



Research Paper

POLYMERASE CHAIN REACTION (PCR) A GENETIC TOOL FOR ASSESSING *CHLAMYDIA* *TRACHOMATIS* IN MID-STREAM URINE OF ASYMPTOMATIC FEMALES

Otoikhian C S O^{1*}, Okoror L E¹ and Ekakitie A O*Corresponding Author: **Otoikhian C S O**, ✉ megabrosy2k@yahoo.co.uk

Mid-stream urine of asymptomatic females was used for this study using Polymerase Chain Reaction (PCR). This study screened a total of fifty seven (57) mid-stream urine samples using PCR. The urine samples were aseptically obtained from patients without symptoms of urinary tract infection (UTI); of the fifty (57) samples 30 (52.63%) were positive while 27 (47.37%) were negative. Hence this concluded that there is a high prevalence of *Chlamydia trachomatis* (about 60% of 70%) in asymptomatic females.

Keywords: Polymerase Chain Reaction, Mid-Stream Urine, *Chlamydia trachomatis*

INTRODUCTION

The word chlamys is a Greek word for “cloak draped around the shoulder”. This describes how the bacterium is “dropped” around the infected cell’s nucleus (Mandell *et al.*, 2000). *Chlamydia* infection is a sexually transmitted disease (STI) in humans caused by the bacterium *Chlamydia trachomatis*. This bacterium is found only in humans and the infection is caused by any species belonging to the bacterial family chlamydiaceae.

Scientific Classification

Domain: Bacteria

Phylum: Chlamydiae

Class: Chlamydiae

Order: Chlamydiales

Family: Chlamydiaceae

Genus: *Chlamydia*Species: *C. Trachomatis*

Chlamydia trachomatis is an obligate intracellular human pathogen of the eukaryotic cell found in the leukocytes and consist of minute particles. They are non-motile, coccoid, ranging from 0.2 to 1.5 µm. They are exquisitely adapted for an intracellular survival and intracellular growth (Okoror *et al.*, 2007). They lack the mechanisms for the production of metabolic energy and cannot synthesize ATP. As a result of this defect, they

¹ Novena University, College of Natural and Applied Sciences, Department of Biological Sciences, PMB 2, Kwale-ogume, Delta State, Nigeria.

are restricted to an intracellular existence (Singleton, 1997, Mandell *et al.*, 2000, Jawetz *et al.*, 2004), where the host cell furnishes energy rich intermediates. Because of their smallness and their inability to multiply outside a susceptible host and their filterability, they were once thought to be viruses and were referred to as large viruses (Okoror *et al.*, 2007). However, they have many common characteristics with other bacterium which is why they are now thought to be special kind of bacteria (Okoror *et al.*, 2007). *C. Trachomatis* is a gram negative bacteria, therefore its cellwall component restrain the counter-strain safranin and appear pink under a light microscope (Budai, 2007). These characteristics are; firstly they contain both DNA and RNA, secondly they divide by binary fusion, thirdly their cell envelope resembles those of other Gram negative bacteria. That is, they have cellwall which lack muramunic acid, fourthly they contain ribosomes similar to those of other bacteria, lastly they are susceptible to various kind of antibiotics; for example penicillin that inhibit cell wall formation and other drugs that inhibit transpeptidation of bacteria peptidology can (Okoror *et al.*, 2007, Davis *et al.*, 1980, Singleton, 1997, Jawetz *et al.*, 2004).

Chlamydia can be seen under high power microscope and the genome is about one third of the size of the *Escherichia coli* genome. Their G+C (Guanosine + Cytosine) ratio is 41-44% (Nester *et al.*, 2001). The organism is extremely temperature sensitive and must be refrigerated at 40 °C as soon as a sample is obtained (Jaschek *et al.*, 1993).

