PREVALENCE OF CHLAMYDIAL AGENTS IN LIVESTOCK IN KERALA

By

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THESIS

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Dedicated

To my parents

DECLARATION

I hereby declare that this thes s entitled **Prevalence of Chlamydial agents in livestock in Kerala** s a bonaf de record of research work done by me dur ng the course of research and that the thesis has not previously formed the bas s for the award to me of any degree diploma assoc ateship fellowship or other s m lar title of any other University or Society

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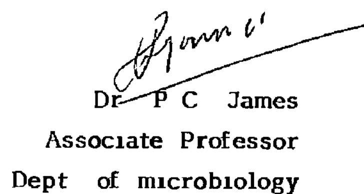
Reji Francis

CERTIFICATE

Certified that this thesis entitled **Prevalence of Chlamydial agents in livestock in Kerala** is a record of research work done independently by Sri **Reji Francis** under my guidance and supervision and that it has not previously formed the basis for the award of any degree fellowship or associateship to him



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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CE	Chicken embryo
CAM	Chorio allantoic membrane
CFT	Complement fixation test
EB	Elementary body
EBA	Epizootic bovine abortion
ELISA	Enzyme labelled immunosorbent assay
IF	Immunofluorescence
IIFA	Indirect immunofluorescence assay
MOMP	Major outer membrane protein
MoCFT	Modified complement fixation test
PHA	Passive haemagglutination test
PBS	Phosphate buffered saline
RB	Reticulate body
SBE	Sporadic bovine encephalomyelitis
SPG	Sucrose phosphate glutamate
SRBC	Sheep red blood cells
W/V	Weight/Volume
YS	Yolk Sac

Introduction

INTRODUCTION

Evolving a generation of livestock with superior germplasm of greater production potential attainable by better breed ng programmes maintenance of good level of nutritional status by better feeding adoption of scientific husbandry practises and provision of efficient health cover age play pivotal role in augmenting the output of livestock products The achievement attained n this respect during the recent past has been re markable Many age old infectious diseases afflicting the animals and causing heavy economic losses have been successfully kept under control by adopting prophylactic measures such as protection of the livestocks employing suitable vaccines timely investigation of outbreaks of infecti ous disease and diagnosis advocating suitable control measures to be adopted to arrest the dissemination and perpetuation of the disease and proper surveillance system In sp te of this health hazards due to var ous infectious diseases pose a grave problem and remain as major im pedements interfering with attainment of anticipated output of animal pro duction because of the emergence of newer and hitherto unknown diseases Among such diseases Chlamydiosis stands as one important disease en tity

Chlaymydiae is notoriously known to be responsible for mortality and morbidity among animals with concomitant economic losses for the farmer Because of its zoonotic nature it becomes all the more import ant involving public health sphere too

Association of chlamydial organisms in the causation of clinical syn drome with sequelae of abortion/still birth/neonatal and perinatal morta

lity in livestock has been reported from many countries (Storz 1971 Pienaar and Schutte 1975 Shewen 1980)

In India also there are a few reports available recording the prevalence of chlamydial agents as proved by serological evidence demon stration of these organisms in the clinical materials by spec al staining techniques and also by cultural isolation from clinical samples (D xit and Kalra 1984 Gupta et al 1985 Krishna et al 1986 a) The research work on chlamydiosis carried out at the All India Institute of Medical Science New Delhi Haryana Agr cultural University Hissar Indian Ve terinary Research Institute Izatnagar has proved the prevalence of chla mydial agents in the northern states of India But the information on this agent regarding its prevalence n the southern states and its status as a clinical entity is very meagre

The pros and cons of the disease syndrome caused by chlamydial infection among livestock in the state of Kerala have not be probed into and hence the information on chlamydial infection in livestocks is pra ctically nil Persual of available literature reveals that there has been no report of documentation of chlamdyiosis in livestocks in Kerala Consi dering its economic importance status as an emerging scourge zoonotic nature and the pausity of even basic information on chlamydial infection in Kerala state it was envisaged to in tiate a pilot study on chlamydial agents The results acquired during the course of work may help unra vel some hitherto unknown facts about this disease entity Moreover the tests for its detection will get standardised and this in turn will be quite useful for the diagnosis and investigational work in future in this

regard

The major aspect of the work envisaged is the assessment of the magnitude of prevalence of chlamydia among the livestock in Kerala state based on serology and isolation of the agent In order to accomplish that the following works were entailed

i) Collection of sera samples from divergent species of domestic animals particularly with history of abortion and pneumonic complications

ii) Detection of antibodies by serolog cal tests

iii) Collection of clin cal mater als particularly from cases of abor tion and pneumonia

iv) Staining the smears prepared out of clinical materials by stan dard procedures and screening for the presence of chlamydia

v) Attempts for the isolation of chlamydia by biological means em ploying chicken embryo/guinea pigs

vi) Study the feasibility of the above experimental animals for the purpose of isolation

Review of Literature

REVIEW OF LITERATURE

Chlamydia are a group of highly specialized ovoid or spherical Gram negative bacteria that undergo a well defined life cycle in the cy toplasm of their host cell This group of pathogens is extremely wide spread in nature with a wide spectrum of hosts

1 1 **Taxonomic Status and Properties**

Inclusion of C trachomatis in infected ocular material were first observed by Halberstaedter and Von Prowazek in 1907 They thought that this agent was a protozoa and conferred the name chlamydozoaceae to these mantled animals The classification and nomenclature of the ch lamydiae were for long unsettled and these organisms have been referred to as Miyagawanella Bedsonia and psittacosis lymphogranuloma trachoma agent Page (1966) proposed the now generally accepted description of the genus Chlamydia

In the infancy of chlamydial research these organisms were consi dered as viruses because they could be propagated only n a living me dia However once the concepts of Stainer and Lwoff on the fundamen tal differences between bacteria and viruses had been formulated and generally accepted it became clear that chlamydia are bacteria (Duguid et al 1978 Merchant and Packer 1983) Unlike viruses chlamydiae possess both the types of nucleic acid DNA and RNA The cell wall is quite analogous in structure to Gram negative bacteria and they multi ply by binary fission They also possess a number of enzymes and have a restricted metabolic activity They are susceptible to many an

tib otics and antimetabolites that do not affect viruses In short there s a fundamental discontinuity between the chlamydiae and even the lar gest animal viruses (Collier 1984)

However chlamydia can be distinguished from other groups of in tracellular bacteria and rickettsiae by their inability to synthesize com pounds for high energy storage and utilization (Weiss and Wilson 1969 Hatch 1975) by their lack of cytochromes and other components of the respiratory cha n They are considered as energy parasites that use the ATP produced by the host cell for their own requirements (Moulder 1966) Moreover the chlamydiae have evolved a developmental cycle that s unique among prokaryotes (Becker 1978 Ward 1983)

Chlamydiae are related to viruses in certain aspects the foremost being that these agents can be cultivated only in a living media More over chlamydiae induce the production of interferons which inhibit their replication The mechanism of interferon med ated inhibition of C psi ttaci replication probably involves an induced depletion of the essential amino acid tryptophan from the cytoplasm c pool and results only in a reversible chlamyd astasis (Byrne 1986)

The possib lity of evolution of chlamyd ae from an ancestral free l ving bacteria is speculated The members of the genus Acinetobacter possess antigens that fix complement with antibodies to chlamydiae(Storz 1971)

The fact that ant gens of Bacterium anitratum (Herella) fixed comp lement in the presence of chlamyd al antibodies but did not provoke an

tibodies reacting with chlamydial antigens was established by Volkert and Matthiesen (1956) and it was confirmed by Shimizu and Bankowsk (1963) Therefore infect on with this beacteria cannot lead to false po sitives in the serodiagnosis of chlamydial infection

For the above reasons a new order chlamydiales was established which consisted of one family chlamydiaceae and one genus chlamydia containing two species Chlamydia trachomatis and Chlamydia psittaci (Storz 1971 Becker 1978 Ward 1983) Some of the features distin guishing the two chlamydial species are summarised n table 1

Kingsbury and Weiss (1968) showed that only 10% of the DNA se quence of C trachomatis and C psittaci are homologous This indicated that these two chlamydia species are d fferent Nevertheless the two species are clearly related by their common developmental cycle common antigens and similar biological and metabolic activity justifying their inclusion within the same genus (Becker 1978 Ward 1983)

The organisms are small non mot le weakly Gram negative obligate intracellular parasites occurring as two types small and large(St orz 1971 Duguid et al 1978 Merchant and Packer 1983 Ward 1983) The small type having 300nm diameter has a compact electron dense nucleoid and this is the highly infectious stable extracellular form the elementary body (EB) of the organism The larger form 800 1200nm in diameter without a dense nucleoid is the initial body or reticulate body (RB) which s intracellular and fragile and constitute the replicating form of the organism The organism grow in the cytoplasm of the host cells for ming characteristic micro colonies or inclusion bodies made up of a mix

Table 1 Features distinguishing between C trachomatis and C psittaci(Becker,1978 and Ward,1983)

Features	C trachomatis	C psittaci
Natural infection	Principally human ocular and urogenital diseases	Respiratory urogenital and systemic infection in a wide variety of animals Man is incidentally infected
Laboratory growth	With exception of LGV agents require centrifugation onto specifically prepared tissue culture cells	Grow readily in tissue culture without centrifugation
Intracytoplasmic replication characteristic	Replicates within cytoplasmic vesicles which have enough rigidity to displace the nucleus of the infected cell	Replicates in cytoplasmic vesicles that spread throughout the cytoplasm of the host cell
Inclusions	Compact glycogen containing stain with iodine	Diffuse non glycogen containing do not stain with iodine
Nucleic acid	Guanosine+cytosine(G+C) 44%	G + C 41 2 %
Sulpha drug sensitivity	Sulphadiazine sensitive	Sulphadiazine resistant

ture of the larger and smaller cells Both the forms of chlamydia are clearly visible by the light microscope

Both forms stain with the Macchiavello Castaneda Gimenez modified Ziehl Neelsen method and also by Giemsas Method(Storz 1971 Cruickshank et al 1975 Buxton and Fraser 1977 Duguid et al 1978 Merchant and Packer 1983) They stain blue by Castanedas method and red by Macchiavelo modified Ziehl Neelsen and Gimenez method The Giemsa s method has the advantage of distinguishing between the small redd sh purple EB and the larger more basophilic RB(Storz 1971)

Chlamydiae are rich in lipid content and all species of the organism are inactivated by ether within 30 minutes They are rapidly inactivated by heat and lose their infectiv ty completely after 10 minutes at 60°C and when stored in glycerol (Buxton and Fraser 1977) C psittaci is reasonably stable when stored at refrigeration temperature but quite labile at higher temperatures

Repeated freezing and thawing is detrimental to the organism and cause a minimum loss of 1 log in infectivity (Clyde 1984 Schachter 1986 a) This aspect has a specific significance during the long term storage of stock cultures

Multiplication of chlamydia is inhibited when treated with adequate concentration of erythromycin chloramphenicol tetracycline or penicillin (Buxton and Fraser 1977 Collier 1984) They are however resistant to a number of antibiotics notably the aminoglycosides polymyxin lincomycin Vancomycin and mycostatin Some of these antibio

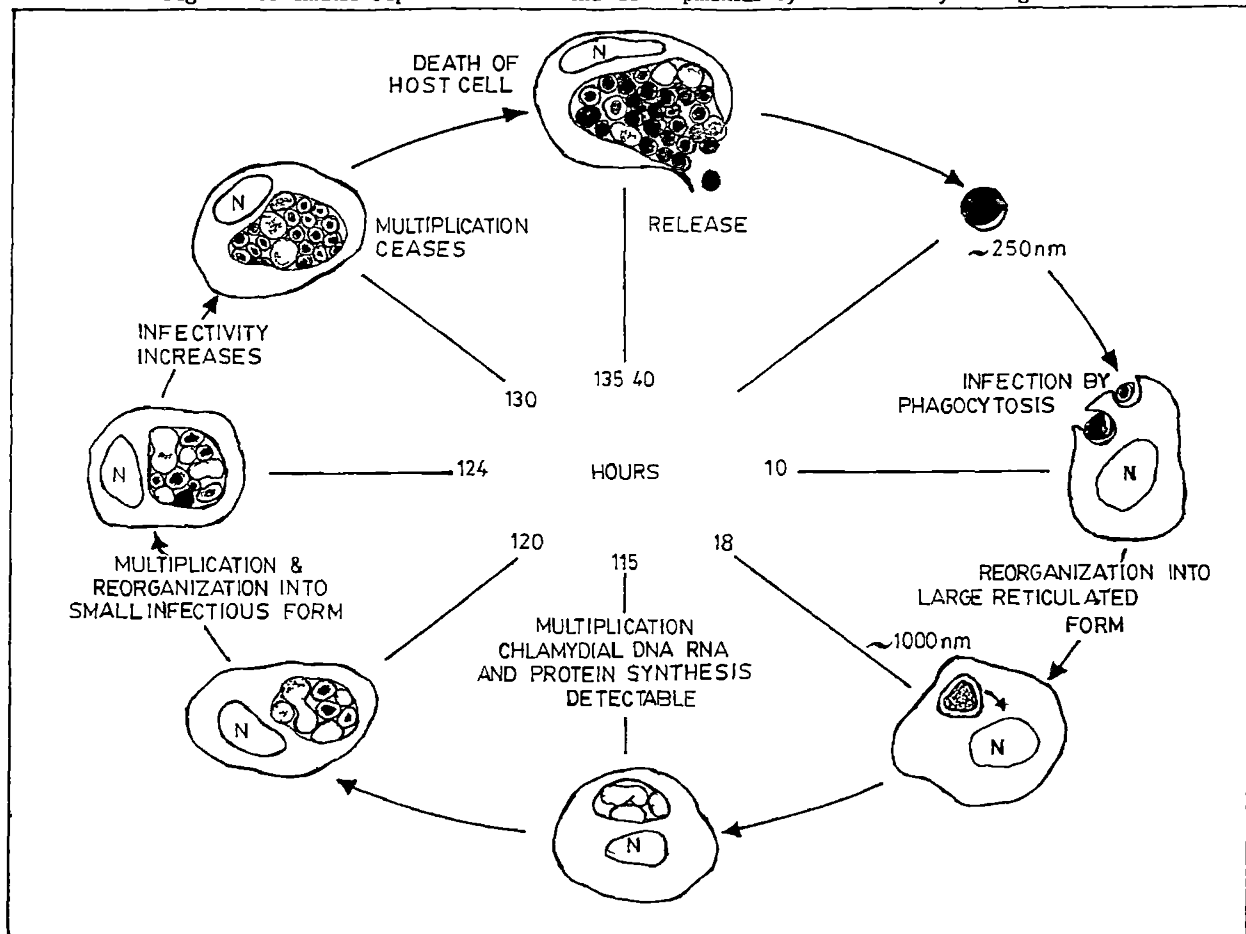
tics alone or in combination are thus useful for suppressing contamina tion by bacteria fungi and yeast dur ng the solation of chlamydia from cl nical specimen or during routine passage n the laboratory

1 2 Multiplication

Chlamydiae have responded to the specialized demands of obligate intracellular life cycle by evolving a developmental cycle that is un que amongst prokaryotes (Buxton and Fraser 1977 Becker 1978 Ward 1983) (Fig 1)

Large number of elementary bodies measuring about 0 2 0 3μm in diameter are adsorbed onto the surface of a susceptible host cell The elementary body adhesins are thought to be cell wall components (Levy and Moulder 1982) Although the major outer membrane protein (MOMP) appears to be a strong candidate for a chlamydial adhesin antibody to MOMP does not affect either attachment or uptake of chlamydia(Caldwell and Perry 1982) It has been proposed that N acetyl glucosamine might be an important component of the host cell receptor (Levy 1979) But th s was refuted by Ward in 1983 Unlike the rickettsiae there s no active mechanism of cell penetration dependent on ATP (Cru ckshank et al 1975) The EB are taken into the host cell by phagocytosis Following phagocytos s the EB lies in a membrane bounded phagosome but is evidently resistant to digestion by the hydrolytic lysosomal en zymes The phagosome contain ng the organism do not for some reason become fused with lysosomes at least until later n the reproductive cycle (Friis 1972 Eissenberg et al 1983) Once inside the cell s

Fig 1 Schematic representation of the developmental cycle of chlamydial agents



cytoplasm the elementary bodies undergo a characteristic sequence of chemical and morphological changes During the lag phase which lasts about 1 10 hours the particles undergoes internal reorganisation and changes from small spherical structures into large less deeply staining initial bodies measuring about 0 7 to 1 2 μm n diameter From about 15 20 hours after infection the number of initial bodies increase rapidly and they aggregate in clusters in a homogenous fluid matrix which appears to digest the surrounding cytoplasm to form a vacuolar structure or vesicle This phase of development is followed by a stage of rapid multiplication when the large bodies appear to break down into smaller bod es with dense centres either by budding or by division in several planes to form four or five particles of equal size Towards the end of the multiplication cycle the cytoplasm of the host cell may be packed with particles of both types but the nucleus is not effected and the cell may continue to grow and divide The particles are ultimately released by lysis of the infected cell The cycle takes about 24 to 48 hours (Cruinckshank et al 1975 Ward 1983)

1 3 Epidemiology

Chlamydiae have a wide spectrum of hosts comprising human be ngs many species of domesticated animals and birds and numerous sylvatic fauna The oroganism does not appear to be very host or tissue specific Many strains produce generalized infections in several host spec es others localize and cause pronounced inflammation in one or more tissue or organs of a specific host Exceptionally some animals may experience severe or even fatal disease as a result of chlamydial

infection A well balanced host parasite relationship represents the common nature of chlamydial infection This long lasting inapparent or or latent state has been documented in several species of animals and birds (Shewen 1980) In some cases chlamydia are excreted by the latently infected host in other cases the organism could be maintained in a non infectious form Under circumstances of stress carrier animals may shed organisms in large numbers or may in fact lapse into clinical disease (Storz 1971 Byrnes and Faubion 1982)

Portals of infection of the susceptible hosts are the oral oropharyngeal conjunctival and respiratory tract when the animal remains active in an environment contaminated with faeces carrying chlamydiae

A scheme outlining the multiplication and spread of chlamydiae as proposed by Eugster and Storz (1971a) is given in the following page (Fig 2)

Other investigators have questioned the capacity of chlamydiae to survive in the forestomachs and abomasum of the adult animal Horsch (1980) has suggested another scheme as shown in the following page (Fig 3)

The transmission of *C psittaci* from birds to man is universally recognized (Shewen 1980) There are also well documented cases of human *C psittaci* infection of mammalian origin Several serological survey of persons in contact with chlamydia infected animals suggest that these animals can be a source of human infection (Storz 1971 Schachter *et al* 1973) Infectious agents with chlamydial property have been isolated from aborted foetus and the placentas of aborting women These

Fig 2 Scheme outlining the proposed multiplication and spread of chlamydia (Eugster and Storz, 1971)

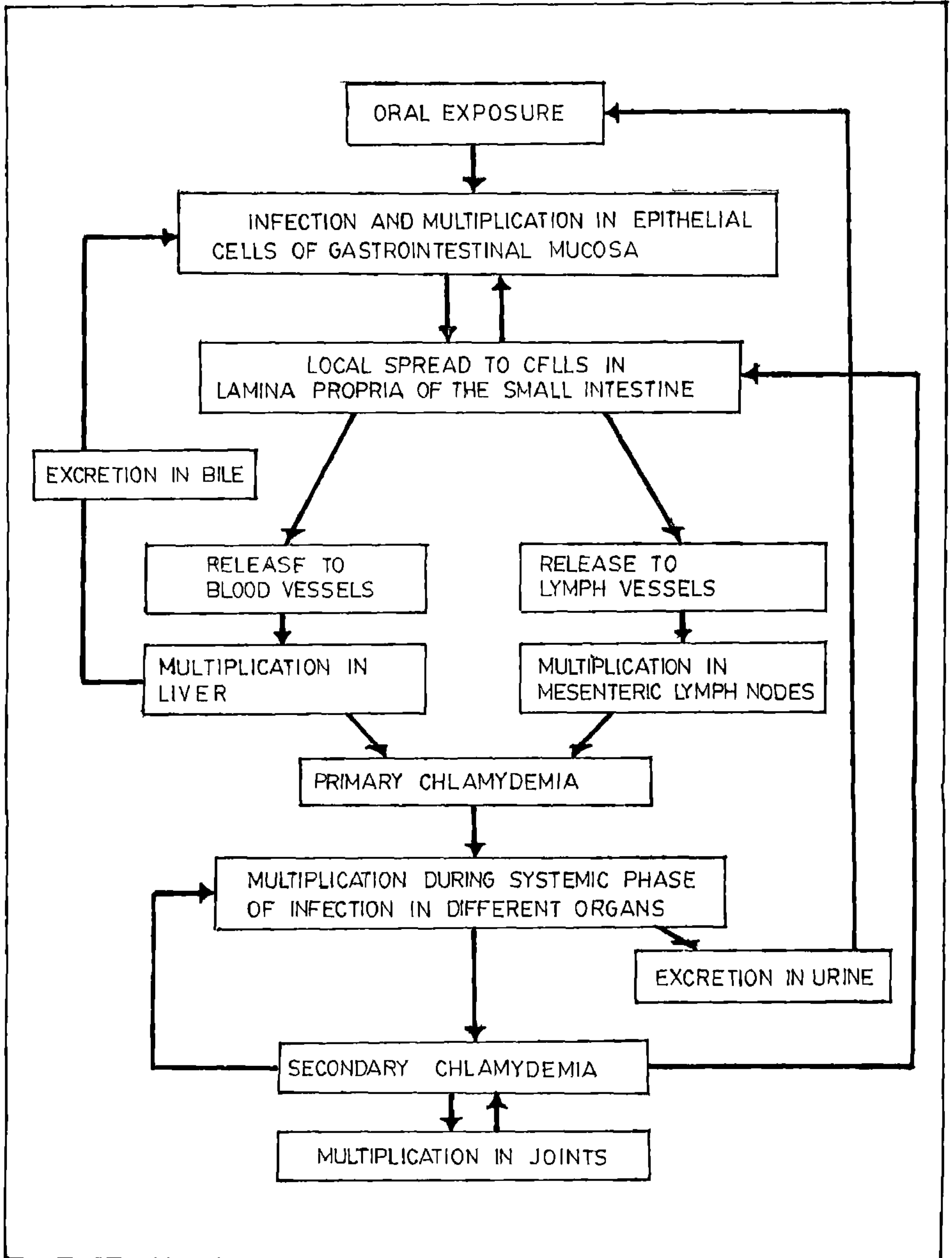
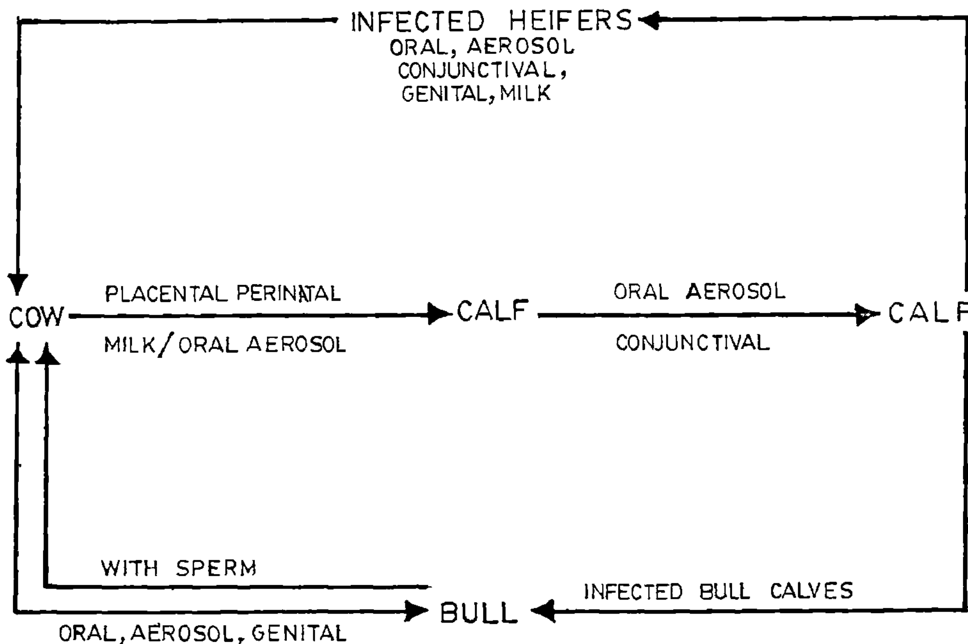


Fig 3 Transmission scheme proposed by investigators who doubt the capacity of Chlamydia to survive in the forestomach and abomasum of the adult cow (Horsch, 1980)



women had contact with sheep goats or cattle suffering from chlamydial pneumonia or abortion (Johnson 1985 Schachter 1986 b)

The natural modes of transmission of C psittaci are insufficiently understood (Johnson 1983) Irrespective of the serotype the intestinal tract is the natural habitat for chlamydia (Eugster and Storz 1971a) Chlamydial organisms have been isolated from the faeces of healthy cattle(W lson 1963 Storz 1968) sheep (Wilson and Dungworth 1963 Storz 1963 Dixit and Kalra 1979) and goats (Omori et al 1957) Upto 60% of animals in a particular herd may shed organisms for several years (Shewen 1980) The quantity of chlamydiae shed through faeces varies from hardly detectable level to a concentration of 10^4 to 10^6 nfectious units per gram of faeces (Storz 1971 Shewen 1980) Infected ruminants excrete chlamyd al agents over a long period of time It has been observed that naturally infected sheep excrete these agents intermittently over several years (Storz 1971) Experimentally infected calves continued to shed chlamydiae in the faeces for more than six months Thus the perpetuation of the infectious cha n s tightly linked to the excretion of infectious faeces

It is ecologically significant that the active intestinal chlamyd al infection does not induce resistance to superinfection with the homologous or other chlamyd al strains nor does parenteral superinfect on n fluence significantly the events of infection in the intestinal tract (Dungworth and Cordy 1962 Storz 1963 Parker et al 1966) Inapparent intestinal infection may play an important role in the maintenance and pathogenesis of ruminant pneumonia and abortion but be less significant in other chlamydial disease (Shewen 1980)

Omori et al (1960) exper mentally induced endometritis in cows Following the induct on he could reisolate the same organism from the milk the uterine and vaginal discharge

