DETECTION OF CHLAMYDIA TRACHOMATIS IN TRACHOMA AMONG EGYPTIAN PATIENTS

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Presented by
Amany Saleh Ahmad Awad
M.B., B.Ch.
M.Sc Bacteriology

Under Supervision of

Prof. Dr. Abla Abdel Salam Haroun
Professor of Microbiology & Immunology
Faculty of Medicine
Ain Shams University

Prof.Dr. Mouchira Fayek Helmy
Professor of Microbiology &
Immunology, Faculty of Medicine
Ain Shams University

Dr. Affaf Shaban Abdel Rahman
Assist. Prof. of Microbiology &
Immunology, Faculty of Medicine
Ain Shams University

Dr. Mohammad Sayed Salamah
Assist. Prof. of Molecular Biology
Faculty of Science
Ain Shams University.

Dr. Mamdouh Hamdy El-Kafrawy
Lecturer in Ophthalmology
Faculty of Medicine
Ain Shams University

Faculty of Medicine
Ain Shams University
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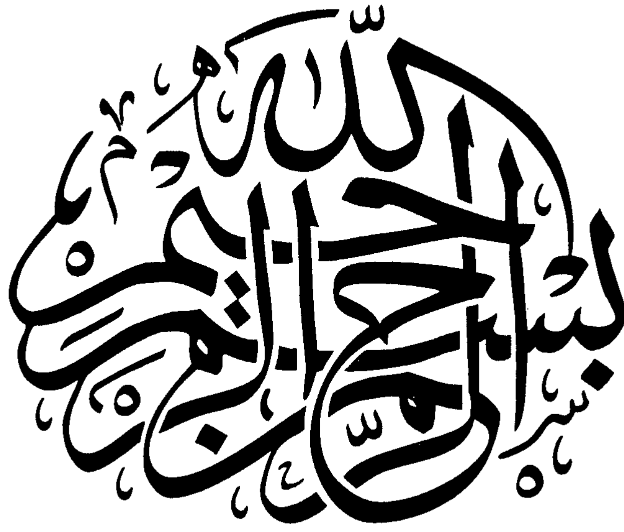

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LIST OF ABBREVIATION

ATP:	Adenosine triphosphate
ADP:	Adenosine diphosphate
bp:	Base pair
C. pecorum:	*Chlamydia pecorum*
C. Pneumoniae:	*Chlamydia pneumoniae*
C. psittaci:	*Chlamydia psittaci*
C. trachomatis:	*Chlamydia trachomatis*
CO2:	Carbon dioxide
dNTPs:	Deoxynucleoside triphosphate
dATP:	Deoxyadenosine triphosphate
dCTP:	Deoxycytosine triphosphate
dGTP:	Deoxyguanosine triphosphate
dTTP:	Deoxythymidine triphosphate
DEPC:	Diethyl pyrocarbonate
DFA:	Direct fluorescent antibody test
DNA:	Deoxyribonucleic acid
DTH:	Delayed type hypersensitivity

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E. coli:	*Escherichia coli*
EB:	Elementary body
EIA:	Enzyme immunoassay
H. influenzae:	*Haemophilus influenzae*
IF:	Immunofluorescence
IL:	Interleukin
INF-γ:	γ interferon
LB:	Luria Bertani medium
LGV:	Lymphogranuloma venerum
LPS:	Lipopolysaccharide
MIF:	Micro-immunofluorescence
MOMP:	Major outer membrane protein
No.	Number
NGU:	Non gonococccal urethritis
Omp-1:	Outer membrane protein gene locus 1
P:	Probability
PHA:	Phytohaemagglutinin
PPD:	Purified protein derivative
PCR:	Polymerase chain reaction
RB:	Reticulate body

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RNA:	Ribonucleic acid
S. aureus:	*Staphylococcus aureus*
S. epidermidis:	*Staphylococcus epidermidis*
SD:	Standard deviation
SDS-PAGE:	Sodium dodecyle sulfate polyacrylamide gel electrophoresis
St. viridans:	*Streptococcus viridans*
STD:	Sexually transmitted disease
Taq Polymerase:	*Thermus aquaticus* polymerase
Th:	T helper
UK:	United Kingdom
USA:	United States of America
UV:	Ultraviolet
VS:	Variable segment
WHO:	World Heath Organization
%	Percentage
+ve:	Positive
-ve:	Negative
X^2:	Chi square

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Introduction

Trachoma is the world's leading cause of infectious blindness. It is estimated that about 500 million people suffer to some extent, of whom 7 million are blind (Dawson et al.,1981). Trachoma was known as Egyptian ophthalmia and is endemic in Middle East since pre-historic times (Miller, 1992).

In Endemic areas, the active stage is seen principally among pre-school children, and is characterized by chronic follicular conjunctivitis. Among older individuals conjunctival scarring replaces the follicles and may lead to blindness (Baily et al., 1994b).

A simplified WHO trachoma grading system has been developed for easy, but reliable clinical assessment of trachoma at the community level (Thylefors et al., 1987).

Trachoma is caused by *Chlamydia trachomatis*, usually serovars A, B, Ba, and C (Grayston and Wang, 1975).

The prevention of blindness due to trachoma depends on the reduction of the transmission of *C. trachomatis* from eye to eye in communities where the disease is endemic. Microbiological techniques for the detection of the organism are an essential adjunct to clinical examination in the attempts to understand the epidemiology of trachoma and hence to formulate rational control measures (Baily et el., 1994b).

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Aim of Work

The aim of this work was to detect *C. trachomatis* in stages of trachoma, and associated bacteria among Egyptian patients and matched control subjects.

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Chlamydiae

History:

Chlamydia trachomatis inclusions were first detected in 1907, by Harlberstaedter and Prowazek, in stained conjunctival scrapings taken from orangutans that had been inoculated with human trachomatous material (Schachter and Dawson, 1987). Miller (1992) stated that, Harlberstaedter and Prowazek termed it '*Chlamydia*' because it seemed to them to have a cloak (*chlamydia*).

Schachter (1992) stated that, shortly thereafter, similar inclusions were identified in conjunctival scrapings taken from trachoma patients and then from infants with inclusion blennorrhea. Inclusions were then found in uterine cervical cells of mothers of affected infants and in urethral cells of fathers. In the first decade of this century, the presence of these inclusions was associated with non-gonococcal urethritis (NGU). He added that, *C. trachomatis* was first isolated from patients with lymphogranuloma venerum (LGV). In the 1930s, the growth cycle of the LGV organism (as seen following intracerebral inoculation in mice and then in eggs) was noted to be similar to that of *Chlamydia psittaci*, which has been isolated during the psittacosis pandemic of 1929 - 1930.

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The trachoma agent proved more difficult to recover, as it is not infective for mice. It was isolated in 1950s by inoculation of embryonated hen's egg yolk sac by T'ang and coworkers (1957).

Jones et al. (1959) isolated *Chlamydia* (other than LGV agent) from the cervix of a mother of an infant with ophthalmia neonatorum. In 1964, chlamydiae were isolated from the urethras of men epidemiologically associated with conjunctivitis cases (Jones, 1964; Rose and Schachter, 1964).

In 1965, the introduction of a tissue culture isolation procedure for *C. trachomatis* made it possible to screen large number of specimens and to obtain the results of an isolation attempt in 48 -72 hours (Gordon and Quan, 1965).

Diseases associated with *C. psittaci* were first recognized in the latter part of the nineteenth century (Schachter and Dawson, 1978). *Chlamydia pneumoniae* had been isolated few years ago (Grayston et al., 1989). Fukushi and Hirai (1993) described another species of the genus chlamydiae; *Chlamydia pecorum* (pathogen of sheep and cattle).

Classification:

Chlamydiae are classified within the kingdom *Prokaryotae*, division (phylum) *Gracilicutes*, class *Scotobacteria*, order *Chlamydiales*, family *Chlamydiaceae,* with one genus, *Chlamydia* (Moulder et al., 1984).

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According to Schachter (1992), apart from the more recently discovered *C. pecorum*, there are three well recognized species, *C. trachomatis, C. psittaci, and C. pneumoniae*. These 3 species differ in morphology, sensitivity to sulfa, and iodine staining. *C. trachomatis* is coccoid, sensitive to sulfa, and stain with iodine, in contrast to *C. pneumoniae* and *C. psittaci* which are sulfa resistant and are not stained with iodine. *C. pneumoniae* is pearl shaped while *C. psittaci* is coccoid. Within each species there are serovars and biovars distinguished on the basis of host range, disease pattern, and antigenic composition.

C. trachomatis species is divided into three biovars; the trachoma, lymphogranuloma venerum, and murine biovar.

The trachoma biovar of *C. trachomatis* affects only humans. It is responsible for many clinical syndromes including: trachoma, inclusion conjunctivitis, ophthalmia neonatorum, urethritis in men, salpingitis, infertility, and ectopic pregnancy in women, and infant pneumonia (Schachter, 1992).

The author added that LGV biovar of *C. trachomatis* affects only humans and is responsible for LGV, while the murine biovar affects mice and there is no known human disease caused by this biovar. It is represented by mouse pneumonitis agent.

The two biovars that are associated with human disease (the trachoma and the LGV biovars) can be differentiated serologically and on the basis of the invasive properties. The

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trachoma biovar does not infect cultured cells efficiently and require mechanical assistance such as centrifugation of the inoculum, in contrast to the LGV biovar which efficiently infects cultured cells. In naturally occurring disease the trachoma biovar seems to infect squamocolumnar epithelium, while the LGV biovar infects endothelial and lymphoid cells (Schachter, 1992).

The natural hosts of *C. psittaci* are birds and lower animals, but in man it causes psittacosis. On the other hand, the natural host of *C. pneumoniae* is human and it is responsible for pneumonia (Schachter, 1992). *C. pecorum* is a pathogen of sheep and cattle causing various diseases including encephalitis, infectious polyarthritis, pneumonia and diarrhea (Fukushi and Hirai, 1993).

There is a strong DNA homology between serovars and biovars within each species, but little DNA homology among the three species of *C. trachomatis* (Smith, 1993).

Characterisation:

The chlamydiae are among the most common pathogens throughout the animal kingdom (Meyer, 1967). They are non-motile, Gram negative, intracellular bacteria. Their unique developmental cycle differentiates them from all other microorganisms (Moulder et al., 1984).

Chlamydiae were once considered viruses, but they differ from viruses by possessing both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and the cell wall is quite

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similar in structure to those of Gram negative bacteria. They are susceptible to many broad spectrum antibiotics, possess a number of enzymes, and have a restricted metabolic capacity that does not result in the production of energy. Thus, chlamydiae have been considered as energy parasites that use the adenosine triphosphate (ATP) produced by the host cell for their own requirements (Schachter, 1991).

Developmental Cycle:

The developmental cycle of chlamydiae sets them apart from all other bacteria. The cycle passes through sequential steps:

1. Attachment:

The attachment of the elementary body (EB), the infectious form of the trachoma biovar of *C. trachomatis* to cells is inefficient, in contrast to that of *C. psittaci* and the LGV biovar (Kuo et al., 1973).

Attachment may be charge dependent. Diethylaminoethyl dextran pre-treatment of cells leads to marked enhancement of attachment and entry of trachoma biovar, but not most *C. psittaci* or the LGV biovar. Treatment with negatively charged molecules such as heparin can inhibit chlamydial infectivity and elute EB from surface of host cells (Becker, 1978). Initial contact of the chlamydial EB with the susceptible host cell may involve a specific receptor-ligand interaction, but no such structure was clearly identified (Schachter, 1992).

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2. Cell entry:

Once attached, the EB is rapidly internalised (phagocytosed) by the host cell. Many of the cells that *Chlamydia* infect are not considered phagocytes. Moulder (1985) differentiated host cells into professional (as macrophages and polymorphnuclear leukocytes) and non-professional phagocytes, and demonstrated that, *Chlamydia* induces phagocytosis by the non-professional phagocytes. The mechanism of chlamydial uptake is controversial. Ultrastructure studies suggested that, *Chlamydia* enter through clathrin-coated pits, via a pathway similar to that of receptor-mediated endocytosis (Hondinka and Wyrick, 1986).

3. Morphological changes and replication:

The infectious EB changes to the metabolically active and dividing form; the reticulate body (RB), within the first 6 to 8 hours after entering the host cell. RBs, using the host cell pool of precursors, synthesize RNA, DNA, and protein (Moulder, 1966). The RBs divide by binary fission from 8 hours after entry into the cell to 18-24 hours. This is the stage of greatest metabolic activity, when the organism is most sensitive to inhibitors of cell wall synthesis and inhibitors of bacterial metabolic activity. At 18 to 24 hours, some of the RBs change into EBs. This entire cycle takes place within the phagosome, which obviously undergoes a large increase in size. At some time between 48 to 72 hours the cell ruptures releasing the infectious EBs (Schachter, 1992).

Phago-lysosomal fusion does not occur until the death of the cell is eminent. This inhibition of phago-lysosomal fusion

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has been attributed to a chlamydial surface antigen, because antibody-treated EBs do not inhibit phago-lysosomal fusion. Inhibition is specific to the chlamydial phagosome, as fusion can take place in other phagosomes in the same cell (Eissenberg and Wyrick, 1981).

Kahane et al. (1993) described a new intracellular *Chlamydia*-like microorganism and they termed it 'Z'. It is an obligate intracellular bacterium that was isolated as a cell culture contaminant of unknown origin. The organism grew in a variety of cultured cells within 5-7 day developmental cycle, within cytoplasmic phagosomes similar to *Chlamydia* and some Rickettsia species. Two alternating developmental forms, EBs and RBs, were observed by electron microscopy. In spite of that, the authors reported that sodium dodecyle sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**), immunoblotting with *Chlamydia* specific antibodies, and polymerase chain reaction (PCR) using chlamydial genus-specific primers provided evidence that this bacterium differs significantly from chlamydiae.

Structure:

Chlamydia trachomatis, as other chlamydiae, is a Gram negative, non motile, non capsulated, non sporulating bacilli. Their cell wall is quite similar in structure to those of Gram negative bacteria (Schachter, 1991).

As a part of their unique growth cycle, *Chlamydia* organisms have evolved two morphologic entities; the

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compact stable EB which successfully persists in the extracellular environment and is responsible for cell to cell and host to host transmission, and the highly labile non-infective RB which is a metabolically active and vegetative form, that does not survive outside the host cell (Schachter, 1992).

The EB is a spherical particle, 350 nm in diameter and has an electron dense centre, while the RBs is 1 μm in diameter and is not electron dense (Moulder, 1966).

EB shows projections of the envelope of unknown function on one hemisphere (Gregory et al., 1979). The electron microscope reveals an outer and inner membranes. No peptidoglycan layer is revealed with muramic acid, but the presence of penicillin-binding proteins suggests a related cross-linked structure. The EB envelop is rigid and relatively impermeable to macromolecules. The outer membrane contains lipopolysaccharide (LPS) and has a major outer membrane protein (MOMP) of 39 to 45 kDa which constitutes 60% of the weight of the outer membrane (Caldwell et al., 1981; Barbour et al., 1982).

The structural rigidity of the EB appears to depend on disulfide cross-linking of MOMP with each other and with other cystein-rich proteins (Newhall, 1987). Soon after the EB enters the phagosome, its envelop loses its rigidity and the subunit layer is disrupted and disappears. This reorganization to the more flexible and fragile structure of the RB probably involves reduction of the cross-linked disulfide bonds. The RB cell envelop is highly permeable (Schachter, 1992).

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Antigens:

The chlamydiae possess group (genus) specific, species specific, and type specific antigens. Although they are antigenically complex, only a few antigens play a role in the diagnosis.

1) Lipopolysaccharide (LPS) antigen:

This LPS is a major heat stable genus-specific complement fixing antigen, extractable from the organism with organic solvents such as ether. The reactive moiety in the LPS antigen is ketodeoxyoctanoic acid (Nurminen et al., 1983).

Chlamydial LPS shares at least 2 antigenic determinants with LPS of certain Gram negative bacteria e.g. *Acinetobacter calcoaceticus* var antiratum. Besides, it has at least one immunodominant epitope unique to chlamydiae as demonstrated by reactivity with monoclonal antibodies (Caldwell and Hitchcock, 1984; Brade et al., 1985).

Acinetobacter LPS can be used as a complement fixing antigen for anti-chlamydial antibodies, but not all sera that react against the chlamydial antigens react with Acinetobacter LPS (Neuramine et al., 1984).

Schachter (1991) stated that, most direct chlamydial detection by enzyme linked immunosorbant assay (EIA) is dependent on antibodies bound to the solid phase against this LPS antigen.

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2) Major outer membrane protein (MOMP):

The MOMP is the principle target for neutralizing antibodies, and may be the target of protective immunity. Antibodies against MOMP were used for typing of chlamydiae by microimmunofluorescence (MIF) test (Wang and Grayston, 1970), and by in vitro neutralization test (Graham and Layton, 1971).

With MIF, Wang and Grayston (1970) recognized 15 serovars of *C. trachomatis* (A, B, Ba, C- K, L1, L2, L3). The A, B, Ba, and C serovars are associated with blinding trachoma, while the D through K are commonly associated with sexually transmitted disease (STD). The L1, L2, and L3 serovars represent the LGV biovar (Grayston and Wang, 1975).

This MOMP contains both species and serotype specific antigens (Caldwell and Schachter, 1982).

Only one serovar has been identified among *C. pneumoniae*. There are many different ones among *C. psittaci,* but they have not been well characterized (Schachter, 1992).

Variations in the MOMP are due to exposed-loop variations in this protein. These surface exposed loops of the MOMP are encoded by variable segments (VS) I, II, and IV of the MOMP gene (Pecharatana et at., 1993). This allelic polymorphism is at the MOMP gene locus 1 (omp-1) (Brunham et al., 1994).

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3) Other antigens:

Two important chlamydial antigens have been implicated in the pathogenesis of eye and female pelvic infections (Morrison et al., 1989; Wager et al., 1990).

The heat shock protein 60 (hsp 60) is a 57 kDa protein. It is triton x 100-soluble antigen, that induces hypersensitivity in ocular model, and was recognized to be a homologue of a heat shock protein found in eukaryotic and prokaryotic organisms (Wager et al., 1990). It is a common chlamydial (genus specific) antigen. Individuals with severe form of chlamydial disease often display immune response to this antigen (Brunham and Peeling, 1994).

The second protein is 60 kDa. It is a sarcosyl-insoluble cystein-rich structural protein (Wager et al., 1990). This protein is synthesized late in the developmental cycle, elicits a major antibody response to chlamydial infection, and has been shown also to be genus specific (Watson et al., 1994).

Nucleic acids:

Chlamydiae, like other bacteria, have a double stranded DNA genome and prokaryotic type RNA. The RBs contain about 4 times as much RNA as DNA, whereas the EBs contain about equal amounts of RNA and DNA (Jawetz et al, 1995).

Chlamydiae have one of the smallest bacterial genomes (6 to 8.5 x 10^5 bp; half the size of DNA of Neisseriae and Rickettsiae (Kingsbury, 1979).

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C. trachomatis, C. psittaci ,and C. pneumoniae can be separated on the basis of DNA homology as the intraspecies homology does not exceed 10% (Smith, 1993).

Chlamydial LPS gene has been successfully cloned into *Escherichia coli*, and is expressed in its outer membrane (Nano and Caldwell, 1985).

Because of its important structural and antigenic role, the genes responsible for MOMP have been of particular interest. The gene is composed of five conserved and four variable segments. These variable sites are responsible for variable domains on MOMP and thus for antigenic reactivity (Stephens et al., 1987). Antibodies to the surface exposed loops on the MOMP, encoded by the variable segments (VS) I, II, and IV of the MOMP, are protective (Pecharatana et al., 1993).

Genotyping of *C. trachomatis* is dependent on those variable segments on MOMP gene (Frost et al., 1991; Sayada et al ., 1991; Hayes et al., 1992; Peharatana et al., 1993).