Chlamydia trachomatis has a high degree of host specificity, being an intracellular pathogen (i.e the bacterium lives within human cells) and causes numerous disease state in both men and

women (Ryan *et al.*, 2004). Both sexes can display gonococcal mucopurulent, proctitis (rectal disease and bleeding) trachoma, and infertility. In men, the bacteria can cause prostatitis, epididymitis, salpingitis, mucopurulent cervicitis, endometritis inclusion conjunctivitis, new born pneumonia, perihepatitis, (fritz-high Curtis syndrome) and later post partum endometritis. In women cervicitis, pelvic inflammatory disease (PID), ectopic pregnancy and acute or chronic pain are frequent. It has been found from studies reported in Italy, Sweden, and USA that about 60% of 70% of *Chlamydia trachomatis* positive cases are asymptomatic (Freund *et al.*, 1992, Stray 1992). It was also reported that the risk is higher for women under 25 years of age who had sex before the age 18 (Freund *et al.*, 1992), this accounts for why samples for asymptomatic females were used for this study. It has also been reported that there is a 5% prevalence among pregnant and non pregnant women and their spouses pre and antenatal clinic in the college of medicine of the university of Lagos Nigeria (Okoror *et al.*, 2010). *Chlamydia trachomatis* could cause pharyngitis in children and new born acquire the infection turning passage through an infected birth canal (hollow, 1985), *Chlamydia trachomatis* similar serotypes has been isolated from the eye of a baby, the vagina of the mother and urethra of the father and the prevalence varies with maternal age (Okoror *et al.*, 2007).

Chlamydia trachomatis is one of the three species (also including *Chlamydia psittaci* and *Chlamydia pneumoniae*) in the genus *Chlamydia*. *Chlamydia psittaci* species causes endemic avian Chlamydiosis, epizootic outbreaks in mammals and respiratory psittacosis in humans. It also causes Orithosis, a Zoonosis which is a pneumonia illness contracted by people working

with parrots and other wild domestic birds (Talaro and Talaro, 1996; and Mandell *et al.*, 2000). Symptoms mimic those of influenza and pneumococcal pneumonia (Nester *et al.*, 2001). Early manifestation are fever, chills, frontal headache and muscle ache and later manifestation are coughing and lung consolidation; when unchecked, infection can lead to systemic complication involving the brain, meninges, heart or liver (Talaro and Talaro, 1996). *Chlamydophila psittaci* is transmitted by inhalation contact or ingestion among birds and to mammals. Psittacosis in birds and in humans often starts with flu-like symptoms and becomes a life-threatening pneumonia (Talaro and Talaro, 1996; and Mandell *et al.*, 2000). It is equally a mild illness in young adults, though it can cause severe reacting in asthmatic patients that is responsible for increase rates of death in this group (Talaro and Talaro, 1996; and Jawetz *et al.*, 2004).

The three species that infect humans have been characterized in Table 1.

Chlamydia trachomatis can be differentiated from *Chlamydia pneumonia* in that *Chlamydia trachomatis* is sensitive to sulphonamides and form inclusion bodies which contain glycogen which allows detection of agent by iodine staining of infected cells cultures. While *Chlamydia psittaci* and *Chlamydia pneumonia* glycogen as seen in Table 1 in addition, *Chlamydia trachomatis* produce single inclusion that displace the nucleus and do not displace it (Davis *et al.*, 1980). The mode of transmission is different among the three species but all can cause systemic disease by haematogenous spread. Respiratory secretions transmit *C. pneumonia* from human to human, whereas infected birds transmit *C. psittaci* to human via the respiratory route through the direct contact or aerosolization.

Therefore, this study was designated to access *Chlamydia trachomatis* in asymptomatic females using Polymerase Chain Reaction using mid-stream urine.

Table 1: Characteristics of *Chlamydia*

	<i>Chlamydia trachomatis</i>	<i>Chlamydia pneumonia</i>	<i>Chlamydia psittaci</i>
Inclusion Morphology	Round, vascular	Round, dense	Large, variable shape,dense
Glycogen in Inclusion	Yes	No	No
Elementary body Morphology	Round	Pear shaped, round	round
Susceptible to Sulphonamides	Yes	No	no
DNA homology to <i>Chlamydia pneumonia</i>	<10%	100%	<10%
Plasmid	Yes	No	yes
Natural host	Humans	humans	birds
Mode of Transmission	Person to person Mother to infant	Airborne, person to person	Airborne, birds excreta to humans
Major diseases	Trachoma, STDs, infant pneumonia, cymphogranuloma Venereum	Pneumonia, bronchitis, Pharyngitis, sinusitis	Psittacosis, pneumonia, fever of unexplained origin.
Source: Jawetz <i>et al.</i> (2004)			