A rap dly fatal disease of newborn calves caused by an overwhel ming chlamydial infection apparently derived from heavily infected colo strum was reported by Blanco Loizelier (1974) Calves not fed with the nfected colostrum remained healthy

A sylvatic cycle involving rodents ticks and fleas has also been suggested (Eddie et al 1969) Chlamydia with pathogenicity similar to Epizootic bovine abortion (EBA) was isolated from the tick Dermacentor occidentalis and mammals from a sylvatic environment(Caldwell and Belden 1973) These investigators were unable to propagate the chlamydia in the viscera of ticks or to transmit the disease by attaching nymph to cattle

Veneral transmission also was proposed by Horsch (1980) which was confirmed by solation of chlamydia from the semen of infected bulls(Storz et al 1968 Boryczko et al 1973 Avzalov and Kurbanova 1987) It is not known how bulls acquire the genital infection under natural condi tions but it is likely that genital infections may be the result of a pre ceding systemic chlamydial nfection It also could be an ascending in fection generated by contamination with chlamydia nfected faeces (Storz 1971)

Adult animals rarely develop enteric disease as a result of intesti nal chlamydial infection However cases of chlamydial enterit s were reported in young animals by York and Baker (1951) & Omor et al (1960)

The onset of signs is rapid but their severity depends on the virulence of the strain of chlamydia age of the animal and protection afforded by colostral antibodies (Eugster and Storz 1971 b)

It is suggested that with contact or aerosol exposure conjunctivitis and pneumonia are the predominant signs (Horsch 1980 Idtse 1984) where as signs of enteritis and polyarthritis predominate with oral exposure (Idtse 1984)

Although chlamydiae are incriminated as the cause of enzootic pneu monia the association of other bacterial and viral agents appear to be essential for the development of a severe disease It is possible that chlamydial infection paves the way for the attack of the lungs by bacte ria and viruses In this respect chlamydia might play a role in patho genesis eventhough by themselves they induce relatively mild s gns(Storz 1971 Shewen 1980 Eugster 1986) Trade and shipping of rum nants probably play an important role in spreading pneumonia(Omori et al 1960) Contact and aerosol appear to be the principal means of transmission of conjunctivitis caused by chlamydia The infection is mild unless a secon dary infection complicates it

Cutlip et al (1972) observed polyarthritis in lambs They noted that the morbidity was high (80%) especially in larger lambs but morta lity was less than 1% In contrast mortality was extremely high in young calves affected with chlamydial polyarthritis though outbreaks was only sporadic (Storz 1971 Shewen 1980)

There are reports of sporadic bovine encephalomyelitis(SBE) parti

cularly ın cattle less than three years of age although older anımals may also be affected (Menges et al 1953 a 1953 b) The dısease may re maın enzootıc ın one herd Sometımes outbreaks follows ıntroductıon of susceptıble cattle ınto establıshed herds

Epızootıc bovıne abortıon(EBA) due to chlamydıa has been reported by several workers EBA ıs also known colloquıally as foot hıll abor tıon because of ıts hıgh ıncıdence among cattle whıch pasture on foot hıll or mountaınous terraın (Kennedy et al 1960 Mckercher 1969 Eugster 1986 K msey 1986) The strıct geographıc dıstr but on suggests the possıbılıty of a vector or a wıld lıfe reservoır ın the epıdemıology of EBA(Kennedy et al 1960 Mckercher 1969)

In epızootıc areas only the natıve heıfers ın the r fırst gestatıon or cattle ıntroduced from an area free of the dısease are affected(Mcker cher 1969) However ın areas where the dısease ıs occurrıng for the fırst t me cattle of all ages are susceptıble The abortıon rate ın such cases range from 25% to 75% or even hıgher among the pregnant anımals ın a herd

Chlamydıal nfectıon of pregnant ewes and does ıs a well documented feature all over the world (Young et al 1958 Storz 1971) On ınıtıal exposure the ıncıdence of abortıons may be as hıgh as 35% of the preg nant stock(Shewen 1980 and Storz 1986) But n enzootıcally ınfected herds only 1 to 5 % of the pregnant anımals abort and losses occur year after year Dams of all ages abort but hıgher rates may be observed among the young ones (Young et al 1958 McCauley and Tıeken 1968 Storz 1986)

Clinical Syndromes

Chlamydiae multiply in cells of the reticuloendothelial system in epithelial cells of the conjunctival genital and intestinal tracts in synoviocytes and in cells of the placenta and foetus Depending on factors such as virulence of the agent species age and sex of the animal environment management practices ecology and physiologic conditions of the host chlamydial infection may result in anyone or a combination of the following clinical manifestations like pneumonia arthritis polyserositis diarrhoea conjunctivitis and abortion/still birth

1 4 1 Respiratory infections

Chlamydial agents have been incriminated as one of the primary causes of pneumonia in small ruminants Some of the earliest reports of enzootic pneumonia in sheep are by Mckercher (1952) and Dungworth and Cordy (1962) Similar condition had also been identified in goats (Ishii *et al* 1954)

Since then this agent has continued to elicit the interest of the scientific community as an etiological agent in pneumonia In India the prevalence and significance of chlamydial pneumonia in small ruminants has been recognized only recently

Purohit and Gupta (1983) processed pneumonic lung lesions from 52 sheep and 55 goats for isolation They were successful in isolating chlamydia from two sheep and one goat

Dixit and Kalra (1984) isolated chlamydia from 50% cases of pneumo

nia in sheep and goat They obtained four isolates from sheep and one from a goat This high rate of isolation was achieved from a local farm where pneumonia was known to be endemic and causing serious problems

Sreeramulu (1984) screened 14 pneumonic lung of sheep for chlamydia by the CE inoculation technique Two samples yielded positive results

Gupta *et al* (1985) processed 102 pneumonic specimens from goats They were able to isolate chlamydia from only one specimen However the same group of workers were able to obtain three isolates of *Chlamydia psittaci* from 69 Sheep with pneumonia The isolation rate in sheep was 4 34% and in goat 0 98% only

Ov ne pneumonia of chlamydial etiology is characterized by fever dyspnoea and anorex a (Stevenson and Robinson 1970) More dramatic signs such as mucopurulent frothy nasal discharge and death occur as a result of secondary bacterial invasion in which *Corynebacterium pyogenes pasteurella multocida P haemolytica* and mycoplasma are frequently implicated (Dungworth and Cordy 1962) Goats affected with chlamydia pneumonia have symptoms similar to sheep (Shewen 1980)

One of the earliest report on chlamydia pneumonia in cattle was by Patolay and Newhall (1958) Although they were of the opinion that it was a new pathogen they were unable to identify it properly

Chlamydial pneumonia n calves may be subclinical detected ncidentally at slaughter or severe with prostration marked dyspnoea and d arrhoea Most cases are of moderate severity characterized by fever mucoid or mucopurulent nasal discharge coughing and depression (Palotay and New

hall 1958 Omori et al 1960 and Storz 1971) Chlamydia have been implicated in the shiping fever syndrome However the concurrent presence of bacteria reovirus or para nfluenza virus were necessary to experimentally reproduce the disease (Storz 1971 Thomas and Collins 1974)

Werdin (1973) isolated C psittaci from five cases of enzootic calf pneumonia He also experimentally reproduced the clinical syndrome in colostrum deprived calves using the isolates obtained from the natural cases

Dingra et al (1980) screened three cases of bovine pneumonia They were able to isolate chlamyd a from one of them

Gupta et al (1985) could obtain only one isolate of C psittaci from a total of 63 pneumonic specimens processed

Chlamydia was also reported as a cause of pneumon a among buffaloes Dingra et al (1980) reported the isolation of chlamydia from two out of nine cases of pneumon a in buffaloes

Sood et al (1986) have confirmed the findings of the former workers By means of the ind rect immunofluorescence technique they established that seven out of the 17 cases of pneumonia in buffaloes was on account of chlamydia

1 4 2 Genital infections

1 4 2 1 Abortion/still birth

In many areas with a good population of cattle sheep and goat in

spite of the eradication of brucellosis and the rarity of trichomoniasis leptospiral and vibrionic abortion the abortions continued unabated This has forced the scientific community to tow a different line of research to pinpoint some etiological agents that could credibly explain the massive epizootics of abortion Their efforts have not been in vain It is now fairly well established that C ps ttaci could explain for many of the epizootics of abortion observed in different parts of the world

The ability to infect the placenta and the foetus in several animal species regardless of the type of placentation is a property of many chlamydial strains In experimental infection this occurs during a secondary bacter aemic phase following replication of the organism in somatic organs

C psittaci was identified as the cause of epizootic bovine abort on (EBA) by Storz et al (1960) in the U S A Since then reports from many countries like the U S A (McKercher et al 1966) the Netherlands(Akkerman and Dinkla 1974) Russia (Kurbanov et al 1978) and Australia (Seaman et al 1986) have established the importance of this agent in the causation of abortions in bovines

Ognyanov and Genchev (1970) could obtain nine isolates of C psittaci from 24 samples of aborted foetuses and placenta

Chlamydial nfection was diagnosed as the cause of 13% of he abortion in a herd of cattle by Schoene in 1971

Kurbanov et al (1978) like other workers isolated chlamydia from

bovine abortion cases They also reproduced the condition experimentally in heifers using their original isolate

Durand et al (1980) could isolate C psittaci from samples in one herd out of the ten with a history of abortion However a much high prevalence was proved serologically The abortion rate varied from 4 10% in the various herds

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

Glavitis et al (1982) reported the isolation of C psittaci from 43 6% of bovine abortions

Chlamydial abort on in a herd of 60 cows in which six cows aborted and twelve had metritis and retained foetal membranes was reported by Wil smore and Dawson (1986)

Epizootic bovine abortion occurs suddenly in a herd without evinci ng any other symptoms prior to abortion (Shewen 1980 and Eugster 1986) Cows appear to be susceptible in the second trimester of gestation with abortion occuring 45 to 120 days after the chlamydemic phase Clinically a good majority of the abortion due to C psittaci occurs during the sixth to the eighth months of gestation (Storz 1971 Shewen 1980 Idtse 1984 Eugster 1986) Earlier abortions occur rarely (Idtse 1984 Eugster 1986) Occasionally the infection results in still birth and neonatal mor tality Reduction of milk yield retained placenta metritis and rebree ding problems can be present post partum(Storz 1971 Shewen 1980 Idtse 1984 Eugster 1986)

In bovines EBA, has also been produced experimentally (Howarth et al 1958 Bassan and Ayalon 1971) Cattle when inoculated with chlamydia develop high fever with n a day or two which persist for two days The time between inoculat on and abortion depends mainly on the route of exposure (McKercher 1969) Intravenous exposure results in abortion three to six weeks later intramuscular inoculation in approximately three months and subcutaneously in four to four and a half months or more (McKercher 1969)

Stamp et al (1950) were the first to identify the etiological agent of enzoot c abortion of ewes (EAE) as C psittaci The d sease is also popularly known as Kebbing (Buxton and Fraser 1977) S nce then ovine chlamydial abortions has been reported from various parts of the world

On microscopic examination of stained impression smears of foetal tissues Frank et al (1962) could discover chlamydial bodies n 30% of the 53 ovine foetuses examined

Chalmers et al (1976) identified chlamydial abortion in four flocks of sheep in Alberta Canada However they were able to isolate chlamydia from the t ssue of aborted lambs from only one flock

Linklater and Dyson (1979) analysed the statistical data collected from twenty sheep flocks in which EAE was d agnosed by examination of the foetal material and maternal blood samples The abortion rate turned out to be 7 6%

C psittac is the major cause of abort on in sheep n the United Kingdom and is widespread throughout flocks of all breeds(Johnson 1983)

The abortion syndrome caused by C psittaci was also identified in goats According to McCauley and Tieken (1968) there was a 12% abortion rate in a flock of goats during the kidding season

Chlamydial abortions in sheep and goats have been reported from India also

Jain et al (1975) examined 77 ovine and three caprine foetuses aborted in late pregnancy They were able to isolate C psittaci from all the cases

Chlamydial abortion in sheep was reported from the State of Rajasthan by Sharma et al (1983) using the chick embryo isolation technique

Gupta et al (1985) were able to obtain three chlamydial isolates from 38 goat foeti and two out of 34 sheep foeti

Purohit et al (1986) subjected 30 clinical specimens from cases of abortion and still birth in goats to isolation and histopathological studies Isolation of chlamydia was effected in two out of the 30 specimens processed yielding a 6 66% isolation rate

Khanna et al (1987) isolated and identified two chlamydial strains from the stomach of two out of the 56 aborted ovine foetuses which were found negative for brucella and mycoplasma

1 4 2 2 Endometritis and repeat breeding

Chlamydial endometritis results in repeat breeding and consequently infertility among animals

Dan eli and Ayalon (1970) demonstrated chlamydial elementary bo dies in the post partum discharge of 23 cows nine of which had endo metritis

Screening to assess the magnitude of the prevalence of chlamydial infection in 28 herds with a total strength of 533 cows carried out by Jahn et al (1972) resulted in the isolation of chlamydia from 42% of the cervical mucus samples from 134 infertile cows Moreover 68% of the 439 animals were found serologically positive for chlamydial antibodies

It is a documented fact that animals inseminated with semen conta minated with chlamydia become repeat breeders

Bowen et al (1978) inseminated ten healthy heifers with semen artificially contaminated with C psittaci They observed that none of the animals were pregnant when examined 40 days later Five out of ten control heifers inseminated with the same semen mixed with the control diluent became pregnant In a second set of experiment normal embryos were recovered from eight heifers 60 to 72 hours after inseminat on with semen containing chlamydia This indicated that fertilization fai lure was not responsible for the infertility Uterine biopsy n this group revealed scattered chlamydia infected cells that were detected by immu nofluorescence in the subepithelial tissues of the uterine horn These find ngs suggest that an alteration in the uterine environment caused by the multiplication of C psittaci could be responsible for repeat breed ing

In India Sharma et al (1982) reported the isolation and characte rization of chlamydia from the genital tract of repeat breeding cattle and

buffaloes The cervicovaginal mucous samples of 45 cows and 160 buffaloes with repeat breeding problems were processed for the isolation of chlamydia Four strains of chlamyd a one from a cow (2 2%) and three (1 8%) from buffaloes were isolated by the yolk sac inoculation technique

Mishra and Mishra (1985) isolated C psittaci from 32 cross bred repeat breeder cows and heifers Using the isolates they contaminated normal semen which was then used to inseminate ten cows Four of the cows iseminated with chlamydia infected semen conceived but aborted after four to f ve months of pregnancy The other six cows repeated more than four t ems They also carried out serological studies on these animals Gel diffusion test was performed on 30 sera samples of the repeat breeders Of these 12 were strongly posit ve while five gave weak prec pitation lines Complement fixation test of the same 30 sera samples yielded titres of 1 8 in 11 and 1 16 in 7 samples

1 4 2 3 Mastitis

Chlamydial agents had also been isolated from a few natural cases of mastitis Wehnert et al (1980) reported the isolation of chlamydia from five out of 34 milk samples from mastitis cases Though the reports on the incidence of naturally occurring chlamydial mastitis are very few l terature on experimentally induced chlamydial mastitis are quite abundant Using an intestinal isolate Ronsholt and Basse(1981) induced a self limiting exudative mast tis with fibrinous secretion in bovines They were also able to record an increase in the CF and agglutinating antibodies in the milk whey and serum of the affected animals

Lactat ng ewes inoculated via the teat canal with C psittaci developed acute mastitis and a system c reaction (Papadopoulos and Leontides 1972)

1 4 2 4 **Other genital infection**

The seminal vesiculitis syndrome (SVS) is a condit on of primarily young bulls characterized by a chronic inflammation of the epididym s testicles and accessory sex glands The ejaculate usually contains large numbers of leukocytes and low number of spermatozoa with poor motility and primary and secondary abnormalities (Eugster 1986) It has been po stulated by Ball (1970) that chlamydia could be one of the primary causes of SV syndrome In the long run secondary bacterial nfection may predominate in long standing cases These secondary infections may further complicate the disease process and may mask the primary cause of SVS C psittaci could cause epididymitis orchitis seminal vesiculit s and infection of the other accessory glands in both bulls and rams It was possible to isolate chlamydia from the infected tissues as well as from the semen of such infected animals The agent isolated could not be differentiated from that causing chlamydial abortion(Storz 1971) In fact many of the cases occurred n herds where abortion was endemic (Shewen 1980)

Storz et al (1968) isolated chlamydia from six samples of semen and the epididymis of ten bulls n which the seminal vesicul tis synd rome was common The agents isolated were found indistinguishable from those isolated from EBA when subjected to specific neutralization test

Balbierz et al (1973) could positively identify chlamydia in the

semen of six bulls out of nine that were suffering from inflammatory conditions of the testes They also demonstrated a significant change in the protein components of the seminal plasma as revealed by starch gel electrophoresis

Sadowski *et al* (1973) from Poland used a comb nation of light and fluorescent microscopy along with isolation techniques to examine 49 se men samples from 42 bulls suspected of chlamydial infection They could confirm the presence of the organism n seven bulls

Storz *et al* (1976) experimentally infected bulls and rams with C *psittaci* to demonstrate the characteristic urogenital infection and excre tion of the organism through semen All rams and three of the four ino culated bulls excreted chlamydiae in the semen for 22 to 29 days The quality of the semen was influenced adversely depending on the stage of infection Secondary abnormalities were highest 17 to 20 days after in fection and the percentage of normal sperm cells was lowest(49%)at this t me The semen also contained increasing amounts of PMN leucocytes

Travnicek *et al* (1980) from Czechoslovakia obtained relatively high antibody titres against *C psittaci* on repeated serological examina tion of five breed ng bulls From the pelleted ejaculates of two of these bulls *C psittaci* was also isolated

Avzalov *et al* (1987) observed that chlamydia infected semen could cause vaginitis abortion and still b rth in cows The surviving calves of the infected cows were liable to develop gastroenteritis bronchopne umonia and polyarthritis

1 4 3 Intestinal infections

Chlamydial infections of the intestine particularly of the leum are not uncommon They are clinically inapparent in adult cattle but can cause diarrhoea and enteritis in young calves This condition has been reported by several workers like McKercher and Wada(1959) and Dougri *et al* (1974) from the U S A Wilson (1963) from U K and French(1959) from Australia

The most severe signs are observed in newborn calves especially when they are deprived of colostrum part ally or totally In such cases death ensures severe enteritis In experimental inoculation of colostrum deprived calves with chlamydia severe diarrhoea as well as pneumonia and polyarthritis are seen In extreme cases death also takes place (Beasley *et al* 1962)

1 4 4 Ocular infection

Chlamydial agents have been associated with keratoconjunctivitis (Surman 1979 Wehr *et al* 1980) and follicular conjunctivitis(Cello 1967) The frequent occurrence of these conditions in conjunction with polyarth ritis or pneumonia may represent a manifestation of a systemic or con current local infection probably by aerosol(Storz 1971 Hopkin *et al* 1973) The conjunctivitis induced by chlamydiae is catarrhal unless a secondary infection supervene resulting in a purulent exudate

1 4 5 Encephalomyelitis

Chlamydial encephalomyelitis is commonly known as sporadic bovine

encephalomyelitis (SBE) or Buss disease (Buxton and Fraser 1977) Wenner *et al* (1953) identified the causative organism of SBE as a chlamydial agent Since the original recognition of SBE in calves this disease has been reported from numerous countries (Storz 1971) Initial depression and inactivity progressing to anorexia fever excessive salivation dys pnoea nasal discharge and mild diarrhoea are character stic Some ani mal may recover at this stage but most develop nervous signs Stiff gait circling and staggering are common Progressive weakness leads to pa ralysis and occasionally opisthotonus Death occurs 10 14 days after the onset of signs Survivors are slow to recover and suffer marked loss of condition (Harshfield 1970 Storz 1971 Blood *et al* 1979)

1 4 6 **Polyarthritis**

Chlamydemia following oral infection in calves and sheep may re sult in subsequent localization of organisms in the joints (Eugster and Storz 1971 a) Mendlowski *et al* (1960) were the first to detect the ability of chlamydial agents to produce polyarthritis in lambs Their observations were subsequently confirmed by other workers as well (Storz *et al* 1963 Pierson 1967)

Calves may also become infected *in utero* Affected calves are born weak become reluctant to move and develop stiff gait Some have fever and m ld diarrhoea Joints grossly enlarged are painful on palpation Death usually follows two to ten days after the appearance of clinical signs (Storz *et al* 1966)

There is another opinion among scientists that polyarthritis is not

a true nfection of the joint but could be an immune complex effect Horsch (1980) suggested the existence of immune complexes in the joint In add tion the gamma globulin fraction is elevated in the synovial fluid from nfected cows

1 5 Isolation of Chlamydiae

By providing reasonable care in the handling of specimens a mini mal loss of infectivity is achieved by decreasing the time between specimen collection and processing in the laboratory (Schachter 1986a) There is no clearly superior transport med um for C psittaci The two most commonly used transport medium are a cell culture medium supple mented with 10 foetal calf serum or a sucrose phosphate glutamate (SPG) medium also supplemented with 10% foetal calf serum (Storz 1971 Spencer and Johnson 1983 Clyde et al 1984 Warford 1984 Schachter 1986a) The foetal calf serum s usually replaced with the cheaper calf serum for routine use In spite of the substitution the results are still good Spencer and Johnson (1983) has claimed that the SPG buffer s capable of maintaining the viab lity of C psittaci for 30 days at room tempera ture and at 4°C for 34 days

Most spec mens for chlamydial isolation are contaminated with unwanted bacteria These samples must be treated with appropriate antibiotics to remove these organisms Broad spectrum antibiotics such as tetracyclines macrolides or penicillin must be excluded as even brief exposure to these antibiot cs may interfere w th the solat on Am noglycosides l ke streptomycin kanamyc n gentamycin and fungic des

like mycostatin are the main stay (Clyde 1984 Schachter 1986a)

The chlamydial specimen should be refrigerated if they can be processed within 48 72 hours after collection if not they should be frozen at 70°C

Since chlamydiae are energy dependent intracellular parasites their isolation can be attempted using only living medium like chicken embryo small laboratory animals and cell cultures

1 5 1 <u>Isolation using chicken embryo (CE)</u>

The most uniform and favourable culture medium for the cultivation and isolation of chlamydial agents was provided when Rake and co workers in 1940 discovered that these agents multiplied in the yolk sac of developing chicken embryos The eggs however should come from a disease free flock fed on an antibiot c free diet (Cruickshank et al 1975) All known chlamydial strains infect and multiply in the endodermal cells of the yolk sac of six to eight day old developing chicken embryo (Storz 1971 Duguid et al 1978 Cottral 1978 Collier 1984) The suceptibility decreases with the age of the embryos (Stewart 1962) After inoculation chick embroys die from three days onwards The time of death of CE after inoculation has an inverse relationship with the chlamydial concentration of the inoculum (Dougherty 1960) The death pattern is also character stic for a given chlamydial strain adapted to the yolk sac of chicken embryos Depending on both these factors the chicken embryos die three to fourteen days after inoculation Deaths are probably caused by a toxin elaborated by the replicating organ sms

(Coll er 1984) Occasionally the infected embroyos develop fully but death in shell occurs sometimes after chipping of the shell (Sreeramulu 1984) Usually inoculated chicken embryos are incubated at 37°C but Page (1965) claimed that the growth rate of C psittaci strains were in creased at 39°C

The chicken embryos and their adnex organs develop rather typical patholog c changes induced by the chlamydial infections The infected yolk sac are thin walled and their blood vessels are deeply in jected (Storz 1971 Collier 1984) The trophoblastic villi of the chlamydia infected yolk sacs are reduced The yolk usually is more liquid than in a normal chicken embryo of the same age and has a br ght yellow colour The chicken embryo that die due to chlamydial infect on may have hyperaemia cyanotic legs and the toes may be deep red in colour with patchy haemorrhages in the skin (Storz 1971 Collier 1984)

For the original isolation of chlamydial agents from samples of di seased animals the chicken embryo inoculated via the yolk sac route with amounts of 0 1 to 0 5 ml has been most valuable In many instances it is one of the most susceptible indicator host provided the inoculum is first freed from contaminating bacteria Chlamydial culture in the yolk sacs of CE is the method of choice for isolation of these infectious agents from trachoma of man and from samples of cattle sheep goats pigs and guinea pigs because these chlamydial agents are as a rule of low pathogenicity for mice (Storz 1971)

The CE also can be nfected by chorioallantoic inoculation (Storz 1971 Collier 1984) However chlamydial agents of different origin vary considerably in their ability to multiply in cells of the chorioallantoic membrane (CAM) Most of them grow poorly and only after careful adaptation Avian and some mammalian strains particularly the ones associated with abortion were propagated successfully on the CAM The death pattern is irregular as compared with yolk sac inocu lated CE Chlamydial infection induces small opaque pock like lesions and oedema in the CAM Some chlamydial agents have been adapted to multiply in the cells lining the chorioallantoic cavity from which the chlamydial agents are released into the allantoic fluid for easy harvest This method of chlamydial propagat on s useful for obtaining relatively pure suspension for research purposes

Infectivity titrations can be performed by inoculating groups of chick embryos with serially diluted suspensions of chlamydiae the time from inoculation to death may be used as a measure of the infecti vity titre but determination of the 50% infective or lethal end points are more accurate

The presence of chlamydiae can be demonstrated in tissue sections or more conveniently in impress on preparations of the yolk sac obtained by either Giemsas Macchiavello G menez modified Ziehl Neelson or immunofluorescence technique

1 5 2 Isolation using guinea pigs

Rivers and Berry (1931) introduced the guinea pig as an experimen

tal animal in chlamydial research But a very important fact to be con s dered while using guinea pigs for the isolation of chlamydia is that some of them harbour natural chlamyd al infections

Guinea pigs are the laboratory animals of choice for bovine and ovine ohlamydial strains because these animals are more prone to pick up infection even with lesser number of chlamydiae than are chicken embryos (Cottral 1978) Intraperitoneal inoculation of guinea pigs is the preferable route to isolate chlamydial agents from faeces and other specimens from cattle sheep and goat

The chlamydial agent might produce in the guinea pigs severe fibrinous peritonitis with fever weakness progressive emaciation and possibly death within seven to fourteen days after intraperitioneal inocu lation Preceding death the animals shows signs of lethargy anorexia and incoordination At necropsy one usually finds splenomegaly necrot c whitish specks in the liver and pulmonary consolidation with a fibri nous yellowish grey mat like covering of the visceral organs (Storz 1971 Page 1981) The exudate contains numerous chlamydiae infected mono nuclear cells (Storz 1971 Page 1981) In attempts to isolate chlamydial agents suspensions of spleen and other organs of infected guinea pigs are passaged in seven day old chicken embryos An increase in comple ment fixing antibodies in guinea pigs noculated with diagnostic specimens also has been used as evidence for the presence of chlamydiae in the test specimen