All *C. trachomatis* serovars contain a plasmid present in multimeric form (Palmer and Falkow, 1986). The restriction endonuclease cleavage patterns of the plasmid for the LGV strain were different from those of the other *C. trachomatis* strains. Functions of the plasmid genes is not known, although some of the gene products are expressed during infection. This cryptic plasmid was used for the detection of chlamydial infection with a DNA probe (Schachter et al., 1988) or by polymerase chain reaction (PCR) (Baily et al., 1994a).

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Presence of a plasmid in *C. psittaci* strain appears to be variable (Schachter, 1992). Morphologic evidence suggests that chlamydiae may have bacteriophages (Richmond et al., 1982).

Most chlamydial RNA exists in ribosomes. The sequence of the 16S RNA genes of *C. trachomatis* and *C. psittaci* has been determined, and there is only 5% difference between the two species (Weisburg et al., 1986).

Metabolic Properties:

Chlamydiae have been termed "energy parasites" because they do not generate their own adenosine triphosphate (ATP). In a sense, the endosome containing the chlamydiae functions as a reverse mitochondrion, taking the host cell produced ATP and releasing adenosine diphosphate (ADP) (Hatch et al., 1982). Typically, chlamydiae require well nourished host cells for replication, and use the pool of host cell metabolites for their own synthesis.

Chlamydiae appear to have an endogenous metabolism similar to that of some bacteria. They can liberate carbon dioxide from glucose, pyruvate, and glutamate; they also contain dehydrogenases. Nevertheless, they require energy-rich intermediates from the host cell to carry out their biosynthetic activity. Some can synthesise folate (Jawetz et al., 1995).

Chlamydiae need specific amino acids. Absence of these amino acids may lead to inhibition of growth which can be

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reversed on the addition of these essential amino acid. This obviously gives the opportunity for latency, since an amino acid-starved infection may lie dormant for a period but reappear on the addition of the essential building block (Smith, 1993).

Relation to Physical and Chemical Agents:

Chlamydiae are rapidly inactivated by heat. They lose infectivity completely after 10 minutes at 60°C. They maintain infectivity for years at -50°C to -70°C. During the process of freeze drying, much of the infectivity is lost. Some air dried chlamydiae may remain infective for long periods. They are rapidly inactivated by ether (in 30 minutes) or by phenol (0.5% for 24 hours) (Jawetz et al.,1995).

Antibiotic Susceptibility:

The replication of chlamydiae can be inhibited by many antibacterial drugs. Cell wall inhibitors such as penicillins and cephalosporins result in the production of morphologically defective forms, but they are not effective in treatment of clinical disease (Jawetz et al., 1995).

Inhibitors of protein synthesis (tetracyclines and erythromycins) are effective in most clinical infections. Aminoglycosides have little inhibitory activity on chlamydiae. *C. trachomatis* synthesize folate and is thus susceptible to inhibition by sulfonamides, but other chlamydiae are not (Jawetz et al., 1995).

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Trachoma

History

The earliest descriptions of trachoma are in ancient Egyptian, Babylonian and Chinese texts, but the word 'trachoma' was first used in Greeco-Roman times. It comes from the Greek word 'rough' which describes the surface appearance of the conjunctiva in chronic trachoma (Sandford-Smith, 1992). It was recognized as a cause of trichiasis as early as the 27th century BC (Schwab and Dawson, 1992).

Trachoma first attracted interest in Europe in the Napoleonic wars, when the British and French armies returned from Egypt. Many of the soldiers had contracted trachoma, and the disease spread in the community. However, it was about 100 years before the organism was first isolated in 1957 by T'ang and co-workers (Sandford-Smith, 1992)

Epidemiology

Trachoma can vary from a mild to a very severe disease in different situations. The most severe form of trachoma is called 'hyperendemic trachoma'. It is seen in some rural village communities, where almost everyone have either active trachoma or scars from an earlier infection. It results in

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visual handicap which, in hyperendemic area, can be as high as 10% (Sandford-Smith, 1992).

Geographical Distribution:

The highest incidence of trachoma is in the dry, hot, dusty climatic zone which stretches from North India, through the Middle East to North Africa and the Sahel Regions of Central and West Africa. It is also common in the rest of Central and South Asia, Indonesia, Northern Australia, Africa, and Central and Southern America (Sandford-Smith, 1992). It is indigenous to the Nile Valley, and has been a major cause of blindness there through recorded history (Miller, 1992; Leherman, 1993; Dawson et al., 1997).

The serotypes of *C. trachomatis* causing trachoma also vary in different countries. When *C. trachomatis* studies were limited to eye disease in trachoma endemic areas, it is true that most isolates from the eye were mainly type A, B, Ba, and C. Ba from American Indians and from Asmara, Ethiopia, and type A only from Middle East and Africa (Grayston and Wang, 1983). However, many serotypes other than A, B, Ba, and C were isolated from trachomatous eyes in endemic and non-endemic countries (Grayston et al., 1977; Mordhorest et al., 1978; Grayston and Wang, 1983; Brunham et al., 1990). The commonest serotype of *C. trachomatis,* causing trachoma, in Kenya is type A followed by type B (Brunhàm et al., 1990). Also in Tanzania (Taylor et al., 1992) and in Gambia (Pecharatana et al., 1993; Baily et al., 1994b; Hayes et al., 1992) only type A and B were demonstrated. In Sudan, serotypes A, B, and C were prevalent (Mahmoud et al.,

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1994). In New Delhi, India, type C appeared to be the most prevalent serotype (Kumar et al., 1991).

Trachoma is always associated with poverty and unhygienic living conditions. It was a significant problem in Europe in the last century, but almost completely disappeared when living conditions improved. This was long before the discovery of any antibiotics, or even before there was any understanding of how trachoma is transmitted (Sandford-Smith, 1992).

Mode of transmission:

Trachoma is not a very infectious disease. It can only spread in poor overcrowded communities where hygiene is bad and conjunctival discharges frequently pass from eye to eye. Most people who examine and treat trachoma patients every day do not themselves contract the disease. This is simply because they follow the basic rules of hygiene and personal cleanliness (Sandford-Smith.,1992).

The disease spreads from a child to a child by direct contact, on the fingers of the children and their mothers, and on clothes, handkerchiefs, and pillows. Poor hygiene, overcrowding, and , in particular, large number of flies all help the disease to spread. In areas where the disease is a major problem, flies are often seen on the faces of young children. They feed on the nasal discharge, and therefore, spread the infection from eye to eye (Sandford-Smith, 1992).

Trachoma is much more severe and blinding disease in areas where heat and dust are a problem, as flies multiply

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rapidly in hot dusty conditions. Also, dust is a direct irritant to the eyes, and so increases the inflammatory reaction produced by trachoma. It also provokes eye and nasal discharge, which are the source of infection (Sandford-Smith, 1992).

Age and Sex Distribution:

In hyperendemic areas, most young children have active trachoma. The main carrier of infection are children below the age of 10, especially the pre-school children (Dawson et al., 1976; Sandford-Smith, 1992). Dawson et al. (1997) reported that, in a small study in Egypt, 75% of children develop some laboratory evidence of trachoma by one year of age. Mahmoud et al. (1994) reported that, in Sudan, 80% of preschool children and 62% of school children showed clinical trachoma.

The incidence of active disease becomes less with increasing age. Nearly all adults show some degree of conjunctival scarring from trachoma. However, some adults, especially women, may still show some active infection. That is because trachoma produces such poor and short lived immunity, and so reinfection is very likely to occur. In hyperendemic areas, there are several cycles of the disease, one after the other. In adults it is not rare to find subconjunctival follicles or papillae on top of scarring (Taylor et al., 1987; Sandford-Smith, 1992).

Trichiasis and entropion, resulting from conjunctival scarring, become an increasing problem over the age of 15, and blindness from corneal scarring is an increasing problem

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over the age of 30 (Sandford-Smith, 1992). Schachter (1992) reported that, the age-specific blinding rate at the age of 60 may be 20% or more.

Reinfections are more common in adult women than in men. This is probably because, women have closer contact with young children than men. It also explains why women are more susceptible to the late complications of trachoma (Sandford-Smith, 1992; Holland et al., 1993).

Pathology of Trachoma

As an intracellular bacteria, *C. trachomatis* is an extraordinarily successful pathogen. Chlamydiae are capable of replicating within macrophages (Kuo, 1978). They specifically inhibit phagolysosomal fusion of chlamydial phagosomes only (Eissenber and Wyrick, 1981).

C. trachomatis appears to be almost exclusively a parasite of squamocolumnar epithelial cells. Because they are obligate intracellular parasites and kill the host cells at the end of their growth cycle, they must cause some cell damage where they persist. The disease process and clinical manifestations probably represent the combined effect of tissue damage resulting from chlamydial replication and the inflammatory response caused by the presence of chlamydiae and necrotic material from the destroyed host cells (Schachter, 1992).

In trachoma, the acute infection results in inflammatory response with infiltration of neutrophils and lymphocytes. These lymphocytes clump together and produce a follicle

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(Smith, 1993). This localized lymphoprolifrative response is produced by all *C. trachomatis* strains inducing follicle formation in the mucous membrane but this is best known in the conjunctiva in cases of trachoma (Schachter, 1992). The follicles are true lymphoid follicles with germinal centres composed of B cells and macrophages surrounded by T cells and all are enclosed in a thin layer of epithelial cells (Schachter, 1992; Smith, 1993).

Typically, the conjunctiva shows diffuse inflammation characterized by congestion, papillary enlargement and the development of follicles, often forming a row along the upper tarsus (Miller, 1992).

Trachomatous infiltration may spread deeply into the subepithelial tissue of the palpebral conjunctiva, and even invading the tarsal plate (Miller, 1992).

Trachomatous infiltration of the cornea manifests itself mainly as a superficial keratitis typically in the upper part of the cornea, where there are numerous epithelial erosions, which later become associated with infiltrated area in the substantia propria. At a later stage, trachomatous pannus develops as lymphoid infiltration and vascularization of the cornea, usually limited to its upper half. Minute superficial vessels, springing from the corneal loops, grow from the upper margin inwards towards the centre. These vessels are superficial, at first lie between Bowman's membrane and the epithelium, carrying with them a short amount of granulation tissue. At the same time follicles like infiltration may appear near the limbus (Herbert's pits). In later stages, Bowman's

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membrane disappears and the superficial layers of substantia propria become involved. In more severe cases, vascularization is not limited to the upper part, but superficial vessels grow in from all sides, and the whole cornea becomes vascularized and opaque (Miller, 1992).

The period of active inflammation may persist for a few weeks, but eventually, healing with scar formation begins (Sandford-Smith, 1992). The follicles become necrotic and epithelial cells enclosing them become sloughed off, and regeneration by fibrotic tissue, stimulated by interleukin-1 (IL-1), leads to scarring (Smith, 1993).

In the cornea, the inflammatory keratitis gradually heals. The infiltrated and hazy area regresses and the blood vessels become obliterated, and pannus may resolve completely leaving the cornea quiet and clear. Sometimes permanent opacity results due to Bowman's membrane destruction (Miller, 1992).

Pathogenesis:

The pathogenesis of trachoma is still not clear, there are many theories explaining the pathogenesis including reinfection, delayed hypersensitivity, autoimmunity, gene susceptibility, and persistence of antigen and nucleic acid in absence of cultivable organisms.

Reinfection, in spite of vigorous immune response to chlamydial infection, in terms of humoral and cell mediated immunity, is an essential feature of trachoma. In non human primate, repeated (weakly) conjunctival instillation of *C.*

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trachomatis produces a disease with many manifestations of trachoma including conjunctival scarring (Taylor et al., 1982). Also, Grayston et al. (1985) suggested that, trachoma is an immunopathogenic disease in which the more severe progressive trachoma infections with pannus and scar formation occur only after reinfection.

Taylor et al. (1987) claimed that, chlamydial diseases result in part from hypersensitivity. A chlamydial heat shock protein (hsp60) has been implicated in delayed type hypersensitivity (DTH) reactions that resemble severe inflammatory trachoma in guinea pig and monkey (Morrisson et al., 1989).

It was speculated that, autoimmune inflammatory response may have a role in the pathogenesis. Individuals with severe forms of chlamydial disease often display immune response to hsp60 antigen. This protein shares nearly 50% sequence with human homologue. It was assumed that, molecular mimicry may result in autoimmune damage that, in turn, causes chlamydial disease sequalae. It was also suggested that, susceptibility genes for chlamydial disease may also exist (Brunham and Peeling, 1994).

Another clue for the pathogenesis of trachoma was based on persistence of infection, as it has been shown that, there is a state in which chlamydial antigen and nucleic acids can be found in human trachomatous material, in absence of cultivable organisms (Schachter et al., 1988). The mechanism of this is not clear. Perhaps γ interferon (INF-γ), a lymphokine active in inhibiting chlamydial replication (Rothermal et al.,

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1983), has a role in this. Other host defence mechanisms such as natural killer cell activity and lymphotoxins, could also result in antigen release in absence of infectivity. This immune modulation is probably, in part, deleterious to the host because it induces an inapparent persistent infection, with a continuous source of antigenic stimulation, which results in persistent inflammation (Schachter et al., 1988).

Coles et al. (1993) suggested that, in vivo local release of INF-γ results, via tryptophan deprivation, in aberrant development of *Chlamydia* and this may account for the presence of the non-cultivable chlamydiae in spite of antigen and nucleic acid persistence.

The mechanism responsible for scarring sequalae of trachoma, that lead to blindness, is unknown. Scarring is seen in older children and adults suggesting that, repeated infection is a prequisite, as in animal model (Grayston et al., 1985). This damage is a feature of *C. trachomatis* infection in other anatomic sites. Analogous process, may therefore, contribute to ectopic pregnancy and infertility following Fallopian tube infection, and rectal stricture in cases of LGV (Holland et al., 1993).

Immune response:

Antibodies against *C. trachomatis* develop both in serum and tears (Jawetz et al., 1995). Antibody production follows the normal sequence of IgM, then IgG, the level of antibody production being dose dependent (Smith, 1993). IgG and

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secretory IgA are found in tears (Taylor et al., 1992), and serum IgA is also produced (Smith, 1993).

Antibodies develop against the genus specific LPS antigen. These are the complement fixing antibodies but have no neutralizing activity (Nurminen et al., 1983).

Antibodies develop also against the MOMP antigens which are species and serotype specific antigens. These antibodies are the basis of immunotyping and are responsible for reactivity seen in microimmunofluorescence (MIF) (Wang and Grayston, 1970; Caldwell and Schachter, 1982), and are neutralizing and protective antibodies (Pecharatana et al., 1993).

Antibodies against hsp60 antigen are found in individuals with severe disease (Morrisson et al.,1989).

Baily et al. (1993) showed that, the presence of anti-chlamydial IgG in ocular secretions of disease free subjects is associated with an increased risk of trachoma while IgA show an opposite trend.

There is a convincing experimental evidence that, T helper 1 (Th1)-like cells play an important role in clearance of intracellular pathogens in vivo, and that preferential activation of T helper 2 (Th2)-like cells may prevent clearance. These two types of helper cells appear to be mutually inhibitory, since INF-γ, secreted by Th1, inhibits the activation of Th2 cells, and IL4, secreted by Th2, inhibits the activation of Th1.

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Th2 clones can trigger resting B cells to secrete IgM, IgG1, IgA, and IgE (Mosmann et al., 1991).

Holland et al. (1993) assumed that, since INF-γ has been shown to inhibit the intracellular development of *Chlamydia* species both in vivo and in vitro, and results in lysis of *Chlamydia* infected cells, it seems that clearance of infection in vivo may depend on up regulation of Th1-like cells and down regulation of Th2-like cells. The authors suggested that, subjects with persistent infection and scarring sequalae mount a predominantly Th 2-like immune response, demonstrated by their high IgG *Chlamydia*-specific serum antibody levels compared to matched control subjects, and were likely to be harbouring ocular chlamydial antigen, or DNA (or both).

Holland et al. (1993) showed that, conjunctival scarring is associated with depressed cell mediated immune response to chlamydial antigens, since peripheral blood mononuclear cells from subjects with scarring sequalae of trachoma have reduced ability to proliferate in response to chlamydial antigens compared to matched controls. They also demonstrated that, this difference was significant to chlamydial antigens (EB, recombinant MOMP, and affinity purified chlamydial hsp60), but not to other common antigens and mitogens (purified protein derivative (PPD), *Candida albicans*, and phytoheamagglutinin (PHA)).

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Clinical Picture

The clinical picture of trachoma varies from a mild condition with hardly any symptoms to severe and blinding disease (Miller, 1992).

Symptoms:

The symptoms are typical of any conjunctivitis, with irritable red eye and discharge which is usually slightly mucopurulent. In severe, cases there may be lid oedema, or pain and photophobia because the cornea is involved (Sandford-Smith, 1992).

Signs:

Trachoma is a chronic disease which progresses slowly in an individual patient. At first, there is only vasodilatation of conjunctival blood vessels. After about 2-3 weeks, the specific changes of active trachoma which affect conjunctiva and cornea become apparent. These changes, as described by Sandford-Smith, (1992), are:

A) Conjunctival Changes:

There are 2 forms of inflammatory response in the conjunctiva which are specific in established active trachoma. These are follicles and papillae. Usually both forms could be seen, but sometimes, one form is more than the other.

Follicles are just visible by the naked eye in the fornices, especially the upper fornix. They are also found on the inside of the upper eyelid, and this is a characteristic feature of

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trachoma. There may be also follicles at the margin of the cornea (the limbus).

Papillae are found on the surface of the conjunctiva, especially on the inside of the upper eyelid. They cause the conjunctiva to look velvety to the naked eye, and obscure the underlying blood vessels. With the slit lamp, it is possible to see each individual papilla with its central blood vessel. In severe cases, the follicles and papillae completely obscure the underlying tarsal conjunctival blood vessels.

B) Corneal Changes:

A punctuate or diffuse superficial keratitis is an early sign of corneal involvement, which is usually only visible with a slit lamp. However, fluorescein dye will stain the individual epithelial defects, so that a faint green haze may be visible to the naked eye.

As the infection becomes more severe, the inflammation in the superficial cornea becomes more obvious. Blood vessels grow from the limbus into the cornea. This is known as progressive pannus, in which the vessels are mostly parallel to each other and directed vertically downwards. They extend to a level which forms a horizontal line, and beyond this line there is a narrow strip of infiltration and haze.

In severe cases, the corneal epithelium may break down forming a shallow ulcer, resulting in much lacrimation and photophobia.

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Signs of healed trachoma:

The period of active inflammation may persist for few weeks, but eventually, healing with scar formation begins. Collagen fibers are found under the conjunctival epithelium, especially in the upper tarsus, as fine white streaks obscuring the underlying blood vessels. In severe cases, the fibrous tissue may form such a dense sheet, that none of the underlying blood vessels can be seen (Sandford-Smith, 1992).

Keratitis gradually heals showing what is called regressive pannus, in which the vessels extend a short distance beyond the area which is infiltrated and hazy. Pannus may resolve completely, leaving the cornea quiet and clear apart from the obliterated vessels (Miller, 1992).

Very often patients get repeated infections, so that both scar tissue and active inflammation may be seen in the same eye (Sandford-Smith, 1992).

Trachoma Grading:

Trachoma is a complicated disease affecting the conjunctiva, the cornea, the eyelids and eyelashes. Specialists have tried to classify the disease in different types in order to get better assessment of clinical picture.

Sandford Smith (1992) reported that, MacCallan, an English ophthalmologist, who worked in Egypt over 50 years ago, was the first person to suggest staging of trachoma. Miller (1992) mentioned that, MacCallan designated trachoma as occurring in four stages. Trachoma I indicated the earliest

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stages of the disease. Trachoma II includes the period between the appearance of typical trachomatous lesions and the development of scar tissue. Trachoma III is the stage when scarring is obvious. Trachoma IV designates the stage when a cure appears or the disease has become quiet but when cicatrization gives rise to symptoms.