MATERIALS AND METHODS

Materials

The materials used for this research work were;

- Urine samples (mid-stream urine containing the template DNA)
- Polymerase Chain Reaction (PCR)
- Eppendorf tubes
- PCR machine
- Primers (CT1, CT2, CT3, CT4) dNTP (deoxynucleoside triphosphate) containing 12.5µl each of 100mM dGTP, 100mM dATP, 100mM dTTP and 100mM dCTP mix
- 10x PCR reaction buffer (contains 100mM KCl)
- 100mM Tris-HCl {Ph 8.3}
- 15mM MgCl₂)
- Taq (Thermophilus aquaticus) polymerase
- Agarose gel
- Universal containers
- Micropipette
- Micropipette tips
- Microcentrifuge machine
- Vortex machine
- Electrophoresis tank
- UV box
- Ethidium bromide (EtBr)
- Sensitive balance
- 1 x TAE buffer
- Gel loading buffer
- Boiling water bath
- Ice (to minimize the chance of primer binding to the DNA template and to prevent the polymerase from working prior to the first denaturing step it is useful to keep the vials in ice while pipetting the ingredients of the reactions).

Methods

Collection of Samples

Early morning mid-stream urine were collected from asymptomatic females who visited the hospital for routine medical check-up, using a clean, dry, and disinfectant free, leak proof universal containers. Outmost care was taken to avoid contamination. They were then labelled indicating the age, time, date of collection etc. The urine samples were refrigerated at 4p c and transported to the microbiological laboratory for analysis.

Examination of Samples

Each of the samples was shaken vigorously to homogenize the urine. A micropipette inserted with sterile micropipette tip was used to measure 1000µl of urine sample and was then dispensed into an Eppendorf tube, which was then labelled to correspond with that on the universal container. The same procedure was repeated for the remaining samples using different micropipette tip to dispense each of the samples. They were then centrifuged using a microcentrifuge machine set at a high speed of about 16000 rpm for 10minutes. After centrifuging the supernatants were decanted to obtain the pellets (residue) which was then redissolved by tapping the bottom of the tube.

The components for PCR where blended in 100 µl portions as follows;

Component	100 µl Portion
10 mm dNTP mix	4 µl
10x PCR buffer plus Mg	10 µl
30 µm primer 1	1 µl
30 µm primer 2	1 µl
200 ng/µl of urine sediment which contain the Template DNA	1 µl
Taq polymerase	0.5 µl
MQ water	82 µl

Then the tubes were spined briefly to collect all droplets. The tubes were loaded into the PCR machine and program was set up as follows:

Preparation of Gel Electrophoresis

Using a sensitive balance, 1g of Agarose was added to 100 ml 1XTAE buffer in a 250 -500ml conical flask.

Place the flask on a boiling water bath to dissolve the agarose and allow the solution to cool to about 50°C or until it was safe enough to be hand-held.

Add 30 µl of a 2.5 mg/ml Ethidium bromide solution.

Pour the molten agarose into the gel holder ensuring that the comb is in place. The gel was allowed to set at room temperature.

Add 5µl of the gel loading buffer to the DNA digest and mix.

I mapped my wells on record and followed plan strictly.

Vortex briefly and the new mixture was placed into the wells.

Marker DNA containing DNA fragments of known sizes were loaded along sides the samples as control.

The order in which the samples were loaded was recorded.

Connect the electrodes to the electric power source and ran the gel at 100V for 1 h. At the of 1 h, the agarose gels were viewed under the U.V box and the photograph of the pattern of DNA movement on agarose were taken.

RESULTS

Results on age distribution of patients infected with chlamydia were shown on Table 3.

It reveals different age distribution. A total of fifty seven (57) mid-stream urinary samples were collected from female patients who where between the ages of 18 and 42 years old. A higher number of positive samples compared to the

Table 3: Result on Age Distribution of Patients Infected With Chlamydia

Age (yrs)	Number of Positive Females	Number of Negative Females	Total Number of Samples	Total Percentage of Positive (%)	Total Percentage of Negative(%)
18-20	6	6	12	50	50
21-23	13	8	21	61.90	38.09
24-26	2	1	3	66.67	33.33
27-29	0	0	0	0	0
30-32	6	4	10	60	40
33-35	0	4	4	0	100
36-38	1	1	2	50	50
39-41	1	3	4	25	75
42-44	1	0	1	100	0
Total	30	27	57		

negative samples; 30(52.63%) positive and 27(47.37%) out of 57 samples for *Chlamydia trachomatis*. Although sample were collected from patients ranging from age 18-42.