1 5 3 **Isolation using mice**

Most chlamydiae strains tested can infect m ce aged three weeks

after intranasal instillation (Storz 1971) This method is useful for the propagation and isolation of chlamydial strains of human and av an origin Attempts to isolate chlamydial agents from field materials of other animal species by intranasal mouse inoculation often give negative results while parallel tests in chicken embryos are successful However all known chlamydial strains infect mice when they are inoculated with a yolk sac propagated agent of high infectivity

Mice develop typical lung lesions within a few days after inoculation with yolk sac propagated chlamyd al agents (Storz 1971) Death may occur in three to seven days after inoculation with highly virulent chlamyd al strains Mice inoculated with less virulent strains may die w thin eight to fourteen days or they may survive and remain latently infected Even when death does not follow intranasal inoculations the mice develop lung lesions wh ch are most regularly detected about seven days after noculation The les ons consist of greyish distinct foci of consol dation involving the hilar region or other portions of the lung or the ent re lung Chlamydial bodies can be demonstrated in stained impression smears of lung taken preferably from the margins of the lesions (Storz 1971)

The susceptibility of white mice to intracerebral inoculat on with chlamydial stra ns is not as uniform as exposure by the respiratory route Strains isolated from human and avian sources usually affect mice after intracerebral injection leading to death within four to seven days The meninges are oedematous and the meningeal blood vessels appear deeply injected Impression smears of meningeal exudates stained with Giemsas

or Gimenez method reveal large number of mononuclear cells with numerous intracytoplasmic chlamydiae With some exceptions mammalian chlamydial strains inoculated intracerebrally into mice fail to cause clinical symptoms (Storz 1971)

Intraperitoneal inoculation of mice with chlamydial strains usually leads to infection and often to death However mammalian chlamydial strains usually have little effect on mice after intraperitoneal inocula tion In cases of active reaction after intraperitoneal inoculation the spleen of the animal will be enlarged the liver mottled with necrotic spots and all visceral organs often are covered with a th n stringy whit sh net of exudative material Some chlamydial strains cause the development of a voluminous ascites (as much as 5 10ml in a 20g mouse) mak ng the mouse so bloated that it cannot move to get food or water (Page 1981) While using mice for isolat on and experimental work with chlamydial agents one has to remember that they can be latently infected with chlamydial agents At least five strains of C psittaci and Nigg s Murine pneumonitis strains of C trachomatis have been so lated from the lungs of mice in various laboratory colonies(Cottral 1978)

1 5 4 Isolation using cell cultures

Several cell lines support growth of a variety of strains of chla mydiae The principal lines used are the L 929 McCoy mouse fibro blasts Hela 229 and baby hamster kidney (BHK 21) These cells may be cultured as monolayers or in suspension Certain spec fic procedures and treatment increase the sensitivity and yield of chlamydiae from cell cultures Stationary adsorption of chlamydia onto cell cultures occurs

randomly and at low frequency Centrıfugatıon of a C psıttacı ınoculum onto cell cultures ıncreases ınfectıvıty upto 1 200 fold Centrıfuged enhanced adsorptıon occurs rapıdly w thın 10 mınutes or less (Spears and Storz 1979b) The chlamydıae and cells have a net negatıve sur face charge at neutral pH(Vance and Hatch 1980) The low spontaneous ınfect vıty of chlamydıae for cultured cells may well be due to theır mutually repulsıve surface charge It ıs lıkely that centrıfugat on en hances nfectıvıty by counteractıng repulsıve forces thereby enhancıng the chance of adsorptıon It ıs also speculated that centrıfugatıon causes conformatıonal changes ın the membrane and ıncreases the contact of chlamydıae wıth the host cell membrane to promote endocytos s (Dennıs and Storz 1982)

Chlamydıal uptake by cell ıs temperature dependent It ıs found that ınfectıvıty ıs hıgher at 37°C than at ambıent temperature and ıt s almost absent below 15°C (Denn s and Storz 1982) Th s phenomenon could be on account of the fact that at hıgher temperatures the memb rane fluıdıty ıs greater

Preınoculatıon treatment of the cells wıth hydrocortısone (Stewart 1960 Dennıs and Storz 1982) Cyclohexımıde (Spears and Storz 1979a Dennıs and Storz 1982) d ethylamınoethyl dextran (Spears and Storz 1979a) and colchıcıne (Dennıs and Storz 1982) ıs found to ıncrease the susceptıbılıty of cells to chlamydıae

The potentıal advantages of a cell culture system over the c cken embryo technıque for the solatıon and propagatıon of chlamydıal agents

consist of greater uniformity in the susceptib l ty of cultured cells avoidance of any seasonal variation in the susceptibility and faster com pletion of infect v ty tests for chlamydial agents (Storz 1971)

1 6 Serodiagnosis

1 6 1 Chlamydial antigens

As techniques for the preparation and pur f cation of chlamydiae im proved it became possible to determine directly the composition of isolated chlamydial components both immunologically and biochemically Such studies confirmed that the parasites possess numerous antigenic com ponents of genus species sub species and serovar spec fic epitopes The increasing availability of monoclonal antibodies to chlamydial epitopes opens up the prospectus of d ssecting the antigenic mosaic of these pathogens further (Stephens et al 1982)

All members of the genus share a common heat stable complement fixing antigen which is sensitive to oxidation by sodium periodate(Allen 1986) This antigenic fraction can be extracted from chlamydial elemen tary bodies with ethyl ether sodium deoxycholate sodium lauryl sulph ate and by procedures used for the isolation of lipopolysacchar des from Gram negative bacteria

Biochemical composition of the genus specific antigen has shown it to contain lipid components which are usually C 17 and C 18 1 fatty acids an acidic carbohydrate ident cal to 2 keto 3 deoxyoctonic acid(KDO) D glucosamine and phosphate (Allen 1986)

By comparison of the antigenic activities of *C psittaci* and *C trachomatis* by two dimensional electrophoresis Caldwell *et al* (1975) have demonstrated the marked antigenic differences between these two species Of 19 distinct antigenic components found in *C trachomatis* only one was strongly cross reactive with *C psittaci* The species specific antigenic act vity is thought to reside in the major outer membrane protein (MOMP)

1 6 2 Serological tests

Most of the serological tests detect the presence of group specif c antibodies in the sera of the infected animals This is a clear indica tion of the presence of chlamydial infections in the population since these antibodies are induced only by chlamydia and no other agent is capable of inducing such antibodies (Ma erhofer and Storz 1969)

Demonstration of at least a four fold rise in the titer of circulating antibodies by any of the several serological tests provides proof of a recent chlamydial infection Therefore obtaining paired sera at the acute and convalescent stages of disease may play an important role in diagno sis if the etiological agent cannot be isolate (Cottral 1978)

1 6 2 1 Complement fixation test

One of the most common serological test used for the diagnosis of chlamydial nfection is the complement fixation test (CF test) This is a relatively reliable test to establish the group specific relationship of chlamydial antigen isolated from cl nical specimens or to detect group specific antibodies in the serum (Storz 1971)

The sensitivity of the commonly used standard CF test can be not ably improved by the addition of bovine complement This feature is exploited n the modif ed complement fixation test (MoCFT) (Peres Martinez et al 1986) The basis for this improvement seems to be the need of species specific heat labile complement components C_1 for fixation of guinea pig complement by some mmunoglobulin isotypes (Knight and Cowan 1961) Although bovine IgG_1 can fix guinea pig complement under conditions of the standard CF test bovine IgG_2 can only be detected in the supplemented test

The microtiter method of the CFT is easily adaptable to chlamydial research Usually CF antibodies appear in mammals within 7 10 days after chlamydial infection (Storz 1971 Cottral 1978) A titre of 1 16 is considered positive (Storz 1971 Gupta et al 1985) Maximal titers may be reached with n thirty days depending on the type and intensity of infection and on the physiologic state of the nfected subject The antibody titres usually declines then over a period of months (Storz 1971) Bassan and Ayalon (1971) reported that the complement fixation t tres appeared and disappeared irregularly and levels of titres were also irregular indicating that the complement fixation reaction is valuable in the diagnosis of epizootic bovine abortion only as a herd test

By means of the CF test Friis (1967) could identify the presence of chlamyd al antibodies in 6 3% of aborted cattle as against 2 6% in nor mal cattle

In 35 cattle farms with the history of abortion 15 6% of the 4177

blood samples were found positive by the CFT (Ognyanov 1970) The serologically positive ones included 62 of 121 cows that had aborted recently and 243 of 1368 cows that had calved normally

In 168 out of 687 cases of abortion in bovines tested during 1969 1970 positive CF titres were obtained indicating 24 5% seropositivity (Schoene 1971)

Jahn et al (1972) examined 28 herds with infertility problems and could find that of 439 animals 68% were serologically positive and 18% suspicious for chlamyd al nfection

Ognyanov et al (1976) screening 359 serum smaples from cattle farms with the history of abortion and pneumonia found that 54% of an mals reacted to chlamydial antigen n the CF test

In Denmark Ronsholt (1977)selected 733 heifers for serological scree ning for chlamyd al antibodies because of the high incidence of abortion Among these 19% of the an mals were serologically positive by means of the CF test

Dixit et al (1980) screened sera samples from 204 sheep and 294 goats for chlamydial ant bodies by means of the complement fixation test A good majority of the sera samples were collected from sheep and goat that were slaughtered for meat However among these there were also sera samples from 11 goats with the history of abort on Besides seven rams used for breeding purpose were also screened since the flock in which they were used for service had a large number of abortions The test revealed that 12 serum samples of sheep and 24 samples from goats

yielded a CF titre of 1 16 and above Ten out of the eleven serum samples from the goats having had a history of abortion were found to be positive Likewise all the sera of rams had significant antibody titre to chlamydia

Purohit and Gupta (1983) screened serum samples from 204 sheep and 294 goats which were slaughtered for food purpose A CF titre of 1 8 and above was detected in the sera of 32 sheep and 27 goats On a percentage basis the incidence rate worked out to 15 7 % in sheep and 9 18% in goats

Sharma and Baxi (1983) screened for the presence of chlamydial antibodies in 370 cattle and 230 buffaloes which were apparently normal The survey revealed that 51 (14 4%)sera of cattle and 26 (11 8%) sera of buffaloes fixed complement at titres of 1 8 and above However at higher base titres of 1 16 the seroprevalence rate was only 8 1% and 6 9% in cattle and buffaloes respectively

Gupta et al (1985) conducted a seroprevalence survey for chlamydial antibodies n the sera of apparently healthy an mals The study revealed that 32 (10 19%) of the 314 sheep 27 (6 99%) of the 386 goats and 9 (3 27%) of the 275 cattle had significant level of antibody A CF titre of 1 8 and above was considered a positive titre The same group of vorkers also screened sera of domestic animals that had the history of abortion Chlamydial antibodies were detected in 15 of the 132 cows 19 of the 138 sheep and 32 of 143 goats that were screened A CF titres of 1 16 and above was taken as a significant titre The incidence of infection was observed to be highest in goats i e 22 7% followed by sheep

13 8% and least among cattle 11 4%

The complement fixation test as a serologic procedure is useful for the diagnosis of chlamydia induced diseases with a systemic phase of in fection but not all immunoglobulin isotypes can be detected Furthermore this test probably does not detect chlamydial infections with localised mucous membrane involvement because they evoke a poor humoral antibody response (Storz 1971)

Only bovine IgG_1 and IgM are able to bind guinea pig complement whereas IgG_2 and IgM have superior agglutinating properties (Mittal and Tizzard 1983) Schmeer et al (1987) using the indirect enzyme linked immunosorbent assay showed that there was a sign ficant IgG_2 dom nance in the bovine humoral immune response to chlamydia infection This being the case a s gnificant number of seropos t ve animals will remain unde tected when screened by the CFT For diagnostic purpose the CFT has been replaced by far more sensitive tests Milon et al (1985) undertook a serolog cal d agnosis of ovine chlamydiosis using three techn ques viz ELISA IF and CFT They concluded that the CFT was the least sensitive

1 6 2 2 Agglutination test

Reports on the use of agglutinat on test for the detection of chlamy dial infections are not many Neal and Davis (1958) undertook a compa rative study on the use of the direct CF test the indirect CF test and the macroscopic agglutinat on test for detecting ornithos s antibodies in turkeys The study revealed that large number of serum samples that gave negative results to the direct and indirect CF test reacted positively

with the agglutination test The correlation between the results of the agglutination test and the indirect CF test was 42 3%

Krishna et al (1986a) performed the agglutination test using the sera of 13 goats and six sheep that had a history of abortion They were able to identify the presence of agglutinins in all the sera samples The test was found to be simple and of high specificity Positive results were obtained even in sera samples that exhibited anticomplementary activity

1 6 2 3 Passive Haemagglutination Test (PHA test)

For the assay of fluids containing small quantities of antibody sensitive methods for antibody detection must be employed One such test is the indirect or passive haemagglutination technique (Carpenter 1975 Garvey et al 1979) The lower limit of antibody detectable by the complement fixation test is 0 01 1 0 μg/ml of test fluid whereas the same for the PHA test it is as low as 0 005 μg/ml (Carpenter 1975)

For reproducible results the concentration of red blood cells to be sensitized with antigen must be standardized since there is an inverse relationship between concentration of cells and antibody titre The agglutination titre doubles when the concentration of cells is halved (Garvey et al 1979) It may happen that reproducible results are not obtained when different batches of red blood cells are used or after storage of cells This can partially be avoided by using fresh cells obtained from one source or by preparing stock suspensions of fixed cells The test can be adapted to a microhemagglutination test as described by Sever

(1962) which has the advantage of using less antigen

Turner and Gordon (1964) undertook some limited studies with trachoma and related organisms The antigen that was used for the sensitization consisted of partially purified elementary bodies treated for one hour in a sonic oscillator at 10 KC and a temperature below 15°C Although only a few tests were performed with heterologous antigens the consistently lower heterologous titres suggested that specific antigens were in part concerned with the reaction

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

Belden and McKercher (1973) studied the feasibility of using PHA test for the serodiagnosis of bovine chlamydial abortion The test was found to be four times more sensitive than the CFT Moreover it was found to be species specific A major drawback was that the soluble antigen was not stable and it lost its antigenic activity in 48 hours

Using sheep erythrocytes sensitized with ether extracted antigens from C trachomatis and C psittaci Yang et al (1984) carried out the indirect HA test In cross reaction experiments they could prove the specificity of the reaction The titres obtained were 8 16 times higher than those of the CF test

Gupta et al (1985) carried out comparative studies on the indirect HA test and the CFT during their seroprevalence survey for chlamydial infection among livestock Fourty five CFT positive serum samples were

subjected to PHA test 17 samples showed one or two fold lower antibody titres while in two cases the PHA titre was detected to be higher than the CF test All the samples tested by PHA test gave positive titres On the basis of these observation they opined that the PHA test could be considered as an alternative test against chlamydial infections

1 6 2 4 **Precipitation test**

The immunodiffusion and immunoelectrophores s techniques though applicable in the diagnosis of chlamydiosis have not gained popularity unlike some other tests (Storz 1971)

Using the technique of double diffusion in gel Barron et al (1972) undertook a study to detect chlamydial antibodies in the serum of animals The ant gen used was a deoxycholate extract of C psittaci Though a good correlation was observed diffusion n gel was considered less sensitive than the CF test

A gel diffusion test was performed on 30 sera samples of repeat breeder cows by Mishra and M shra (1985) Among these 12 were strongly positive and five samples gave weak precipitation lines CF tests of the same 30 serum samples yielded a titre of 1 8 in 11 and 1 16 in 7 samples Their findings indicated that the gel diffusion technique was slightly superior to the direct CF test

Krishna et al (1986a) undertook a comparative study to evaluate the efficacy of various serological tests in chlamydiosis among sheep and goats They could find no significant difference between the sensitivity of the CF test and the agar gel precipitation test

1 6 2 5 Immunofluorescence (IF)

The fluorescent antibody (FA) techniques have been appl ed to study different problems posed by chlamydial infections The fluorescent antibody technique possesses advantages in its specificity sensitivity reliability and rapidity compared with other methods for the diagnosis of chlamydiosis (Baumann 1974) However isolation of the agent in cell culture is more confirmatory than IF (Schachter 1986a) More recently fluorescein conjugated monoclonal antibodies are being used to identify *C trachomatis* (Stephens *et al* 1982)

The antigens reacting in this test has not been identified but they probably represent the majority of chlamydial antigens ncluding species and group fractions (Donaldson *et al* 1958 Storz 1971) Thus by the IF studies posit ve results can be obtained either when the antigens and antiserum are homologous or when they are different as in cross tests (Feteanu and Popovici 1973)

Donaldson *et at* (1958) used the d rect fluorescent antibody techni que for the detection of the ornithosis agent in the yolk sac explant tissue culture They found it to be as effective as the indirect CF test for the diagnosis of ornithosis n turkeys

Ross and Borman in 1963 developed direct and indirect fluorescent antibody techniques for the detection of group antigens in infected tissue cultures They also made an interesting observation that rooster conju gates showed brighter staining and higher antibody titres than the guinea pig or human conjugates Besides the rooster conjugate was more effective in detecting minimal amounts of chlamydial antigens

Blanco Loizelier (1971) carried out extensive diagnosis of acute chlamydial infection among livestock using the direct IF technique The direct FA staining of smears prepared from the lungs spleen and liver revealed typical granular fluorescence In the macrophages they identified three types of fluorescing inclusions type one was seen as dense grouping identified as a polar cap type two were half moon or letter C form and the third type was seen as a simple ring around the periphery of the nucleus

Sharma et al (1982) processed the cervicovaginal mucous samples of 45 cows and 160 buffaloes for the isolation of chlamydia Four isolates of chlamydia one from a cow and three from buffaloes were obtained by means of isolation in the chicken embryo The isolates were characterized by the CF test and the direct fluorescent antibody staining technique The direct fluorescent antibody staining of the YS smears revealed specific apple green fluorescence

Krishna and Rajya (1985) could obtain 48 chlamydial isolates from 80 cases of perinatal mortality in lamb and kids Isolation was achieved in the yolk sac of developing CE The yolk sac smears stained by different methods revealed extra and intra cytoplasmic elementary bodies in clusters Confirmation was done by performing the direct and indirect FA staining which revealed bright greenish yellow fluorescence

Chlamydia was isolated from 32 cross bred repeat breeder cows and heifers by Mishra and Mishra (1985) When YS smears stained with conjugated chlamydial antisera were examined under the fluorescent microscope they were able to identify specific fluorescence They correlated this

findings along with the other test results to allocate the isolate to the genus chlamydia

Krishna (1986b) reported spontaneous cases of chlamydial abortions in sheep at an organised farm in Himachal Pradesh Diagnosis was possible by conducting solation and histopathological studies In add tion further confirmation was done by employing the direct FAT on yolk sac smears and placental impression smears

1 6 2 5 Other tests

The application of modern techniques such as microimmunofluorescence enzyme linked immunosorbent assay and the radioimmuno assays have significantly improved the serodiagnosis of chlamydial nfection in human subjects birds and laboratory animals Of late these tests have also started to gain popularity in the serodiagnosis of chlamydial infection among an mals

Milon et at (1985) undertook comparative serological diagnosis of ovine chlamyd os s using three tests namely the complement fixat on immunofluorescence and the ELISA From their studies they could infer that ELISA was the most sensitive while complement fixation was the least sensitive It is belived that the superiority of the ELISA and IF was due to the detection of non complement fixing immunoglobulins

Peres Martinez et al (1986) demonstrated that it was possible to mprove measurably the serodiagnosis of bovine chlamydial abortions and other chlamydial infections of cattle using two non conventional tests like the ELISA and the indirect inclusion fluorescence assay (IIFA) They

noted that n spite of the super ority of the modif ed complement fixation test over the standard complement fixat on test the IIFA and the ELISA surpassed the react vity of the mod f ed complement fixat on test

Material and Methods

2 MATERIALS AND METHODS

The nature of work involved both solation and serological aspects The methods and materials employed were chosen accordingly

The clinical history managemental practises and agroclimatic conditions of certain organized farms besides the clinical history of other individual animals were collected These informations vere utilised for discuss on in corelation with the results accrued in this study

2 1 Nature of specimens collected

Specimens for the isolation work were collected from clinical cases in which chlamydial agents have been implicated They included the visceral organs of the aborted foetuses or st ll born young ones the placenta and uterine discharge from cases of abortion and still birth Pneumonic lung lesions were also collected from carcases examined at post mortem and slaughter Frozen semen samples with poor motility and sperm abnormalit es were also subjected to isolation studies Sim larly the seminal vesicular secretions collected by manual stimulation per rectum from bulls suspected to have seminal vesicultis was also screened Likewise isolation of chlamydial agents was also attempted from cases of conjunctivitis and polyarthritis In these cases swabs were used to collect materials from the respective sites When specimens were taken from visceral organs the regions bordering the active lesion and the normal tissues was taken

In order to assess the magnitude of seroprevalence serum samples from cattle goats and sheep were collected by venipuncture All the

amples were from animals which manifested clinical conditions such as abortion/still birth retention of placenta infert lity and respiratory tract infection/pneumonia To the serum after harvest sodium azide was added at the concentration of 0 1% and stored at 20°C unt l used

2 2 **Transportation of specimens**

Specimens were collected with all asceptic precautions possible If it was not possible to transport the specimen to the laboratory within a per od of one hour t was placed over ice and transported in a ther mocole box However if the time required for the transportation was less than one hour the materials were transported as such without any refri geration

The tissue specimens were collected in sterile PBS (pH 7 2) supplemented with streptomycin and kanamyc n @ 500 µg/ml and 500 IU/ml respectively and nystatin @ 50 IU/ml

Transportation of d luted semen and seminal vesicular secretion was undertaken in sucrose phosphate glutamate(SPG) buffer fortified with 15% seronegative calf serum and supplemented with streptomycin @ 500 µg/ml Kanamycin @ 500 IU/ml and nystatin @ 50 IU/ml

2 3 **Staining of smears from clinical specimens and experimental animals**

Smears were prepared from the clinical mater als and exper mental animals and stained by the following techniques

2 3 1 **Giemsa s staining** The slow method of staining advocated by Cruickshank *et al* (1975) was adopted however with slight modifications

0 5ml of the stock solutıon of Gıemsa s staın was dıssolved ın 80 ml of dıstılled water The smears were then left ımmersed n thıs dılute staın solutıon for 12 16 hours Fınally the smears were washed drıed and then examıned under the oıl ımmersıon

2 3 2 <u>Gımenez staınıng</u> The staınıng was done as per the procedure laıd down by Storz (1971)

2 3 3 <u>Modıfıed Zıehl Neelsen staınıng</u> The method advocated by Paık and Suggs (1974) wıth slıght modıfıcatıons was adopted The smears were staıned wıth a 1 10 dılutıon of the stock carbol fuchsın for 10 mınutes It was then washed ın runnıng tap water Decolourızatıon was done wıth 0 5% acetıc acıd for 2 5 seconds only The smears were ımmedıately washed agaın n runnıng water Counterstaınıng was done wıth 1 % methy lene blue for ½ to 1 mınute Washed agaın ın runnıng tap water drıed and examıned under the oıl ımmersıon

2 3 4 <u>Macchıavello staınıng</u> The staınıng was done as per the proce dure laıd down by Stroz (1971)

2 4 <u>Storage of specımens for ısolatıon studıes</u>

Tıssue specımens brought to the laboratory were washed two to three tımes ın sterıle PBS then suspended ın PBS contaınıng antıbıotıcs and were kept for a perıod of 6 hours at 4°C Thereafter the specımens were washed agaın ın sterıle PBS Fınally the larger chunks of tıssue were cut ınto smaller pıeces and dıspersed ınto ster le vıals contaınıng PBS supplemented wıth antıbıotıcs

When the specımens processed as above could not be processed

further within 24 hours they were preserved at 20°C On the other hand if processing could be undetaken within a period of 24 hours the speci mens were maintained at 4°C until used

Fluid specimens such as semen or uterine discharge were transported to the laboratory in SPG buffer and either immed ately processed or stored at 20°C if a delay of more than 24 hours was antic pated

2 5 **Processing of the samples**

Tissue pieces were cut into small bits by means of a sterile scissors These were then suspended in PBS containing antibiotics to obtain a 40% (w/v) suspens on Sterile silica gel was added to this sus pension to facil tate proper homogenization of the specimen in a tissue grinder Following this the tissue homogenate was transferred to properly labelled sterile test tubes This was left aside in a refrigerator for 15 to 20 minutes to facilitate the coarse tissue particles and the silica gel to settle down by gravity

Thereafter the supernatant was collected from these test tubes and subjected to three steps of centrifugation each at different rpm All the centrifugation was carried out using refrigerated centr fuge The tissue homogenate cleared of its heavier particles by simple sed mentation was centr fuged first at 600g for half an hour The supernatant was collected and stored at 4°C for s x to seven hours after which it was centrifuged a second time for half an hour at 1100 g The supernatant was collected and kept in a refrigerator for another six hours Then it was centrifuged at 1700 g for half an hour and the supernatant was collected and refri gerated overnight

The sterility of the emulsion was tested by streaking it onto blood agar/serum agar plates After overnight incubation of the inoculated plates at 37°C they were scanned for bacterial contam nation

The emulsion established to be free of bacterial contamination was used for inoculation into chicken embryos/experimental animals

2 5 1 Inoculation of chicken embryos (CE)

Prior to the inoculation of the sample into CE the temperature of the emulsion was raised to that of room temperature by placing it outside the refrigerator

Eggs from hens fed on antibiot c free ration were pre ncubated for six to eight days n a hum d atmosphere at 37°C were candled Viable and healthy embryonated eggs were inoculated w th 0 25 ml of the emul sion by the yolk sac (YS) route us ng a 21 gauge needle The hole in the shell was sealed and incubat on was cont nued at the same temperature and hum dity

2 5 2 Inoculation of guinea pigs

Sero negative healthy guinea pigs aged around f ve to six weeks of age were used for the isolation attempts The animals were anaesthe tized using anaesthetic ether prior to all manipulations After steril zing the ventral aspect of the abdomen with alcohol three millilitre of the sterile emulsion was inoculated intraper toneally These animals were housed in separate cages and given identification numbers