According to Thylefors et al. (1987), a simple grading system for trachoma, based on the presence or absence of five selected "key" signs, have been developed and recommended by the World Health Organization (WHO). This facilitates the assessment of trachoma and its complications by non-specialist health personnel working at the community level. These grades are:

1) **Active trachoma with follicles (TF):** There must be at least 5 follicles on the upper tarsal conjunctiva. There may be also some papillae, but the conjunctival blood vessels must be visible through the follicles and papillae. This represent active moderate infection.

2) **Active trachoma intense (TI):** There are many follicles and /or papillae so that the conjunctival blood vessels can not be seen. This represents a severe infection with a high chance of developing severe complications.

3) **Trachomatous scarring (TS):** White scars are present on the upper tarsal plate, showing that

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the patient has previously suffered a trachoma infection.

4) **Trachomatous trichiasis (TT):** There are some eyelashes rubbing against the cornea.

5) **Corneal opacities (CO) :** There are corneal opacities present blurring some of the pupil margin and lowering the vision to less than 6/18. These represent eyes with a significant visual handicap from trachoma

Sequalae and Complications:

Trachoma is the world's leading cause of infectious blindness. Apart from the results of pannus and corneal ulceration, the most severe effects are caused by the scarring and distortion of the eyelid (Miller, 1992).

Also, the course and sequalae are largely determined by the presence or absence of a complicating bacterial infection and by repeated reinfections from flies and infected relatives. In many countries where the disease is endemic, secondary bacterial infection results in acute and incapacitating condition, liable to relapses, with severe gross cicatricial sequalae. These cicatricial changes, as described by Sandford-Smith (1992), are:

Cicatricial entropion of the upper eyelid results in trichiasis with eyelashes rubbing against the cornea. On the other hand, fibrosis around individual hair follicles results in trichiasis

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without entropion, and the tarsal plate may be thickened and deformed.

Meibomian glands may be obstructed or even destroyed. Similarly, glands secreting mucous, present mainly in the fornices, are also lost as a result of obliteration of the fornices due to subconjunctival fibrosis.

Keratinization of the conjunctival epithelium results in loss of the moist wettable surface, while obstruction of the lacrimal passages causes watering of the eyes and recurrent lacrimal sac infections. Occasionally, there may be damage to the lacrimal gland and this reduces tears production.

As regards the cornea, trachoma itself may cause little scarring in the upper part of the cornea, however, it does not extend to the pupil, and so causes no damage to vision. Unfortunately, damage of the eyelid, conjunctiva, and lacrimal apparatus may have serious effects upon the cornea. The ingrowing eyelashes constantly rub against the corneal epithelium causing irritation, inflammation, and discomfort. They may also cause recurrent corneal ulceration and scarring. Damage of the conjunctiva and lacrimal apparatus also weakens the defence of the eye. The risk of secondary corneal infections and further scarring therefore increases.

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Prevention of Trachoma

To prevent blinding trachoma, it is not necessary to eradicate the disease altogether. However, it is necessary to prevent the severe forms of trachoma which can so easily lead to blindness (Sandford-Smith, 1992).

Improving personal and public hygiene is obviously the best way to eradicate blinding trachoma. However, for economic and cultural reasons, it is not usually possible to persuade people to change their traditional way of life. It is probably much better to aim for a few specific changes in the community which can significantly reduce the incidence of infection and reinfection with trachoma. This can be achieved by supplying piped water to encourage personal hygiene and the washing of clothes, removing rubbish and faeces to control the fly population, and teaching personal hygiene to primary school children and young mothers (Sandford-Smith, 1992).

The author suggested that, antibiotic can be an effective measure in preventing blinding trachoma by destroying the organism in the individual patient and helping the infection to resolve earlier thus reducing the risk of scarring. Also, most seasonal conjunctivitis which aggravates trachoma responds to antibiotic treatment. If the whole community is treated, there would be fewer active carriers to spread trachoma.

In hyperendemic areas, all pre-school children and children in the first years at school are 'at risk'. It is either possible to treat them all, or preferably to examine them all and treat

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those with active trachoma. It is much cheaper to pay for drug prevention scheme now than to look after large number of blind people after (Sandford-Smith, 1992).

The author also recommended that, early surgical correction of trichiasis and entropion is a valuable way to prevent blindness in the community. When patients first develop trichiasis or entropion, they have already suffered quite severe trachoma, however, the vision is still good, and the cornea is fairly unscarred. If no treatment is given, progressive corneal scarring and blindness is likely to develop within 10 years.

Vaccination:

There are many trials for vaccine development. Experimental vaccines have induced a short lived immune response, but in some vaccinees the disease was exacerbated, suggesting hypersensitivity to the organism (Grayston and Wang, 1975; Schachter and Dawson, 1978).

Experimentally, many chlamydial antigens have been tried including hsp60 (Rank et al., 1995), MOMP extract (Campos et al., 1995), and a 12 amino acid peptide from a conserved region in MOMP (Knight et al., 1995). Also different routes were tested; intradermal, mucosal, and systemic routes (Knight et al., 1995; Campos et al., 1995; Rank et al., 1995).

Knight et al. (1995) showed that, a 12 amino acid peptide from the conserved region of MOMP is a primary T cell epitope in human and rodents. This peptide stimulated dendritic cells in vitro which, then, stimulated proliferation of

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syngenic T cells. In vivo, this peptide initiated cell mediated immunity only when injected intradermally and conferred some protection against *C. trachomatis*. The authors suggested that this peptide may be useful in the development of vaccines.

Campos et al. (1995) extracted and tested MOMP as a vaccine in Cynomolgus monkey models of trachoma by mucosal and systemic routes. Although this resulted in both *Chlamydia*-specific local and systemic immunity to MOMP and to whole organism, ocular disease was not significantly reduced after challenge, compared to control group. The authors demonstrated that, despite the resulting vigorous MOMP-specific and other *Chlamydia*-specific humoral and cell-mediated immunity, as well as, anamnestic serologic response to *Chlamydia*, vaccination was only partially protective against chlamydial ocular disease. They suggested that alternative chlamydial antigens may have to be considered in future vaccine development.

Rank et al. (1995) used a recombinant hsp60 for immunization with both subcutaneous and combined subcutaneous and ocular routes. They showed that, immunization with hsp60 did not produce exacerbated disease on challenge with viable organism. They demonstrated the presence of high level of IgA and IgG antibody titers to hsp60 in tears and suggested that, the response to challenge have been modified by the presence of blocking antibodies that either may have removed the antigen quickly or prevented the interaction with sensitized T cells. They also demonstrated

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that, the combined immunization regimen resulted in no gross pathology after reinfection in contrast to immunization by the subcutaneous route only.

Treatment of Trachoma

The organism is sensitive to tetracyclines (tetracycline, chlortetracycline, doxycyline, and oxytetracycline), the sulphonamides, and erythromycin. To a lesser extent, it is also sensitive to penicillin and chloramphenicol. However, the trachoma organism lives inside the body cells, and so it is protected from the full effects of these antibiotics. Antibiotic treatment may be given locally or systemically, but local treatment is usually better because it is cheaper and most trachoma patients are poor, and there is no risk of systemic side effects. Tetracycline can discolour and damage the teeth of children, and cause gastrointestinal disturbances., while sulphonamides can cause severe hypersensitivity reactions. Also, local antibiotic treatment is effective against bacterial conjunctivitis, which may be present as well as trachoma (Sandford-Smith, 1992).

The author added that, tetracycline seems to be more effective than sulphonamides and it is also more effective against other bacteria. Both tetracycline ointment and oily suspension are available usually in 1% strength. Greater strengths are available up to 3%, but they sting and irritate the eye. He stated that, two different dose schedules are recommended:

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1)Continuous treatment requires drops or ointment 4 times a day for 6 weeks. This is probably better for treatment of an individual patient.

2)Intermittent treatment requires drops or ointment 2 times a day for one week each month. This is probably better for treating the community.

If the infection is very severe, local and systemic treatment may be necessary. Even then, a two weeks course of systemic treatment is probably adequate. Longer periods of treatment increase the risk of side effects.

Recently azithromycin in a single oral dose of 20mg/kg was described as an effective treatment for trachoma. The drug is detectable in urine for, as long as, 14 days after a single dose (Baily et al., 1993). In Egypt, Dawson et al. (1997) compared azithromycin with topical oxytetracylcin/polymyxin ointment, and reported that, even single oral dose of azithromycin was equivalent to 30 days of topical ointment, but the compliance with azithromycin was expected to be better, and they suggested that, oral azithromycin may offer an effective alternative means of controlling endemic trachoma.

Patients with trachoma need antibiotic and steroid mixtures, such as tetracycline and hydrocortisone, as it is possible that much of tissue damage is caused by an immunological reaction rather than by direct toxins from the organism.

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Local steroids should only be given under the direct supervision of a specialist. Surgical correction of trichiasis and entropion may be needed to prevent blindness (Sandford-Smith, 1992).

conjunctival flora and conjunctivitis.

Miller (1992) and Sandford- Smith (1992) mentioned that, pure trachoma is relatively a mild disease, so mild and symptomless, indeed, as to excite little or no attention until perhaps cicatrization manifests itself later in life. Miller (1992) stated that, the course of trachoma is determined mainly by the presence or absence of a complicating infection.

Microbial flora of the conjunctiva:

Rather indigenous flora exist in the conjunctival sac. *Staphylococcus epidermidis*, and *Lactobacillus* species are most frequently encountered organisms; *Propionibacterium acnes* may also be present. *Staphylococcus aureus* is found in less than 30% of people, and *Haemophilus influenzae* colonizes 0.4% to 25%. *Moraxella catarrhalis,* various *Enterobacteriaceae*, and various streptococci (*Streptococcus pyogenes*, *Streptococcus pneumoniae*, and other alpha haemolytic and non haemolytic forms) are found in very small percentage of subjects (Baily and Scott's, 1994).

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Conjunctivitis:

Baily and Scott's (1994) mentioned that, *C. trachomatis* is responsible for one of the most important types of conjunctivitis, trachoma, one of the leading causes of blindness of the world. The authors added that, *C. trachomatis,* acquired by neonates during passage through an infected vaginal canal, is also one of the causes of acute conjunctivitis in newborn.

Bacterial conjunctivitis is the most common type of infectious conjunctivitis (Seall et al., 1982). Giglrottie et al. (1981) reported that, in adults, the most common organisms cultured are *St. pneumoniae, S. aureus*, and *S. epidermidis*, however, the author added that, there is some dispute as to the significance of the isolates of the latter two organism, since they are often recovered from non-infected eyes. The author mentioned that, the most common causes of bacterial conjunctivitis in children are *Haemophilus influenzae, St. pneumoniae,* and perhaps *S. aureus*.

St. pneumoniae and *H. influenzae* (specially sub sp. *aegyptius*) have been responsible for epidemics of conjunctivitis. Gonococcal conjunctivitis may be quite destructive. Diphtheritic conjunctivitis may occur in conjunction with diphtheria else where in the body. *Moraxella lacunata* produces a localized angular conjunctivitis with little discharge from the eye. Distinctive clinical picture is caused by *Mycobacterium tuberculosis, Francisella tularensis,*

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Treponema pallidum, and *Yersinia enterocolitica* (Baily and Scott's, 1994).

The authors added that, fungi may also be responsible for this type of infections as well, often with association with a foreign body in the eye or an underlying immunological problem.

According to Chou and Malison (1988), viruses are an important cause of conjunctivitis; 20% of such infections result from adenoviruses type 4, 3, and 7A. Worldwide enterovirus 70 and Coxsackievirus A24 are responsible for outbreaks and epidemics of acute haemorrhagic conjunctivitis.

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Laboratory Diagnosis of Trachoma

Specimen Collection:

For demonstration of the organism, its antigen, or nucleic acids, specimens are collected from the upper subtarsal conjunctiva by scraping or by vigorous swabbing. Metal spatula could be used for scraping with the possibility of topical anaesthesia application (Schachter et al., 1988). The Swabs could be either paper-stick swabs (Mabey et al., 1987), dacron (Schachter et al., 1988; Bobo et al., 1991; Taylor et al., 1991) or cotton swabs (Mabey et al., 1987; Baily et al., 1994a).

Serum or tears are collected for antibody detection. Tears are collected by cellulose sponges (Graham, 1989) or dacron swab and then eluted from it with phosphate buffered saline (Taylor et al., 1992).

I-Direct Cytological Examination

Because chlamydiae are large enough to be seen by light microscopy and the intracytoplasmic inclusions are pathognomonic, much of the diagnostic methodology depends

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on microscopic identification of the organism (Schachter, 1991).

1)Iodine staining:

Fully formed mature intracellular inclusion of *C. trachomatis* are compact masses near the cell nucleus with glycogen-like matrix which when stained with diluted Lugol's iodine solution, some of these inclusions appear brown (Jawetz et al., 1995).

According to Gordon et al. (1969), smears are air dried, fixed in absolute methanol or in 10% (v/v) formalin saline (4% formaldehyde) and stained with 5% iodine in 10% potassium iodide for 5 minutes.

This technique is rapid and effective with heavily infected materials. Its speed and simplicity have made it a popular test for examining *C. trachomatis* infected cell cultures, although some cells such as HeLa 229 may have accumulations of glycogen that lead to problems in interpretation (Graham, 1989).

However, Schachter (1991) stated that, this technique is the least sensitive cytological procedure and it is not recommended with clinical specimens.

2)Giemsa staining:

The smears are dried, fixed with absolute methanol for at least 5 minutes, and dried again. It is then covered with diluted (2%) Giemsa stain for one hour, rinsed rapidly in 95% ethyl alcohol, dried and examined microscopically under the

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oil immersion lens. Positive slides contain typical intracytoplasmic inclusion bodies. EBs stain reddish purple, while the RBs are more basophilic, staining bluish, as do most bacteria. The examination of Giemsa smears from suspected ocular infections is both time consuming and insensitive (Schachter et al., 1988; Schachter, 1991).

3)Immunofluorescence staining:

According to Schachter et al. (1988), the conjunctival smear is air dried, fixed by flooding with 100% acetone or methanol and air dried again. They can be stored, if needed, at 4°C, but before staining the slides should be brought to room temperature (~23°C). Slides are then overlaid with the fluorescent antibody reagent, incubated for 30 minutes in moist chamber, rinsed with distilled water, and air dried. A drop of mounting fluid is applied to the smear and a coverslip is added. Slides are examined with fluorescent microscopy.

Schachter (1991) stated that, most of the early experience with immunofluorescence (IF) procedures used polyclonal antibodies in either direct or indirect fluorescent antibody procedures, and that, these represented efforts to detect typical chlamydial inclusions within epithelial cells. There were no commercial sources, and laboratories had to prepare their own reagents. Then later on, fluorescein-conjugated antibodies have been made available and the test is based on detection of EBs in smears, in contrast to previous efforts to detect inclusions.

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In the early commercial direct fluorescent antibody (DFA) reagents, false positive reactions were a problem due to binding of Fc portion of the monoclonal antibodies with Protein A of many *Staphylococcus aureus* strains, or due to cross reaction with certain Gram negative bacteria, but these reagents have been improved (Graham, 1989; Schachter, 1991).

Several commercial kits are available using monoclonal antibodies against the LPS or the MOMP. Monoclonal antibodies against LPS antigen stain all *Chlamydia* species, and are not specific for *C. trachomatis*, but the quality of fluorescence is somewhat mitigated by uneven distribution of LPS on chlamydial particle, while the anti-MOMP monoclonal antibodies prepared against *C. trachomatis* are species specific and the quality of fluorescence is better because MOMP is evenly distributed on the chlamydial particle (Schachter, 1991).

Schachter et al. (1988) considered samples positive when slides are identified as having 10 or more smooth, evenly fluorescing, bright apple green disks consistent with chlamydial EBs, while Taylor et al. (1991) and Bobo et al. (1991) defined specimens positive if 5 or more EBs are present.

DFA tests offers the possibility of rapid diagnosis, since the technique takes approximately 30 minutes to perform (Schachter, 1991). However, DFA is not a simple test for diagnosis of trachoma and requires a fluorescence

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microscope, expensive bulbs, and well trained examiners (Bobo et al., 1991).

Methanol fixation is superior to acetone fixation, as shown by Schachter et al. (1988), with marked improvement of the sensitivity, as it acts as a solvent, partially solubilizing the LPS, exposing more of the MOMP, to which the fluorescein labelled antibody is directed.

Methanol fixed slides show a good sensitivity (78%) compared to isolation (76%), EIA (73%), and DNA probing (84%) (Schachter et al., 1988). Similar results were given by Taylor et al. (1991) who showed that, there is good agreement between DFA and EIA (38% and 36% respectively).

On the other hand, Dean et al. (1989b) have shown a low sensitivity of DFA compared to isolation. Besides, Bobo et al. (1991) demonstrated that, DFA sensitivity is markedly lower than polymerase chain reaction/enzyme immune assay (PCR-EIA).

Dean et al. (1989a) reported that, DFA specificity versus tissue culture was 96.9%, while Taylor et al. (1991) stated that, specificity of DFA in correlation to clinical trachoma was 95%.

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II- Other Non-Culture Diagnostic Tests

1) Enzyme immunoassay:

Chlamydia antigens can be detected by solid phase antigen capture methods using specific monoclonal or polyclonal antibodies (Graham, 1989).

Schachter (1991) stated that, most of enzyme immunoassays (EIA) detects chlamydial LPS, which is more soluble than MOMP, thus can detect all types of chlamydiae.

A novel EIA for specific detection of *C. trachomatis*, utilizing monoclonal anti-idiotypic antibody against a *Chlamydia*-specific epitope on 60 kDa heat shock protein (hsp60), was developed by Lafferriere et al. (1993). The basis of this assay is the inhibition of idiotype anti-idiotype binding by the antigen, if present, in the tested sample. The authors described the assay as being sensitive and highly specific.

Schachter (1991) reported that, although EIA takes several hours to be done, it is suitable for batch processing, and can be used to test many specimens in one setting.

EIA is comparable in terms of sensitivity to isolation of *C. trachomatis* for the diagnosis of trachoma in trachoma-endemic communities. Mabey et al. (1987) considered isolation of *C. trachomatis* in tissue culture as a golden standard for the detection of *C. trachomatis* from trachomatous patients, and mentioned that in relation to culture, EIA had a sensitivity of 70.6%. However, the authors

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reported that, the overall detection rate of EIA among trachomatous patients was 25% (56/228).

Schachter et al. (1988) selected 100 Egyptian trachomatous children, and used for detection of *C. trachomatis*, Giemsa stain, EIA, DFA, DNA probe, and isolation in tissue culture. The authors defined, for statistical analysis, a true positive as any child who yielded a positive tissue culture, a positive Giemsa stained specimen, or a specimen that was positive in two of the other three noncultural methods (i.e. EIA, DFA, and DNA probe). By these criteria, 45 out of the 91 fully assessable children (49%) had demonstrable infection. Sensitivity of EIA in relation to the defined true positive children was 73% (33/45). On the other hand, 36 were EIA positive among 99 children that were assessed by EIA, i.e. the detection rate in relation to clinical trachoma was 36%.

Taylor et al. (1991) stated that, there was good agreement between their results of DFA and EIA in terms of sensitivity in relation to clinical trachoma (38% and 36% respectively).

Baily et al. (1994b) showed a low sensitivity of EIA against clinical signs (25%), but 100% specificity.

Similarly, specificity of EIA in detection of *C. trachomatis* from trachoma patients, as reported by many authors, was high. Mabey et al. (1987) reported that only 49 subjects among 997 (5%) subjects who did not have active (follicular) trachoma were EIA positive for *C. trachomatis*. According to

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Schachter et al. (1988), specificity of EIA, in relation to their defined true positive cases, was 93%.

2) Nucleic acid detection:

a- Nucleic acid probing:

Nucleic acid probe is a sequence of single stranded nucleic acids that can hybridize specifically with its complementary strand by way of nucleic acid base pairing. A probe may be constructed to detect either single stranded DNA or single stranded RNA. Many of the commercially available nucleic acid probes are directed against sequences found in the single-stranded RNA, that along with ribosomal proteins, make up the ribosome (Koneman et al., 1992).

