



TO STUDY A NOVEL CHSP 60 (CHLAMYDIAL HEAT SHOCK PROTEIN) ANTIBODY AS A POTENTIAL MARKER FOR C.TRACHOMATIS INDUCED COMPLICATIONS ESPECIALLY INFERTILITY.

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Abstract:

Most genital Chlamydial infections tend to be asymptomatic or present with minimal symptoms coupled with the occurrence of severe sequela in untreated patients makes the laboratory evaluation of great importance in the diagnosis of the disease. Chlamydial heat shock protein 60 (Chsp60) may be an important antigen involved in the immunopathogenesis of *C. trachomatis* associated complications especially in chronic infection.

The present study was designed to determine the prevalence of anti Chlamydial heat shock protein 60 (Chsp60) antibodies in women attending a OBGY outpatient department in a tertiary care hospital in Navi Mumbai and whether it could be used as a potential marker for detection of *C.trachomatis* induced complications A total of 100 female patients were enrolled for the study. Blood/serum samples were collected from all the patients. The patients were investigated for the presence of Anti Chsp60 antibody of *Chlamydia trachomatis* with the help of the Enzyme Linked Immunosorbant Assay (ELISA). Our study showed that use of Anti Chsp60 antibody could be considered as a potential marker for detection of *C.trachomatis* induced complications.

Keywords C.trachomatis, Chlamydial heat shock protein 60 (Chsp60), Enzyme Linked Immunosorbant Assay (ELISA).

Introduction

Chlamydia trachomatis infections account for some of the most prevalent sexually transmitted bacterial infections recognized in the world, with 80 to 90 million new chlamydial infections being detected annually worldwide.^[1] India alone, a high chlamydial prevalence rate (up to 30%) among symptomatic women has been reported.^[2] Chlamydial infection in females has been associated with several complications such as infertility, ectopic pregnancy, pelvic pain and pelvic inflammatory disease (PID).^[3] Chlamydial infections tend to be usually mild or asymptomatic which are generally self-limiting, but repeated or persistent infections can cause severe damage to the inflamed tissues.^[4]

Chlamydial infections which are asymptomatic, not recognized or insufficiently treated may lead to persistence of the pathogens. It is at this point that Chlamydial heat shock protein 60 (cHSP60) is continuously over expressed such that the human immune system has to deal with this foreign antigen. Both cellular and humoral immune responses are triggered and upregulated. Furthermore the human heat shock protein (hHSP60) is also over expressed in order to protect the human cells from chlamydial attacks. The antibodies that are directed towards the

conserved epitopes of the cHSP60 may than cross-react with the conserved regions of the hHSP60 which could be responsible for autoimmune responses and consequent complications.^[5]

Heat shock proteins

Heat shock proteins (hsps) are among the most abundant proteins in nature and are highly conserved amongst both eukaryotes and prokaryotes. The heat shock response is an important survival mechanism that safeguards the cell or microbe from conditions of stress.

Heat shock proteins (HSPs) are highly conserved cellular stress proteins; their amino acid composition has not changed very much during evolution and they are expressed in both prokaryotes and eukaryotes with pronounced homology.^[5]

When cells infected with *C.trachomatis* are subjected to heat shock there is an increased synthesis of at least 20 heat shock proteins (HSPs), the most extensively studied were HSP60 (approximate molecular weight 57kDa), and HSP70 (approximate molecular weight 75kDa). Both Chlamydial cHSP60 and cHSP70 remain confined inside the inclusion during the path of the chlamydial developmental cycle in vitro.

Both Chlamydial cHSP60 and cHSP70 are constitutively expressed, and cHSP60 transcription is upregulated under heat stress, in IFN- γ treated cells, penicillin exposed cells, and under iron limiting conditions. It has been suggested that the chlamydial dnaK (encoding HSP70) and groE (encoding HSP60) operons may be regulated by a mechanism similar to that described in *Bacillus subtilis* in which, when the transcription of HSP60 is turned off, the dnaK operon is activated, and if HSP60 is over produced there is decreased expression of the dnaK operon. Chlamydial HSP70 is found in both elementary and reticulate bodies as a dithiothreitol extractable protein, and shares 46% amino acid sequence identity to the mammalian homologue. Recent studies have suggested that membrane associated cHSP70 may influence entry into host cells. It has also been shown that polyclonal antibody to recombinant chlamydial HSP70 can neutralize chlamydial infectivity in vitro.^[6]

Chlamydial cHSP60, like cHSP70 is found in both developmental forms, and is extractable with sarkosyl or dithiothreitol from elementary bodies. Within the Chlamydial genus cHSP60 amino acid sequence homology is greater than 80%, but possibly more importantly the Chlamydial cHSP60 protein shares 48% homology to human HSP60.