2 5 3 Inoculatıon of mıce

Mıce of two dıfferent age groups were employed for studyıng the pathogenıcıty of three ısolates The fırst group comprısed eıght mıce aged 3 4 weeks and the second group of e ght mıce aged sıx to eıght weeks The former group was ınoculated ntraperıtoneally wıth 0 2 ml of the YS homogenate prepared whıle the latter receıved o 4 ml The YS homogenate was prepared out of yolk of CE ınoculated wıth double passa ged ısolates

2 6 Harvestıng from ınoculated embryonated eggs

The noculated eggs were candled daıly Death of embryos wıthın the fırst 48 hours was consıdered nonspecıf c and they were dıscarded Embryos whıch dıed on subsequent days were collected and placed ın a refrıgerator overnıght The noculated embryos found vıable even after ten days were kılled by plac ng them n the ref gerator The next mornıng harvestıng was done After dısınfect ng the shell wıth 70% ethyl alcohol t was opened by cuttıng and remov ng the shell over the aır sac usıng ster le scıssors and forceps Subsequently the shell membrane and the chorıoallantoıc membrane n the area of the aır sac were also removed Wıth a paır of sterıle forceps the ınner contents were emptıed ınto a sterıle petrıdısh The YS and the embryo were washed ın ster le PBS (pH 7 2) Small pıeces of the yolk sac blotted dry on a fılter paper were used to prepare mpressıon smear onto clean grease free slıdes

The yolk sacs and embryos so obtaıned were then suspended ın SPG buffer fort fıed wıth f ve per cent serum and ant bıot cs at the rate of

500 μg/ml of streptomycin 500 IU/ml of kanamycin and 50 IU/ml of nystatin These material were then dispersed into sterile vials labelled and kept at 20°C until further use

Even if the yolk sac smears failed to reveal the typical chlamydial bodies by the common staining techniques it was subjected to a m nimum of three ser al blind passages before t was discarded as negative The yolk sac were processed as mentioned earlier The only difference was that the PBS (pH 7 2)was replaced with SPG buffer fortified with 10% calf serum supplemented with antib otics The inoculation techniques adopted was identical to that already described

2 7 Tracking of guinea pigs inoculated with clinical specimens

The guinea pigs inoculated with the clinical specimens were daily observed for the development of any clinical signs of the disease When death is imminent the blood samples of such animals were collected by intracardiac puncture Once death of the animal had occurred the post mortem examination was performed immediately to note pathological changes and to collect tissue specimens and exudate

The animals which rema ned apparently healthy for three to four weeks following the inoculation of the clinical specimens were bled by ntracardiac puncture and serum was collected for serolog cal tests Such animals were then sacrificed to carry out further studies

2 7 1 Post mortem examination of guinea pigs

The abdominal and thoracic cavities of the dead animals were cut open observing all sterile precautions The serosanguineous fluid accumu

lation in the abdom nal cavity if noted was collected by aspiration A careful note of all the gross pathological changes in the various organs was made Following th s the various organs were removed ascept cally into sterile petridishes and washed thrice in sterile PBS (pH 7 2)

Impression smears of the peritoneum and v sceral organs were also prepared The remaining portions of the various organs were cut into smaller pieces and suspended in SPG buffer fortified with 5% nactivated calf serum and antibiotics They were then dispersed as aliquots into sterile vials and preserved at 20°C until used

The tissues whose impression smears revealed on staining the granu lar bodies were again emulsified and processed as narrated earlier These tissue homogenates were then inoculated into the yolk sac of chicken embryos so as to re solate the same agent

The study of the sacrificed guinea pigs was also undertaken as done above Smears were prepared from organs revealing lesions and looked for chlamydiae after staining Thereafter they were suspended in SPG forti fied with 5Y inactivated calf serum and antibiotics and stored at 20°C Attempts to reisolate the same agent in CE from these organs were also made

2 8 Tracking of mice inoculated with highly infectious yolk sac suspension

The inoculated animals were observed daily for the development of any clinical signs of the disease Once death of the animal had occurred the post mortem examination was performed immediately to note patholo

gical changes and to collect tissue spec mens and exudate

The animals which remained apparently healthy for three weeks following the inoculation of the infectious material were sacrificed by cer vical dislocation They were also subjected to a close study and appro priate materials were also collected

2 8 1 <u>Post mortem examination of mice</u>

The abdominal and thoracic cavities of the dead animals were cut open observing all sterile precautions Ascitic fluid if present was collected by aspiration A careful note of all the gross pathological changes on the various organs was done Following this the various or gans were removed asceptically into sterile petridishes and washed thrice in sterile PBS (pH 7 2) Impression smears of the peritoneum and v s ceral organs were also prepared and stained The remaining portions of the various organs were cut into smaller pieces and suspended in SPG buffer fortified with 5% inactivated calf serum and antibiotics They were then dispersed as aliquots nto sterile vials and preserved at 20°C unt l used

The tissues whose impression smears revealed on staining the gra nular bodies were aga n emulsified and processed as narrated earl er These tissue homogenates were then inoculated into the yolk sac of CE so as to reisolate the same agent

The study of the sacrificed mice was done just as mentioned earl er

2 9 Testing the sensitivity of the isolates for sodium sulphadiazine

The procedure recommended by Storz (1971) was used The titration was made using two sets of CE one of which was noculated with the isolate treated with sodium sulphadiazine for 24 hours at 4°C and the other (Control) without treatment with sulphadiazine

2 10 Serological screening

Chlamydial antibodies were detected in the serum collected from various sources by performing passive haemagglutination test (PHA) as per the procedure recommended by Talwar(1983) with slight modifications

2 10 1 Collection of serum

The serum samples were collected from animal which manifested clinical conditions such as abortion/still birth retention of placenta infertility and pneumonia/respiratory tract infection The serum samples were preserved at 20°C after the addition of sodium azide at the concentration of 0 1%

2 10 2 Preparation of sheep red blood cells (SRBC)

Sheep blood was collected in Alsevers solution from the slaughter house Trichur This suspension was then maintained at 4°C for 24 hours The SRBC in Alsevers solution was washed thrice in sterile PBS (pH 7 2) and finally a 50% suspension was prepared in sterile PBS

2 10 3 **Gluteraldehyde stabilization of SRBC**

The gluteraldehyde SRBC mixture was prepared as described below

Neutral Gluteraldehyde	1 5 ml
Normal Saline solution	5 0 ml
Phosphate buffer(pH 8)	1 0 ml
Sheep RBC (50%)	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% even suspension was made in PBS (pH 7 2) and stored at 4°C

2 10 4 **Preparation of antigen for the passive haemagglutination test**

Chlamydia psittaci of ovine origin ** was propagated in the yolk sac of developing chicken embryo Yolk sacs reveal ng high concentra tation of the chlamydial agent were pooled together after washing them thoroughly in sterile PBS (pH 7 2) A 40% w/v emulsion of the YS was prepared in plain sterile PBS (pH 7 2) After having cleared the emul sion of its larger debr s it was centrifuged at 1100 g for half an hour using refrigerated centrifuge The supernatant so obtained was centri fuged twice at 1700 g for half an hour each The supernatant was collected and centrifuged again at 3500 g for half an hour The super natant obtained was decanted and fresh sterile PBS was added to resus pended the pelleted mass of organism to its original volume This suspension was centrifuged once again at 3500 g for half an hour After

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discarding the supernatant the pelleted mass was carefully resuspended in PBS(pH 7 2) so as to obtain a moderately turbid uniform suspension The concentration of the organism in the suspension was verified by examining a stained smear under the oil immersion A relatively uniform distribution of the bodies free from extraneous tissue debris was considered ideal for the preparation of antigen

2 10 5 Sonication of the antigen

The suspension of the semi purified C psittaci was held on an ice bath and then disrupted by sonication The procedure adopted was the one recommended by Pillai (1986) Sonitron(IMECO Ultrasonics) an ultrasonicator was used for the purpose of disrupting the organism Sonication was car ed 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 a total of four minutes This sonicated antigen was used to sensitize the SRBC

2 10 6 Procedure for the sensitization of stabilized as well as fresh SRBC

The procedure adopted was that recommended by Talwar (1983) however with slight modifications A 10% suspension of SRBC was washed thrice in PBS (pH 7 2) and then resuspended to a 2 5% suspension in PBS (pH 7 2) Equal volumes of 2 5% SRBC and 1 20 000 tannic acid 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% Three

m lliltre al quots of this suspension was dropwise mixed with 0 25 ml 0 5 ml and 0 75 ml of the son cated antigen These m xtures were then ncubated at 37°C for 1 hour Follow ng the sensitization the cells were centr fuged and washed twice with sterile PBS (pH 7 2) The cells were resuspended in PBS conta ning 1% BSA to provide a 1% suspension Finally 0 1% sodium azide was added and it was stored at 4°C until used

2 10 7 Titration of antigen

A checker board titration was carried out to determine the optimum concentrat on of the sonicated antigen required for use in the passive haemagglutination test Hyperimmune sera** prepared in roosters and raised against a C psittaci isolate of ov ne origin was used for the titration The hyperimmune sera was inactivated at 56°C for half an hour This inactivated sera was m xed with an equal volume of 1% SRBC in PBS (pH 7 2) to remove the heterophile antibodies Th s serum of 1 2 dilut on was serially diluted with PBS BSA to provide two fold dilu tions ranging from 1 4 to 1 1024 Fifty microlitre of 1% sensit zed SRBC carrying different concentrat ons of chlamyd al antigen sensit zed SRBC were added ndividually to each serum d lution 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 haemagglu t nation t tre with the highest serum dilut on was taken as the optimum concentration of antigen required for the sensitization of SRBC

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2 10 8 <u>Procedure for the bulk sensitization of SRBC with the sonicated antigen</u>

A 2 5% suspension of tanned SRBC was prepapred n PBS (pH 6 4) To 3 ml of 2 5% tanned SRBC 0 5 ml of sonicated antigen of optimum con centration as assessed prev ously was mixed SRBC antigen m xture was incubated for 1 hour at 37°C The sensitized cells were washed twice in PBS (pH 7 2) and then resuspended in PBS BSA to obtain a 1% suspen sion Finally 0 1% sodium azide was added and it was stored at 4°C till further use

2 10 9 <u>Protocol for the passive haemagglutination test (PHA test)</u>

All test sera samples were first inactivated in a water bath at 56°C for half an hour Following this the sera samples were mixed with an equal volume of 1% unsensitized SRBC in PBS (pH 7 2) and incu bated at room temperature for one hour The unsensit zed SRBC was sedimented by centr fugation and the supernatant serum column which was diluted to 1 2 was harvested Two fold serial dilutions of this serum (50 μl)were prpepared in PBS BSA to provide a dilution rang ng from 1 4 to 1 512 in V bottom microtitre plate Fifty microlitre of the sen sitized SRBC was added to all the dilutions Gently agitated the plates and incubation was carried out at room templerature 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

2 11 Elucidation of identity of the isolates

In order to establish the identity of the isolates the following tests were done

2 11 1 PHA using SRBC sensitized with the unknown isolate

The different unknown isolates suspected to be chlamydia were purified from infected yolk sac as mentioned Such purified isolates were sonicated as described in the section for the preparation of anti gen The SRBC was sensitized with this antigen and PHA test was performed as per the protocol mentioned previously using hyperimmune sera raised in rooster against a chlamydial isolate of ovine origin

2 11 2 PHA using antiserum raised against the unknown isolates in guinea pigs

It became necessary to separately raise antibodies in guinea pigs against two isolates one from bov ne abortion and another from semen because sera samples could not be collected from the guinea pigs inocu lated with the clinical specimens prior to their death Seronegative guinea pigs three months of age were used to raise antibodies The isolates were purified from highly infectious suspensions of yolk sac and this purified suspension of the isolate in PBS (pH 7 2) was admini stered intraperitoneally to the guinea pigs Intracardiac bleeding was done 25 days post inoculation The serum obtained was used to perform the PHA test In this case the antigen used to sensitize SRBC was the ovine isolate of C psittaci supplied by Dr Lal Krishna

2 11 3 Fluorescent antibody staining

The YS and peritoneal impression smears were fixed in ch lled acetone for 10 15 minutes prior to staining Fluorescein isothiocyanate labelled hyperimmune sera which was raised in roosters against a *C psittaci* isolate of ovine origin ** was used for the staining After fixing the smears they were washed thrice with cold PBS (pH 7 2) Following this the antichlamydial conjugate was layered over the slides These slides were then incubated in a humidified chamber at 37°C for 45 minutes The stained slides were washed thoroughly with cold PBS five times to remove excess of the conjugate Finally they were mounted in 50% glycerol saline and examined under the fluorescent microscope

2 11 4 Electron microscopy

2 11 4 1 Fixation of the isolate for electron microscopy

One isolate from bov ne abort on another from bull semen and a third from goat pnemonia were sedimented by high speed centrifugation from suspensions of the yolk sac The procedure adopted was identical to that mentioned previously However the pelleted mass of organism was resuspended in sterile dist lled water This suspension was sub jected to another round of centrifugation at 3500 g for half an hour under refrigeration The supernatant was decanted and the pelleted mass of organism was fixed as per the method of Kellenberger (Cruickshank *et al* 1975) as follows The pelleted mass of organism was resuspended in 3 ml of the Kellenberger f xative to which had been

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added 0 1ml of tryptone med um The mixture was allowed to stand at room temperature for 16 hours Subsequently it was mixed with 24 ml of Kellenberger buffer centrifuged the suspension to obtain a pellet and it was resuspended in sterile distilled water This suspension was used for direct electron microscopy

2 11 4 2 **Negative staining of the isolate**

A small drop of the f xed sample was charged onto a carbon coated grid Immediately a small drop of 2% phosphotungstic acid (pH 6 4) was also added onto the grid and allowed to act for two minutes The excess fluid remaining on the grid was carefully absor bed off using a filter paper The grids were screened in a Hitach Hu IIF Transmission electromicroscope

Results

3 RESULTS

3 1 Detection of Chlamydiae by staining

The presence of chlamydiae in the smears stained by the various staining procedures could be discerned n positive cases The details in respect of the specimens screened by staining the direct smears are furnished in **table 2** Out of the total 71 smears examined one abortion material of bovine origin, two samples of bull semen one sample from caprine abortion and three pneumonic lesions of caprine origin were found positive for structures akin to chlamydia The organism appeared as having two forms viz small granular compact bodies about 300 400 nm in diameter and large plaque like form measuring about 800 1200nm in diameter Infected cells revealed in their cytoplasm an adm xture of both the developmental forms of the organism

Both the developmental forms when present intracytoplasm cally stained equally well by the different methods of staining But this was not the case when the organisms were found extracellularly The larger plaque like forms stained less intensely than the smaller forms The smaller forms which stained more intensely could be easily identified particularly in smears containing less number of infected cells

3 1 1 Staining techniques

3 1 1 1 Modified Ziehl Neelsen method

This technique gave consistent and reliable results Both the

Table 2. Showing the details of clinical materials screened by staining

Species of animal	Nature of sample	Number screened	Number positive	% age positive	Remarks
Bovine	1 Uterine discharge placenta, foetal liver spleen lungs	17	1	5 9	Chlmydia like bodies could be seen in the foetal liver impression smears
	2 Pneumonic lung	2			
	3 Semen	15	2	13 3	Chlamydia like bodies amidst many artifacts could be seen in two semen samples
	4 Seminal vesicular scecretion	6			
	5 Synovial fluid	1			
Bubaline	1 Conjunctival exudate	1			
Caprine	1 Uterine discharge placenta foetal liver spleen lungs	5	1	20	Chlamydia like bodies were detected in placental smears amidst too many artifacts
	2 Lungs liver spleen of dead kids	5			
	3 Pneumonic lung	15	3	20	Chlamydia like bodies were detected in impression smears Some of them lacked the characteristic arrangement
Ovine	1 Pneumonic lesion	4			

developmental forms stained deep red when detected ntracytoplasmically However extracellularly the recticulate bodies took a paler reddish pink colour (Fig 4)

3 1 1 2 Macchiavello method

The chlamydial elementary bod es stained a deep red colour against a blue background The recticulate bodies stained less intensely The stain used had to be relatively fresh Staining solutions older than 3 weeks tended to precip tate and thereby interferred with the results

3 1 1 3 Gimenez method

This staining technique also proved to give good results The chlamydial elementary bodies stained deep red The background had a bluish green colour Chlamydial incusion of the earlier developmental forms did not stain well with this procedure

3 1 1 4 Giemsa's method

The Giemsa s method of staining had a distinct advantage over the other methods in that it was able to distinguish between the two deve lopmental forms The smaller elementary bodies took on a reddish purple colour while the ret culate bodies tended to be basophilic i e bluish tinged

Fig 4

Direct caprine pneumonic lung impression smears stained by modified Ziehl Neelsen s method, revealing chlamydial bodies among artifacts (1000 X)

3 2 Isolation of chlamydiae

Attempts were made aiming at the isolation of chlamydiae adopting biological means by inoculating cl n cal samples suspected to be posi tive for the presence of chlamydiae into chicken embryos and guinea pigs

3 2 1 Preliminary processing of the samples

Mere treatment of the emulsion of clinical material w th antibiotics combined with slow speed centrifugation at 600 g for half an hour was found not effective in totally eliminating the bacterial load When the above procedure was adopted it was observed that bacteria resistant to both streptomycin and kanamycin survived in the inoculum and such organisms could be isolated when seeded onto bacteriological media

The repeated centrifugation of the antibiotic treated tissue homoge nate at 600g 1100g and 1700g for 30 minutes each made the supernatant totally free of contaminant bacteria retaining the chlamydiae in the supernatant Inoculation of CE with such supernatant carrying chlamydiae produced death of the CE on account of the specific infection

3 2 2 Inoculation of chicken embryo

The results of the attempt to isolate chlamydia from the clinical specimens by CE inoculation method are given n **tables 3a, 3b, 3c& 3d** Out of the total of 71 samples employed two of 17 bovine aborted material four of 15 bull semen and two of 15 pneumonic lesion from goat

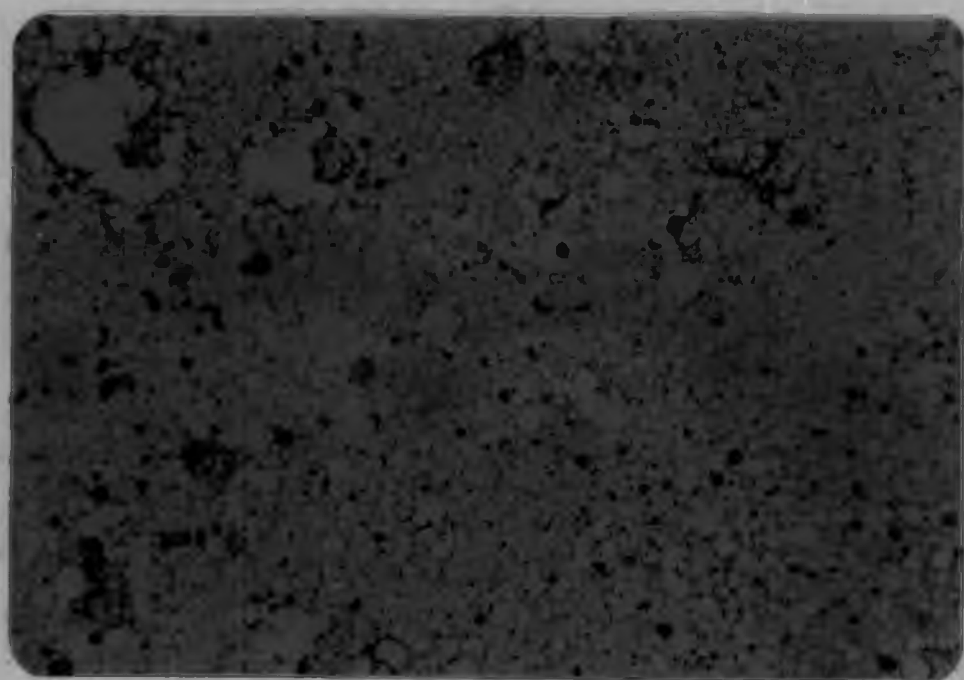


Table 3a **Tissue wise isolation rates**

Sl No	Tissue/specimen processed	Species of animal	Number screened	Number positive	Percentage positive
1	Placenta uterine discharge foetal lungs liver and spleen	Bovine	17	2	11 8
		Caprine	5		
2	Lungs liver spleen and kidney of dead kids	Caprine	5		
3	Pneumonic lesion	Bovine	2		-
		Caprine	15	2	13 3
		Ovine	4		
4	Synovial fluid	Bovine	1		
5	Conjunctival exudate	Bubaline	1		
6	Semen	Bovine	15	4	26 7
7	Seminal vesicular secretion	Bovine	6		

Table 3b. Number and rate of isolations in relation to the topography of the terrain

Species of animal		Nature of specimen	Nature of terrain	Number Screened	Number positive	% age positive	Remarks
Bovine	1	Placenta uterine dis charge lungs liver spleen of foetus	a) Hill tract	2	2	100	Two samples yielded chlamydia like bodies in the YS smears
			b) Plains	15			
	2	Diluted semen	a) Hill tract	15	4	26 7	Four samples yielded chlamydia like bodies in the YS smears
	3	Seminal vesicular secretion	a) Plains	6			
	4	Pneumonic lesion	a) Plains	2			
	5	Synovial fluid	a) Plains	1			
Bubaline	1	Conjunctival exudate	a) Plains	1			
Caprine	1	Placenta uterine dis charge,lungs liver spleen of foetus	a) Plains	5			
	2	Pneumonic lesion	a) Plains	15	2	13 3	Two samples yielded chlamydia like bodies in the YS smears
	3	Lungs,liver spleen Kidneys of dead kids	a) Plains	5			
Ovine		Pneumonic lesion	a) Plains	4			

Table 3c. **Number and rate of isolations in relation to species of hosts**

Sl No	Species of animal	Number of samples screened	Number of samples positive	Percentage positive	Remarks
1	Bovine	41	6	14 6	C psittaci could be isolated from two cases of abortion and four semen samples
2	Bubaline	1			
3	Caprine	25	2	8	C psittaci could be isolated from two cases of pneumonia
4	Ovine	4			
	Total	71	8	11 3	Eight strains of C psittaci could be isolated from a total of 71 samples screened

Table 3d **Number and rate of isolations in relation to the clinical entity**

Species		Clinical manifestation	Number screened	Number positive	Percentage positive	Remark
Bovine	1	Abortion/still birth	17	2	11 8	_C psittaci_ isolated from two cases of abortion
	2	Pneumonia	2			
	3	Polyarthritis	1			
	4	Poor quality semen	15	4	26 7	_C psittaci_ isolated from four samples of semen
	5	Seminal vesiculitis	6			
Bubaline	1	Conjunctivitis	1			
Caprine	1	Abortion/still birth	5			
	2	Pneumonia	15	2	13 3	_C psittaci_ islolated from two cases of pneumonia
	3	Perinatal mortality	5			
Ovine	1	pneumonia	4			

yielded chlamydial growth as d scerned by staining of impress on smears of YS of CE died after three days of inoculation

The two isolates from bovine aborted material were from an organised herd in the high ranges of Kerala wherein the climate is cool and humid throughout the year unlike the hot and humid climatic conditions prevailing in the plains of Kerala In this particular herd 11 8% (13/110) of pregnant animals had aborted dur ng 1986 87 Generally no clinical signs were evident prior to abortion The records available in the farm revealed that the abortion rate was greater in heifers than in cows Following abortion many animals had problems such as retention of placenta metritis and/or repeat breeding The practice on the farm was to allow the cows to graze on the lush green pastures found on the foot hills surrounding the farms which was greatly infested with ticks

The f rst chlamydial isolate was obtained from the liver of an aborted foetus four months of age The liver revealed diffused areas of necrosis and a slight degree of congestion No bacterial organism could be isolated from this organ The second solate was obtained from the placenta and utrine discharge from a heifer that had aborted at the fourth month of pregnancy

Chlamydiae could not be isolated from the remaining 15 cases of bovine abortions All the samples were obta ned from veterinary institutions located in the plains of Kerala

Fifteen diluted semen samples preserved in liquid nitrogen were

screened for chlamydial agents All the samples exhibited poor moti lity and were of low sperm concentration Besides the sperms had primary and secondary sperm abnormalities It was possible to isolate chlamydia from four of the 15 samples

The seminal vesicular secretions from s x bulls suspected to have seminal vesiculitis were screened Large number of inflammatory cells were present in the samples Chlamydia could not be isolated from any of these samples However corynebacterium could be isolated from all the samples when it was seeded on blood agar prior to antibiotic treat ment

The other samples of bovine and bubaline origin were all found negative for chlamydial agents

The attempts to isolate chlamydiae from five cases of caprine abortion was without any positive results

In an attempt to isolate chlamydia from pneumonic lesions of goats 15 samples were screened Two isolates of *C psittaci* could be recove red The two goats from which chlamydia could be isolated were apparently normal except for that they were slightly run down in condi tion and had a rough hair coat No respiratory distress was manifested by these animals

The gross lesions in the lungs were mainly confined to the apical and to some extent the cardiac lobes About 80% of the apical lobes and

40% of the cardiac lobes were consolidated and greyish Granularity of the consolidated areas was not observed Gram negative bacilli could also be isolated from both the pneumonic lesions

Other samples of caprine and ovine origin did not reveal the presence of chlamydiae

The positive samples almost invariably produced death of the embryo in a spread over period Following the inoculation the organisms were found to produce death of the embryo as early as the third day post inoculation and as late as the 21st day of incubation

The chicken embryos and their adnex organs developed rather typical pathologic changes after they were inoculated with the isolates When death of the embryos occurred within 3 7 days post noculation the yolk sac was thin walled and their blood vessels were deeply injected The yolk was usually found to be more liqu d than in normal chicken embryos of the same age and had a bright yellow colour The chicken embryos that died due to the infection had hyperaemic cyanotic legs and patchy haemorrhagic areas all over the body (Fig 5 & 6) The liver of the dead embryos showed marked necrotic changes