Schachter et al. (1988) used ^{32}P-labelled probes for detecting cryptic plasmid of *C. trachomatis*. They prepared the probes and applied it to the extracted nucleic acids of *C. trachomatis*. Hybridization was done on nylon filter. Autoradiographic exposures of 4 days were then obtained. The authors compared DNA probing with other non-cultural detection methods (Geimsa staining, EIA and DFA). They reported that DNA probing was the most sensitive (83%) with high specificity (96%).

Dean et al. (1989a) compared DNA probe and DFA against tissue culture of *C. trachomatis* from trachoma patients. They found that DNA probe was much more sensitive than DFA (86.6% and 47.8% respectively) and specific (86.9%). In another study Dean et al. (1989b) compared DFA, DNA probe and PCR with serial tissue

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culture passage for detection of *C. trachomatis* in conjunctival specimens. Again, they found that, ^{32}P-probes and PCR are more sensitive (87% and 90% respectively) than DFA (84%). They suggested that DNA probe for *C. trachomatis* might be considered as a valuable epidemiological tool in screening trachoma-endemic populations for ocular chlamydiae.

Detection of *C. trachomatis* ribosomal RNA, as a target for molecular hybridization, was also used by Hudson et al. (1992) for conjunctival swabs from trachoma patients, as well as, experimental animals. They reported some of DFA negative samples giving a positive signal for chlamydial RNA.

b- Polymerase chain reaction (PCR):

Polymerase chain reaction (PCR) is a method of amplifying a target sequence of DNA. Specificity is based on the use of two oligonucleotide primers that hybridize to complementary sequences on opposite strands of DNA and flank the target sequence. The DNA sample is first heated to separate the two strands (denaturation), the primers are allowed to bind to DNA (annealing), and each strand is copied by a DNA polymerase (extension), starting at primer site. Each strand serves as a template for the synthesis of new DNA from the two primers. Repeated cycles of heat denaturation, annealing, and extension of the annealed primers result in exponential amplification of DNA segments of defined length (Garner, 1993).

Early PCR reactions used an *E-coli* DNA polymerase that was destroyed by each heat denaturation cycle. Substitution

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with a heat-stable DNA polymerase from *Thermus aquaticus*; an organism that lives and replicates at 70 - 80°C, obviated this problem and allowed for automation of the reaction, since the polymerase reactions can be run at 70°C. This has improved the specificity and yield of DNA (Garner, 1993).

For detection of *C. trachomatis* causing trachoma by PCR, two primers are designed to hybridize two DNA strands aiming at amplifying a *C. trachomatis* specific chromosomal or cryptic plasmid sequence (Bobo et al., 1991; Hayes et al., 1992; Baily et al., 1994b).

The chromosomal sequence is based on the MOMP gene (Omp1 gene). This gene consists of 4 variable segments (VS); VS I, VS II, VS III, and VS IV, and these are flanked by sequences that are highly conserved (Hayes et al., 1992; Pecharatana et al., 1993). That is why primer selection is very critical for PCR-based diagnosis. For detection of all *C. trachomatis* serovars (genotypes), the primers should be chosen from the conserved sequence. This allows detection of the 3 serovars responsible for trachoma. However, genotyping is dependent on selecting primers specific for each serovar depending on the variable segments in the Omp1 gene (Hayes et al., 1992; Pecharatama et al., 1993).

Cryptic plasmid can also be a target for PCR-based detection of *C. trachomatis*, and primers are derived from sequence of this plasmid (Baily et al., 1994a).

Baily et al. (1994a) compared plasmid-based PCR with MOMP gene-based PCR. They reported a higher sensitivity

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of plasmid-based PCR which was able to detect DNA corresponding to 1 -10 EBs compared to MOMP-based PCR which detected only 10 -100 EBs.

Many PCR protocols are followed. The DNA is first extracted from the cellular material in the sample. Crude DNA can be kept at -20°C till use. Amplification is then done using the appropriate primers, DNA polymerase, the 4 deoxynucleoside triphosphate (dNTPs), and the crude DNA. Different number of amplification cycles are described by many workers. Each cycle consists of denaturation, annealing and extension. The time and temperature of the 3 steps are different in different protocols, except for the extension temperature which is always 72°C; the optimum temperature for Taq DNA polymerase. Great care should be taken to avoid false positive results. Both positive and negative controls should be included in each PCR run (Bobo et al., 1991; Pecharatana et al., 1993; Baily et al., 1994a).

The amplified PCR produced is looked for by detecting a band with a specific length using silver stained polyacrylamide gel electrophoresis (Bobo et al., 1991) or agarose gel electrophoresis (Hayes et al., 1992; Baily et al., 1994a).

Fluorochrome labelled primers were used by Pecharatana et al. (1993). These primers result in fluorochrome labelled PCR product that can be detected after gel electrophoresis.

PCR-EIA is another method for amplified sequence detection that was described by Bobo et al. (1991). A sample

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of the PCR product is hybridized to a biotinylated RNA probe that is complementary to nucleotide sequences internal to the selected primers. The hybridization is performed in a solution for 30 minutes at 78°C. The nucleic acid mixture is then incubated in antibiotin antibody coated wells of a microtitre plate. Hybrid bound to the solid phase can be detected by β-d-galactosidase conjugated monoclonal antibody specific for DNA-RNA hybrid. The amount of solid phase enzyme is then assessed by measurement of fluorescent production from the reaction of the enzyme with 4-methylmbelliferyl β-d-galactoside. The authors reported that, PCR-EIA has the advantage of being semiquantitative, and this is useful for monitoring a decrease in chlamydial DNA after treatment.

The result of their study revealed that 65% of subjects with clinical trachoma who were negative by DFA were DNA positive suggesting that these subjects may indeed have been infected with *Chlamydia* at levels below the detection ability of DFA. Additionally, 24% of their subjects with no signs of trachoma were PCR positive. They concluded that PCR is the most sensitive test for detection of *C. trachomatis* in active trachoma.

Hayes et al. (1992) demonstrated a higher sensitivity of PCR when compared with EIA (51% and 25% respectively). Similar results were obtained by Baily et al. (1994a); PCR was more sensitive in detection *C. trachomatis* from active trachoma patients than EIA (72% and 62% respectively). Also, In another study, Baily et al. (1994b) reported that,

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against clinical signs, PCR appeared to be more sensitive than EIA (51% and 25% respectively).

In spite of detecting chlamydial DNA in 24% of clinically normal eyes, Bobo et al. (1991) described PCR-EIA detection of *C. trachomatis* as being very specific. On the other hand, specificity of PCR against clinical cases was reported to be about 95% by other workers (Hayes et al., 1992; Baily et al., 1994b).

III- Isolation of Chlamydia trachomatis

For isolation of chlamydiae, 3 main systems are considered; tissue culture, yolk sac of embryonated egg, and mice inoculation. Schachter (1991) stated that, mice are of no use in recovering isolates of the trachoma biovar, and that isolation of *C. trachomatis* from trachomatous specimens depends on either tissue culture or yolk egg inoculation.

1) Isolation in tissue culture:

Schachter (1991) recommended tissue culture for primary isolation of chlamydiae. But, its disadvantage is that, it detects only viable organisms, and specimens should be send quickly to the laboratory in suitable transport medium at 4°C or after freezing the sample in liquid nitrogen (Smith, 1993).

Graham (1989) suggested that, because *Chlamydia* spp. are bacteria, the selection of antibiotic to prevent other bacterial contamination is restricted and that, specimens must not be put into viral or mycoplasma transport medium which

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contains penicillin. Schachter (1991) stated that, broad spectrum antibiotics such as tetracyclines, macrolides and penicillins must be excluded, and that aminoglycosides and fungicides are the mainstay. On the other hand, Gilbert (1996) reported that, vancomycin, gentamycin, and amphotericin B are frequently added to the transport medium.

According to Gilbert (1996), specimens for culture placed in the transport medium can be stored at 4°C if processed within 24 hours of collection, but if there will be delay, the specimen should be stored at -60°C, and that, repeated thawing and freezing should be avoided. The author stated that, the most commonly used transport media for chlamydiae is 2-sucrose phosphate saline.

Mycoplasma free, untreated, irradiated, or metabolically inhibited McCoy cell line is the most common cell line used for *C. trachomatis* isolation (Gordon et al., 1972; Ripa and Mardh, 1977; Schachter et al., 1978; Evans and Taylor-Robinson, 1979; Yoder et al., 1981; Holland et al., 1992).

Many other cell lines can be used for isolation of chlamydiae including BHK cells (Taverne and Blyth, 1971), HeLa 229 cells (Croy et al., 1975), 'Buffalo' green monkey (BGM) cells (Hobson et al., 1982), Chang conjunctival cells (Moffa et al., 1989), and HaK line monolayer cells (Byrne et al., 1993).

According to Darougar et al. (1974), the clinical specimen should be shaken with glass beads before inoculation. Standard inoculation procedure involves removing medium

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from the cell monolayer and replacing it with the inoculum in a volume of 0.1-1ml. The specimen is then centrifuged onto the cell monolayer at approximately 3,000 x g at 35°C for 1 hour to achieve the maximum infectivity with *C. trachomatis* serotypes A-K.

Schachter (1991) recommended that, the vials should be held at 35°C for 2 hours before the cells are washed or the medium is changed to medium containing 1-2μg of cycloheximide per ml. The cells are then incubated at 35°C for 48-72 hours and then stained. He stated that, the most common technique involves inoculation of clinical specimens into cycloheximide-treated McCoy cells. Cycloheximide prevents continued multiplication of host cells, which allows maximum multiplication of EBs and therefore the ease of identification of chlamydial inclusions.

Staining of infected monolayers for identification of chlamydiae is done by different stains. Iodine is used for detecting *C. trachomatis* inclusions only, but in HeLa 229 cells they can be confused with glycogen granules. Iodine staining is a simple procedure although it is less sensitive than other methods (Schachter et al., 1988, Graham, 1989; Schachter, 1991). Inclusions appear as dense collections of small rounded deep brown or black particles within a defined area of cytoplasm (Graham, 1989).

Giemsa stain is more sensitive than iodine, but microscopic evaluation is more difficult. Slide reading can be facilitated by examining the Giemsa stained cover-slips by dark field rather than the bright field microscopy (Darougar et al., 1971;

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Mabey et al., 1987; Schachter, 1991). Inclusions appear as bright yellow particles under darkfield illumination and deep purple under bright field illumination (Graham, 1989).

Immunofluorescence staining with fluorescein-conjugated monoclonal antibodies may allow earlier detection of inclusions and represents the most sensitive method for detecting *C. trachomatis* inclusions in culture, and it might have a slightly increased isolation rate (Stamm et al., 1983; Schachter, 1985; Schachter, 1991). Slides are examined with immunofluorescense microscope. *Chlamydia* inclusions are identified with their apple-green staining (Graham, 1989; Holland et al., 1992).

Other staining methods as immunoperoxidase, Hoechst 33258, and the nuclear staining part of the three part blood staining kit, 'Diff-Quik', that was described by Hardy and Nell in 1985 were also used (Graham, 1989).

The isolation of *Chlamydia* in cycloheximide-treated McCoy cell has been considered the most sensitive method for laboratory diagnosis of trachoma (Jones, 1974; Mabey et al., 1987; Schachter et al., 1988).

Mabey et al. (1987) mentioned that, this technique is not positive in more than 50% of clinically active cases. The authors demonstrated that there was no statistical significant difference in sensitivity between *Chlamydia* isolation in tissue culture and antigen detection by EIA, although EIA was slightly more sensitive than isolation. They mentioned the practical advantages of EIA over isolation as a diagnostic

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technique in this field. EIA, since it depends on detection of heat-stable glycolipid (LPS) antigen, its specimens need not be refrigerated immediately after collection, moreover, it is more simple to carry out, and if plate reader is available, provides an entirely objective results. Besides, its biggest advantage over isolation is that, samples from several hundred individuals can be assayed for *Chlamydia* antigen in a single day; this is not possible by culture.

Schachter et al. (1988) showed that, there was no significant differences between culture and non-cultural methods, i.e. EIA and DFA, in detecting *C. Trachomatis* (76%, 73% and 78% respectively), but DNA probe was the most sensitive (84%).

2) Isolation in chick embryos:

A suspension of the specimen, treated with 500 µg/ml gentamycin and 100 units/ml nystatin, is prepared. Of this suspension, 0.2 - 0.5 ml is inoculated into the yolk sac of 6 - 8 day developing embryo. Embryo is then incubated at 35°C. Eggs are candeled daily and dead ones within 48 hours are discarded. Yolk sac from embryos which die subsequent to the second day and from those alive 13 days post inoculation are harvested. Impression smears of yolk sacs are fixed and stained by Macchiavello, Castaneda, Giemsa, IF, or Hoechst 33258, and examined for EBs. The distinction between EBs and yolk sac granules requires substantial experience. If the yolk sac is negative, it should be homogenized and reinoculated into a fresh batch of embryos (Graham, 1989).

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According to Schachter (1991), after 2 blind passages, attempts are terminated as negative.

The generally acceptable criteria for positive isolation are finding of EBs in the impression smears, serially transmissible egg mortality, the presence of a group antigen in the yolk sac, and absence of contaminating bacteria (Schachter, 1991).

IV-Serodiagnosis

Although *C. trachomatis* provokes powerful humoral immune response to its antigens in both serum and secretions in forms of IgG, IgA, and IgM, serological tests for diagnosing individual cases of trachoma are not useful (Schachter, 1992; Smith, 1993; Jawetz et al., 1995).

In hyperendemic areas of trachoma, the great majority of inhabitants have serum antibodies to *C. trachomatis*, i.e. high prevalence of chlamydial antibodies. The finding of antibodies to *C. trachomatis* in a single serum sample and /or secretions may indicate current infection in certain individual, such antibodies in absence of isolation of *C. trachomatis* are generally of little diagnostic value. However, the finding of specific antibody of the IgM class is probably of greater predictive value for infection than that of IgG (Treharne and Forsey, 1983; Mahmoud et al., 1994).

Although the complement fixation test is the most widely used serological test for diagnosing chlamydial infection, it is not particularly useful for diagnosing trachoma. Trachoma may be diagnosed by microimmunofluorescence (MIF)

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technique; the most sensitive procedure for measuring anti-chlamydial antibodies, if appropriately timed paired acute and convalescent sera can be obtained. However, it is often difficult to demonstrate rising titre (Schachter, 1991).

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Materials and Methods

Subjects:

The present study was done on 90 eyes of 90 patients with active trachoma, and 45 control subjects.

The patients were among those attending the ophthalmology outpatient clinic in Ain Shams University Hospital during June and July, 1996, and from April to June, 1997.

Patients' eyes were examined by the slit lamp, and only cases showing signs of active trachoma (either trachoma with follicules (TF) or trachoma intense (TI)), according to the WHO grading system (Thylefors et al., 1987), were included in the study.

From each patient data as regards, age, occupation, residence, complaints, antibiotic eye drops application, past history of similar complaint (at least one month before), family history of simultaneously complaining household subject, and the clinical stage of active trachoma were recorded.

Matched control subjects during the same period were included. Their eyes were examined by the slit lamp and were

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free of any signs of active trachoma (According to the WHO classification, at least five follicles should be found in the conjunctiva to consider the stage as TF).

Patient and control subjects were divided according to their ages into three groups (school children, middle age adults, and older age groups). The age range of the patients was 5-72 years. They were 46 females and 44 males. The age of control subjects ranged from 5 to 66 years. They were 22 females and 23 males.

Specimens:

From all subjects (patients and controls) of the present study, two conjunctival swabs were taken from the upper tarsal conjunctiva of one eye (the one showing more pathogenic features in patients). Sterile disposable cotton tipped swabs with plastic stem (Hoechst) were used for both groups.

According to manufacturer instructions, the first swab was used to remove any excess exudate from the surface of eyes before sampling for EIA. It was also tested for the detection of other bacteria.

The second collected swab was immersed immediately in 1ml transport medium of EIA (Dako Diagnostics Ltd, UK), and the stem was broken off.

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Each transport medium tube, with the swab, was vortex mixed for 15 seconds. Of this inoculated medium, 250 µl were transferred into 1.5 ml microfuge tube and were kept at -20°C till tested by PCR. The remaining 750µl were placed in water bath (100°C) for 15 minutes, then stored at -20°C for maximum 1 month prior to testing with EIA.

Conjunctival scraping was done, with the blunt edge of a sterile surgical blade, for 6 patients after the second conjunctival swabbing. This was used to compare conjunctival scraping with swabbing as regards detection of *C. trachomatis* antigen by EIA. The scrape was done after the application of topical anaesthetic eye drops (Benoxenate hydrochloride ophthalmic solution 0.4%).

I. Bacteriological Examination

Reagents:

1) Media:

Chocolate blood agar plates, blood agar plates, nutrient broth, sugar media (glucose, lactose, maltose, mannite, and sucrose), gelatin, litmus milk, nutrient agar slope, and soft agar.

All media were prepared according to Collee and Marr, (1996).

2) Anaerobic jar and CO_2 producing gas pack (Oxoid, England).

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3) Optochin disc (Oxoid, England).

4) Hydrogen peroxide (H_2O_2).

5) Sterile paraffin oil.

6) Citrated human plasma.

7) Gram's stain.

Method:

The first conjunctival swab collected from each subject was immediately inoculated onto chocolate agar plate. Then a smear was prepared and stained with Gram's stain (Collee et al., 1996).

The plates were transported, as soon as possible, to the laboratory. They were incubated at 37°C overnight with 5-10% CO_2 (Collee et al., 1996).

The growing colonies were examined by the conventional bacteriological methods.

According to Barid (1996), for the differentiation of *Micrococcus* which is strict aerobe from *Staphylococcus* and *Stomatococcus* that are facultative anaerobes, a glucose tube was inoculated with the suspected strain and then covered with few drops of sterile paraffin oil for anaerobic conditions.

Catalase production test using H_2O_2 reagent was done to differentiate staphylococci (catalase positive) from

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Stomatococcus (weak catalase) (Baird, 1996) and streptococci (catalase negative) (Ross, 1996a).

Gram positive diplococci strains were subcultured on blood agar plates with the application of optochin discs to differentiate *Streptococcus pneumoniae* (sensitive to optochin) from *Streptococcus viridans* (optochin resistant) (Ross,1996b).

Staphylococci were examined for their ability to produce coagulase by slide coagulase test and, when negative, the tube coagulase method was done (Barid, 1996).

II. Enzyme Immunoassay (EIA)

EIA was used to detect *C. trachomatis* antigen in the conjunctival swabs using DAKO IDEIA Chlamdyia kit (Dako Diagnostics Ltd , UK).

Principle of the test

It is a qualitative enzyme-linked immunoassay for detection of *Chlamydia* specific LPS antigen and utilizes monoclonal antibodies and an enzyme amplified system to enhance the test signal.

If there is a chlamydial antigen in the ophthalmic specimen, it gets bound by monoclonal antibody to the surface of the plastic wells. Enzyme monoclonal antibody binds to the captured antigen and subsequently the enzyme catalyses the

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conversion of substrate to a product. This product participates in a second enzyme reaction which results in a colour change. The colour development process is stopped by the addition of acid. A colour intensity significantly above background levels is indicative of *Chlamydia* antigen present in the specimen.

Materials

Reagents provided by the kit:

1. Microtitration plate coated with *Chlamydia* specific monoclonal antibody. The plate was presented in a strip format i.e. twelve 8x1 well strips in a frame, inside a sealed pouch.

2. Transport medium (X10): non-ionic detergent in a solution of organic buffer salt containing metal chloride, reducing sugar, and antimicrobial agent.

3. Positive control: formalin inactivated *Chlamydia* antigen in buffered solution containing antimicrobial agent and coloured dye.

4. Conjugate: alkaline phosphatase conjugated *Chlamydia* monoclonal antibody in buffer containing detergent, protein, inorganic salts, coloured dye, and antimicrobial agent.