Positive results showed specific age range showing varying number of infection rates and/or levels. Ages 21-23 had the highest number of infection which was 13(24.70%) followed by ages 18-20 which was 6(11.40%), 30-32 which was also 6(11.40%), 24-26 had values of 2(3.80%), 36-38, 39-41, 42-44, 27-29, and 33-35 which were 1(1.90%), 1(1.90%), 1(1.90%), 0(0) and 0(0) respectively.

Negatively results showed a higher percentage at age 21-23 which was 8(16.89%) followed by 18-20 which was 6(12.67%), 30-32 had 4(8.44%), 33-35 which was also 4(8.44%), 39-41, 36-38, 24-26, 42-44, 27-29 had 3(6.33%), 1(2.11%), 1(2.11%), 0(0) and 0(0) respectively.

Note that, in both positive and negative percentages, age 27-29 had 0(0) percent although. This was because no sample was collected from patients within this age range.

DISCUSSION

Chlamydia trachomatis has been reported by various researchers to be the major cause of sexually transmitted diseases and studies reported in Italy, Sweden and USA have shown that about 60% of 70% of *Chlamydia trachomatis* positive cases are asymptomatic (Okoror et al., 2007). *Chlamydia trachomatis* is also responsible for infection on female genital tract (Paukku et al., 1997). They are found in the cervix in relatively high percentage of women around the world (Oh et al., 1996), and often not associated with any symptoms (Talaro and Talaro, 1996; and Paukku et al., 1997), with rates as high as 2% of pregnant

women having routine examination are reported (Davis et al., 1980; and Rews et al., 1997). This is one of the reasons why asymptomatic females who visited the hospital for various reasons asides urinary tract infection related cases were used for this test to ascertain the prevalence rate in our local community. Pelvic Inflammatory Disease (PID) is an infection of the fallopian tubes, ovaries and/or uterus characterized by lower abdominal pain, painful sex, increased pain during menstruation, fever and chills. Scarring from PID can cause infertility and ectopic pregnancy (Oh, 1997; and Okoror et al., 2007).

The result of this study showed a high prevalence rate of *Chlamydia trachomatis* infection between the ages of 21-23 which corresponds to reports that the risk is higher for women under 25 years of age who had sex before the age of 18 (Okoror et al., 2007). It has been estimated that *Chlamydia trachomatis* is carried in the reproductive tract of up to 10% of all people with even higher rates among the promiscuous (Talaro and Talaro, 1996). According to my study, at age 21-23, the total negative percentage was relatively high. This shows that women who start having sex at age 21-23 were less prompt to the infection *Chlamydia trachomatis*. Statistics has shown that one *Chlamydia* infection can lead to an 80% chance of infertility (Domets, 1998; Okoror et al., 2006). Therefore, control is by reduction in promiscuity, use of condoms and early diagnosis and treatment of infected individuals (Prescott et al., 2005)

In this study urine samples were screened for *Chlamydia trachomatis* using Polymerase Chain Reaction (PCR) techniques. Urine was used because it is a non-invasive source thereby making collection easier and facilitates its

acceptance by patients. And in cases where samples require transportation to the laboratory, urine is also relatively stable to refrigeration temperature (Jaschek *et al.*, 2001). The specificity and sensitivity of PCR technique is very high because it saves time, rapid and easily regulates its water bath temperature and it is also cost effective.

CONCLUSION AND RECOMMENDATION

This study establishes the prevalence rate of *Chlamydia trachomatis* in asymptomatic females visiting the hospital for various reasons.

This study would help clinicians in proper administration of treatment in such asymptomatic cases.

It would also help government to decide whether to embark on community screening of Chlamydia, which so far one may refer to as "silent endemic" because in women, it may not cause any symptoms in 75% of cases, (Okoror *et al.*, 2007).