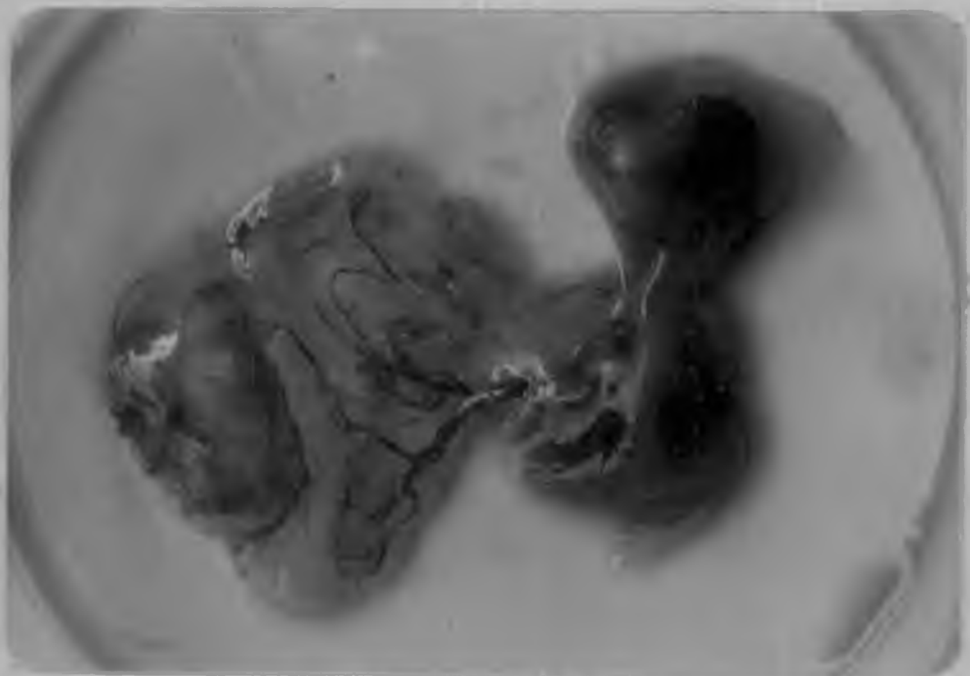
In cases where the chicken embryos died only after eight days of inoculation the typical changes mentioned above were not observed There was only moderate congestion of the blood vessels supplying the yolk sac The yolk showed a tendency for inspissiation The embryos did not exhibit the typical patchy haemorrhagic areas on the sk n

Fig. 5

Chick embryo died 3 days post inoculation showing haemorrhagic changes and injected yolk sac vessels

Fig 6

Chicken embryo(left) died 7 days post inoculation revealing stunting, haemorrhagic lesions with unabsorbed yolk Control chick embryo(right)showing normal development



The liver of the dead CE revealed only mild damage in the form of a few patches of necrosis

3 2 2 1 Impression smears from the yolk sac

Following staining of the YS smears by any one of the specific methods the presence of chlamydial bodies could be discerned in posi tive cases **(Fig 7, 8 & 9)** The organism appeared as having two developmental forms viz small granular compact bodies about 300 400nm in diameter that were usually found extracellularly and larger plaque like forms measuring about 800 1200nm in diameter which were usually found intracellularly The cytoplasm of the infected cells was found to contain an admixture of both the developmental forms of the organism Within the cytoplasm the larger forms seems to break down into smaller spherical bodies Although the cytoplasm of the host cell was found to be packed with the organism the nucleus did not reveal any appreci able change In many smears it was also possible to observe the gra nular bodies being released by lysis of the infected cells

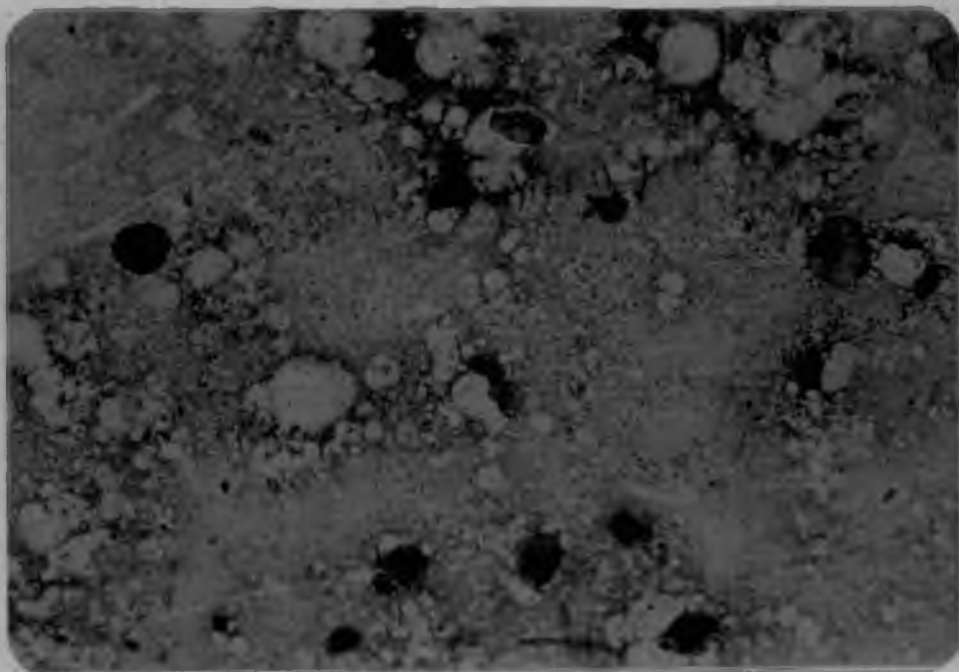
Impression smears of the yolk sac taken from sites which exhi bited marked congestion revealed moderate concentration of chlamydial bodies However if smears were prepared from sites with mild conge stion they revealed very few organisms Sometimes the smears were totally devoid of the typical granular bodies if they were prepared from regions on the yolk sac without much appreciable congestion Thus localization of the infection to specific regions on the yolk sac was

Fig 7

Yolk sac smear stained by modified Ziehl Neelsen s staining technique revealing numerous chlamydial bodies (1000 X)

Fig 8

Yolk sac smear stained by Giemsa s method revealing chlamydial bodies (1000 X)



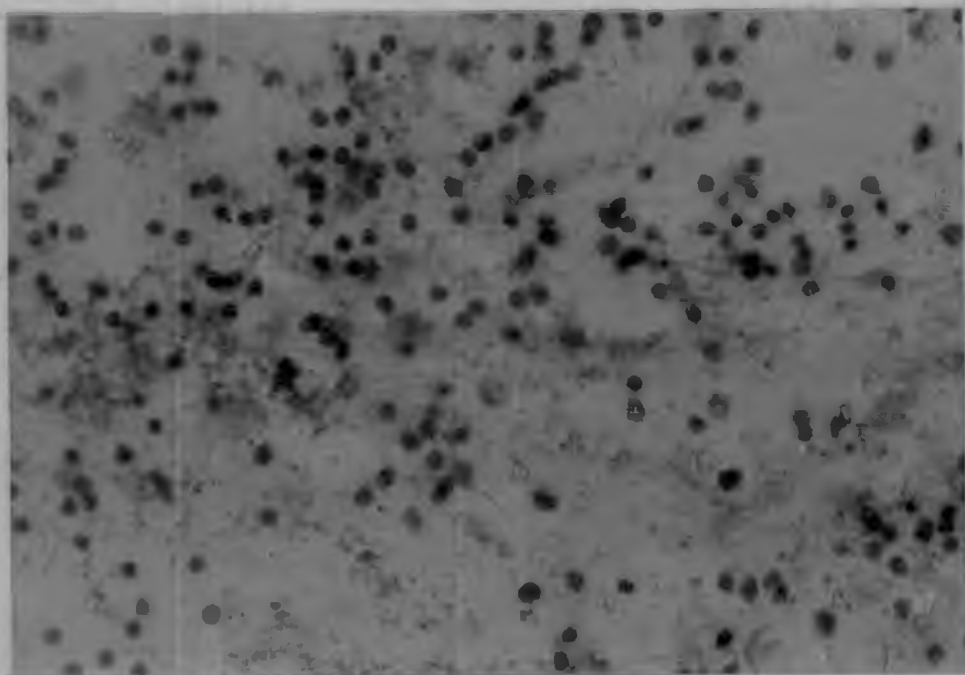
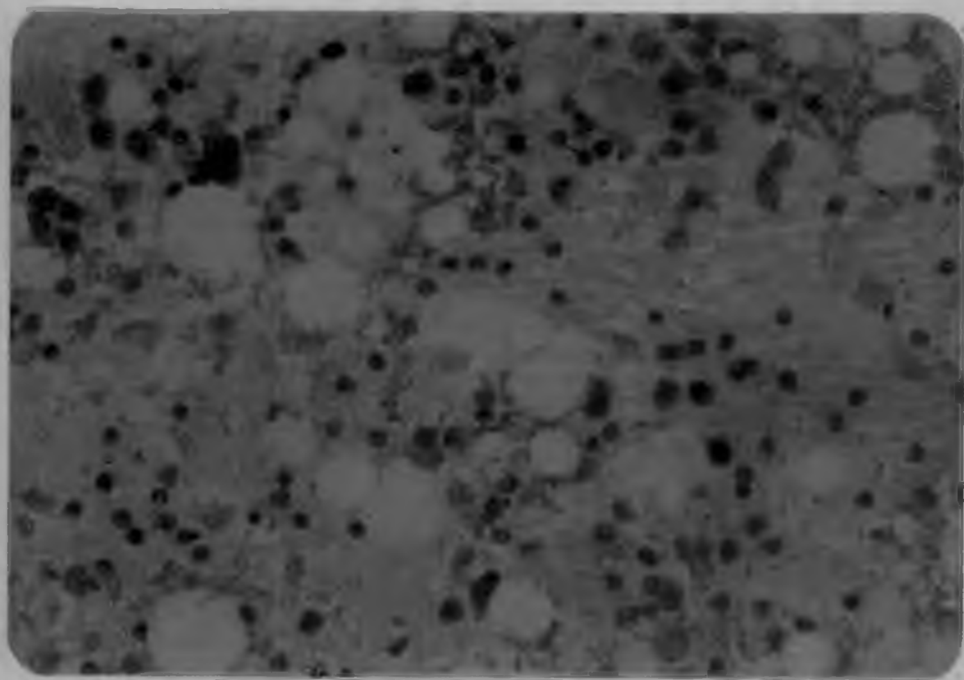


Fig.9

Yolk sac smear stained by Macchiavello's method revealing chlamydial bodies (1000 X).



observed. Keeping this in mind it was essential that smears were prepared from at least three different sites of the same yolk sac. This ruled out the possibility of making a false negative diagnosis.

Smears prepared from the YS of embryos that died five to seven days post-inoculation of the infectious material were found to contain the maximum concentration of organisms per microscopic field. In such cases the smears displayed more or less equal concentration of the smaller and larger forms of the organism. Yolk sac smears prepared from embryos that died in three to four days revealed lesser number of organisms per microscopic field than in the former case. The deeper staining smaller forms appeared to predominate in such smears. The concentration of chlamydial bodies in smears prepared out of the yolk sac of embryos that died between the eight and tenth day was markedly low. The larger pale staining forms tended to predominate over the smaller form of the organism in such smears.

3.2.3 Inoculation of guinea pigs

Guinea pigs aged four to six weeks were found to be suitable for the primary isolation of chlamydiae from clinical specimens. The results are furnished in **table 4.**

3.2.3.1 Morbidity and mortality

Among the eight isolates recovered in this study four were able to cause death of the guinea pigs. Death of the animals was found to take place in 12-15 days time after inoculation of the infectious material.

Table 4 **Presentıng the results of the ısolatıon usıng guınea pıgs**

Spec es of an mal	Nature of the samples	Number screened	Number pos tıve	percentage pos tıve		Remarks	Conc of chlamydıal bodıes n smears
Bov ne	Foetal v sceral organs uterıne d scharge placenta	6	2	33 3		Both ısolates caused death of the guınea pıgs Chlamydıal bodıes dentıf ed ın ımpressıon smears prepared from the spleen lıver lungs and per toneal cav ty Reısolatıon of the same agent was possıble n CE	+++
	Semen	9	4	44 4	1	Two ısolates caused death of the guınea p gs Chlamyd al bod es ıdentıfıed n mpress on semars prepared from the spleen l ver lungs and perıtoneal cavıty Reısolatıon of the same agent was poss ble n CE	++
					2	Two solates produced latent nfect on n the gu nea p gs Chlamydıal bodıes dentıf ed wıth some dıff culty ın mpressıon smears prepared from the pneumon c les ons of such latently nfected anımals A four fold ıncrease ın the PHA tıtre was demonstrated Chlamyd al could be re solated from the pneumonıc lesıons	+
	Sem nal vesıcu ar secret on	6					
Capr ne	Pneumon c lung	10	2	20		Two solates produced latent ınfect on n the guınea p gs Chlamydıal bodıes ıdentıfıed wıth some dıffıculty n ımpressıon smears prepared from pneumonıc les on of such latently ınfected anımals A four fold ncrease ın the PHA tıtre was demonstrated Chlamydıal could be reısolated from the pneumon c les ons	
	V sceral organs of dead kıds	2					
	Foetal v sceral organs uterıne d scharge	3					

Both the ısolates obta ned from bovıne abort on were able to cause death of the guınea pıgs However only two ısolates from semen produced death of the anımals The remaınıng two ısolates each from semen and caprıne pneumonıa produced latent ınfectıon of the guınea pıgs

After ınoculatıon of the ınfectıous materıal ınto guınea pıgs they appeared to be normal for the fırst three to four days However by the f fth day the anımal started show ng anorexıa droopıness cuddlıng up ın a corner of the cage mıld dıarrhoea hypertherm a and congestıon of the conjunctıva Followıng thıs the ınfectıon had eıther an acute course or a chronıc one

3 2 3 1 1 Acute ınfectıon ın guınea pıgs

Wıthın 11 14 days tıme the anımals became ıncapacıtated They are completely off feed exhıbıted staggerıng gaıt when forced to move showed marked shıverıng and remaıned cuddled up ın one corner of the cage In two cases marked congestıon of the skın overlyıng the scrotal regıon was a promınent feature Death occurred w thın the next 12 24 hours Two to three hours prıor to the death the anımals lapsed ınto a coma whıch was frequently ınterrupted by marked convulsıons Such guınea pıgs on postmortem revealed typıcal changes ın the ınternal organs **(Fıg 10)**

Among the two ısolates obtaıned from bovıne abortıon one of them produced severe lesıons ın the v sceral organs There was moderate ascıtes wıth accumulatıon of a serosanguıneous fluıd ın the perıtoneal cav ty The other gross lesıons ıncluded splenomegaly focal nodular

Fig.10

Guinea pig died of acute infection manifesting pneumonia, necrotic patches on the liver & mild fibrinous peritonitis.

areas of necrosis on the spleen **(Fig.11)** and patchy necrotic areas on the liver. Pneumonia with acute congestion of 50% of the lungs was also observed **(Fig.12)**. The second isolate also from bovine abortion produced milder lesions in the visceral organs. A stringy fibrinous exudate was present in the peritoneal cavity. All the visceral organs revealed marked congestion. There was severe pneumonia with involvement of about 80% of the lungs.

The first isolate from semen produced severe fibrinous peritonitis with marked fibrinous perihepatitis **(Fig. 13)**. The entire spleenic tissue was converted into a yellowish white necrotic mass **(Fig. 14)**. Patchy necrosis of the liver, mild enteritis and acute pneumonia was also observed. The second isolate from semen was found to produce a stringy fibrinous exudate in the peritoneal cavity. Besides this severe congestion of the visceral organs also was observed. Eighty per cent of the lungs was acutely congested.

Smears prepared from the visceral organs of such guinea pigs revealed large number of the organisms. The lungs were found to be the most ideal organ to demonstrate the presence of the organism **(Fig.15)**. Impression smears prepared from the spleen also revealed the presence of the chlamydial bodies, however their concentration was markedly lower than in the lungs. Smears prepared from the other visceral organs revealed exceedingly few organisms. A very efficient method of identifying the presence of chlamydial agents in the dead animal was by preparing impression smears from the peritoneal cavity. Large number of the organisms could be demonstrated in macrophages intracytoplasmically as well as excracellularly **(Fig.16)**. This could also be

Fig.11

Guinea pig spleen revealing raised
nodular necrotic lesions.

Fig.12

Guinea pig lungs manifesting acute pneumonia.

Fig.13

Guinea pig liver manifesting perifibrinous hepatitis

Fig.14

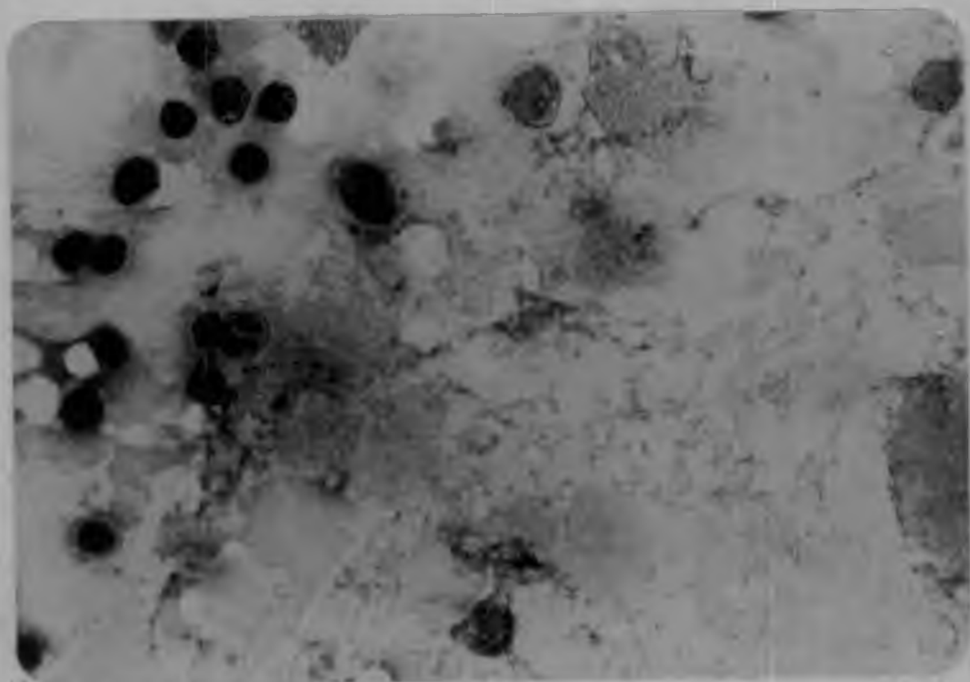
Guinea pig spleen the entire parenchyma which was converted into a yellowish white necrotic mass.

Fig.15

Guinea pig lung impression smear stained by modified Ziehl-Neelsen's method revealing chlamydial bodies (1000 X).

Fig.16

Guinea pig peritoneal impression smear stained by modified Ziehl-Neelsen's method revealing chlamydia within the macrophages (1000 X).



confirmed by the fluorescent antibody staining technique.

From the visceral organs of the dead guinea pigs it was possible to reisolate the infectious agent in the yolk sac of developing CE. The reisolated agent had the morphological and tinctorial properties of the original isolate.

3.2.3.1.2 **Latent infection in guinea pigs**

Inoculation of the infectious material into guinea pigs produced latent infection in four cases **(Table 4).** Following inoculation of the infectious material the animals manifested clinical symptoms by the fifth or sixth day. The animals exhibited mild fever, anorexia, droopiness and sometime diarrhoea. However, the infection seems to progress no further, and by the end of one week they are in the process of a slow recovery. By the second week they became normal. However, such chronically infected animals had a rough hair coat and were cachetic compared to normal guinea pigs. It was possible to demonstrate a four fold increase in the antibody titre in all these chronically infected animals by means of the PHA test. When such chronically infected animals were sacrificed and examined 25-28 days post-inoculation no gross pathological changes could be detected in the visceral organs. However in all the cases the lungs revealed signs of chronic pneumonia. About 15-40% of the lungs was consolidated, greyish in colour. In spite of these lesions in the lungs the guinea pigs appeared apparently healthy.

Impression smears prepared from the pneumonic lungs of chronically

infected guinea pigs revealed very few organisms per microscopic field. Likewise the pale staining RB predominated in the smears.

Reisolation of an agent identical to chlamydia in all aspect was possible from the pneumonic lungs of the chronically infected guinea pigs. A feature observed was that the death pattern of the reisolated agent in CE was identical to the chlamydial strains that had undergone six serial passages in CE. The smears prepared from the YS of such CE revealed extremely few organisms per microscopic field.

3.3 Passage of chlamydial isolates in heterologous system

A few chlamydial isolates recovered from bovines and caprine species were passaged in chicken embryo and mice in order to understand the effect of passage.

3.3.1 Passage in chicken embryo

3.3.1.1 Mortality pattern

The data on the influence of the number of passage of chlamydial isolate in CE on the mortality pattern are furnished in **table 5.**

It was observed that a few embryos died within 1-2 days due to non-specific reasons. Though death specific to infection was seen within 3-4 days the maximum rate of mortality was found to occur during the period 5-7 days post-inoculation during the early passages.

The strain undergone two passages caused greater mortality rate than the one that had undergone six serial passages in CE. The per-

Table 5. **Mortality pattern of chicken embryos inoculated with chlamydial isolates passaged two times and six times.**

Identity of the isolant	Animal of origin of the	No.of passage undergone	No. of embryos inoculated	Number of embryos died in					Percentage of embryos died due to specific infection			
				1 - 2 days	3 - 4 days	5 - 7 days	8 - 10 days	After 10 days	3 - 4 days	5 - 7 days	8 - 10 days	After 10 days
EBA 1	Bovine	2nd passage	18	2	5	10	1	-	31.3	62.5	6.3	-
		6th passage	18	3	1	5	5	4	6.6	33.3	33.3	26.6
EBA 2	Bovine	2nd passage	24	1	6	14	2	1	26.1	60.8	8.7	4.3
		6th passage	24	3	1	5	9	6	4.8	23.8	42.9	28.6
Semen 1	Bovine	2nd passage	24	3	5	14	2	-	23.8	66.6	9.5	-
		6th passage	24	2	2	7	9	4	9.1	31.8	40.9	18.2
Semen 2	Bovine	2nd passage	24	5	4	13	2	-	21.1	68.4	10.5	-
		6th passage	24	3	1	6	9	5	4.8	28.6	42.8	23.8
Semen 3	Bovine	2nd passage	18	1	4	11	1	1	23.5	64.7	5.9	5.9
		6th passage	18	2	-	5	7	4	-	31.3	43.8	25.0
Semen 4	Bovine	2nd passage	18	4	4	9	1	-	28.6	64.3	7.1	-
		6th passage	18	1	1	5	8	3	5.9	29.4	47.1	17.6
Pneumonia I	Caprine	2nd passage	24	-	5	13	2	-	25.0	65.0	10.0	4.8
		6th passage	24	1	2	7	9	5	8.7	30.4	39.1	21.7
Pneumonia II	Caprine	2nd passage	18	1	3	12	2	-	17.6	70.6	11.8	-
		6th passage	18	3	-	6	7	2	-	40.0	46.6	13.3

centage of mortality, due to twice passaged strain within 3-4 days post-inoculation ranged from 17.6-31.3% as against the range of 60.8 - 70.6% during 5-7 days period. The corresponding values in the case of strains that had undergone six serial passages were 0 - 9.1% and 23.8 to 40% respectively.

Greater death rate of the embryos during the later half of the incubation was seen when they were inoculated with isolates passaged six times than with two times.

3.3.1.2

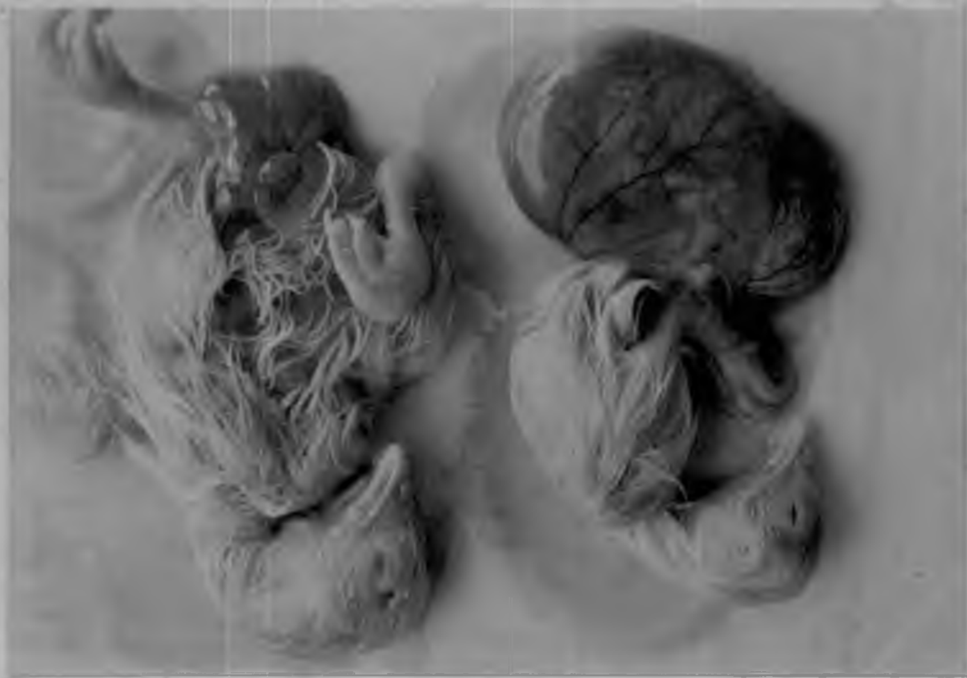
The isolates exhibited maximum virulence for the CE during the second and third passages. The lesions observed were identical to that produced during the primary isolation of the agent; however, it was more severe in nature. From the fourth passage onwards there was a gradual reduction in the virulence of the isolates for CE. This reduction in the virulence of the isolates for CE was distinctly evident from the sixth passage onwards. Such isolates tended to produce chronic infection in the CE with majority dying eight days post-inoculation. In cases where the chicken embryos died between the 8th and 10th day post-inoculation the characteristic changes in the YS and CE were not observed **(Fig.17)**. There was only moderate congestion of the blood vessels supplying the yolk sac. The yolk became more inspissiated than in control chicken embryos. The yolk sac tended to be bigger in size and contained more yolk. In many cases the embryos underwent marked stunting compared to that of control embryos. Majority of the

Fig.17

Chick embryo(left) died 9 days post inoculation
showing dwarfing, congested yolk sac, unabsorbed yolk
Control (right) chick embryo showing
normal development

Fig.18

Chick embryo (left) died on the 21st day of incubation
showing dwarfing, congested yolk sac, unabsorbed yolk
Control (right) chick embryo showing
normal development.



embryos did not exhibit the typical patchy haemorrhagic areas on the skin. The liver of the dead CE revealed only mild necrotic changes. In some cases the CE did not die even 10 days post-inoculation. In such cases if incubation was continued the CE survived to break open the shell partially. However they failed to come out and died in the shell itself. Even if such embryos were assisted in coming out of the shell they failed to survive and died in about two hours time. Such embryos showed poor feathering and were markedly underdeveloped. There was herniation of the yolk sac which contained a substantial amount of inspissiated yolk. Imbibition of bile into the yolk sac was also seen **(Fig.18).** Mild necrosis of the foetal liver was observed in many cases.