5. Washing buffer concentrate (X20): tris buffered solution containing detergent, inorganic salts, and an antimicrobial agent.

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6 Substrate diluent: diethanolamine buffered solution containing inorganic salts, ethanol, and antimicrobial agent.

7. Lyophilized substrate: NADPH in a stabilising inert matrix.

8. Amplifier diluent: phosphate buffered solution containing tetrazolium, stabiliser, and inorganic salts.

9. Lyophilized amplifier: Alcohol dehydrogenase and diaphorase in a stabilising matrix containing protein, carbohydrate, detergent and inorganic salts.

10. Stopping solution (0.46mol/L dilute sulphuric acid).

- The kit was stored at 4°C until used.

Additional reagents and equipment:

1. Distilled water (Milli-pore distiller).
2. Vortex mixer (Vortex Genie 2).
3. Moist chamber.
4. Water bath (Fisher Scientific. Versa bath).
5. Pipettes (Ependorff) and disposable tips to deliver 50µl, 100µl, 200µl, and 1000µl.
6. Automated plate washer washing 8x1 well microtitration strips (SLT).

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7 Spectrophotometer reading the absorbance at 492nm and reference wave length at 620 nm (SLT. Spectra).

Preparation of reagents

1.All reagents were brought to room temperature before used.

2.The stored inoculated transport medium tubes were allowed to thaw to room temperature and vortex mixed vigorously for a minimum of 1 minute prior to testing.

3. Positive control preparation: The positive control concentrate of the kit was vortex mixed for 1 minute. One hundred (100) µl of it was added to 1ml of working strength transport medium in a heat resistant vial. The working strength positive control was then vortex mixed for 1 minute and heated to 100°C in a water bath for 15 minutes. Immediately before use, it was vortex mixed for a further 15 seconds.

4.Negative control was prepared of 1ml of working strength transport medium which was treated as the positive control.

5.Washing buffer was freshly prepared by diluting the washing buffer concentrate with fresh

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distilled water: 1 part concentrate to 19 parts distilled water.

6. The substrate was freshly reconstituted by adding 6ml of substrate diluent to one bottle of lyophilised substrate .

7. Amplifier was freshly reconstituted by adding 6ml of amplifier diluent to one bottle containing lyophilised amplifier.

Procedure

1. Negative control (200µl) was added to 3 wells, 200µl of positive control and 200µl of each sample were then added to the wells, i.e. 3 negative control and one positive control wells were included with each batch of specimens tested.

2. The used strips of wells were incubated in a moist chamber at room temperature overnight.

3. The enzyme conjugated monoclonal antibodies (50µl) was added to each well. The contents were mixed gently by tapping the edge of plate.

4. The strips were then incubated for further 1 hour in moist chamber at room temperature.

5. The contents of the wells were aspirated and washed 5 times, including 2 minutes soak during the second wash, using the freshly prepared

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washing buffer and the automated washing machine.

6. Reconstituted substrate (100μl) was added to each well. The wells were incubated at room temperature in a moist chamber for 40 minutes.

7. Freshly reconstituted amplifier (100μl) was added to each well. Wells were then incubated at room temperature in a moist chamber for 10 minutes.

8. Stopping solution (50 μl) was added to each well.

9. The absorbance value of each well was read with a spectrophotometer at 492 nm with reference filter of 620 nm wave length. The blank was air.

The cut off value = the mean of the three negative controls + 0.05

In the present study, the mean absorbance value of the 3 negative controls was 0.059

The cut off value was 0.109

All specimens having an absorbance value greater than 0.109 were considered positive, and those less than 0.109 were considered negative.

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III. Polymerase Chain Reaction:

PCR was used for the detection of the DNA of *C. trachomatis*. This involved three main steps; DNA extraction, amplification, and detection of specific DNA product.

A. DNA Extraction

Instruments:

1. Microfuge (Beckman, Microfuge E).
2. Dry bath (Thermolyne, type 16500 Dri bath).
3. pH meter (Jenco Electronics, Model 671p).
4. Disposable bacteriological filter (Nagle).
5. Shaker incubator (ROSI 1000 Reciprocating orbital shaking incubator).
6. Cooler centrifuge (Beckman, C5-15 R).
7. Vortex (Genie 2).

Reagents:

1.Lysing buffer

0.5% tween 20 (Amresco, USA).
0.5% (v/v) Nonidet (NP-40) (Amresco, USA).
100μg/ml proteinase K (Sigma, USA).

2.Luria Bertani (LB) broth and agar.

1% trypton (Oxoid, England).
0.5% yeast extract (Oxoid, England).
1% sodium chloride (Adwic, Egypt).
3% agar powder for LB agar (Oxoid, England).

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100μg/ml ampicillin (Sigma, USA).

3.STET:
0.1M sodium chloride (Adwic, Egypt).
0.1M Tris HCl (pH8) (Pharmacia, Biotech, Denmark).
1mM EDTA disodium salt (pH8) (Pharmacia, Biotech, Denmark).
5% triton X-100 (Amresco, USA).

4. Lysozyme (10mg/ml 10mM in tris HCl, pH 8) (Sigma, USA).

5. Sodium acetate (2.5 mM, pH 2.5) (NTL, UK).

6.Isopropanol (Amresco, USA).

7.Ethanol, 70% (Adwic, Egypt).

8.Diethyl pyrocarbonate (DEPC) (Amresco, USA).

Method:

Sample preparation:

This was done according to Baily et al. (1994a).

The stored microfuge tubes of the inoculated transport medium were left to thaw. Tubes were centrifuged at 12,000 rpm to pellet cellular material. After removal of supernatant, the pellet was resuspended in 40μl of lysing buffer, incubated at 60°C for 1 hour in the dry bath to provide the optimum temperature for proteinase K, then at 100°C for 10 minutes to

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inactivate proteinase K. The resulting crude DNA preparations were stored at -20°C until used.

Positive control preparation:

Deep agar culture of *E. coli,* transformed with TA recombinant plasmid carrying a functioning copy of MOMP gene of *C. trachomatis* (serovar A-1) and ampicillin resistance gene (Fig. 1), was kindly supplied by Dr. Linda Bobo, Ph.D. (Assistant professor, Johns Hopkins University, School of Medicine, Division of Pediatric Infectious Diseases).

E. coli was propagated on ampicillin containing LB medium (according to Dr. Bobo instructions). Agar powder (3%) was added to LB broth for the preparation of LB agar plates. The pH was adjusted to 7.5 and the medium was autoclaved. Ampicillin was filtered and added in a concentration of 100 μg/ml to the medium at 55°C.

•In the present study two methods for positive control preparation were tried.

1.Bacterial DNA preparation:

The deep agar culture of the transformed *E. coli* was subcultured on LB agar plates and incubated for 24 hours. Dense suspension of the growing *E. coli* was made in 500 μl sterile distilled water. Care was taken not to scrape off any agar since it can inhibit the PCR. The suspension was boiled in

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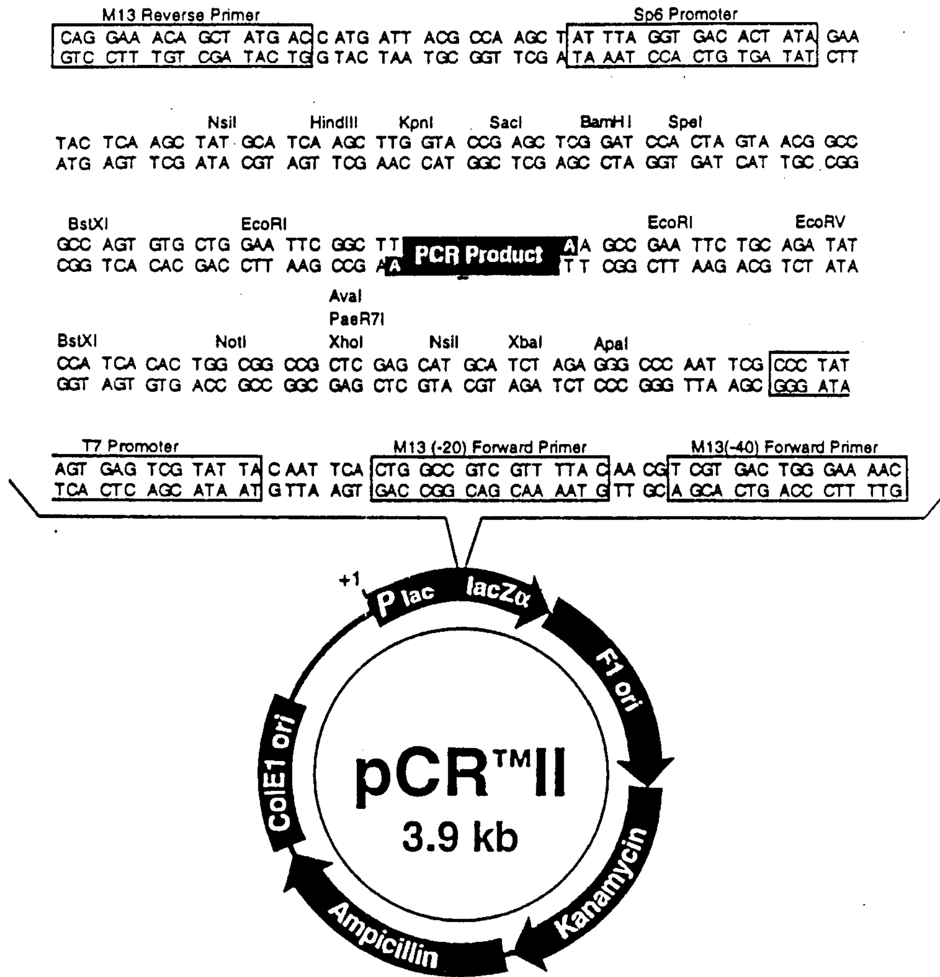

Fig. 1. RCRII cloning plasmid (Invitrogen, USA) designed for cloning PCR product directly from PCR reaction. This cloning plasmid was used for cloning of PCR copy of the MOMP gene.

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a water bath for 10 minutes, left to cool, and then kept at -20°C until used.

In the present work, 2μl of this suspension were used for a PCR final reaction volume of 50μl.

2.Plasmid DNA preparation:

According to Sambrook et al. (1989) for small scale preparation of plasmid DNA (miniprep), bacterial lysis was performed by boiling method.

A single bacterial colony was transferred from ampicillin containing LB agar plate into 3 ml of ampicillin containing LB broth which was then incubated overnight at 37°C in the shaker incubator to aerate the culture and increase the growth of the incubated bacteria. This overnight culture (1.5 ml) was transferred into a new microfuge tube and centrifuged at 14,000 rpm for 30 seconds at 4°C in the cooler centrifuge. The remainder of the culture was stored at 4°C.

The supernatant was aspirated leaving the bacterial pellet as dry as possible. The pellet was then resuspended in 350μl of STET buffer as a step for preparation of bacterial lysis which was achieved by adding 25μl of lysozyme to the suspension and vortex mixing for few seconds. The tube was then placed in the dry bath at 100°C for exactly 40 seconds.

The bacterial lysate was then centrifuged at 12,000 rpm for 10 minutes at room temperature. The supernatant, containing

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the DNA, was transferred to another microfuge tube leaving the bacterial debris.

To precipitate the plasmid DNA, 40μl of 2.5 M sodium acetate (pH 5.2) and 420μl of isopropanol were added to the supernatant and mixed by vortex. The tube was kept for 5 minutes at room temperature.

Nucleic acids were pelleted by centrifugation at 14,000 rpm for 5 minutes at 4°C. The supernatant was removed by gentle aspiration and discarded. The tube was then inverted on a paper towel to allow all the fluid to drain away.

One ml of 70% ethanol was added and the tube was recentrifuged at 14,000 rpm for 2 minutes at 4°C. Again all the supernatant was aspirated and discarded, the tube was inverted over a paper towel, and then left opened at room temperature until ethanol was completely evaporated and no fluid was visible in the tube (2-5 minutes).

DNA was stored as such at -20°C until used.

Before use, DNA was dissolved in different volumes of double distilled DEPC-treated water (nuclease free) (0.5ml, 0.8ml, & 1ml) to obtain different concentrations of DNA. Vortex and slight heating at 50°C were done to completely dissolve the DNA. The three different concentrations were tried as positive control and the three were equally effective.

In the present study, 2μl of plasmid positive control (the 0.8ml volume) were used /50μl PCR total volume.

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B. DNA amplification

Instruments:

1. Laminar flow (Labconco. Purifier class II biosafety cabinet).
2. Vortex (Genie 2).
3. Microfuge (Beckman E).
4. Thermal cycler (PCR machine) (Biometra, UnoII).

Reagents:

1. Diethyl pyrocarbonate (DEPC) (Amresco, USA).

2.Primers (Research Genetics, USA).

The sequence of the primers was chosen from the common (conserved) sequence of the MOMP of chlamydiae.

Primer sequence:

Sense (154-176, 5`-3`):
`ACC ACT TGG TGT GAC GCT ATC AG`
Antisense (1187-1166):
`CGG AAT TGT GCA TTT ACG TGA G`
The expected PCR product is 1,034 bp

These primers were supplied as dry powder (lyophilized). Each primer was dissolved in DEPC treated water to a final concentration of 5 pmol/µl. Primers were stored in aliquots at -20°C to avoid contamination. In PCR total volume of 50 µl, 20 pmol of each primer were added (4µl each).

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3. DNA polymeras (Advanced Biotechnology, UK).

Super-Therm DNA polymerase is a thermostable DNA polymerase isolated from *Thermus sp.* supplied in a concentration of 5U/µl. It was added to the reaction in a concentration of 1U/50µl total volume (0.2µl).

The polymerase was provided with its reaction buffer (10X, 1ml volume, containing 15mM $MgCl_2$), (5µl/reaction were used).

4.Deoxynucleoside triphosphate (dNTPs) (Advanced Biotechnology, UK).

These were supplied as a pack of 4 vials containing dATP, dTTP, dGTP, and dCTP (10 mM each, pH 7.0).

dNTPs mix was prepared by adding equal volume of each dNTP in one tube to give a final concentration of 2.5 mM each.

In PCR final volume 50µl, 200 µM of each was added (4µl of the mix).

Method:

The work was done in a laminar flow using nuclease free aerosol resistant tips (ART). Disposable latex gloves were used to avoid the contamination with DNase.

PCR was performed in 15 settings; each included in addition to the samples a negative and a positive control.

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A PCR mix (Table 1) was prepared, containing the DNA polymerase and its buffer, the 2 primers, the dNTPs mix and distilled water (the water volume was calculated to give the PCR a final volume of 50μl).

Table 1.The final preparation of PCR mix (total volume 50μl).

Reagent	Original Concentration	Final Concentration	Volume in Microlitres
DNA polymerase buffer	10X	1X	5
Sense primer	5pmol	20pmol	4
Anti-sense primer	5pmol	20pmol	4
dNTPs mix	2.5mM	0.2mM	4
DNA polymerase	5U/μl	1U	0.2
DEPC treated water			12.8
DNA			20

The mix was then distributed in the PCR tubes (0.2ml thin wall tubes); 30μl of mix per tube. Then 20 μl of each sample were added, giving 50μl per tube.

In the negative control tube, 20μl of DEPC treated water were added to the PCR mix instead of the sample.

As regards the positive control, both the extracted plasmid DNA and the total transformed bacterial DNA were compared. In the first 5 settings, 2 μl of the prepared plasmid DNA and 18 μl of DEPC treated water were added to the PCR

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mix to complete the final volume to 50 μl. In the other settings, the boiled transformed *E. coli* was used (2μl + 18μl DEPC treated water).

The prepared PCR tubes were vortex mixed followed by momentary spinning to bring all fluid to the bottom.

The tubes were then placed in the PCR machine without the addition of mineral oil, since the PCR machine has a lid that can be heated to avoid evaporation of the mix in the tubes.

The PCR machine was programmed first to heat the lid to 105°C then 95°C for 5 minutes (hard denaturation), followed by 35 cycles each consists of: denaturation at 95°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes. Prolonged extension at 72°C for 5 minutes was programmed to be followed by cooling to 4°C and holding at this temperature until the tubes were taken out of the machine to be examined by agarose gel electrophoresis.

The optimum annealing temperature for the primers (52°C), in the present work, was determined by trying different temperatures using the positive control.

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C. Detection of the specific DNA product

Instruments:

1. Hot plate with magnetic stirrer (Fisher Scientific, Thermix Hot Plate Model 210T).
2. Electrophoresis unit (Easy Cast Electrophoresis System, Model # K2).
3. Power supply (Titan).
4. Ultraviolet (UV) transilluminator (Cole Parmer).
5. Polaroid Camera and Polaroid films (DS43 Direct screen instant camera).

Reagents:

1. Tris acetate EDTA buffer *(TAE):*
   Tris HCl (pH8) (Pharmacia, Biotech, Denmark).
   Glacial acetic acid (Adwic, Egypt).
   5mM EDTA disodium salt (pH8) (Pharmacia, Biotech, Denmark).

2. Molecular biology grade agarose (Amresco, USA).

3. Ethidium bromide (Amresco, USA).

4. 10X stock of DNA loading dye:
   15% Ficol 400
   0.25bromophenol blue.

5. DNA molecular size marker: ϕx174/Hae III, 250μg/ml (72 bp to 1353 bp) (Advanced biotechnology, UK).

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Method:

The procedure was done according to Harris et al. (1996).

Molecular biology grade agarose was used to prepare 1% agarose gel. The agarose powder was added to TAE buffer (pH 8).

The agarose was allowed to dissolve in the buffer by heating using a hot plate with magnetic stirrer with a magnetic bead in the buffer agarose mixture. The agarose was left to cool to about 50°C at which ethidium bromide was added in a concentration of 5 mg/ml. Ethidium bromide was used to stain the DNA to be visualized when exposed to ultraviolet rays.

Casting the agarose gel was done in the tray (mould) of the electrophoresis unit. The comb, supplied with the tray, was placed at one end of it. In this unit, the comb is separated from the floor of the tray by a suitable distance to allow the development of wells when pouring the gel. The gel was poured in the tray and was allowed to solidify (usually in 20 minutes), then the comb was drawn up carefully leaving the wells in the solid gel.

The gel was then placed in the electrophoresis unit (tank) which was filled with TAE buffer to cover the agarose gel by about 5 mm. By this, the gel was ready for loading the DNA.

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Loading of the gel:

On parafilm, the DNA loading dye was mixed with the PCR product of samples, the positive control, the negative control, and the DNA marker, and each was loaded in a well in the solidified agarose.

The agarose was located, so that the wells were at the negative pole, to allow the negatively charged DNA to migrate in the gel towards the positive pole.

The power supply was turned on at 100 volts for 30-45 minutes to allow separation of the DNA marker bands. Then the gel was visualized on the UV transilluminator and photographed by Polaroid camera loaded with Polaroid films.

Interpretation of the results:

The positive control gave a sharp band (1034 bp) nearly opposite to the second band (1078 bp) of the ϕx147/Hae III DNA molecular size marker.

The negative control lane was free of any band.

The sample was considered positive when there was a band at the same level of the positive control band and the second band of the marker (Fig. 7 & 8).

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Statistical Methods:

The results were analysed statistically by using the chi square (X^2) test.

$$X^2 = \sum_{sum} x\ (O-E)^2/E$$

O: observed value.
E: expected value.

This test was done for the association between two variables (qualitative).

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Results

The results of the present study are tabulated as follows:

I. Collected data of the subjects of the present study *(Tables 2 to 12).*

II. Rate of detection of *C. trachomatis (Tables 13 to 24).*

III. Clinical presentation of infected subjects *(Table 25).*

IV. Results of bacteriological examination *(Tables 26 to 29).*

I. COLLECTED DATA OF THE SUBJECTS OF THE PRESENT STUDY.

The present study involved 90 patients, complaining of symptoms of conjunctivitis and with clinical signs of active trachoma (TF or TI), and matched 45 control subjects.

Table 2. Sex distribution of the studied subjects and their ages.