This study reaffirms the use of PCR and mid-stream urine sample in diagnosis of *Chlamydia trachomatis* since it is a cost effective, specific and sensitive technique.

I recommend that more research work should be conducted on *Chlamydia trachomatis* in mid-stream urine of asymptomatic females using Polymerase Chain Reaction (PCR), and emphasis should be made on females within the ages of 27-29 since samples of this age range was not included in this research work. Recent phylogenetic studies have revealed that *Chlamydia* shares a common ancestor with modern plants and retains unusual plant-like traits (both genetically and physiologically). In particular,

the enzyme *L,L-diaminopimelate aminotransferase*, which is related to lysine production in plants, is also linked with the construction of chlamydia's cellwall. This unexpected discovery may help scientists develop new treatment avenues: if scientists could find a safe and effective inhibitor of *L,L-diaminopimelate aminotransferase*, they might have a highly effective and extremely specific new antibiotic against chlamydia.

Since little reports exists regarding the relationships between *Chlamydia trachomatis* and infertility cases (although they appear to show no symptoms) generally in this part of the world (Okoror *et al.*; 2007).

REFERENCES

1. Belland R, Ojcius D and Byrne G (2004), "Chlamydia", *Nat Rev Microbiol.*, Vol. 2, No. 7, pp. 530-531.
2. Budai I (2007), "Chlamydia Trachomatis: Milestones in Clinical and Microbial Diagnostics in the Last Hundred Years: A Review", *Acta Microbiologica et immunologica Hungarica*, Vol. 54, No. 1, pp. 5-22.
3. Buimer M, Van Doorum G J, Cling S, Peerbooms D G, Plier P K and Ram D (1996), "Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by Ligase Chain Reaction-Based Assays with Clinical Specimens from Various States.
4. CDC (1993), "Recommendation for the Prevention and Management of *Chlamydia trachomatis* Infections", MMWR42 (RR-12).
5. Chernesky MA, Jang D, Sellors J, Coleman P, Bodner J and Hrusovsky I (1995), "Detection of *Chlamydia trachomatis* Antigens in Male Urethral Swabs and Urine

- with Microparticle Enzyme Immunoassay”, *Sexually Transmitted Disease*, Vol. 22, pp. 55-59.
6. Chernesky M A, Lee H, Schachter J, Burczak J D, Stamm W E and McCormack, W M (1994), “Diagnosis of *Chlamydia trachomatis* Urethral Infection in Asymptomatic men by Testing First-void Urine in a Ligase Chain Reaction Assay”, *Journal of Infectious Disease*, Vol. 170, pp. 13087-11.
 7. Davis D B, Dulbeco R, Essein N H and Ginsberg H (1980), *Chlamydia in: Microbiology: Including Immunology and Molecular Genetics*, 3rd Edition, Harper and Row Publishers, Philadelphia, pp. 776-784.
 8. Domeika M A, Bassiri M and Mardh P A (1994), “Diagnosis of Genital *Chlamydia trachomatis* Infection in Asymptomatic Males by Testing Urine by PCR”, *Journal of Clinical Microbiology*, Vol. 23, pp. 402-406.
 9. Freund K M, Buttlar C A, Giampaolo C, Philips R S and Aronso Moskowitz M A (1992), “The Use of Cervical Cytology to Identify Women at Risk for Chlamydia Infection”, *Am. J. Prev. Med.*, Vol. 8, pp. 292-297.
 10. Gaydos C A, Ngeow Y F, Lee H H, Canavaggio M, Welsh L E and Johansson J (1996), “Urine as a Diagnostic Specimen for the Detection of *Chlamydia trachomatis* in Malaysia by Ligase Chain Reaction”, *Sexually Transmitted Diseases*, Vol. 23, pp. 402-406.
 11. Gene M and Mardh A (1996), “A Cost Effectiveness Analysis of Screening and Treatment for *Chlamydia trachomatis* Infection in Asymptomatic Women”, *Annals of International Medicine*, Vol. 124, pp. 4-7.
 12. Hatch T P (1996), “Disulfide Cross Linked Envelope Proteins; The Functional Equivalent of Peptidoglycan in Chlamydia?”, *Journal of Bacteriology*, Vol. 178, pp. 1-5.
 13. Higgins S P, Klapper P E, Struthers J K, Bailey A S, Gough A P and Moore R (1998), “Detection of male genital infection with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using an automated multiple PCR system (Cobas Amplicor)”, *International Journal of Sexually Transmitted Diseases, AIDS*, Vol. 9, pp. 21-24.
 14. Hills S (1995), “Impact of a Comprehensive Chlamydia Prevention Program in Wisconsin”, *Fam. Plann. Pers.*, Vol. 27, pp. 108-111.
 15. Hollow F C (1985), “Community Based Action for the Control of Trachoma”, *Rev. Infect. Dis.*, Vol. 7, p. 777.
 16. Howell M R, Quinn T C, Brathwaite W and Gaydos C A (1998), “Screening for *Chlamydia trachomatis* in Asymptomatic Women Attending Family Planning Clinics. A Cost Effectiveness of DNA Amplification Assays”, *Sexually Transmitted Diseases*, Vol. 25, pp. 108-117.
 17. Howell M R, Quinn T C and Gaydos C A (1998), “Screening for *Chlamydia trachomatis* in Asymptomatic Women Attending Family Planning Clinics, A Cost Effectiveness Analysis of Three Strategies”, *Annals of International Medicine*, Vol. 128, pp. 227-284.
 18. Jawetz A, Brooks G F, Butel J S and Morse S A (2004), *Chlamydia in Medical Microbiology*, 23rd Edition, McGraw-Hill Publishers, pp. 357-363, New York.
 19. Mandell G L, Bunnett J E and Dolin R (2000),