The yolk sac smears revealed the maximum number of chlamydial bodies during the second and third passages. From the fourth passage onwards a gradual reduction in the infectivity was observed. Thus earlier passages of the organisms in CE resulted in a significant increase in the infectivity while continued passage resulted in a gradual loss of infectivity.

The characteristic features of smears prepared from the YS of CE that died at different stages post-inoculation were identical to that of primary isolation. An additional observation was that YS smears of embryos that failed to die even after ten days revealed extremely few organisms per microscopic field. A majority of these chlamydial bodies were the larger plaque like form which stained poorly.

3.3.2 Passage in mice

The results of the study on the effect of passage of three chlamydial isolates in mice are presented in **table 6.**

The inoculation of the infectious YS material, passaged twice in CE, into mice aged 3-4 weeks produced mortality of 25-50% of the animal within 10-15 days. At postmortem the gross lesions observed were a stringy, yellowish fibrinous excudate on the peritoneal surface and acutely congested liver, spleen and kidneys. Extensive pneumonia was a consistent finding. Around 70-80% of the lungs manifested severe congestion and signs of red hepatization. Smears prepared from the body cavities and the lungs revealed large number of chlamydial bodies (++++).

The animals, which did not die of infection even after four weeks on postmortem revealed no gross pathological lesions but for the pneumonia observed invariably in all cases. In these animals 20-40% of the lungs showed signs of grey hepatization and consolidation. Smears prepared from the lungs of such mice though positive,revealed fewer number of organism (++).

The inoculation of the same infectious material into mice aged 6-8 weeks produced low mortality ranging from 0-12.5% of the inoculated animals. On postmortem a stringy yellowish fibrinous exudate was detected on the peritoneal surfaces. The liver, spleen and kidney were moderately congested and pneumonia involving 60% of the lungs was also observed. Smears prepared from the visceral cavity and the lungs re-

Table 6. Mortality pattern and morbid anatomy of mice inoculated with chlamydial strains double passaged in chicken embryos.

Isolate	Material used	Age of mice (weeks)	No. of mice used	Number dead	%age mortality	Period of death	Postmortem findings	
							Dead	Latent infection
EBA I	YS 2nd passage	3-4	8	3	37.5	12-15	Stringy yellowish fibrinous exudate on peritoneal surface. Congested liver, spleen and kidneys. Extensive pneumonia 70-80%	Pneumonia 20-30 %
		6-8	8	1	12.5	18	Stringy yellowish fibrinous exudate on peritoneal surface. Congested liver, spleen and kidneys. Pneumonia 60%	Pneumonia 5 - 10%
Semen I	YS 2nd passage	3-4	8	2	25	12-14	Stringy fibrinous exudate on the peritoneal surface. Congested liver,spleen and kidneys. Pneumonia 70-80%	Pneumonia 20 - 30 %
		6-8	8	-	-	-	-	Pneumonia 5 - 10 %
Pneumonia I	YS 2nd passage	3-4	8	4	50	10-13	Stringy fibrinous exudate on the peritoneal surface. mild spleenomegaly, congested liver and kidneys. Pneumonia 70-80%	Pneumonia 30 - 40 %
		6-8	8	1	12.5	16	Stringy fibrinous exudate on the peritoneal surface, congested liver, spleen and kidneys. Pneumonia 60%	Pneumonia 15 - 20 %

vealed large number of organisms (+++).

When the latently infected animals aged 6-8 weeks were sacrificed after four weeks the only lesions observed was pneumonia. This was a consistent finding in all the cases. The condition was mild involving only 5-20% of the lungs. The lesions were consolidated and greyish in colour. Smears prepared from the lungs of such animals revealed stray organisms (+) per microscopic field.

3.4 Detection of chlamydial antibodies by PHA test

3.4.1 Field samples

A total of 169 serum samples from animals with a history of either abortion/still birth, retention of placenta, repeat breeding, respiratory tract infection/pneumonia were screened by the PHA test. The results are furnished in **tables 7, 8 & 9.**

3.4.1.1 Bovine sera

A total of 18 cows with the history of abortion were screened. Except for five sera samples all the others were obtained from two organised farms A and B. Both these farms are located in the high ranges of Kerala. In farm A six animals with a history of abortion were screened by the PHA test and three of them were found positive with titres ranging from 1:16 to 1:32. In farm B about eight years back there was a massive epizootic of abortion and 60% of the pregnant animals had aborted. Numerous attempts were made to identify the etiological agent but nothing conclusive could be established. However,

Table 7. The results of PHA test giving the antibody titres and percentage positive sera samples from the field.

Species	Number screened	Number positive at					percentage positive (1 : 16 & above)
		1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	
Bovine	92	3	7	7	5	2	22.8
Caprine	67	5	6	5	2	-	19.4
Ovine	10	1	1	-	-	-	10.0
Total	169	9	14	12	7	2	20.7

Table 8. Results of PHA showing terrain wise seroprevalence

Species	Clinical history	Nature of terrain	Number tested	Number positive	Percentage positive
Bovine	1. Abortion/still birth	a) Hill tract	13	6	46.2
		b) Plains	5	-	-
	2. Retention of placenta	a) Hill tract	3	1	33.3
	3. Repeat breeding	a) Hill tract	5	3	60
		b) Plains	60	9	15
	4. Respiratory tract infection	a) Hill tract	6	2	33.3
Caprine	1. Abortion/still birth	a) Plains	14	2	14.3
	2. Repeat breeding	a) Plains	25	5	20.0
	3. Pneumonia	a) Plains	28	6	21.4
Ovine	1. Pneumonia	a) Plains	10	1	10.0

Table 9. Species wise and clinical history wise seropositivity rates in respect of sera from the field.

Clinical History	Species	Number screened	Number positive at					percentage positive (1:16&above)
			1:8	1:16	1:32	1:64	1:128	
Abortion/still birth	1. Bovine	18	–	2	3	1	–	33.3
	2. Caprine	14	1	1	1	–	–	14.3
Retention of placenta	1. Bovine	3	–	–	–	1	–	33.3
Repeat breeding	1. Bovine	65	3	5	3	2	2	18.5
	2. Caprine	25	2	3	1	1	–	20.0
Pneumonia/Respiratory	1. Bovine	6	–	–	1	1	–	33.3
	2. Caprine	28	2	2	3	1	–	21.4
	3. Ovine	10	1	1	–	–	–	10.0

the abortion rate in the herd had dwindled gradually. When the present seroprevalence study of the herd was undertaken the farm had an annual abortion/still birth rate of 8-10% during the last three years. Most of the abortions were found to occur during the second trimester of pregnancy. The aborted animals had complications like retention of placenta, metritis and repeat breeding problems. Seven animals with a history of abortion were screened from this herd and three of them had a significant antibody titre ranging from 1:16 to 1:64.

The remaining five samples obtained from various veterinary institutions located in the plains of Kerala were found to be negative for chlamydial antibodies.

Thus, out of the 18 bovines with a history of abortion/still birth that were screened serologically by the PHA test six were found positive. All these positive sera were from the hill tracts.

Two cows from farm A and one from farm B manifesting the placental retention syndrome were screened serologically for chlamydial antibodies. Only one animal from farm A was found to be positive, with a titre of 1:64. The cow had calved 15 days prior to the date of collection of the blood sample.

Sixty sera samples were collected from repeat breeders presented at the venue of various infertility camps. Nine of them had a significant antibody titre of 1:16 and above.

Two repeat breeders from farm A and three from farm B were

also screened serologically. One animal from farm A and two from farm B were found to be positive.

Thus out of the total of 65 repeat breeders that were screened serologically 12 (18.5%) were found to possess an antibody titre ranging from 1:16 to 1:128.

Among the six sera samples that were collected from cattle with high fever from farm B, two had significant antibody titres 1:32 and 1:64. All the animals had an attack of foot and mouth disease and had recovered two to three weeks prior to the collection of the sera samples. The animals with a PHA titre of 1:64 had a marked mucoid nasal discharge and severe panting. The other animal with a titre of 1:32 showed occasional panting with a slight mucoid discharge from the nostrils.

3.4.1.2 <u>Caprine serum</u>

Fourteen sera samples from goats with the history of late abortion / still birth were screened by the PHA test. Two sera samples were found to be positive and both of them were from cases of stillbirth. Out of the 14 sera that were screened 10 were from an organised goat farm C. Both the seropostive cases identified were from this farm. The annual abortion/still birth rate was 10% in this farm.

A serosurvey of 25 repeat breeding goats was also undertaken. Among them, five animals had an antibody titre of 1:16 and above i.e., 20% of the animals were found to be serologically positive. Eighteen

samples of the sera were from an organised goat farm C among which four were found to be serologically positive.

The prevalence of chlamydial antibodies among 28 goats with pneumonia was also studied. All the sera samples were collected from animals with pneumonic lesion that was detected at the time of slaughter. A significant antibody titre of 1:16 and above was detected in 21.4% of the sera i.e., in six samples. It was also possible to obtain an isolate of C. psittaci from the pneumonic lung of a goat whose sera yielded a PHA titre of 1:32.

3.4.1.3 Ovine sera

Ten sera samples from sheep with pneumonia were also subjected to the PHA test to detect the presence of chlamydial antibodies. Only one sera sample yielded a significant titre of 1:16.

3.5 Elucidation of the identity of the isolates

The isolates provisionally identified as chlamydiae were subjected to further confirmatory tests in order to establish their complete identity.

3.5.1 Detection of chlamydial antibodies in guinea pigs inoculated with field samples

The results are summarised in **table 10**. In six guinea pigs it was possible to demonstrate a four-fold increase in the PHA titre after the clinical material was inoculated intraperitoneally. The seropositive animals included one guinea pig inoculated with specimen from a case of

Table 10. **Results of the PHA test using guinea pig sera from animals inoculated with the clinical material**

Sl. No.	Species	Nature of inoculated material	Pre-inoculation PHA titre (Reciprocal)	post-Inoculation PHA titre (Reciprocal)
1	2	3	4	5 *
1	Bovine	Uterine discharge, placenta	2	16
2	Bovine	Placenta, foetal liver and lungs	2	2
3	Bovine	Uterine discharge, placenta,foetal lungs, liver and spleen	2	2
4	Bovine	Foetal lungs, liver,spleen	4	4
5.	Bovine	Foetal lungs, liver, spleen	2	2
6	Bovine	Semen	2	16
7	Bovine	Semen	4	32
8	Bovine	Semen	2	32
9	Bovine	Semen	2	2
10	Bovine	Semen	2	2
11	Bovine	Semen	4	4
12	Bovine	Semen	2	2
13	Bovine	Semen	2	2
14	Bovine	Seminal vesicular secretion	2	2
15	Bovine	Seminal vesicular secretion	4	4
16	Bovine	Seminal vesicular secretion	2	2
17	Bovine	Seminal vesicular secretion	2	2
18	Bovine	Seminal vesicular secretion	2	2
19	Bovine	Seminal vesicular secretion	4	4
20	Caprine	Pneumonic tissue	2	32
21	Caprine	Pneumonic tissue	2	64
22	Caprine	Pneumonic tissue	2	2

(contd on page 98)

Table 10 continued from page 97

1	2	3	4	5*
23	Caprine	Pneumonic tissue	2	2
24	Caprine	Pneumonic tissue	4	4
25	Caprine	Pneumonic tissue	2	2
26	Caprine	Pneumonic tissue	4	4
27	Caprine	Pneumonic tissue	4	4
28	Caprine	Pneumonic tissue	2	2
29	Caprine	Pneumonic tissue	4	4
30	Caprine	Lungs, liver, spleen of dead kids	2	2
31	Caprine	Lungs, liver, spleen of dead kids	2	2
32	Caprine	Placenta, uterine discharge	4	4
33	Caprine	Foetal lungs, liver, spleen	2	2
34	Caprine	Uterine discharge, foetal lungs, liver, spleen	4	4

* Animals were bled 25 - 28 days post-inoculation

Table 11. Results of the PHA test on guinea pigs inoculated with two isolates tentatively identified as chlamydia.

Sl. No.	Source of isolate	Pre-inoculation PHA titre (Reciprocal)	Post-inoculation PHA titre (Reciprocal) *
1.	Bovine abortion	4	128
2.	Bull semen	2	64

* Animals were bled 25 days post-inoculation

Table 12. Results of the PHA test performed using the various isolates against a known positive chlamydial hyperimmune sera

Sl.No.	Source of the isolate	PHA titre (Reciprocal)
1.	Bovine abortion I	256
2.	Bovine abortion II	128
3.	Bull semen I	256
4.	Bull semen II	256
5.	Bull semen III	128
6.	Bull semen IV	128
7.	Caprine pneumonia I	256
8.	Caprine pneumonia II	256
9.	Ovine pneumonia(Control)*	512

* The isolate against which hyperimmune serum was raised in roosters.

bovine abortion, three animals inoculated with bull semen and two with caprine pneumonic tissue.

3.5.2 Results of the PHA test on guinea pigs inoculated with two isolates tentatively identified as chlamydiae

Both the purified isolates were able to induce antibody production in the inoculated animals. A four fold increase in the PHA titre was demonstrated in both the cases. The results are given in **table 11.**

3.5.3 Results of the PHA test performed using the isolates against a known positive chlamydial hyperimmune sera

The amount of antigen that was used to sensitize SRBC in each case was not the same. In spite of this it was seen that the various isolates yielded significant titres with the control hyperimmune sera. The results are tabulated in **table 12.**

3.5.4 Immunofluorescence

All the isolates tentatively identified to be chlamydia revealed specific apple green fluorescence when they were stained with the known FITC conjugated chlamydial antisera. The various isolates could be confirmed as chlamydia in smears prepared out of the YS in which they were propagated **(Fig. 19)**. The characteristic feature of the specific fluorescence was that it blended perfectly into the black background. Non-specific fluorescence on the other hand could be identified as larger irregular masses which seems to project out from the dark background. YS smears stained by this technique revealed fluorescent bodies with

Fig.19

Yolk sac smear stained by fluorescent antibody staining technique, revealing a single speck of specific fluorescence. (432 X).

Fig.20

Yolk sac smear stained by fluorescent antibody staining technique, revealing fluorescent bodies with two distinct morphological features. (432 X).

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two distinct morphological features **(Fig. 20).** The smaller form identified were granular in nature and tended to be more numerous. The larger form were irregular, plaque like and fewer in number.

Two distinct fluorescing inclusions could be detected in smears of macrophages prepared from the peritoneal cavity of acutely infected mice and guinea pigs. Type one was seen as dense groupings of granular bodies **(Fig.21).** Type two was diffused polar immunofluorescence **(Fig.22).**

3.5.5 Electron microscopy

Negative staining with PTA revealed two types of structures with distinct morphological features **(Fig. 23).** The first one was a large irregular plaque like form while the other one was smaller and more spherical in its feature. Some of the larger forms were found to have a few notches on their periphery. A few larger bodies observed also gave an impression that they are in the process of undergoing fission **(Fig.24).**

3.5.6 Sulphadiazine sensitivity

None of the isolates were found to be sensitive to sodium sulphadiazine. There was no significant difference in the mortality rate between the control and treated sets of CE. The results are furnished in **table 13.**

Fig.21

Peritoneal impression smear of acutely infected guinea pig stained by fluorescent antibody staining technique, revealing dense grouping of granular bodies in macrophages (432 X).

Fig.22

Peritoneal impression smear of acutely infected mice stained by fluorescent antibody staining technique, revealing diffused polar immunofluorescence in macrophages (432 X).

Fig.23

Electronmicrograph showing negatively stained chlamydial bodies with two distinct morphological features. (37,000 X).

Fig.24

Electronmicrograph showing negatively stained reticulate bodies with notches on their surface while another has undergone multiple fission.

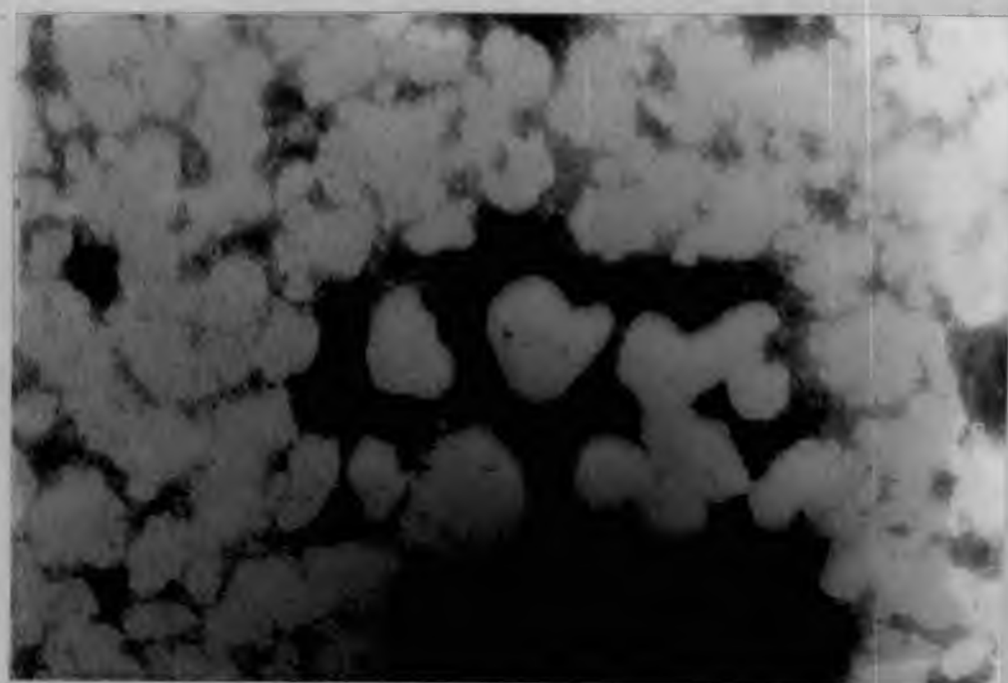

Table 13. **Showing results of sulphadiazine sensitivity test**

Identity of the isolate	Number of CE inoculated		No.of CE dying on account of the specific infection	
	Sulphadiazine treated	Control	Sulphadiazine treated	Control
EBA I	10	10	8	7
EBA II	10	10	7	7
Semen I	10	10	8	9
Semen II	10	10	6	7
Semen III	10	10	9	9
Semen IV	10	10	9	8
Goat pneumonia I	10	10	8	7
Goat pneumonia II	10	10	9	8

Discussion

DISCUSSION

Chlamydial infections results in a wide array of clinical sequelae causing heavy economic losses to the farmers. But there is not even a single documented report on chlamydiosis in Kerala with respect to its prevalence and the extent of losses as a result of chlamydial infection.

The present study envisages mainly to understand the magnitude of the prevalence of this emerging disease among the livestock in Kerala.

Detection of chlamydiae by staining the smears

For detecting the presence of structures tinctorially akin to chlamydia in clinical samples, a few staining techniques are in vogue as adopted by several workers in chlamydial research. The usefulness of this staining techniques had been proven by the findings of many workers (Storz, 1971; Page, 1981; Krishna _et al_., 1986b). The advantages or disadvantages of one method over the other has also been studied by them.

In this study all the four staining methods facilitated recognition of chlamydial structures in the stained smears. Out of 71 specimens collected from different species of animals one abortion material of bovine origin, two samples of bull semen, one sample from caprine abortion and three pneumonic lesions of caprine origin revealed structures suggestive of chlamydial morphology and the remaining were negative. In the positive cases the chlamydial bodies were found sparsely and

unevenly distributed and sometimes showing too many artifacts making the detection of chlamydia difficult. Moreover, the comparison with results of isolation using the same material as used for direct smear examination revealed that direct examination of stained smears was not very efficient to detect all the positive cases. Only one among the two specimens from bovine abortion that was culturally positive revealed chlamydial bodies on direct examination of smears. Like wise of four culturally positive semen only two revealed chlamydial bodies, that too, with many artifacts.

Chlamydia like bodies were detected in impression smears from three cases of caprine pneumonia out of a total of 15 cases examined. Chlamydia could be isolated from only two cases of caprine pneumonia although smears from three specimens revealed chlamydia like bodies. Out of five cases of caprine abortion that were screened by direct examination only one revealed chlamydia like bodies. However parallel isolation attempts in CE/guinea pigs were not successful in isolating any chlamydial agent out of these samples.

Thus the disadvantage of using this technique is the possibility of obtaining false positive results. The possibility of making a false positive conculsion becomes greater especially if the specimens contains purulent material and inflammatory exudate. This feature was observed in specimens collected from cases of caprine abortion and pneumonia where smears positive by direct microscopic examination did not yield chlamydial by isolation.

It was observed that positive specimens might give false negative results as observed with specimens from cases of bovine abortion and bull semen. This could probably be on account of the extremely few organisms that was present in the smears as well as the uneven distribution of the agent in the smears.

For the above reasons, it is obvious from the present study that direct microscopic examination of clinical specimen, though generally considered as a rapid mode of diagnosis, cannot be relied upon as a single fool proof method. It would be appropriate to subject all specimens meant for diagnosis to the isolation technique which is more sensitive and confirmatory to arrive at an accurate diagnosis as advocated by several other workers like Storz (1971), Cottral (1978) and Page (1981).

In positive stained smears two distinct developmental forms could be detected. The smaller compact spherical bodies measuring about 200-300 nm were usually found extracellularly. This developmental form was inferred to be the 'elementary bodies' of chlamydia described by Storz (1971), Buxton and Fraser (1977), Becker(1978) and page (1981). The other developmental form also had all the characteristic features of the 'reticulate bodies' of chlamydia, described by the same group of workers. Its large plaque like features with a size of 800 -1200 nm and its usual intracellular location confirmed that they are the reticulate bodies. These two distinct developmental forms could also be identified in fluorescent antibody stained smears as well as in electron micrographs of negatively stained organism.

It was also possible to conclude that all the isolates had a intracellular developmental cycle. Infected cells especially from the YS and the RE cells of experimentally infected animals revealed the typical intracellular clustering of the agent. Such culsters were found to contain an admixture of both the developmental forms. In such clusters of the organism it was also possible to observe the large reticulate bodies breaking down into the smaller elementary bodies. Fluorescent antibody stained YS cells and peritoneal macrophages also revealed the presence of the organism intracytoplasmically. Electron microscopy revealed the larger plaque like recticulate bodies with numerous notches on their surface giving an indication that they have been initiated to undergo multiple fission. Besides this many reticulate bodies that had undergone partial multiple fission was also observed by electron microscopy. This gave adequate proof that it was the reticulate body that fragmented and gave rise to the elementary bodies.

Thus the isolates obtained during the present study had two developmental forms in its life cycle. Both these are characteristic features of chlamydial agents (Storz, 1971; Buxton and Fraser, 1977; Duguid *et al.*, 1978; Page, 1981; Collier, 1984).

All the isolates took a characteristic red colour, which is typical of chlamydial agents when they were stained by the Macchiavello, Gimenez and modified Ziehl-Neelsen staining techniques. Among these three different staining techniques, the modified ZN method was found to be simple to carry out, reliable and gave consistent results. Krishna (1984) had observed the very same features with this technique and

recommended the same for routine use.

When the developmental forms were located extracellularly, the EB stained deeper compared to the RB which took a paler colour. The difference in the staining reaction between the EB and the RB has also been documented by Storz (1971) and Krishna (1984). This difference in the staining reactions between the two developmental forms could be possibly due to the structural difference between the two forms. The RB has a porous, non-rigid cell wall that facilitates the easy absorption of nutrients from the host's cytoplasm (page, 1981). The RB is more porous than the EB since the peptidoglycan layer of the former is not extensively cross linked by peptide chain unlike that of the EB (Duguid et al., 1978). This 'loosing of the stays' will definitely increase the permeability of the RB for all agents including dyes.

Both the developmental forms when present intracytoplasmically stain equally well probably because the integrity of the RB is still intact in the protected environment. Thus, there is minimum loss of the primary stain on decolourization. However, when RB are found extracellularly they become easily damaged as they cannot withstand the harsh extracellular environment (Storz, 1971; Duguid et al., 1978; page, 1981). Thus all RB which are detected extracellularly might have undergone some extent of damage. The partially damaged RB with its inherently porous cell wall could probably explain why the RB partially lose their primary stain on decolourization. The EB with its extensively cross-linked cell wall is a stage that has been evolved to face the harsh extracellular environment. This being the case the

EB can easily withstand the effect of decolourization and stain more intensely than the RB.

The Giemsa's staining technique was able to clearly distinguish between the two developmental forms. The EB stained reddish-purple while the RB tend to be more bluish in colour. Storz (1971) and Hanna et al.,(1974) have recommended this technique to differentiate between the two developmental forms of chlamydia. The difference in the staining reaction is attributed to the quantitative difference in the nucleic acid composition of the two developmental forms. The abundance of ribonucleoprotein in the reticulate bodies results in giving it a blusih colour. The elementary bodies, on the other hand, being rich in deoxyribo-nucleo-proteins stain reddish-purple in colour. This type of differential staining reaction was observed in the present study corraborating the similar observation made by the previous workers.

Isolation of chlamydiae

Since chlamydiae are obligate intracellular parasites their isolation can be effected by using living systems like chicken embryos, guinea pig, mice or tissue culture. For the successful isolation the inoculum should be essentially free of other microbial agents. In order to totally eliminate the unwanted microbial load from the tissue homogenate used as inoculum, a combination of centrifugation and pretreatment with antibiotics which can selectively knock away the microbial contaminants retaining the viability of chlamydiae is a highly mandatoryprocedure.