Group	No.	%	Mean age in years	± SD	Minimum age in years	Maximum age in years
Patients	90	100	33.8	20.1	5	72
Females	46	51.1	33.4	18.8	5	70
Males	44	48.8	34.2	21.6	6	72
Controls	45	100	32	19.5	5	66
Females	22	48.9	31.4	19	6	65
Males	23	51.1	32.5	20	5	66

Table 2 shows that, the subjects were selected to the present study to be nearly in equal number of both sexes with matched ages.

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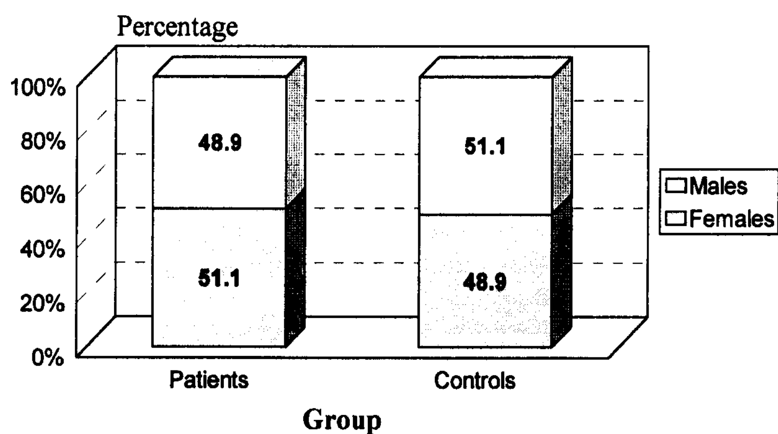

Fig. 2. Sex distribution of the studied subjects.

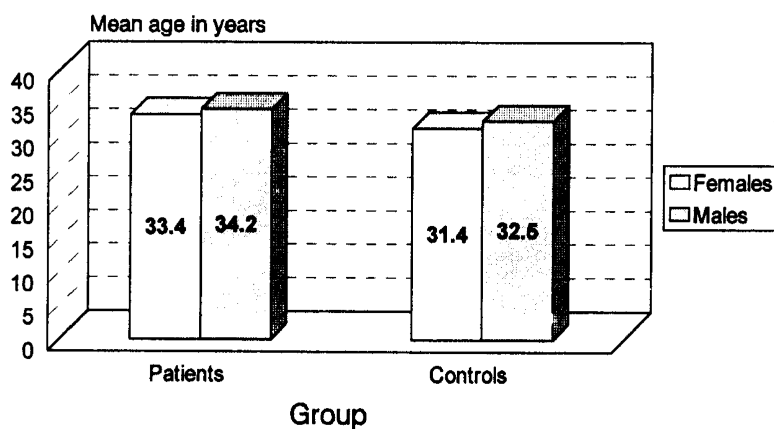

Fig. 3. The mean age of males and females of subject of the present study.

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Table 3. Age distribution of the studied subjects.

Subjects	Total	Age groups*					
		I		II		III	
		No	%	No.	%	No.	%
Patients	90	27	30	40	44.4	23	25.6
Controls	45	13	28.9	20	44.4	12	26.7

*According to the age, subjects in the present study were divided into 3 age groups: I (school age), II (middle age), and III (older age).
Table 3 shows similar percentages of patients in each group which match with that of the controls.

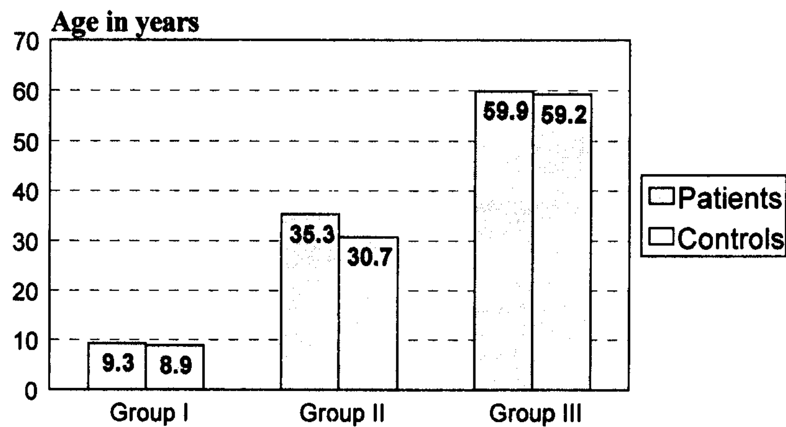

Fig. 4. The mean age of subjects in the three age groups.

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Table 4. Distribution of subjects as regards their residence.

Residence	Patients		Controls	
	No.	%	No.	%
Cairo	63	70	37	82.2
Others*	27	30	8	17.8
Total	90	100	45	100

* Other areas were Kalubia, Suis, Menoufia, Menia, Souhage, Aswan, Sharkia, Dakahlia, Gizza, and Bani-sweef.

In table 4, there was no much difference in the percentage of patients and controls living in Cairo.

Table 5. Occupation of the subjects of the studied sample.

	Patients		Controls	
	No.	%	No.	%
No job	3	3.3	0	0
Housewives	30	33.3	6	13.3
Students	19	21.1	11	24.4
Workers	16	17.8	7	15.6
Employee	10	11.1	7	15.6
Below school age	5	5.6	3	6.7
Retired	5	5.6	3	6.7
Physicians*	1	1.1	8	17.8
Soldiers	1	1.1	0	0
Total	90	100	45	100

In table 5, there were 9 physicians included in the present study; one of them was in the patient group, the others were in the control group. Among these, this patient and 6 of the controls were in direct and continuous contact with patients' eyes in ophthalmology clinics.

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Table 6. Complaint(s)* of patients of the present study.

Complaints	No. (90)	%
Burning sensation	26	28.9
Itching	26	28.9
Redness	17	18.9
Foreign body sensation	15	16.7
Lacrimation	15	16.7
Discharge	14	15.6
Blurring of vision	9	10
Photophobia	6	6.7
Heaviness of eyelids	5	5.6
Pain	4	4.4
Lid oedema	1	1.1

*Some of the patients were complaining of more than one complaint.

Table 6 shows that, the commonest two complaints were burning (28.8%) and itching (28.8%). Pain was mainly given by children.

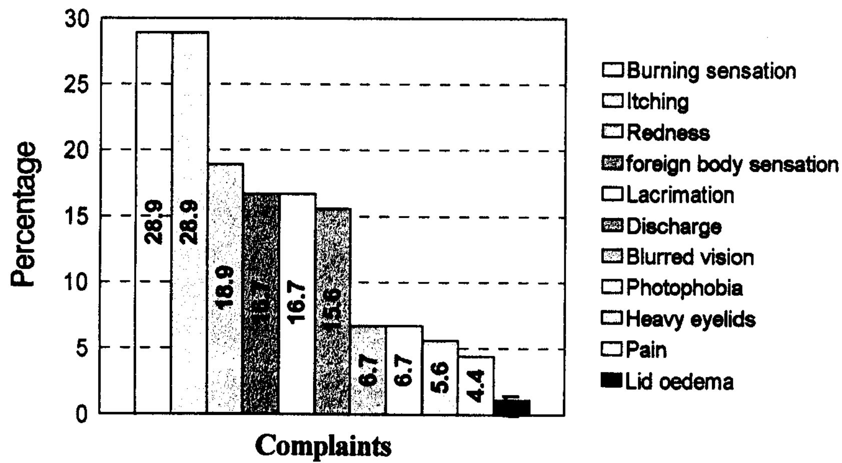

Fig. 5. The different complaints given by patients.

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Table 7. Distribution of studied subjects in relation to their past history of trachoma.

Past history	Patients		Controls	
	No.	%	No.	%
With	34	37.8	2	4.4
Without	56	62.2	43	95.6
Total	90	100	45	100

In table 7, the percentage of patients giving past history of trachoma (37.8%) was less than those without past history (62.2%). The same applies for the controls.

Table 8. Distribution of studied subjects as regards family history of trachoma.

Family history	Patients		Controls	
	No.	%	No.	%
With	8	8.9	0	0
Without	82	91.1	45	100
Total	90	100	45	100

In table 8, none of the controls had family history of trachoma, while 8.9% of patients had family history.

Table 9. Subjects using topical antibiotics by time of samples taking.

Topical antibiotics	Patients		Controls	
	No.	%	No.	%
Using	8	8.9	0	0
*Not using	82	91.1	45	100
Total	90	100	45	100

*No application of antibiotics 3 - 4 days before sampling.

In table 9, none of the controls, but 8.9% of patients, were using topical antibiotics.

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Table 10. Types of antibiotics used by patients of the present study.

Antibiotic	No.	%
Sulphonamides	5	62.5
Tetracycline	2	25
Chloramphenicol	1	12.5
Total	8	100

In table 10, the commonest antibiotic used by patients before asking medical advice was sulphonamides (62.5%).

Table 11. Stages of active trachoma in the 90 patients of the present study.

Stage*	Patients	
	No.	%
TF	60	66.7
TI	30	33.3
Total	90	100

* Stages of active trachoma were recorded according to the WHO classification (Thylefors et al., 1987).

Table11 shows that, TF (active trachoma with follicles) was more presented clinically (66.7%) than TI (active trachoma intense) (33.3%).

Table 12. Stage of active trachoma in the different age groups of patients.

	I		II		III	
Stage	No.	%	NO.	%	No.	%
TF	20	74.1	28	70	12	52.2
TI	7	25.9	12	30	11	47.8
Total	27	100	40	100	23	100

In table 12:

-The percentage of TF was higher in group I (5-15 years) than in both II (16-50 years) and III (> 50 years).

-The percentage of TI was highest in group III.

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II. Rate of Detection of C. trachomatis.

A) Enzyme Immune Assay:

Detection of *C. trachomatis* antigen (MOMP) by EIA was negative for all subjects involved in the present study (Fig. 6).

Six conjunctival samples were collected by both swabbing and scraping. These two methods were compared by EIA in the same setting and both gave the same negative result.

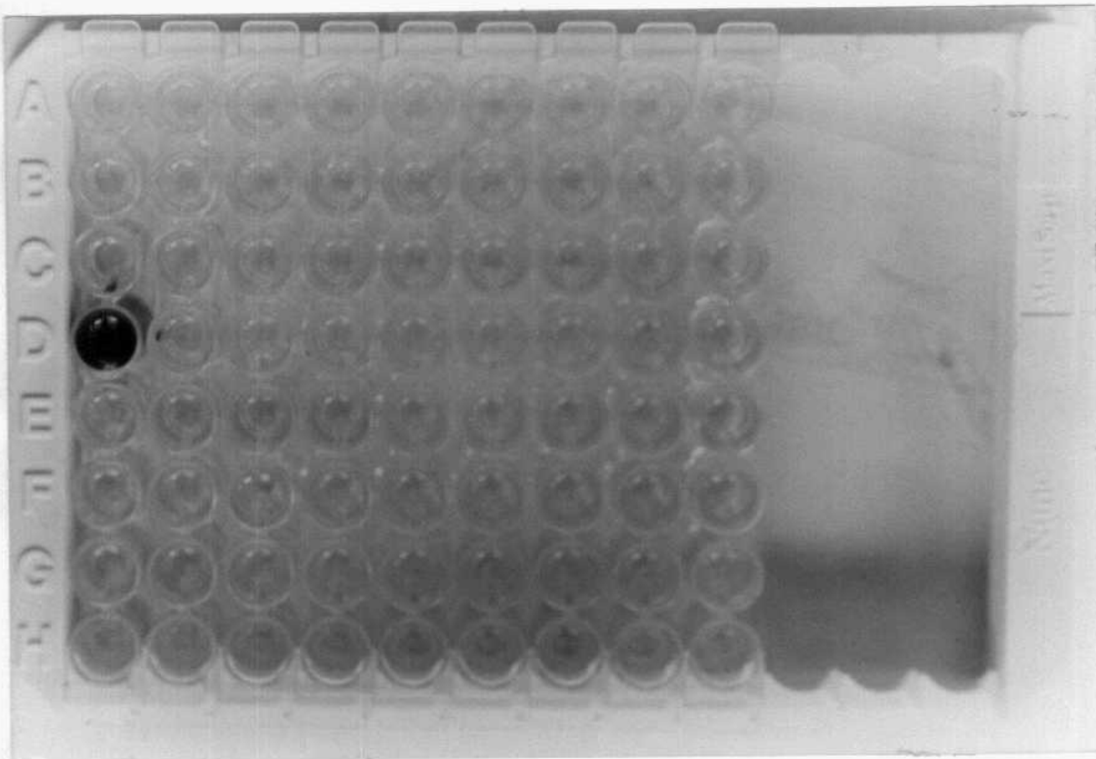

Fig. 6. EIA results in one of the settings. The first 3 wells (A, B, & C) in the first column were for the negative controls. The next well (D) was for the positive control. None of the samples tested were positive.

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B) Polymerase Chain Reaction:

Results of detection of *C. trachomatis* DNA by PCR are shown in figures 7 to 11 and tables from 13 to 24.

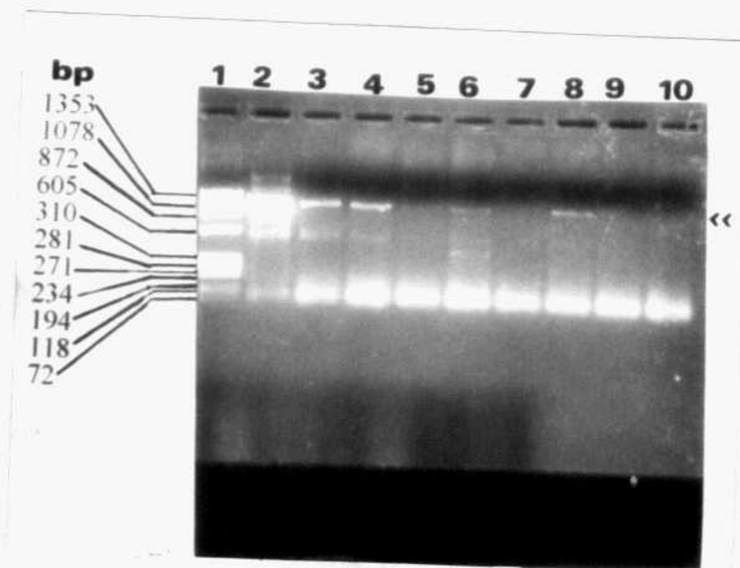

Fig.7. Electrophoretic separation of PCR amplified products of the MOMP gene of *C. trachomatis* using 2 specific primers. The PCR product (1034 bp, right arrow) is separated on 1% agarose gel and stained with ethidium bromide. Lanes 3, 4, 6, & 8 gave positive PCR (specific product is present), while lanes 5, 7 & 9 were negative samples. ϕx174/Hae III DNA molecular size marker (lane 1) was used for size determination. Lanes 1 & 10 represent the +ve control (plasmid DNA) and the -ve control (no DNA) respectively.

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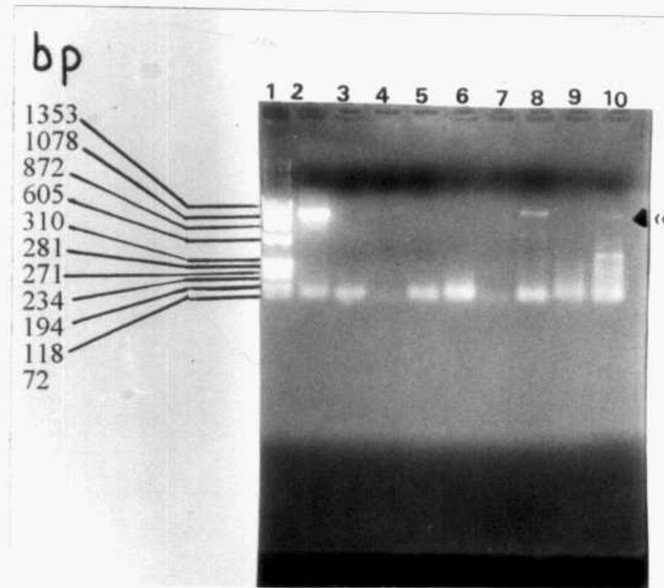

Fig. 8. Electrophoretic separation of PCR amplified products of the MOMP gene of *C. trachomatis* using 2 specific primers. The PCR product (1034 bp, right arrow) is separated on 1% agarose gel and stained with ethidium bromide. Lanes 8 &10 gave positive PCR (specific product is present), while lanes 4, 5, 6, 7, & 9 were negative samples. ϕx174 DNA molecular size marker (lane 1) was used for size determination. Lanes 2 & 3 represent the +ve control (plasmid DNA) and the -ve control (no DNA) respectively.

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Table 13. Rate of detection of *C. trachomatis* by PCR in the subjects of the present study.

	Total	PCR +ve		PCR -ve	
		No	%	No.	%
Patients	90	13	14.4	77	85.6
Controls	45	4	8.9	41	91.1

In table 13, the percentage of PCR +ve patients (14.4%) was higher than that of the controls (8.9%), but the difference was not statistically significant ($X^2 = 0.84$, $P > 0.05$).

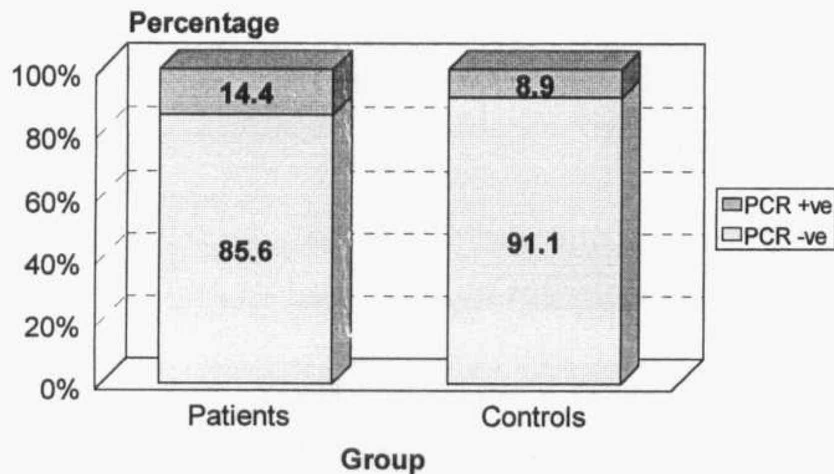

Fig. 9. Rate of detection of *C. trachomatis* in the studied subjects.

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1) In patient group:

Table 14. Rate of detection of *C. trachomatis* in the patients (90) of the present study in the three age groups.

Age Group	Total	PCR +ve		PCR -ve	
		No.	%	No.	%
I	27	0	0	27	100
II	40	9	22.5	31	77.5
III	23	4	17.4	19	82.6

In table 14, the percentage of PCR +ve patients was higher in group II (22.5%) than groups I (0%) and III (17.4%). The difference was statistically significant ($X^2 = 6.8$, $P < 0.05$).

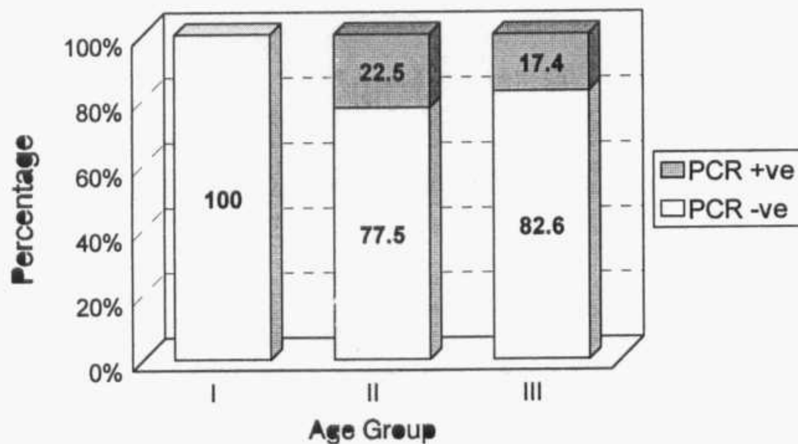

Fig. 10. Rate of detection of *C. trachomatis* in the three age groups of patients.