- "Chlamydia Diseases Vol. 2 Section C, in Mandell Douglas and Bunnetts's Principles of Infectious Diseases, 5th Edition, Churchill Livingstone, Edinburgh.
20. Marrazo J M, Celeum C L, Hillis S D, Deliste S and Handsfield H H (1997), "Performance and Cost Effectiveness of Selective Screening Criteria for *Chlamydia trachomatis* Infection In Women. Implications for a National Chlamydia Control Strategy", *Sexually Transmitted Disease*, Vol. 24, pp. 131-141.
21. Morre S A, Van Valkangoed I G M and De Jong A (1999), "Mailed Home Obtained the Specimen; A Reliable Screening Approach for Asymptomatic *Chlamydia trachomatis* Infection", *Journal of clinical Microbiology*, Vol. 14, pp. 676-680.
22. Nester E W, Anderson D G, Robert C E, Pearsall N N and Nester M T (2001), "Chlamydia Genital System Infections", in *Microbiology: A Human Perspective*, 3rd Edition, McGraw-Hill, New York, pp. 646-648.
23. Nettleman M D and Bell T A (1991), "Cost Effectiveness of Prenatal Testing for *Chlamydia trachomatis*", *American Journal of Obstetrics and Gynecology*, Vol. 164, pp. 1289-1294.
24. Ngondi J, Onsarigo A, Matthews F et al. (2006). "Effect of 3 years of Surgery, Antibiotics, Facial Cleanliness, And Environmental Change (SAFE) Strategy For Trachoma Control in Southern Sudan: A Cross-sectional Study", *Lancet*, Vol. 368, No. 9535, pp. 589-595.
25. Oh M K, Richey C M, Pale M S, Brown P R and Hook E W (1997), "High Prevalence of *Chlamydia trachomatis* Infections in Adolescent Females not Having Pelvic Examinations; Utility of PCR based Urine Screening in Urban Adolescent Clinic Setting", *Journal of Adolescent Health*, Vol. 21, pp. 80-86.
26. Oh M K, Cloud G A, Fleenor M, Sturdevant, M S, Nesmith J D and Feinstein RA (1996), Risk for Gonococcal and Chlamydia Cervicitis in Adolescent Female; Incidence and Reoccurrence in a Prospective Cohort Study", *Journal of Adolescent Health*, Vol. 18, pp. 270-275.
27. Okoror L E, Omilabu S A, Fadojutimi J and Nsongkhai V (2000), "Seroepidemiological Survey of Chlamydia in Patients Attending Pre- and Post-natal Clinic at the College of Medicine of the University of Lagos Nigeria", in *Book of Abstract of the 24th Annual Conference of the Nigerian Society for Microbiology*.
28. Okoror L E, Agbonlahor D E, Esumeh F I and Umolu P I (2007), "Prevalence of Chlamydia in Patients Attending Gynaecological Clinics in Southern Eastern Nigeria", in *African Health Science*, Vol. 7, pp. 18-24.
29. Paukku M, Puolakhainen M, Apter D, Hirvonen S and Paavonen J (1997), "First Void Urine Testing for *Chlamydia trachomatis*: by Polymerase Chain Reaction in Asymptomatic Women", *Sexually Transmitted Diseases*, Vol. 24, pp. 343-346.
30. Prescott L M, John P H and Donald A K (2005), "Gram Negative Bacteria", in *General Microbiology*, 6th Edition, McGraw – Hill, pp. 884-887, New York.

