERENT EXPERIMENTAL VACCINES AGAINST Chlamydophila abortus (Chlamydia psittaci) IN RABBITS

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ABSTRACT OF THE THESIS

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ABSTRACT

Two isolates of *Chlamydia psittaci* viz., M-430 and M-28, maintained in the Department of Microbiology, were used in the study. M-430 was used for the preparation of inactivated yolk sac and elementary body vaccines and M-28 was used for challenge experiment.

These isolates were revived by inoculating to six to eight day-old chicken embryo through yolk sac route. Both isolates produced characteristic lesions in the embryo and yolk sac membrane.

M-430 was also propagated in Mc Coy cell line for high yield of elementary bodies for vaccine preparation.

Homogenous suspensions of the yolk sac and elementary body vaccines were inactivated with formalin to a final concentration of 0.4 per cent. Pure and safe preparations were used for vaccination trial.

Immunogenic potential of the vaccines were tested in rabbits by giving two doses of each of the vaccines. The first dose was given at three months of age and second dose was given 14 days after the first. Three vaccinated and control rabbits were challenged with M-28 isolate on 28^{th} day post vaccination and rest three on 70^{th} day post vaccination.

The clearance of elementary bodies from the tissues (lung, liver lymphnode and spleen) of vaccinated rabbits was an indication of the protection conferred by the vaccines. Better response was noticed with EB vaccine than yolk sac vaccine.

The sera were collected from rabbits at regular intervals of 0, 7, 14, 21, 28, 42, 56 and 70 for passive haemagglutination and serum neutralization test. Both vaccines elicited good immune response. The greater humoral immune

response of the rabbits that received EB vaccine suggests its slight superiority over the yolk sac vaccine.

More evaluation and elaborated field trials on target species are required before advocating the vaccine for field use.