Different workers had used different speed and time of centriguga-tion and different periods for the action of antibiotics in order to

eliminate the microbial contamination

Gupta <u>et al</u> (1985) reported that single low speed centrifugation at 600g for 15 minutes of the clinical materials treated with antibiotics and incubated for 30 minutes at 37°C could eliminate all the contaminants But in the present study centrifugation at 600g for 30 minutes followed by incubation for as long as 24 hours at 4°C was found insufficient to eliminate the contaminants The reason attributable for this is the development of resistant forms of these bacteria which were possibly susceptible to streptomycin and kanamycin during earlier periods

Three centrifugations of the tissue homogenate at 600g 1100g and 1700g for 30 minutes each time and incubation for 24 hours at 4°C was found efficient to eliminate the contaminants totally Efficiency of high speed centrifugation at 1800g three times for half an hour each giving a contact time with antibiotics only during the period of centrifugation for the total elimination of contaminants had been proved by Storz(1963) and Dixit and Kalra (1979) Thus from the present study also it was observed that only a combination of antibiotic treatment and high speed centrifugation would effect the total elimination of the contaminants

<u>Isolation using chicken embryos</u>

The isolation of chlamydiae from clinical specimens has been remarkably improved by replacing the commonly used chicken embryo inoculation techniques by the faster and more sensitive cell culture methods but the process remains technically difficult and relatively

few laboratories offer this service (Peres Martinez et al 1986) In the absence of such tissue culture facil ties the CE is still a reliable method for the isolat on of chlamyd ae from clinical materials (Storz 1971 Hanna et al 1974 Collier 1984) The present study also found the CE to be a favourable medium for the cultivation and isolation of chlamydial agents provided the noculum was first freed of contamina ting bacteria All the eight chlamyd al strains isolated in this study were found to infect and multiply in the cells of the yolk sac of six to eight day old developing CE Following inoculation of the infectious material into the YS death of embryos occuring within the first 48 hours was found to be nonspecif ic Identical observat ons have also been made by other workers like Kr shna and Rajya (1985) and Purohit et al (1986) while isolating chlamyd ae CE dying from the third day post inoculat on was found to be on account of the specific infect on The chlamydial agent replicating in the YS produced typical lesions in the YS and embryo on account of th s When compared with uninocu lated YS the infected YS were thin walled, severely congested and its yolk tended to be more liquid in nature The typical changes observed in the embryo included the hyperaemic cyanotic legs and toes which were deep red in colour and patchy haemorrhages on the skin The characteristic les ons in the YS and embryo were exactly identical to that reported by Storz (1971) page (1981) Sreeramulu(1984) and Gupta et al (1985)

Page (1981) also noted the local zation of the infection to specif c regions on the YS where there was marked congestion A similar obser vation was also made in this study It was observed that smears pre

pared from sıtes whıch were mıdly congested revealed extremely few organısms On the other extreme end smears prepared from areas whıch were free of congestıon faıled to reveal the presence of typıcal chlamy dıal bodıes not ınfrequently Thıs observatıon establıshed the ımportance of preparıng smears from a number of congested sıtes to avoıd the possıbılıty of makıng a false negatıve dıagnosıs

Idtse (1984) observed that ıdentıfıcatıon of chlamydıae durıng prımary ısolatıon attemepts ıs quıte a dıffıcult task unless the clınıcal specımen contaıns a heavy concentratıon of the organısm He opıned that most clınıcal specımens mıght requıre two to three passages ın CE before chlamyd ae can be ıdentıfıed However ın the present study chlamydıal bodıes could be easıly dıscerned ın the YS smears from posıtıve cases ın the prımary passage ıtself Any of the samples whıch was negatıve ın the prımary passage dıd not turn out to posıtıve ın the subsequent passages

Isolatıon usıng guınea pıgs

Intraperıtoneal ınoculatıon of guınea pıgs has been used ın many ınstances as a means to ısolate chlamydıal agents from faeces and other specımens taken from cattle sheep and goats (York and Baker 1951 Pıenaar and Schutte 1975 page 1981 Dıxıt and Kalra 1984) The present study also found thıs anımal ıdeal for the ısolatıon of chlamydıae from clınıcal specımens Cottral (1978) observed that these anımals were more prone than CE to pıck up ınfectıon even wıth lesser number of chlamydıae However from the present study ıt was found that guınea pıgs are not superıor to the CE for the ısolatıon of chlamydıae

from clinical specimens since the isolation rate achieved in guinea pigs and CE were the same and moreover some of them were chronically infected without showing clear symptoms A total of eight strains of Chlamydia psittaci were isolated in guinea pigs by the intraperitoneal inoculation of clinical specimens and only four among the eight strains produced acute infections in the guinea pig Such acutely infected animals died within 12 15 days after the inoculation of the clinical material The remaining four strains produced chronic infection among the animals

Following inoculation of the positive clinical specimens the animals started manifesting clinical signs only by the fifth or sixth day post inoculation An identical observation was made by Sreeramulu (1984) The clinical signs included lethargy anorexia hyperthermia incoordination a mucoid nasal discharge and occasionally loose faeces These symptoms were more or less identical to that described by Omori et al (1960) and Page (1981) Dixit (1977) on the other hand observed only a hyperthermic reaction which reached its peak by the fifth day and occasionally loose faeces in guinea pigs inoculated with chlamydial strains Sreeramulu (1984) also observed orchitis eight days post inoculation However orchitis was not a consistent finding in this present study Only two strains one isolated from a case of bovine abortion and another from semen produced orchitis in the guinea pig within 10 days post inoculation The remaining strains did not produce orchitis This observation proves that orchitis is not a consistent invariably occurring condition in chlamydial infection

In acutely infected animals their conditions continued to worsen day after day until it terminated in death In the chronically infected

animals on the other hand after the in tial phase of illness they recovered completely by the end of the second week

At necropsy guinea pigs that had died of an acute infection revealed splenomegaly with nodular yellowish white areas of necrosis on the spleen patchy necrotic foci on the liver pneumonia and a stringy fibrinous exudate in the peritoneal cavity These were also the classical postmortem lesions described by Storz (1971) and page (1981)

Chlamydiae could be reisolated from the internal organs revealing such ac ute lesions There was no significant change n the virulence of the re solated chlamydial strains for CE

Numerous chlamyd al bodies could be discerned in impression smears prepared from the internal organs as reported by Storz (1971) and page (1981) In the present sdtudy it was noted that the choicest tissue revealing maximum number of chlamydiae was pneumonic lung eventhough other organs like spleen and liver were positive for chlamy dial bod es In the spleen chlamydial bod es could be dentified intra cytoplasmically in macrophages and PMN cells Smears prepared from spleen with milder lesions revealed fewer bodies and sometimes gave negat ve results also The organism could also be identified in smears prepared from the liver although they were extremly few in number The other visceral organs were not found su table for the demonstration of the organism

Another method which gave reliable and cons stent results was demonstration of chlamydial agents n smears prepared from the fibrinous exudate of the peritoneal cavity The organism could be detected both

extracellularly as well as intracytoplasmically in mononuclear cells This mode of diagnosis has also been recommended by Storz (1971) and page (1981) The above observation also had another important implication The chlamydial agents were seen intracytoplasmically in macrophages at the end of two weeks by which time the macrophages should have actually destroyed the organism However stained smears revealed that the organism were actually found to replicate w th n the macrophages This observation was n concurrence with that of Friis (1972) Eissenberg et al (1983) and Ward (1983) They have attributed this to the failure of the phagosome containing the organism to fuse with the lysosomes This feature ensured that the organism is able to replicate and continue its life cycle although in the midst of powerful hydrolytic enzymes The macrophages being highly mobile RE cells thus might actually aid in the dissemination of the infectious agent within the body of the host

The present study found that two chlamydial strains isolated from bull semen and another two strains from caprine pneumonia produced chronic infection in the guinea pigs The presence of chlamydiae in the test specimens was diagnosed by demonstrat ng a four fold increase in the specific antibody titre in these animals 25 to 28 days post inoculation Storz (1971) has also recommended this technique to prove the presence of chlamydiae n clin cal spec mens This serological ev dence could be further conf rmed by two other means also The organism although few in number could be identified in smears prepared from the consolidated lungs of the guinea pigs sacrificed on the same day of collection of the serum Besides this chlamydiae could also

be reisolated in CE from the chronic pneumonic lesions

Direct solation of the organism is the conclusive and confirmatory method of diagnosis of chlamydiosis in any animal species (Johnson 1983 Idtse 1984) Accordingly a total of 71 clinical specimens from bovines bubaline caprine and ovines were screened with the intention of isolating chlamydia

Out of the 41 clinical specimens screened from bovines six strains of Chlamydia psittaci could be isolated This worked out to a prevalence rate of 14 6% as assessed by the CE and guinea p g isolation techniques Among the six strains of C psittac obtained two were from cases of bovine abortion while the other four isolates were from semen samples of poor quality

It was also possible to isolate two strains of Chlamydia psittaci from 25 clinical samples screened from capr nes On a percentage basis the prevalence rate in caprines turned out to be 8% only

The attempts to isolate chlamydiae from samples of ovines and bubaline were not fru tful probably because of the fact that less number of samples were processed for isolation

The CE and guinea pig isolation techniques although still useful for the isolation of chlamydia is not as sensitive as the cell culture method for the solation of chlamydiae (Johnson 1983 Collier 1984) Thus the prevalence rate as assessed in the present study by isolation technique could be an underestimate of the actual situation

Passage of chlamydiae in chicken embryo

The effect of repeated passage of all the chlamydial strains in CE was also studied Repeated passage n CE resulted in the ncrease in number of chlamydia in the second and third passages The most severe lesions in the YS and embryo were observed during the second and third passages Above all the maximum percentage of death occurring within 7 days post inoculation was found to take place in these two passages The mortality in the second passage worked out to 17 6 31 3% during the first 3 4 days and 60 8 70 6% during the next 5 7 days post inoculation These findings were in concurrence with that reported by Storz et al (1968)

Continued serial passages of the same isolates in CE resulted in gradual reduction in the intensity of infection and severity of lesions Moreover there was a gradual shift in the mortality pattern These changes were observed as a gradual process from the fourth passage onwards and were well established by the sixth passage The isolates after six passages produced very mild lesions in the CE and the YS Likewise smears prepared from the YS revealed very few chlamydial bodies Above all there was a substantial decrease in the number of embryos dying within 7 days post inoculation The embryos dying within 3 4 days and 5 7 days post noculation dropped to 0 9 1% and 23 8 40% respectively Concurrently there was an appreciable increase in the number of CE dying beyond the eight day post inoculation During the second passage the number of CE dying within 8 10 days was 5 9 11 8% only The same isolate passaged 6 times in CE produced mortality

ranging from 33 3 47 I within 8 10 days post inoculation Another significant observation was that there was a significant increase in the number of CE dying 10 days post inoculation An isolate passaged six times in CE produced mortality in 13 3 28 6% of the inoculated CE While the same was 0 5 9% only for the isolate passaged twice in CE

Beyond the sixth passage the mortality pattern was extremely erratic and irregular and the organism tended to produce very mild infection in the CE which allowed then to develop fully very often though they died in the shell after chipping it

The observations of Sreeramulu (1984) on the effect of repeated passage of chlamydial strains in CE was distinctly different from that of the present study He found that during the early passages of the isolate the mortality pattern of the CE was erratic and irregular Unlike in the present study the ovine chlamydial strain was able to produce the characteristic lesions in the YS and the embryo as late as the fifth passage He also observed a regular mortality pattern from the sixth passage onwards But in the present study it was found that the mortality pattern of the CE was erratic and irregular beyond the sixth passage

The present study could establish that early passage of the isolates produced acute infection in the CE while repeated passages resulted in low grade infections Inspite of this mild infection the CE were markedly underdeveloped and failed to hatch It is postulated that this low grade infection severely interferred with the utilization of

the yolk by the developing CE This was clearly evident since the quantity of yolk in the yolk sac was substantially more in comparison to control uninoculated embryos

The repeated passage of the isolate in the CE which is an unnatural host could have reduced its pathogenicity for the same Another factor which could have contributed to this phenomenon was that there was a distinct reduction in the concentration of chlamydial bodies in the YS smears after the fourth passage Thus the number of infectious particles that were inoculated into the YS could have been progressively reduced in each subsequent passage resulting in the accompanied changes as mentioned above

Passage of chlamydiae in mice

The present study did not use mice for the primary isolation of chlamydiae from clinical specimens However the study was primarily directed at determining the pathogenicity of three different chlamydial strains for mice Besides this the effect of age of the mice on the pathogenicity was also studied The strains used included one each from bovine abortion bull semen and caprine pneumonia

Inoculation of infectious YS material into mice aged 3 4 weeks produced mortality in 25 50% of the animals The same material inoculated into mice aged 6 8 weeks resulted in a mortality of only 0 12 5% of the inoculated animals This observation points to the possible influence of age as a factor deciding the susceptibility of host and pathogenicity of the agent thereby conceding with the observations

made by Storz (1971) and Page (1981)

Thus younger age group of an mals are preferred when isolation of chlamydia s the objective The older group of mice could serve as ideal models to study the effect of latent or inapparent infections among animals

Intraperitoneal inoculation of mice with virulent strains produced marked splenomegaly and hepatomegaly which frequently is associated with a serofibr nous peritonitis focal liver necrosis and occasional patches of pneumonia (Storz 1971 Pienaar and Schutte 1975)

The present study observed only some of the classical lesions that are produced by virulent toxigenic strains Only the strain isolated from caprine pneumonia produced mild splenomegaly after intraperitoneal inoculation The remaining two strains merely produced severe congestion of the spleen All the three strains failed to produce the characteristic mottling of the liver with necrotic foci The only gross lesions that the liver and kidney revealed was severe congestion However a consistent finding at necropsy was that all the visceral organs were covered with a thin whitish mesh of exudative material Numerous organisms could be identified n smears prepared from the fibrinous exudate The organism could be discerned intra and extra cellularly in the peritoneal macrophages This gave adequate proof that the macrophages were not effective in destroying the organism even after two weeks time As in the case of guinea pigs they might actually support the replication of the agent and play an important role in the dissemination of the organism within the body Some chlamydial strains cause the develop

ment of a volum nous ascites mak ng the mouse so bloated that it cannot move to get food or water (Page 1981) This classical wasp l ke appearance could not be observed n the present study P enaar and Schutte (1975) have stated that only occas onal patches of pneumon a are detected in nfected mice However pneumon a was a consistent finding n all the infected animals irrespective of whether they produced an acute or a chron c infection

From the present study it could be inferred that the three strains of C psittaci had low v scerotropism and were of moderate v rulence for mice Storz (1971) had also observed that chlamydial isolates from ruminants were of low pathogenicity for mice

Latent infection

Although chlamydial agents produce severe and often fatal diseases n man and many an mal species this state of the host parasite relation ship is the exception rather than the rule A well balanced host parasite relationsh p in which pers stence of the chlamydial agent causes no obvious harm to the host is more common These long last ng napparent infect ons n vhich the paras te remains in the host in a potent ally v rulent state represents the vell known latent chlamydial infect on (Storz 1971 Hanna et al 1974 Jawetz et al 1980 Page 1981) Antibod es to several antigens of chlamyd ae are regularly produced by the infected host however these ant bodies appear to have little protective effect Commonly the infectious agent persist n the presence of high titres of antibody (Hanna et al 1974) It is possible that chlamydial agents may exist in the latently infected host in a non infect

ious stage or one that has an extremely low infectivity (Storz 1971)

This long lasting inapparent or latent state has been documented in several species of animals including sheep (Storz and Thornley 1966) guinea p gs (Murray 1964 Cottral 1978) and mice (Cottral 1978 Page 1981)

The present study was also able to identify this character st c feature of chlamydial infection among mice gu nea pigs and goats

Following intraperitoneal inoculation of the infectious material into mice a majority of them developed the latent infection Such animals failed to exhib t any clinical signs of the disease after the initial period of illness The only indicat on of infection n these an mals was the presence of pneumonia Smears prepared from such chronic lesions revealed chlamydial bodies besides chlamydia could also be reisolated from the lesions

Latently infected guinea pig failed to exhibit any cl n cal sign of the disease after the initial illness as in the case of mice The chronic infection in these an mals could be confirmed serologically by demonstrating a four fold ncrease in the antibody titres Inspite of the presence of specific ant bodies n the serum they seem to afford little protection against this agent This was evident because chlamydia could be reisolated from the chronic pneumonic lesions that persisted n these animals

Likewise the two goats from which chlamdia could be isolated were apparently normal prior to slaughter Clinical signs associated

with pneumonia were totally absent It was only during the postmortem that the presence of pneumonia could be identified In one case it was also possible to demonstrate a significant antibody titre in the serum As in the case of guinea pigs it was evident that the organism can persist in the presence of specific antibodies This gives a probable indication that the antibodies might not be protective in nature

When smears prepared from the pneumonic lesions of the chronically infected guinea pigs and mice were examined a consistent finding was that the number of chlamydial bodies observed were few in number Likewise a preponderance of the pale staining reticulate bodies were also observed Besides it was also noted that when these agents were re isolated from such chronic pneumonic lesions they were of low pathogenicity for CE The reisolated agent tended to produce a chronic infection in the CE with death predominantly taking place eight days post inoculation

The above observations gave an indication that the immune system of these animals were only partially effective in checking the chlamydial infection It is possible that the immune system could have eliminated a majority of the extracellular forms of the organism the elementary bodies The immune system of the host would not have ventured out to destroy all the intracellular forms of the agent since that would have involved destruction of a large amount of host tissue thereby placing the very life of the animal at stake Probably on account of this the immune system of the animal would have found it more appropriate to stall the intracellular replication of the agent by certain

specific means without actually destroying it totally These partially damaged organisms found intracellularly could have contributed to the state of latency Storz (1971) also suggested the possibility of this phenomenon in latently infected animals

Inspite of the absence of typical clinical signs associated with pneumonia some of the latently infected animals were cachetic and had a rough hair coat The two goats from which chlamydia could be iso lated had a history of gradually diminishing milk yield and were run down in condition with a rough hair coat

It could be postulated that inspite of the absence of overt cl nical symptoms the chlamydial infection has an adverse effect on the host although mild n nature The only problem is that we are unable to quant fy it in absolute terms This has got a significant economic implication especially when large flocks of animals are infected The chronic nfection will not attract the attention of the farmer or even the veterinarian due to the absence of clinical signs At the same time animals with excellent genetic potentials fails to perform to the expected standards Identification of chronic chlamydial infect on among l vestock should help a great deal in optimising production

Genital infections

Two chlamydial strains could be isolated from 17 cases of bovine abortion yielding an isolation rate of 11 8% It requires special mention that both these isolations were made from the farm A located in the high ranges of Kerala Chlamyd a could not be isolated from the remain

ing fifteen samples collected from the plains The results of the present isolation study indicate the possibility that chlamydial abortions in bovines could be confined to the high ranges mainly This agent might not be a significant etiological agent in bovine abort ons in the plains The endemicity of bovine chlamydial abortions to the mountai nous tracts has also been observed by McKercher (1969) Storz (1971) and Kimsey (1986)

Different workers have reported different rates of chlamydial abortion among different bovine herds at different locations The isolation rate reported by Wilsmore and Dawson (1986) who obtained a 10% isolat on rate s significantly lower than that reported by other workers Ognyanov and Genchev (1970) reported an isolation rate of 37 5% while Glavitis et al (1982) found the same to be 43 6% Wehner and Wehr(1980) from Germany reported an extremely high rate of isolation which worked out to 80% In all these cases the screening was confined to endemic herds which guaranteed a higher isolation rate If the isolation rate from farm A alone was taken into account a 100% rate of isolation was obtained But this will not be the true picture of the situation since the number of samples screened was very low

The remaining 15 samples which were culturally negative were from the plains of Kerala Th s could probably account for the relati vely low isolation rate based on total number screened

The present study failed to solate chlamydial organisms from

any of the five cases of caprine abortion This could probably be due to the fact that the number of specimens processed was relatively low Gupta et al (1985) were able to obtain only three chlamydial strains after examining 38 goat foeti Likewise Purohit et al (1986) succeeded in obtaining only two isolates even after screening 30 samples

In the present isolation study four chlamydial strains could be isolated from 15 diluted semen samples containing penicillin @ 500 IU/ml On a percentage basis the isolation rate worked out to 26 7% This was higher than that reported by Boryczko et al (1973) and Sadowski et al (1973) who recorded an isolation rate of 15% and 16 6% respectively Storz et al (1968) who were the first to report the isolation of chlamydia from semen obtained a 60% isolation rate Similarly Balbierz et al (1973) obtained a high isolation rate of 66 6%

Incubation of chlamydia contaminated semen with penicillin at a concentration of 500 IU/ml for six to eight hours was not found to be deterimental to the organism Tamura and Manire (1968) interpreted the action of penicillin on chlamydia to be merely limited to the prevention of maturation of the EB They also pointed out that penicillin did not interfer with the reproduction of the RB nor did it affect the formation of their cell wall The same group of workers have also reported that removal of penicillin before 15 hours from infected cell cultures maintained at 37°C did not affect the final yield of the organism This being the case the incubation of semen with penicillin for 6 to 8 hours at refrigeration temperature would not have been successful in controlling the chlamydial infection of semen This fact

may be attributed for the success n solat ng chlamyd ae from four samples which already contained penicillin The sera from bulls whose semen had been culturally screened were not available for serolog cal screening Hence seroevidence could not be corraborated

When minute volumes ($\leq$ 1ml) of infected semen is used for insemination the effect of pen cillin will be rapidly removed as it get diluted in the cervical and uterine mucous secretion Once the effect of penicillin is removed these organism can continue with their developmental cycle and establish an intrauterine infection Avzalov et al (1987) has specif cally observed that chlamydia infected semen can cause vaginitis abortion and still birth in cattle Thus chlamydial contamination of semen has got a very great economic implication

The semen from which chlamyd a could be solated contained a large number of inflammatory cells and had low sperm concentration Besides this some of the spermatozoa exhibited primary and secondary abnormalities These typical features of chlamydia infected semen was also reported by Storz et al (1976) and Eugster (1986)

Although six samples of seminal vesicular secretions were screened from bulls suspected to have seminal vesiculitis chlamyd a could not be solated from any of them However corynebacterium could be iso lated from all the samples It has been postulated by Ball (1970) that chlamydia could be one of the primary causes of the seminal vesiculitis syndrome In the long run secondary bacterial nfection may predominate in chronic cases These secondary infect ons may further complicate the d sease process and may mask the primary cause

of the SVS

Respiratory infection

Two chlamydial isolates could be obtained from 15 pneumonic tissue processed for isolation The isolation rate of 13 3% achieved in this study was far greater than that reported by other workers Purohit and Gupta (1983) processed pneumonic lesions from 55 goats however they were successful in isolating chlamydia from only one animal resulting n an isolation rate of 1 8% only Likewise Gupta et al (1985) processed 102 pneumonic specimens from goats They were able to isolate chlamydia from a single specimen only The isolation rate achieved in their study was as low as 0 98% only However Dixit and Kalra (1984) isolated chlamydia from 50% cases of pneumonia in goats although they screened only two specimens

One of the goats from which chlamydia was isolated had a significant antibody titre of 1 32 also which was again supportive serological evidence

Both these isolates were obtained from cases in which the gross pathological lesions were confined to the anterior portions of the apical and cardiac lobes only The remaining lobes of the lungs were free from gross pathological changes This feature ie the localization of the lesions to the apical and cardiac lobes has also been reported by other workers (Purohit and Gupta 1983 Dixit and Kalra 1984) The typical greyish granular areas of consolidation reported by these workers was not observed in this particular case The lesions compri

sed completely consolidated greyish raised areas without any granular features Dixit and Kalra (1984) has clarified that such lesions are found in cases complicated with bacterial infection This was found to be true since bacteria could be isolated from both the pneumonic lesions

Storz(1971) and Shewen (1980) were of the view that although chlamydia might play a primary role in the pathogenesis of rum nant pneumonia they by themselves induce relatively mild signs of the disease The presence of other agents like bacteria and viruses was essential for the development of an acute clinical condition

Another feature noted was that the presence of pneumonic les ons in the lungs could not be corelated with any clinical manifestations prior to the slaughter of the animal The animals seemed to be apparen tly normal in all aspects The infection seemed to have lapsed into a state of latency in the animal This sort of a long lasting latent or napparent infection has been documented in several species of ani mals (Storz and Thornley 1966) According to Jawetz et al (1980) and Shewen (1980) this could represent a well balanced host parasite relationship result ng in prolonged often life long persistence of the infection Jawetz et al (1980) has generalized that subcl nical infection is the rule and overt disease the except on in the natural host of these agents

The present study could not mplicate chlamydia as a cause of perinatal mortality in kids which were screened Krishna and Mathur

(1979) processed 10 samples for the isolation of chlamydia and they were successful in isolating four strains of the same. In another extensive study, Krishna and Rajya (1985) recovered 48 strains of chlamydia from 80 samples that were processed. At the same time they were not able to isolate any bacteria from the specimens from which chlamydia was recovered. However in the present study bacteria could be isolated from all the specimens processed. It is likely that if at all a chlamydial infection was present that could have been overcome by the severe bacterial infection present.

Although four cases of pneumonia in sheep were screened, chlamydia could not be isolated from any of the cases. Purohit and Gupta (1983) processed pneumonic lesions from 52 sheep and were successful in isolating chlamydia from two cases only. Likewise Gupta et al. (1985) were able to obtain only three isolates of chlamydia from 69 cases of pneumonia. Eventhough both these workers screened a considerable number of samples the isolation rate was relatively low. Viewed from this context the possibility of isolation a chlamydial agent from four samples was quite remote and isolation would have been successful had more number of samples been processed.

Gupta et al. (1985) could obtain only one isolate of chlamydia from a total of 63 bovine pneumonic specimens processed. Considering this extremely low rate of isolation it could not have been possible to achieve an isolation from just two samples processed as in the present study.

Other infections

A case each of conjunctivitis and polyarthritis that were screened for chlamydial agents, were found to be negative, probably due to the fact that the number of cases screened were too low to achieve an isolation.

Serodiagnosis

The diagnosis of chlamydial infections of cattle, goats and sheep is based mainly on serological procedures because the isolation of the agent remains a difficult and time consuming task (Peres-Martinez et al., 1986; Schmeer et al.,1987). This is because of the fact that the detection of the specific antibodies in the sera of infected animals is a clear indication of the prevalence of chlamydial infections in the population, since these antibodies are induced only by chlamydia and no other agent is capable of inducing such antibodies (Maierhofer and Storz, 1969). In the present study the serological survey was carried to determine the magnitude of chlamydial infection among the livestock population of Kerala.