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Table 15. Rate of detection of *C. trachomatis* in the 90 patients of the present study according to sex.

	Total	PCR +ve		PCR -ve	
		No.	%	No.	%
Females	46	10	21.7	36	78.3
Males	44	3	6.8	41	93.2

In table 15, the rate of detection of *C. trachomatis* in females (21.7%) was significantly higher than that of males (6.8%) in the patient group ($X^2 = 4.1$, $P < 0.05$).

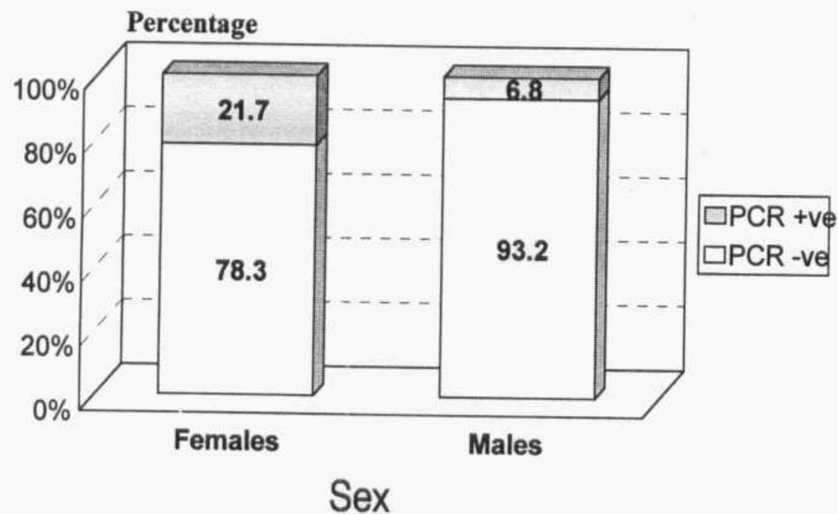

Fig. 11. Rate of detection of *C. trachomatis* in both sexes in the patient group.

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Table 16. Rate of detection of *C. trachomatis* in the 90 patients of the present study as regards their residence.

Residence	Total	PCR +ve		PCR -ve	
		No.	%	No.	%
Cairo	63	9	14.3	54	85.7
Others*	27	4	14.8	23	85.2

Table 16 shows that, the rate of detection of *C. trachomatis* in patients living in Cairo (14.3%) was similar to that of those living in other areas (14.8%).

*The 4 PCR +ve patients were living in: El-Kalubia (2 = 50%), Aswan (1 = 25%), and El-Menoufia (1 = 25%).

Table 17. Rate of detection of *C. trachomatis* in the 90 patients of the present study in relation to their occupation.

Job	Total	PCR +ve		PCR -ve	
		No.	%	No.	%
No job	3	0	0	3	100
Housewives	30	10	33.3	20	66.7
Students	19	0	0	19	100
Workers	16	1	6.3	15	93.7
Employee	10	0	0	10	100
Below school age	5	0	0	5	100
Retired	5	2	40	3	60
Physicians	1	0	0	1	100
Soldiers	1	0	0	1	100

Table 17 shows that, the rate of detection of *C. trachomatis* was higher in the retired patients (40%), followed by housewives (33.3%), and then workers (6.3%).

The Physician in the patient group was PCR -ve, in spite of his close and continuous contact with patients' eyes in the ophthalmology outpatient clinic. This physician was using sulphonamides 4 days before sample taking.

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Table 18. Rate of detection of *C. trachomatis* in the 90 patients of the present study according to their complaints.

Complaint	Total No.	PCR +ve No.	PCR +ve %	PCR-ve No.	PCR-ve %	X^2	P	S
Burning	26	6	23.1	20	76.9	2.2	> 0.05	NS
Foreign body sensation	15	4	26.7	11	73.3	2.18	> 0.05	NS
Lacrimation	15	4	26.7	11	73.3	2.2	> 0.05	NS
Discharge	14	1	7.1	13	92.9	0.72	> 0.05	NS
Blurred vision	9	1	11.1	8	88.9	0.1	> 0.05	NS
Photophobia	6	1	16.7	5	83.3	0.02	> 0.05	NS
Pain	4	1	25	3	75	0.4	> 0.05	NS
Lid oedema	1	0	0	1	100	0.2	> 0.05	NS
Itching	26	0	0	26	100	6.1	< 0.02	S
Redness	17	0	0	17	100	3.5	> 0.05	NS
Heavy eyelid	5	0	0	5	100	0.9	> 0.05	NS

In table 18, PCR +ve patients were mostly complaining of lacrimation (26.7%), foreign body sensation (26.7%) and burning (23.1%), but the difference between PCR +ve and -ve patients was statistically non-significant ($P > 0.05$). Absence of itching in PCR +ve patients was statistically significant ($X^2 = 6.1$, $P < 0.02$).

(S: significant, NS: non significant).

Table19. Rate of detection of *C. trachomatis* in patients (90) of the present study in relation to their past history.

Past history	Total	PCR +ve		PCR -ve	
		No.	%	No.	%
With	34	5	14.7	29	85.3
Without	56	8	14.3	48	85.7

In table 19, the percentage of PCR +ve patients with past history of trachoma (14.7%) was similar to that of those without past history (14.3%).

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Table 20. Rate of detection of *C. trachomatis* in the 90 patients of the present study in relation to family history.

Family history	Total	PCR +ve		PCR -ve	
		No.	%	No.	%
With	8	2	25	6	75
Without	82	11	13.4	71	86.6

In table 20, the percentage of PCR +ve patients with family history of trachoma (25%) was higher than that of without family history (13.4%), but this was not statistically significant ($X^2 = 0.8$, $P > 0.05$).

Table 21. Rate of detection of *C. trachomatis* in the 90 patients of the present study in relation to their use of topical antibiotics.

	Total	PCR +ve		PCR -ve	
		No.	%	No.	%
Using antibiotics	8	0	0	8	100
Not using antibiotics	82	13	15.8	69	84.2

In table 21, none of the PCR +ve patients was using antibiotics by the time of sample taking, while 100% of those using antibiotics were PCR -ve.

Table 22. Rate of detection of *C. trachomatis* in patients (90) of the present study in relation to the stage of trachoma.

Stage	Total	PCR +ve		PCR -ve	
	No.	No.	%	No.	%
TF	60	10	16.7	50	83.3
TI	30	3	10	27	90

In table 22, *C. trachomatis* was detected in 16.7% of patients with TF. This rate was higher than those with TI (10%), but the difference was not statistically significant ($X^2 = 0.72$, $P > 0.05$).

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2) In controls:

Age:

All the PCR +ve controls were in group II (16-50 years old).

Table 23. Rate of detection of *C. trachomatis* in controls (45) of the present study in relation to sex.

	Total	PCR +ve		PCR -ve	
		No	%	No.	%
Females	22	2	9.1	20	90.9
Males	23	2	8.7	21	91.3

In table 23, the rate of detection of PCR +ve females (9.1%) was slightly higher than that of males (8.7%). This difference was not statistically significant ($X^2 = 0.002$, $P > 0.05$).

Occupation:

All the 4 PCR +ve control subjects were among the physicians included in the present study and all were working in close and continuous contact with patients' eyes. Therefore, they were considered, in the present study, as infected subclinical patients. The control subjects of all other occupations were PCR -ve. The difference was statistically significant ($X^2 = 28.5$, $P > 0.001$).

Residence:

All the PCR +ve controls (100%) were living in Cairo.

Table 24. Rate of detection of *C. trachomatis* in the 45 control subjects of the present study in relation to their past history.

Past history	Total	PCR +ve		PCR -ve	
		No	%	No.	%
With	2	1	50	1	50
Without	43	3	7	40	93

In table 24, the percentage of PCR +ve controls with past history of trachoma was higher (50%) than those without past history (7%). Statistically this was of a significant difference ($X^2 = 4.37$, $P > 0.05$).

Family history:

None of the controls gave family history of trachoma.

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Antibiotics:
None of the controls was using topical antibiotics.

III. Clinical presentation of C. trachomatis infected subjects of the present study.

Table 25. Data of the PCR +ve subjects of the present study.

Data		PCR +ve			
		Patients (13)		Controls (4)	
		No.	%	No.	%
Age group	I	0	0	0	0
	II	9	69.2	4	100
	II	4	30.8	0	0
Sex	Females	10	76.9	2	50
	Males	3	23.1	2	50
Occupation	Physicians	0	0	4	100
	Others	13	100	0	0
Residence	Cairo	9	69.2	4	100
	Others	4	30.8	0	0
Complaint(s)	Burning	6	46.2	-	-
	Foreign body sensation	4	30.8	-	-
	Lacrimation	4	30.8	-	-
	Discharge	1	7.7	-	-
	Blurred vision	1	7.7	-	-
	Photophobia	1	7.7	-	-
	Pain	1	7.7	-	-
Past history	With	5	38.5	1	25
	Without	8	61.5	3	75
Family history	With	2	15.4	0	0
	Without	11	84.6	4	100
Antibiotics	Using	0	0	0	0
	Not using	13	100	4	100
Trachoma stage	TF	10	76.9	-	-
	TI	3	23.1	-	-

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From the foregoing analysis of the results of the present study, it was found that:
Most of *C. trachomatis* positive patients were in the second age group (15-50 years old), females, living in Cairo, complaining of burning, foreign body sensation, and / or lacrimation, without past history of trachoma or family history of infected household relative, not using topical eye antibiotics, and in TF stage of trachoma. The same applies for the 4 infected subclinical cases except for absence of complaints and clinical signs of active trachoma.

IV. RESULTS OF BACTERIOLOGICAL EXAMINATION.

Table 26. Rate of detection of bacteria in conjunctival swabs of studied subjects.

Bacterial culture	Patients		Controls	
	No.	%	No.	%
Growth	69	76.7	30	66.7
No Growth	21	23.3	15	33.3
Total	90	100	45	100

In table 26, the rate of isolation of bacteria among patients (76.7%) was higher than that in control group (66.7%), but this difference was not statistically significant ($X^2 = 1.5$, $P > 0.05$).

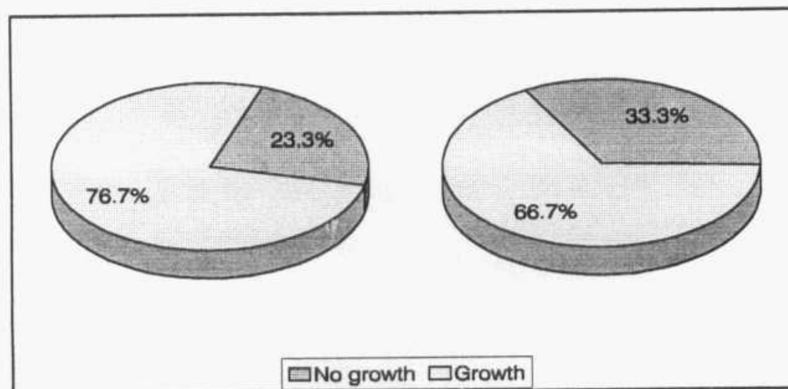

Fig. 12. Rate of isolation of bacteria from conjunctival swabs from patients (left) and controls (right).

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Table 27. Isolated bacteria in studied subjects.

Growth	Patients		Controls	
	No.	%	No.	%
Growth of single Organism	58	64.4	29	64.4
S. epidermidis	52	57.8	27	60
Diphtheroids	4	4.4	2	4.4
St. viridans	2	2.2	0	0
Growth of mixed Culture	11	12.2	1	2.2
S. epidermidis & Diphtheroids	6	6.7	0	0
S. epidermidis & *Micrococcus*	2	2.2	0	0
S. epidermidis & *St. viridans*	3	3.3	0	0
S. epidermidis & *S. aureus*	0	0	1	2.2
No Growth	21	23.3	15	33.3
Total	90	100	45	100

In table 27, the most common encountered organism was *S. epidermidis* (57.8%).

Table 28. Rate of detection of different bacteria in the 90 patients of the present study in relation to the results of PCR.

Growth	PCR +ve		PCR -ve	
	No.	%	No.	%
Single	8	61.5	50	64.9
S. epidermidis	6	46.2	46	59.7
Diphtheroids	1	7.7	3	3.9
St. viridans	1	7.7	1	1.3
Mixed	2	15.4	9	11.7
S.epid. & Diphtheroids	2	15.4	4	5.2
S.epid. & *St. viridans*	0	0	3	3.9
S.epid. & *Micrococcus*	0	0	2	2.6
No growth	3	23.1	18	23.4
Total	13	100	77	100

In table 28, there was no statistical difference between PCR +ve and -ve patients as regards bacterial growth ($X^2 = 0.001$, $P > 0.05$).

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Table 29. Rate of isolation of bacteria in infected subjects of the present study.

	PCR +ve			
	Patients		Controls	
Growth	No.	%	No.	%
Single	8	61.5	3	75
S. epidermidis	6	46.2	3	75
Diphtheroids	1	7.7	0	0
St. viridans	1	7.7	0	0
Mixed	2	15.4	0	0
S.epid. & Diphtheroids	2	15.4	0	0
No growth	3	23.1	1	25
Total	13	100	4	100

In table 29, the rate of bacterial growth in PCR +ve patients (76.9%) was similar to that of controls (infected subclinical cases) (75%).

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Discussion

Trachoma was known as Egyptian ophthalmia and is endemic in Middle East. It is indigenous to the Nile Valley, and has been a major cause of blindness there through recorded history (Miller, 1992; Leherman, 1993; Dawson et al., 1997).

The infectious agent responsible for trachoma is *C. trachomatis* of which serovars A, B, Ba & C have a propensity for the conjunctiva (Dolin, 1994).

The aim of the present study was to detect the causative agent of trachoma and to detect the associated bacteria among Egyptian patients in order to evaluate the diagnostic procedures and the epidemiology of the trachoma.

Therefore, conjunctival swabs collected from 90 patients, presented with symptoms of conjunctivitis and signs of active trachoma (TF or TI), and matched control subjects were collected for the detection of *C. trachomatis* and associated bacteria. EIA was used for detection of the LPS genus specific antigen of chlamydiae. On the other hand, PCR was used to detect DNA of MOMP of *C. trachomatis*.

For the detection of the LPS antigen of *C. trachomatis*, EIA was done for all the 135 studied subjects of the present

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study (90 patients and 45 controls), but the result was negative.

In order to insure that the negative result was not due to inappropriate collection of the conjunctival swabs, conjunctivial scraping was done as well for 6 patients and examined by EIA in the same setting. Both methods of sampling gave the same negative result.

Since the positive control of the test gave positive result, and the three negative controls of the test gave negative result, and no positive result was detedted in all subjects, EIA was 0% sensitive and 100% specific against clinical backgrounds (patients complaints and signs of active trachoma). This high specificity might be partially explained by the low sensitivity of the test.

Evaluation of EIA as a method of detection of *C. trachomatis* was variable in different reported studies.

In Egypt, Schachter et al. (1988) reported that, 36 out of 99 (36%) of rural children, one to 10 years old, with signs of active trachoma, were positive for antigen detection by EIA. They defined true positive subjects as children who yielded a positive tissue culture, a positive Giemsa stained specimen, or a specimen that was positive in 2 of the other 3 nonculture methods (DFA, EIA, or DNA probing). By all these criteria, 45 of the children only had demonstrable infection. Sensitivity of EIA in relation to this defined group was 73%, and the specificity was 93% (43/45).

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Mabey et al. (1987), working in The Gambia, reported that, the sensitivity of EIA was 25% (56/228 clinically active cases), and specificity was 95% (5% were positive in 997 subjects with no disease). On the other hand, when they took isolation as the "gold standard", the EIA had a sensitivity of 70.9% and specificity of 90%.

In correlation with clinical trachoma, Taylor et al. (1991) in central Tanzania reported that, EIA had a sensitivity of 36% and specificity of 97%. Also, Hayes et al. (1992) in The Gambia reported 25% sensitivity and 100% specificity. Bailey et al. (1994 a), in the same country, reported that EIA was 62% sensitive and 93.5% specific, and in another study, Baily et al. (1994b) showed a 25% sensitivity and 100% specificity of EIA.

Although isolation of *C. trachomatis* on tissue culture has long been the "gold standard" for the diagnosis of chlamydial infections, many studies reported EIA to be equally, or more, sensitive than culture (Mabey et al., 1987; Schachter et al.,1988). But, on the other hand, Holland et al. (1992), working on Cynomologus monkey, suggested that the "gold standard" of tissue culture may need to be reassessed, since host-dependent factors in the immune animal can inhibit tissue culture (elaboration of antibodies, IL-1, γ-interferon, and tumour necrosis factor).

These previous studies show up the controversy in the evaluation of EIA as a diagnostic method of *C. trachomatis* eye infection. This can involve the recorded low sensitivity of the assay in the present work.

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Besides, Schachter et al. (1988) stated that, tests for *C. trachomatis* are negative in a substantial proportion of clinically active cases, even when a variety of diagnostic tests are used.

Also, the negative results of EIA of the present study could be explained by the report of Taylor et al. (1991) that, there is a considerable variation in the shedding of *C. trachomatis* by people living in trachoma endemic areas. In a longitudinal study on endemic trachoma in Tanzania, they found a discordance rate of 10% in DFA specimens collected 2-5 minutes apart, and 25% discordance rate in DFA specimens collected 2 days apart. Also, Taylor (1992) in the same area, were able to detect *C. trachomatis* early in the course of recently acquired infection, but not later in resolving infection, and that during disease resolution the shedding of *Chlamydia* is reduced, to levels below the detection by EIA, 3 to 6 months before clinical recovery is observed. They explained the persistence of disease in spite of absence of detectable infectious agent by the role of cell mediated hypersensitivity in response to chlamydial antigens.

In the present work, PCR was used for the detection of the MOMP DNA of *C. trachomatis*. Two methods for extraction of the recombinant plasmid from the transformed *E. coli* were used for the preparation of the positive control for the PCR test. The first method involved extraction and purification of the plasmid (Miniprep), the other required just boiling of the transformed *E. coli* and using its whole DNA. Both methods

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were equally effective but the latter method was found to be easier, cheaper, and more rapid.

In the present study, PCR was able to detect the DNA of the MOMP of *C. trachomatis* in 14.4% of patients (13/90) and 8.9% of controls (4/ 45), but there was no statistical significant difference in the detection rate between patients and controls (P> 0.05). The PCR was 14.4% sensitive and 91.1% specific against clinical signs.

In Evaluation of PCR technique for detection of *C. trachomatis* in trachoma patients, Bobo et al. (1991) working in Tanzania, reported 48% sensitivity of PCR amplifying the MOMP DNA, but chlamydial DNA was detected in 24% of clinically free subjects. They reported that, PCR was more sensitive but less specific than antigen detection (DFA).

In The Gambia, Hayes et al. (1992), and Baily et al. (1994b) stated that, PCR used for direct amplification of MOMP was 51% positive against clinical background of active disease and 95% specific. They concluded that, PCR was more sensitive but less specific than EIA. In the same country, Baily et al. (1994a) reported PCR sensitivity and specificity of 72% and 92.5% respectively, but the target DNA was the cryptic plasmid. They reported that, using the cryptic plasmid as a target for PCR amplification is more sensitive (detecting 1-10 EBs) than using the MOMP (detecting 10 - 100 EBs).

In the present study, PCR specificity (91.1%) was in the range reported in other studies (76% - 95%), and similarly it

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was less specific than EIA. Baily et al. (1994b) suggested that, the lower specificity of PCR might be due to an extremely high level of clinically inapparent infection, or alternatively, that the increased sensitivity of PCR is achieved at the expense of the specificity. The former explanation is more likely to be applied in the present study, since the 4 PCR positives detected in the 45 controls were physicians and in direct and continuous contact with patients eyes in ophthalmology clinics ($P < 0.001$).