-
31. Resnikoff S, Pascolini D and Etya'ale D et al. (2004), "Global data on Visual Impairment in the Year 2002". *Bull World Health Organ*, Vol. 82, No. 11, pp. 844-851.
 32. Resnikoff S, Pascolini D and Etya'ale D et al. (2004), "Trachoma", *Lancet*, Vol. 362, No. 9379, pp. 223-229 <http://linkinghub.elsevier.com>
 33. Rewe W W, Lee H H, Roden W J and Mott C W (1997), "Detection of Genitourinary Tract Chlamydia Trachomatis Infection in Pregnant Women by Ligase Chain Reaction Assay", *Obstetrics and Gynecology*, Vol. 89, pp. 556-560.
 34. Singleton P (1997), "Appendix Mini Description of Some Genera, Families, Orders and Other Categories of Bacteria" in *Bacteria In Biology, Biotechnology and Medicine*, 4th Edition, John Wiley and sons, Ltd., New York, pp. 368-378.
 35. Stry A (1992), "Public Health Aspects with Emphasis on European Perspective", in *Proceedings of the European society for the Chlamydia Research Asculapio*, Stoccoloma, pp. 275-277.
 36. Stry A, Choueiri B, Horting-Muller I, Halisch P and Teodorowicz L (1996), "Detection of *Chlamydia trachomatis* in Urethral And Urine Samples from Asymptomatic Male Patients by the Polymerase Chain Reaction" *European Journal of clinical Microbiology of Infectious Diseases*, Vol. 15, pp. 465-471.
 37. Steingrimsson O, Olatsson J H, Thorafinnsson H, Ryan R W, Johnson R B and Tilton R C (1994), "Single Dose Azithromycin Treatment of Gonorrhoea and Infections Caused by *Chlamydia trachomatis* and *Ureaplasma urealyticum* in men", *Sexually Transmitted Diseases*, Vol. 21, pp. 43-46.
 38. Stine G J (1992), *The Biology of Sexually Transmitted Diseases*, A Wiley Medical Publication, New York, p. 24.
 39. Talaro K and Talaro A (1996), "Chlamydia", in *Foundation in Microbiology*, 2nd Edition, McGraw-Hill Publisher, pp. 736-742, New York.
 40. Thylefors B, Négrel A D, Pararajasegaram R and Dadzie K Y (1995), "Global Data on Blindness", *Bull World Health Organ*, Vol. 73, No. 1, pp. 115-121.
 41. Van Valkengoed I G M, Postma M J and Morre S A (2004), "Cost Effectiveness Analysis of Population Based Screening Program For Asymptomatic Chlamydia Trachomatis Infections in Women by Means of a Home Obtained Urine Specimens", *Sexually Transmitted Infection*, Vol. 77, pp. 276-282.
 42. Wagenlehner F M, Naber K G and Weidner W (2006), "Chlamydial Infections and Prostatitis in Men", *BJU Int.*, Vol. 97, No. 4, pp. 687-690, <http://www.blackwell-synergy.com>
 43. Witkin S S, Inghs S R and Polaneczky M (1996), "Detection of *Chlamydia trachomatis* and *Trichomonas vaginalis* by Polymerase Chain Reaction in Intraital Specimens from Pregnant Women", *American Journal of Obstetrics and Gynecology*, Vol. 175, pp. 165-167.
-