Among the 18 serum samples from bovines with a history of abortion/still birth six of them revealed a significant antibody titre in them. As per the present study the seroprevalence rate was 33.3%. This was significantly higher than that reported by Friis (1967) who by means of the CF test detected the presence of chlamydial antibodies in 6.3% of cattle that had aborted. Similarly Schoene (1971) also reported a lower seroprevalence rate of 24.5% after he screened 687

cases of abortion by the CF test. Ognyanov (1970) on the other hand could detect the presence of chlamydial antibodies in 62 of the 121 cows that had aborted.

The high rate of seroprevalence in mountainous tracts endemic for EBA have been reported by several workers (Kennedy et al., 1960; McKercher, 1969; Eugster, 1986). The results of the PHA test proved that this feature was very much the same in the state of Kerala. Among the 13 serum samples collected from the hill tract six of them had a significant antibody titre. At the same time all the five serum samples screened from the plain were negative for chlamydial antibodies.

Out of 14 serum samples from goats with a history of abortion/ still birth screened by the PHA test two had positive antibody titre giving the seroprevalence rate of 14.3% in the present study. Gupta et al. (1985) reported a high seroprevalence rate of 22.7%. Dixit et al. (1980) also reported an extremely high seroprevalence rate of 90.9% after screening 11 sera samples from goats with a history of abortion. The present study thus gave an indication that chlamyida could also be an etiological agent responsible for the caprine abortions reported from the plains of Kerala.

Only three serum samples from bovines with a history of retention of placenta were screened by the PHA test. In spite of this small number screened a significant antibody titre could be detected in one of the animal screened. The lone seropositive animal had calved normally 15 days prior to the collection of the serum sample. The results of the present study proves that intrauterine chlamydial infection

need not always result in an acute syndrome like abortion/still birth. Milder clinical conditions like retention of placenta could also result from such infection although they might not have the same magnitude and economic importance as that of EBA.

The present study could prove serologically that chlamydial agents is associated with the repeat breeding problems encountered in the ruminants of Kerala. Although the condition is not an acute one which warrants an immediate attention it has great significance in the long term utility of the animal. Jahn et al. (1972) examined 28 herds of cattle with infertility problems and could find that of 439 animals, 68% were seropositive while another 18% were suspicious cases. However, the present study could not establish such a high seroprevalence rate among the repeat breeding cattle of Kerala. Among 65 repeat breeders screened, 12 had a significant antibody titre giving a seroprevalence rate of 18.5%. The results of the present study was more or less in agreement with that reported by Mishra and Mishra (1985) who screened 30 sera samples from repeat breeders and reported a seroprevalence of 23.3%.

There is every likelyhood that repeat breeding on account of chlamydial infection might be of greater relevance in the cattle of the hill tracts than in the plains. Among the animals in the hill tract three out of five (60%) of them had a significant antibody titre while in the plains only nine out of 60 (15%) animals were positive.

Twenty percent of the repeat breeding goats were found to have significant antibody titres against chlamydia. The results of the present

study indicate that this organism could definitely have a role to play in repeat breeding among goats just as in the case of cattle.

The importance of chlamydia as an etiological agent in respiratory tract infection among cattle could be established serologically in the present study. Among six cows screened with respiratory tract infection two had positive antibody titres of 1:16 and above. The seroprevalence rate of 33.3 % detected in this study was far lower that reported by Ognyanov (1976). He screened 359 serum samples from cattle farms with a history of abortion and pneumonia and 54 % of the animals reacted to the chlamydial antigen in the CF test.

The present study could establish the prevalence of chlamydial antibodies in the serum of goats and sheep suffering from pneumonia. A total of 28 serum samples from goats having had pneumonia were screened by means of the PHA test and six of them were found to have positive antibody titres ranging from 1:16 to 1:64. The seroprevalance rate obtained in this study was 21.4% as against 9.2% reported by Purohit and Gupta (1983). They screened sera samples from 294 goats with pneumonia and found that 27 of them had a CF titre of 1:8 and above. In the present study if the positive base titre was fixed at 1:8 the seroprevalence rate was as much as 28.6%. The concurrent results of isolation obtained in this study substantiated the significance of the higher seroprevalence rate obtained in this study. In the present study chlamydia could be isolated from 2 out of the 15 samples processed giving an isolation rate of 13.3%. On the other hand Purohit and Gupta (1983) who carried out concurrent isolation attempts along

with serological studies could report the isolation of a single chlamydial strain from 55 samples processed. The isolation rate achieved by these workers was as low as 1.8% only.

The present study was able to isolate chlamydia from the pneumonic lesion of a goat whose serum had a significant antibody titre against this organism. The concurrent isolation of chlamydial along with the demonstration of an significant antibody titre was adequate proof that this agent is an important etiological agent in caprine pneumonia.

Only 10 serum samples from sheep with pneumonia were screened by means of the PHA test. Inspite of the small number of serum samples screened one of them had a significant antibody titre of 1:16. However if the lower base titre of 1:8 was taken as the positive titre there was yet another positive sample. Purohit and Gupta (1983) tested 204 sera from sheep with a history of pneumonia by means of the CF test and reported that 32 samples had a significant titre of 1:8 and above. The seroprevalence rate of 15.7% reported by these workers was lower than the 20% obtained in the present study.

The result of the PHA test revealed that the seroprevalence rate was significantly higher than the prevalence rate determined by the isolation techniques. Among 92 sera samples of bovines that were screened 21 samples had a significant antibody titre of 1:16 and above giving a seroprevalence rate of 22.8%. However the isolation rate was only 14.6%.

In caprine out of a total of 67 samples tested by the PHA techni-

que 13 were positive exhibiting antibody titres of 1:16 and above indicating a 19.4% seropositivity. But the isolation rate was only 8%.

Thus both in bovines and caprines seroprevalence was found to be greater than the isolation rate and hence isolation cannot be effected from all seropositive animals.

The seroprevalence rate of chlamydiosis in bovines in the hill tracts was found to be significantly greater than in the plains. Among 27 sera samples of bovines obtained from the hill tracts of Kerala 12 had a positive titre giving a seroprevalence rate of 44.4% as against the corresponding value, of 13.8% for the sera collected from the plains. The greater seroprevalence rate in hill tract is corraborative to the higher isolation rate also from hill tracts as evidenced by 35.3% isolation from 17 samples of bovine origin from hill tract and 0% isolation from 24 samples from the plains.

A strict geographic limitation of EBA to the mountainous tracts is a well documented fact (Kennedy et al., 1960;McKercher, 1969; Eugster, 1986; Kimsey, 1986). The results obtained and the observations made in the present study were in concurrence with those stated by the above workers. The present study also indicated that problems like retention of placenta, repeat breeding and respiratory tract infections on account of *C. psittaci* could be more prevalent among bovines of the hill tracts than in the plains as was evidenced by the greater seroprevalence correlated with the history of the two organised farms A and B located in the high ranges of Kerala.

The prevalence of chlamydial infection in farm A could be proved by both isolation and serological means. The isolation of chlamydia from both the cases of abortions in this farm was absolute proof of the same. The fact that the farm is free from some of the other commonly encountered etiological agents responsible for a high rate of abortion in cattle points to the possibility that this agent could be responsible for the high rate of abortion encountered in the farm. The classical features of EBA like the sudden onset without any premonitory signs, high incidence among heifers which grazed on the mountainous terrain and late abortions as described by other workers (McKercher, 1969; Storz, 1971; Shewen, 1980; Kimsey, 1986) were also observed in this farm. The colloquially used term for EBA namely "Foothill abortion" was found to be most appropriate in this case also. Likewise the significant PHA titre among three of the six aborted animals was an additional evidence for the incrimination of chlamydial agents for the high rate of abortion in the farm. The chlamydial infection on the farm was not merely confined to cases of abortion alone. As reported by workers like Storz (1971), Jahn _et al._ (1972) and Eugster (1986) it was also responsible for causing retention of placenta and repeat breeding problems. A seropositive animal was detected among two animals with the problem of retention of placenta. Of the two sera samples from repeat breders one was positive with a relatively high titre of 1:64.

The peculiarity of the topography of the terrain, agro-climatic conditions and managemental practices might be conducive for the perpe-

tuation of the infection within the herd. The cool, relatively humid climatic condition prevailing throughout the year could have favoured the prolonged survival of the organism outside the animals body. It is also possible that many of the animals might be excreting the organism in large numbers especially through the dung as chlamydial organisms have been isolated from the faeces of normal cattle by Wilson (1963) and Storz (1968). The practice of allowing the animals to pasture on the foothills would have facilitated the rapid and massive contamination of the pasture with the infected dung. Shewen (1980) is also of the view that inapparent intestinal infection may play a role in the maintenance and pathogenesis of chlamydial abortion among ruminants. The ticks belonging to the genus Rhipicephalus found on these animals could have also maintained the infectious cycle. Blanco-Loizelier(1971) was able to isolate chlamydia from Rhipicephalus ticks found in infected farms.

A veneral transmission of the infection is also proposed because chlamydia could be isolated from four of the fifteen semen samples obtained from bulls that were maintained in this farm. Although only good quality semen was consistently used for insemination it was possible that semen from mildly infected bulls without any significant change in its quality might have been used for insemination. Shewen (1980) observed that in many herds where chlamydial abortion was endemic it was also possible to isolate chlamydia from the semen used for inseminating the animals.

The presence of a significant antibody titre in five out of the

ten serum samples screened indicates an overall seroprevalence rate of 50% among the animals of this farm.

Although no attempts for the isolation of chlamydia from the animals of farm B was made, the prevalence of this infection was proved serologically. Three out of seven serum samples screened from animals with the history of abortion were found positive. Repeate breeding problems was also prevalent in this farm and two of three such animals screened were found to be positive. Likewise a significant antibody titre could be detected among two of the six animals with respiratory tract infection. The overall seroprevalence among the animal screened was 43.8 %

Both the climatic conditions as well as the managemental practices adopted in this farm were identical to those of farm A. Thus the perpetuation of the chlamydial infection in farm B could be attributed to the same reasons as that on farm A.

An important point that could be inferred from the history of the farm B was that the infection had become enzootic in the herd over a period of time. Eight years back when the abortion rate in the herd was as high as 60%, that could have been when the infection was newly introduced into the herd. Later on the infection seems to have gradually established as a chronic one as evident from the fact that at the time of the present screening the abortion rate among the pregnant animals had been reduced to less than 10%. McKercher (1969) and Eugster (1986) have observed that in fresh epizootics, the abortion rate among pregnant animals ranged from 25% - 75% or even

higher Another important observation confirming that the infection was enzootic in the herd was that only heifers of the herd aborted If cows aborted they were the ones that were newly introduced into this herd from an outside source McKercher (1969) has also observed that in enzootic areas only the native heifers in their first gestation or cattle introduced from an area free of the disease would abort

Attempts to isolate chlamydia from five cases each of abortion and perinatal mortality in kids of the goat farm was not successful But seroprevalence could be demonstrated in this farm Chlamydial antibodies could be detected in two out of the ten sera samples of abortion/still birth cases Besides among the 18 sera samples collected from repeat breeders four had significant antibody titres The total prevalence rate among the goats screened was 21 4% The present study revealed that chlamydial infections in goats could be one of the impor tant factors contributing to the economic losses

Elucidation of the identity of the isolates

Immunofluorescence

Sharma et al (1982) Kr shna and Rajya (1985) and Mishra and Mishra (1985) used the direct fluorescent antibody staining technique to confirm the r isolates as chlamydia obtained during their investiga tions In the present study also the same technique was used for the confirmation of the isolates which had other characteristic features of chlamydia All the isolates revealed the specific apple green fluore sence when they were stained with the known FITC conjugated chlamydial

hyperimmune sera Thus the direct fluorescent antibody staining technique was able to confirm the identity of all the isolates as chlamydia

In addition to this the fluorescent antibody staining technique was able to dist nguish the two distinct developmental forms of this organism The smaller spherical fluoresc ng bodies which were more numerous were the elementary bodies The reticulate bodies were identified as large plaque like fluorescing masses which were fewer in number

Following fluorescent antibody staining the characteristic intracellular location of these agents could be identified in the peritoneal macrophages of infected mice and guinea pigs Within the macrophages the organism could be identified as two d stinct types of fluorescencing inclusions type one as dense groupings of granular bodies at the polar region and type two as diffused polar fluorescence Blanco Loizelier (1971) had reported the presence of a third type of fluorescing inclusion also in infected macrophages ie a hallow like fluorescence around the nucleus of infected macrophages But this was not observed in the present study

Demonstration of group spepcific antigen by PHA

The demonstration of group specific chlamydial antigens in the CF test using serum w th known chlamyd al antibody is one of the statutory obligation that has to be fulfilled for the identification of an infectious agent as chlamydia (Storz 1971) In the present study it was possible to demonstrate the presence of group specific chlamydial antigen n all the isolates by means of the passive haemagglutinat on

test performed against the known chlamyd al hyperimmune sera

Demonstration of specific antibodies by PHA

Another technique was also used for the confirmation of the isolates dentity as chlamydia The PHA test was used to find out if these isolates could induce a humoral immune response in the infected guinea pigs and if so whether those antibodies are specifically directed against the chlamydial group specific antigens A four fold increase in the chlamydial antibody titre could be established in the serum samples of all the infected guinea pigs by means of the PHA test per formed with a known C psittaci strain

Electronmicroscopy

The nature of the chlamydial isolates were also studied using an electronmicroscope Two distinct developmental forms could be identi fied using this technique The smaller spherical compact bodies were inferred to be the elementary bodies The reticulate bodies were recog nised as larger irregular plaque like bodies Some of the RB had numerous notches on their periphery giving an indication that they have been just initiated to undergo multiple fission Sometimes it was also possible to observed RB that had partially undergone multiple fission Both these features gave adequate proof that it was the RB which under went multiple fission and gave rise to the EB Thus the electronmicro scopic studies was able to confirm the morphological details of the developmental forms as well as the life cycle of the organism observed

by light and fluorescent microscopy Gutter *et al* (1973) also used the negative staining technique and recorded the very same features in the developmental cycle of *C trachomatis*

Sensitivity to sulphadiazine

To establish the differential identity of chlamydiae at species level test of sensitivity to sodium sulphadiazine is one important criterion (Storz 1971 Becker 1978 Ward 1983) Accordingly the isolates recovered during the course of this study were subjected to one important criterion sensitivity to sulphadiazine All the strains were found resistant to sulphadiazine indicating that the isolates are *C psittaci* and not *Chlamydia trachomat s*

Thus the results of the present investigation irrevocably proved the prevalence of *Chlamydia psittaci* infection among the livestock of Kerala

Summary

SUMMARY

The magnitude of the prevalence of chlamydial infection in live stock in Kerala was assessed by

1) Screening the stained smears of various clinical materials collected from divergent species of animals

2) Isolation of chlamydiae by inoculating the materials into chicken embryo by the yolk sac route and guinea pigs by intraperitoneal route

3) Detecting chlamydial antibodies in the serum by performing passive haemagglutination test

Four methods viz Gimenez Macchiavello modified Ziehl Neelson and Gimesa s staining techniques were used for staining the smears In positive cases two developmental forms the elementary bodies and the reticulate bodies could be discerned The elementary bodies were compact spherical bodies measuring about 200 300 nm and were usually found extracellularly The reticulate bodies on the other hand were mostly intracellular plaque like structures with a size of 800-1200nm

The modified Ziehl Neelsons staining technique gave more consistent and reliable results than other staining methods The chlamydial bodies were stained red in colour against a blue background

The Macchiavello and Gimenez staining techniques were also found to be good for the demonstration of chlamydial bodies which were stained red in colour

The Giemsa s staining technique was able to clearly distinguish bet ween the RB and the EB The RB were stained reddish purple while the EB more bluish tinged

The direct examination of stained smears prepared from clinical specimens from 71 animals revealed structures akin to chlamydia n one abortion material of bovine origin two samples of bull semen one sample from caprine abortion and three pneumonic lesions of caprine origin This method though a rapid mode of diagnosis cannot be relied upon by itself because certain samples found negtive in smear examination were proven culturally positive

The tissue homogenate prepared to be inoculated into chicken embryo and guinea pigs for isolation purpose could be made free of contaminant bacteria by treating the same with streptomycin @ 500 /ml and kanamycin @ 500 IU/ml followed by repeated centrifugation at 600 g 1100 g and 1700 g for half an hour each using refigerated centri fuge Mere treatment with the above antibiotics followed by low speed centrifugation at 600 g was found not effective to totally eliminate the contaminant bacteria from the tissue homogenate The tissue homogenate prepared as above were used for isolation by inoculating 6 7 day old chicken embryo by yolk sac route and guinea pig by intraperitoneal route

A total of 71 clinical specimens from bovines bubal ne capr ne and ovines were culturally screened resulting in the recovery of C psittaci from eight samples The overall isolation rate achieved in this study was 11 3%

Among these total specimens screened 41 were from bovines Six strains of *C psittaci* could be isolated from them the isolation rate being 14 6%

Two strains *C psittaci* was also isolated from the 25 clinical samples screened from caprines The isolation rate achieved was 8%

Although four clinical specimens from ovines and a lone specimen from bubaline were screened chlamydia could not be isolated from any of them

Out of 71 samples screened two out of 17 bovine aborted material (11 8%) four out of 15 bull semen (26 7%) and two out of 15 pneumonic lesions from goats (13 3%) yielded chlamydial growth as discerned by staining of impression smears of YS of CE that died after three days post inoculation

The mortality pattern extent of mortality and tissue changes in chicken embryo and guinea pigs used for isolation were also studied

The samples pos tive for chlamydiae produced death of the CE within three days post inoculation and as late as the twentyfirst day of incubation Maximum mortality was found to occur within 5 7 days post incubation

The chicken embryo which died within 3 7 days exhibited maximum lesions and tissue changes The chicken embryos that died due to the infection had hyperaemic cyanotic legs and patchy haemorrhagic areas all over the body The yolk sac was thin walled and their blood

vessels were deeply injected while the yolk in it was usually found to be thin and watery

The infected YS cells revealed in their cytoplasm an admixture of both the developmental forms of the organism which confirmed their obligate intracellular life cycle

The localization of the infection in certain regions on the YS was observed Smears prepared from sites exhibiting marked congestion revealed maximum number of chlamydial bodies

YS smears of embryos that died five to seven days post inoculation revealed the maximum concentration of chlamyd al bodies while that prepared from embryos that d ed three to four days PI revealed lesser number of organisms per microscopic f eld The concentration of chlamy dial bod es in smears prepared out of the YS of embryo that died beyond the eighth day post inoculation was extremely less

All the eight isolates recovered by CE inoculation technique could be isolated in guinea pig also Both the isolate recovered from bovine abortion as well as two isolates obtained from bull semen produced an acute infection in the guinea pig which resulted in death of the animal 12 15 days after the inoculation of the clinical specimens The remaining two isolates each from bull semen and caprine pneumonia pro duced latent infection of the animals

The necropsy findings in guinea pigs that died of an acute infection included fibrinous peritonitis splenomegaly focal nodular necrosis of the spleen patchy necrosis of the liver and extensive pneumonia

The lungs was found to be the most ideal organ to demonstrate the presence of the organism while the spleen was next in order Likewise impression smears prepared from the peritoneal cavity revealed a number of organisms intracytoplasmically in peritoneal macrophages as well as extracellularly Reisolation of the same agent was possible from the visceral organs of the dead guinea pigs

When the chronically infected guinea pigs were sacrif ced and examined 25 28 days post inoculation the only gross pathological change observed was pneumonia Although 15 40% of the lungs was consolidated these animals were apparently normal Impression smears prepared from such chronic les ons revealed extremely few organisms Chlamydia could be reisolated from these pneumonic lesion

The chlamydial strains undergone two passages caused greater mortality within seven days post inoculation while the stra n passaged s x times produced greater death rate of the embryos beyond the eighth day post inoculation

The isolates exhibited maximum virulence for the CE during the second and third passages From the fourth passage onwards there was a gradual reduction in the v rulence and nfectivity of the isolates for CE which became distinctly evident by the s xth passage

The three chlamydial isolates one from bovine abortion another from bull semen and a third from cparine abortion were found moderately v rulent for mice aged 3 4 weeks in wh ch it produced mortal ty in 25 50% of the animals after intraperitoneal inoculation with nfectious

YS material In the animals which died of an acute infection the lesions detected at necropsy included a stringy yellowish fibrinous exudate on the peritoneal surface severe congestion of the visceral organs and extensive pneumonia An mals which became latently infected had signs of pneumonia involving 20 40% of the lungs

The same isolates were less pathogenic to mice aged 6 8 weeks and produced mortality only in 0 12 5% of the animals The necropsy lesions were almost identical as in the younger age group of mice that died of an acute infection The latently infected mice exhibited mild pneumonia only involving 5 20% of the lungs

Among 169 serum samples of bovines caprines and ovines screened by means of the PHA test 35 of them had a significant ant body titre ranging between 1 16 1 128 The overall seroprevalence was 20 7%

The seroprevalence of chlamydiosis as determined in this study was higher than the prevalence rate determined by the isolation technique

Twentyone out of 92 (22 8%) bovine sera 13 out of 67 (19 4%) caprine sera and one out of 10 (10%) ovine sera were found positive for chlamydial antibodies

The present study ndicated that there was a higher prevalence of chlamydial infect on among the bovines in the hill tract Among the 17 specimens screened from the hill tract six of them yielded a chlamydial isolates ie a 35 3% isolation rate At the same time 24 clinical specimens from bovines that were screened for isolation did

not result in any isolation The seroprevalence rate of chlamydiosis in bovines in the hill tracts was also significantly greater than in the plains Among 27 sera samples of bovines from the hill tracts of Kerala 12 of them had significant antibody titres giving a seropre valence rate of 44 4% At the same time 65 sera samples from the plains had s gnificant antibody titre in 9 sera indicating a seropre valence of 13 8% only

Six out of 34 guinea pigs exhibited significant level of chlamydial antibodies in the PHA test The seropositive animals were the one inoculated with bovine aborted material three with bull semen and two w th caprine pneumonic tissue

Two guinea pigs inoculated with purified suspension of the C psittaci isolated from a case of bovine abortion and another from bull semen revealed high level of specific antibody titres of 1 64 and 1 128

By means of the PHA test the presence of group specific chlamydial antigens in all the isolates could be demonstrated using a known positive chlamydial hyperimmune sera

The fluorescent antibody staining technique was used to confirm the identity of the isolates in YS smears stained by this technique revealed two types of specific apple green fluorescencent bodies The organism could also be identified as fluorescing inclusions in the peri toneal macrophages of acutely infected mice and guinea pig

Negative staining with PTA revelaed structures analogous to the RB & EB under the electron microscope Various stages of multiplication

were also observed

None of the chlamydıal solates were sens tıve to sodıum sulphad a zıne and hence they were ıdentıfıed as Chlamydıa ps ttac

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PREVALENCE OF CHLAMYDIAL AGENTS IN LIVESTOCK IN KERALA

By

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ABSTRACT OF A THESIS

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ABSTRACT

The magnitude of the prevalence of chlamydial infections in the livestock in Kerala was assessed by screening the smears of various clinical materials after staining, isolation using chicken embryos and guinea pigs and serologically by passive haemagglutination test.

The results obtained were discussed correlating to the managemental practises in the organised herds and agroclimatic conditions.

A total of 71 biosample comprising 17 bovine abortion materials, 15 bull semen, 6 seminal vesicular secretion, one synovial fluid from a calf, 5 caprine abortion material, 15 caprine pneumonic lungs, 5samples from perinatal mortality in kids, 4 ovine lung tissue and one conjunctival washings from a buffalo were utilised for screening the smears stained by Gimenez,Macchiavellos, modified Ziehi-Neelsen and Giemsa's methods and for isolation purpose.

On screening the stained smears, one bovine abortion material, two bull semen, one caprine abortion material and three caprine pneumonic lesions were found positive for developmental forms of chlamydiae which could be discerned intra and extracytoplasmically. The overall prevalence rate by this method was 9.9% and species wise prevalence rates were 7.3 % among cattle and 16 % among goats.

Attempts for isolation resulted in the recovery of chlamydiae from two of 17 bovine abortion materials, four of 15 bull semen and two of 15 caprine pneumonic lungs. The overall prevalence based

on isolation rate was 11.3% and species wise prevalence rates were 14.6% and 8% respectively for cattle and goats.

A total of 169 serum samples consisting of 92 from cattle, 67 from goats and 10 from sheep were screened serologically. Of these 21 from cattle, 13 from goats and one from sheep were found positive showing titres of 1:16 and above. The overall seroprevalence rate was found to be 20.7 % and species wise rates were 22.8%, 19.4% and 10% for cattle, goat and sheep.

On comparison the seropositivity rate was found greater than the cultural positivity rate and the rate of positive cases detected by stained smear examination of clinical materials was the least.

Among bovines the prevalence was found greater in the hill tracts than in the plains.

The mortality pattern and the extent of mobidity due to chlamydial infection were also studied by inoculating 6-7 day old chicken embryos and mice of two age group using strains that had undergone passages in chicken embryo two times and six times. In the case of chicken embryos it was observed that with increase in number of passage the pathogenicity of the strains got reduced, the time of mortality shifted from early death (within 3-7 days) to late death (beyond 8 days) and the double passaged strains caused greater mortality.

The lesions and tissue changes in the dead chicken embryos were developmental retardation, haemorrhages, necrotic lesions in

the liver, intense congestion of the yolk sac membrane which invariably contained unabsorbed watery yolk.

In the case of mice, younger age group (3-4weeks) suffered greater mortality than older group (6-8 weeks). The lesions were mainly extensive pneumonia, congestion of the visceral organs and a yellowish fibrinous exudate on the peritoneal surface.

Latent or inapparent infection could be noted in some mice infected with the isolates and in some of the guinea pigs inoculated with the clinical materials which were, later on, proved positive for chlamydia. The animals on autopsy, revealed low degree of pneumonia.

The electron microscopic studies of three isolates revealed structures analogous to the chlamydial developmental forms.

The identity of the isolates was confirmed by fluorescent antibody staining technique and test of sensitivity to sulphadiazine. Following fluorescent antibody staining (FAT) two distinct developmental forms of the organism could be identified. The intracellular location of this agent could also be identified in the peritoneal macrophages following FAT, which revealed two distinct types of fluorescencing inclusions -type one as dense groupings of granular bodies at the polar region and type two as diffused polar fluorescence. None of the isolates was sensitive to sulphadiazine indicating that the isolates are Chlamydia psittaci.