These 4 controls could be actually infected and either in the incubation period (2-3 weeks) (Sandford-Smith, 1992), since the presence of demonstrable organism can antedate recognizable disease (Taylor et al., 1992), or the infection was so mild to pass with no complaints by infected patients and with minor clinical signs (Miller, 1992). According to the WHO classification, the conjunctiva of the controls of the present study was not considered trachomatous if having follicles less than 5. These positive controls were considered, in the present study, as actually subclinically infected cases, therefore, the PCR was sensitive in detecting these subclinical cases and also a specific tool for the diagnosis of *C. trachomatis* infection.

Besides, Bobo et al. (1997) stated that, the reaction and clinical response to *C. trachomatis*, in the infected subject, is influenced by host genetic factors, resulting in severe long term sequalae and scarring disease in some people, and mild unnoticed one in others.

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As regards the low sensitivity of PCR in the present study (14.4%), it may be due to the presence of inhibitors in the clinical specimens as suggested by Wilde et al. (1990).

Bobo et al. (1991) reported that, 4 specimens, from *C. trachomatis* actively infected children, were PCR negative but DFA positive. When these 4 samples were diluted (1/10) with water, 3 (75%) became PCR positive, suggesting the presence of inhibitors in the undiluted samples. They further supported this observation when chlamydial DNA was added to these three samples, and this resulted in complete inhibition of PCR giving negative result.

The presence of inhibitors was not tested in the present study, but could be expected in the specimens since DNA was not completely purified (only lysing buffer was used).

In the present study, subjects were divided into 3 age groups; I: school age (5 - 15 years), II: middle age group (16 - 50 years), and III: older group (more than 50 years).

The rate of detection of *C. trachomatis* by PCR in patients was 0% (0/27) in group I, 22.5% (9/40) in group II, and 17.4 % (4/23) in group III. The high level of detection of *C. trachomatis* among the adult group was of statistical significance in relation to that in school children and older age groups. In controls, all the 4 PCR positive subjects were in the adult age group.

The detection rate of *C. trachomatis* in patients in group I (5 - 15 years) was 0%, however, generally the age-specific

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prevalence of trachoma is higher in pre-school age (Sandford-Smith, 1992; Taylor et al., 1992; Mahmoud et al., 1994). This could be seen if children less than 5 years of age were included in the present study.

The rate of detection of *C. trachomatis* in females was 21.7% (10/46) and was significantly higher than that in males (6.8%, 3/44) (P > 0.05). Similarly Sandford-Smith (1992) and Dolin (1997) reported that, females are more affected than males and explained this by their close contact with children; the main reservoir of infection.

Most of the patients in the present study were living in Cairo (63%), while 27% were living in other governorates. This is because the location of the Ain Shams University Hospitals is in Cairo, but has no actual relation to disease distribution.

The rate of detection of *C. trachomatis* in Cairo residents (14.3%) was equal to that for those living in big cities of other governorates (14.8%). Among the PCR positive patients, 69.2% (9/13) were living in Cairo. Previous studies (Sandford Smith, 1992; Dolin et al., 1997) reported that, trachoma is specially common in rural areas, but this high rate of detection of *C. trachomatis* in Cairo, in the present study, shows that the infection with *C. trachomatis* is not related to the residence, it is mostly affected by the socioeconomic status.

Most of the 45 controls were living in Cairo (82.2%). All the PCR positive controls were living in Cairo, and all were of high socioeconomic level and highly educated, but their work

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was in close relation with eyes of patients in ophthalmology clinics, and most probably this was the main risk factor for them.

As regards the occupation, the rate of detection of *C. trachomatis,* in the present work, in retired patients was 40% (2/5), followed by housewives (33.3%, 10/30), then workers (6.3%, 1/16). This can be explained by the high load of infection in the housewives and the retired patients due to their close relation with children; the main shedders of *C. trachomatis* (Sandford Smith, 1992).

There was one physician included in the patient group who was PCR negative. This could be due to the use of sulphonamides eye drops 4 days before sampling.

The control group included 6 subjects in close and continuous contact with patients' eyes in the ophthalmology clinic. All the PCR positive controls (4/6) were belonging to this group with a detection rate of 66.7%. This rate was significantly higher than that for the other 39 controls (0%) ($P < 0.001$).

Different complaints given by the patients of the present study were related to symptoms of conjunctivitis. The commonest were burning sensation and itching (29% each), followed by redness (19%), foreign body sensation and lacrimation (17% each), discharge (10%), photophobia (7%), heavy eyelids (5.5%), pain (4%), and lid oedema (1%).

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Sandford-Smith (1992) and Schwab et al. (1992) reported that, the clinical picture of trachoma varies from a mild condition with hardly any symptoms at all, to a severe disease. They also mentioned that, patients complain of tearing, photophobia, pain, exudation, oedema of the eyelids, and redness.

The 13 PCR positive patients in the present study were complaining of burning sensation (46.2%), foreign body sensation and lacrimation (30.8% each), and discharge, blurring of vision, photophobia, and pain (7.7% each).

As regards the past history, 37.8 % of patients (34/90) and 4.4% (2/45) of the controls, of the present study, gave past history of similar condition. Among the PCR positive subjects, 61.5% of patients (8/13) and 75% of controls (3/4) did not give past history.

The relatively low percentage of past history given by subjects, in the present study, is probably because infection occurred early in childhood, as suggested by Dawson et al. (1997), or because infection may be inapparent.

As regards family history, in the present study, 8.9% of the patients (8/90), and none of the 45 controls (0%) had family history of a simultaneously complaining household. The rate of detection of *C. trachomatis* among the patients with family history (25%), was higher than among those without family history (14.3%). Although the difference was not statistically significant ($P > 0.05$), still positive family history is related to *C. trachomatis* infection. This rate of detection of *C.*

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trachomatis in subjects with family history of trachoma, in a trachoma endemic community like Egypt, is very low, since trachoma has long been known as an infectious disease transmitted mainly within the household (Barenfanger, 1975; Baily et al., 1989; Dolin et al., 1994 and Dolin et al., 1997).

Munoz et al. (1989) showed that, most transmission is probably by direct spread of infected ocular material from one child to another and that mothers and other women involved in child care also share their children's pool of infection and an increase risk of infection. West et al. (1990) reported that, the presence of a sibling with trachoma greatly increases the risk of a given child also having trachoma.

This low percentage of family history of trachoma among patients and controls of the present study could be due to the mild nature of trachoma infection that may pass completely unnoticed, or that the household may be in the incubation period or late in the disease and passed the severe acute stage but is still shedding *C. trachomatis.*

As regards the use of antimicrobials in the present study, 8.9% of patients (8/90) used antibiotic eye drops 3-4 days before sampling without seeking medical advice. On the other hand, none of the controls was under antibiotic treatment. The antibiotics used were: sulphonamides (62.5%), tetracycline (25.5%), and chloramphenicol (12.5%). *C. trachomatis* was reported to be sensitive to tetracylcines, sulphonamides and erythromycins (Jawetz, 1995; Dawson, 1997). Miller (1992) added to the above mentioned antibiotics rifampicin, while Sandford Smith (1992) reported also chloramphenicol and

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penicillins as being effective against *C. trachomatis* but to a lesser extent.

In the present work, it has been noticed that, none of the PCR positive subjects (patients and controls) was using antibiotics before sampling (the detection rate of *C. trachomatis* in patients and controls using antibiotics was 0%). This indicated that *C. trachomatis* is still sensitive to sulphonamides, tetracyclines and chloramphenicol, and that they are rapidly capable of eliminating the infectious agent.

As regards the stage of trachoma, the 90 patients of the present study were in active trachoma with follicles (TF) (66.7%) stage or in active trachoma intense (TI) (33.3%) stage. It was noticed that the percentage of TF patients decreases with the increase in the age (74.1%, 70%, and 52.2% in groups I, II ,and III respectively), and the reverse with TI.

Trachoma is known to be a disease with allergic element and has long been considered a disease of hypersensitivity and one in which repeated exposure to chlamydial antigen , through either reinfection or persistent infection, is involved in the pathogenesis (Grayston and Wang, 1975).

Since reinfection is very common in trachoma, the older the age the commoner the probability of reinfection. So patients in group III probably suffered more bouts of infection than younger ages. This can in part explain the increase in the severity of active trachoma in the older group.

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The rate of detection of *C. trachomatis* by PCR in the patient group of the present study was 16.7% in TF stage (10/60) and 10% in TI stage (3/10), but the difference was not statistically significant.

Most of the previous studies reported that, the rate of detection of *C. trachomatis by PCR* increases with the increase of the severity of the of trachoma (Bobo et al., 1991; Hayes et al., 1992; Baily et al., 1994b), except Baily et al. (1994a) who reported results similar to that of the present study.

Bacteriological examination for the detection of other bacteria than *C. trachomatis* was done in the present study.

Miller (1992) and Sandford-Smith (1992) mentioned that, pure trachoma is relatively a mild disease, so mild and symptomless, indeed, as to excite little or no attention until perhaps cicatrization manifests itself later in life.

Miller (1992) added that, the course of trachoma is determined mainly by the presence or absence of a complicating infection. He also reported that, in many countries where the disease is endemic, particularly in North Africa and the Middle East, secondary infection (as by *Haemophilus aegyptius*, *Gonococci* or other organisms) results in acute and incapacitating condition leading to gross cicatricial sequalae which often ends in blindness.

Sandford Smith (1992) mentioned that, the same conditions which help trachoma spreading also help spreading

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of other infections specially bacterial conjunctivitis and adenovirus, also resulting in scarring.

In the present study, the rate of isolation of bacteria in conjunctival swabs of patients was 76.6% (69/90), and in the controls it was 66.7% (30/45). Although the rate of isolation of bacteria in conjunctival swabs of the patients was higher than that of the controls, this difference was not statistically significant ($P > 0.05$).

The most commonly encountered organism in the conjunctival of patients and controls, of the present study, was *S. epidermidis* (57.8% and 60% respectively), followed by Diphtheroids (4.4% for both). *Streptococcus viridans* and *Micrococcus* were detected only in the patient group (22% and 3.3% respectively), while *S. aureus* was detected only (2.2%) in the controls.

According to Baily and Scott's (1994), all the detected organisms in the eyes of patients and controls, of the present study, are microbial flora of the conjunctival sac. The authors reported that, *S. epidermidis* and *Lactobacillus species* are the most frequently encountered organisms, and that *S. aureus* is found in less than 30% of people.

Schachter and his colleagues (1988) were able to detect possible bacterial pathogens (*S. aureus, Streptococcus pneumoniae, and Haemophilus aegyptius*) in the clinical specimens of 99 rural Egyptian children with sings of active trachoma. They collected their specimens in winter and reported that, bacterial conjunctivitis in Egypt is common in

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winter. Dawson et al. (1997) reported that, annual epidemics of purulent conjunctivitis in Egypt occur each autumn, and that it spreads by transfer of eye discharges by eye-seeking flies. Mazloum et al. (1986) isolated a *Neisseria* like species from conjunctival cultures in rural Egypt. However, none of these pathogens was detected in the present study.

Absence of bacterial pathogens in the present study can be explained, in part, by the difference in the time of sampling between the present study (in spring and summer) and the other studies. The use of antibiotics before sampling also plays a role. Subjects presenting in the ophthalmology clinic with mucopurulent conjunctivitis are diagnosed without giving much attention to the possibility of a concomitant *C. trachomatis* infection. It may be worthy to do the reverse i.e.; to detect the infection rate with *C. trachomatis* in cases presenting with bacterial conjunctivitis. Care should, also be taken during clinical examination of bacterial conjunctivitis to avoid missing a concomitant *C. trachomatis* infection, so as to give the patient the necessary treatment.

The rate of detection of bacterial growth in the 90 patients of the present study in the PCR positive subjects (76.9%) was nearly the same as for the PCR negatives (76.6%). The most commonly isolated bacteria in both groups was *S. epidermidis*.

Bacterial isolates from swabs of the 13 PCR positive patients were *S. epidermidis, Streptococcus. viridans,* and Diphtheroids, while the only bacteria isolated from the PCR positive controls was *S. epidermidis*.

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Although all bacteria detected, in the eyes of the PCR positive subjects, were normal flora of the conjunctival sac, they may have a role in increasing the severity and sequalae of trachoma by acting as opportunistic pathogens for the already diseased conjunctiva, since damage of the conjunctiva and lacrimal apparatus resulting from *C. trachomatis* infection weakens the defence of the eye (Sandford Smith, 1992).

From the above discussed items it is concluded that, the *C. trachomatis* positive subjects, in the present study, were characterized by being mainly middle age females, living in Cairo, with occupations mainly related to the source of infection (housewives and retired are in contact with children, and physicians in contact with patients' eyes in ophthalmology clinics). Some of the subjects were with past and concomitant family history of trachoma, but none of them was using topical antimicrobial agents, while all who were using topical antibacterial agents were PCR negative indicating their rapid clearing effect on *C. trachomatis*. The most common complaints given by the PCR positive patients were burning, foreign body sensation, and lacrimation, and most of them were presenting in TF stage. Besides, attention should be paid for the presence of other bacteria that may act as opportunistic pathogens for the C. *trachomatis*-infected conjunctiva aggravating the condition.

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Summary and Conclusions

Trachoma is an infectious preventable blinding disease endemic in Egypt since pre-historic time. In order to provide suitable preventive measures against trachoma, the epidemiology of the disease has to be clear.

The aim of the present work was to detect *C. trachomatis* in conjunctival swabs of patients with different stages of trachoma and matched control subjects, and to find out the relation between trachoma and associated bacteria.

The present study included 90 patients and 45 controls of both sexes and different ages (5-72 years). A full clinical sheet was taken from all subjects and they were examined clinically to determine the stage of trachoma. Conjunctival swabs were collected for bacteriological examination, detection of *C. trachomatis* LPS antigen (by EIA), and detection of the DNA of the MOMP gene of *C. trachomatis* (by PCR).

In the present study, the EIA was not sensitive in the detection of *C. trachomatis* against clinical signs and symptoms (0%). On the other hand, PCR was more sensitive

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in detecting *C. trachomatis* in patients with active trachoma (14.4%), and subjects who were subclinically infected in the control group (physicians who were in direct and continuous contact with patients in the ophthalmology outpatient clinic). EIA and PCR were specific (100% and 91.1% respectively).

C. trachomatis was detected by PCR in subjects in the middle age of both sexes (but more in females). Most of them were living in Cairo. As regards their occupation, they were housewives, physicians in ophthalmology clinics, retired subjects, and a worker. Some of them had family history of simultaneously complaining household or past history of trachoma, but none was using topical antibiotics. The patients were complaining of different symptoms of conjunctivitis, with sings of active trachoma (TF or TI).

The bacteriological examination of the conjunctival swabs of all subjects of the present study did not reveal any bacterial pathogen; all detected bacteria were normal conjunctival flora, especially *S. epidermidis,* followed by Diphtheroids. There was no difference in the bacteria isolated from both PCR positive and negative subjects, but these conjunctival flora may act as opportunistic pathogens to the *C. trachomatis* infected conjunctiva in the PCR positive subjects aggravating the clinical condition.

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Conclusion:

PCR is the most sensitive diagnostic method for the detection of *C. trachomatis* in trachoma, and can be a useful epidemiological tool since it is getting cheaper. However, its results are not very satisfactory, and more work and study are needed to improve its sensitivity. Special attention should be made to the presence of associated bacteria that can aggravate the condition.

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الملخص العربى

الرمد الحبيى من الأمراض المتوطنة فى مصر منذ ما قبل التاريخ, و هو مرض معد يؤدى إلى فقدان البصر, وعلى الرغم من كون أعراضه المرضية قد تكون أحيانا بسيطة غير ملحوظة إلا أن تكرار العدوى أو مصاحبة العدوى بنوع آخر من الميكروبات قد يسبب أعراضا و مضاعفات خطيرة.

و يتسبب فى الإصابة بهذا المرض نوع من البكتيريا يسمى الحراشف البرعمية و بخاصة الأنواع المصلية أ و ب و ج.

هدف البحث:

كان الهدف من البحث الكشف عن الحراشف البرعمية فى مرضى الرمد الحبيي فى مراحله النشطة فى المصريين وعزل البكتريا المصاحبة لها.

طريقة البحث:

شمل البحث ٩٠ مريضا ممن شخصوا إكلينيكيا بالرمد الحبيى النشط و ٤٥ من المجموعة الضابطة من كلا الجنسين ممن تتراوح أعمارهم من ٥ إلى ٧٢ عاما. وقد سئل الجميع عن تاريخهم المرضي و تم فحصهم إكلينيكيا و أخذ من كل منهم مسحتان من الملتحمة إحداهما لعزل البكتريا المصاحبة و الأخرى للكشف عن مولد الأنتجن (إليزا) و الحامض النووى (اختيار تفاعل البلمرة المتسلسل PCR) الخاصين بالحراشف البرعمية.

نتائج البحث:

كان اختبار الإليزا غير حساس فى الكشف عن الحراشف البرعمية فى كل من شملهم البحث, أما اختيار تفاعل البلمرة المتسلسل فقد كشف عن الحراشف البرعمية فى مرضى الرمد الحبيى بنسبة ٤,١٤%, و فى غير ذوى الأعراض المصابين من المجموعة الضابطة, وكان كلا الاختبارين متخصصا.

وقد تميز الأشخاص الذين أمكن الكشف عن الحراشف البرعمية فى عيناهم بالآتى:

كانوا من الجنسين إلا أن معظمهم كان من متوسطى العمر من النساء المقيمين فى القاهرة و كانوا إما رّبات مترل أو ممن يعملون فى عيادة أمراض العيون أو المحالين إلى المعاش. وقد ذكر بعضهم سابق إصابته بالرمد الحبيى أو كون أحد أفراد العائلة يشتكى فى الوقت الحاضر من شكوى مماثلة و لكنهم نفوا جميعا تعاطيهم مضادات البكتريا الموضعية. وكان المرضى يعانون من أعراض التهاب الملتحمة و بالكشف الإكلينيكى تبين إصابتهم بالرمد الحبيى النشط (إما فى صورة التهاب حويصلى أو التهاب شديد).

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أما البكتريا التى تم عزلها من المرضى و المعافين فجميعها من النوع غير الضار المتعايش فى ملتحمة العين و أغلبها من المكورات العنقودية البيضاء تليها شبيهات العصيات الدفتيريه وهى نفس ما تم عزله فيمن أمكن الكشف عن الخراشف البرعمية فيهم. و على الرغم من أن هذه البكتيريا التى تم عزلها غير ضارة إلا أنها قد تلعب دورا مرضيا من خلال مهاجمة الملتحمة المصابة بالخراشف البرعمية.

و يستنتج مما سبق أن اختبار تفاعل البلمرة المتسلسل هو أكثر طرق الكشف عن الحراشف البرعمية حساسية فى حال الإصابة بالرمد الحبيبى مما يجعله وسيلة ملائمة للدراسات الوبائية إلا أنه يحتاج لمزيد من الدراسة لزيادة كفاءته.

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الكشف عن الحراشف البرعمية الرمدى الحبيببى
فى مرضى الرمد الحبيببى المصريين

رسالة مقدمة من

الطبيبة / أمانى صالح أحمد عوض

بكالوريوس الطب و الجراحة العامة

ماجستير بكتريولوجى

توطئة للحصول على درجة الدكتوراه فى

العلوم الطبية الأساسية (الكائنات الدقيقة و المناعة)

تحت إشراف

أ.د/ عبلة عبد السلام هارون

أستاذ علم الكائنات الدقيقة و المناعة

أ.د/ منيرة فايق حلمى

أستاذ علم الكائنات الدقيقة و المناعة

كلية الطب - جامعة عين شمس

د/ عفاف شعبان عبد الرحمن

أستاذ علم الكائنات الدقيقة و المناعة المساعد

كلية الطب - جامعة عين شمس

د/ محمد سيد سلامه

أستاذ علم البيولوجيا الجزيئية المساعد

كلية العلوم - جامعة عين شمس

د/ ممدوح حمدى الكفراوى

مدرس طب و جراحة العيون

كلية الطب - جامعة عين شمس

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