Chlamydial heat shock proteins cHSP60 and cHSP70 have both been shown to be highly immunogenic during the course of natural infection.

In women the presence of anti-chlamydial cHSP60 antibodies has been correlated with pelvic inflammatory disease (PID), tubal factor infertility, and ectopic pregnancy. In a recent study immunity to a specific chlamydial cHSP60 epitope was associated with autoimmunity to human HSP60, and with a history of two or more spontaneous abortions.^[7] Conversely, anti-chlamydial cHSP70 antibodies have been associated with protective immunity in women against ascending infection and tubal disease.

Immunopathology

It is believed that immune pathogenesis is the underlying mechanism of Chlamydial disease but it is still not clear if specific antigens coupled with cellular immune responses are directly responsible (eg. delayed-type hypersensitivity [DTH]), or if other inflammatory or immune regulatory processes contribute to disease. Chlamydial infection has been shown to evoke a DTH response, and that re-infection is required to induce disease. Evidence suggests that Chlamydial heat shock protein 60 (cHSP60) may be the mediator of pathogenesis. Many studies have shown an association between the detection of anti-chlamydial cHSP60 antibodies and pelvic inflammatory disease, tubal factor infertility, ectopic pregnancy, and trachoma.^[8]

It has been proposed that increased levels of chlamydial HSP60 production may result in the initiation of self anti-HSP60 antibodies inducing autoimmunity which ultimately results in tissue damage and scarring.

Thus this study was undertaken to evaluate the role of cHSP 60 (Chlamydial Heat Shock Protein 60) antibody as a potential marker for *C.trachomatis* induced complications.

MATERIALS AND METHODS

Type of study: Prospective and Experimental

Period of study: 3 years (Aug 2014 to July 2017)

Place of study: Hospital Microbiology Laboratory (NABL Accredited), MGM Medical College and Hospital, Navi Mumbai and Department of Infectious Disease & Biology, ICMR-National Institute of Research in Reproductive Health (NIRRH), Parel, Mumbai.

Ethical Aspects: The study was submitted to research and ethics committee of the institute and approval was obtained on 27th March 2015.

Sample size: 100 samples were taken and analyzed.

Specimens: Blood/Serum.

Selection of Cases:

Inclusion criteria – Women attending the OBG Dept. of MGM Hospital, Navi Mumbai with an age limit of 16-45 years. The group comprised of women with histories of Spontaneous Abortions, Infertility, Lower Genital Tract Infections and Pregnant Women attending ANC

Exclusion criteria- Women having per vaginal bleeding and or on recent antibiotic use. HIV positive subjects.

A total of 100 subjects were included in this study over the time period from April 2015 to May 2017. The clinical history and presentation in each group was as follows:

- 1. Bad Obstetric History (BOH):** Women having history of one, two or more than two spontaneous abortions or Recurrent Spontaneous Abortion (RSA), those operated for ectopic pregnancies and pre term delivery cases were included in this group.
- 2. Infertility:** Women who were living with their husbands but not conceiving and desirous of having a child were enrolled in this group. Infertile women were subdivided into Primary/1st degree Infertile i.e. who did not conceive and Secondary/2nd degree Infertile having one full term delivered child but not conceiving subsequently.
- 3. Lower Genital Tract Infections (LGTI):** Women with signs and symptoms of cervicitis and vaginitis on per speculum examination or having complaints like burning micturition, leucorrhoea, abdominal pain and lower backache were included in this group.

4. **Asymptomatic/Control:** Women attending Ante Natal Care(ANC) clinic without any of the above mentioned conditions and with a confirmed pregnancy of more than 12 weeks gestation were enrolled in this group. In the ANC/Control group the gestational period of the pregnant women varied from 2 to 4 months.

Specimen Collection and Processing:

Collection of Blood/Serum

Blood was collected by venepuncture and placed in Red top (Plain) blood collection tubes. Serum samples were then stored at -25°C until they were processed. Thawed samples were mixed well before antibody testing.

For detection of serum IgG antibodies to *Chlamydial* heat shock protein (cHSP 60)(Medac Diagnostika, GMBH, Germany)

Test Principle

The plate is coated with recombinant heat shock protein 60 from *Chlamydia trachomatis*(cHSP60).The antibodies from the specimen that are directed towards cHSP60 are bound to the antigen. Peroxidase-conjugated anti-human IgG antibodies bind to the IgG. Incubation with TMB-substrate. The reaction is stopped by the addition of sulfuric acid. The absorption is read photometrically.

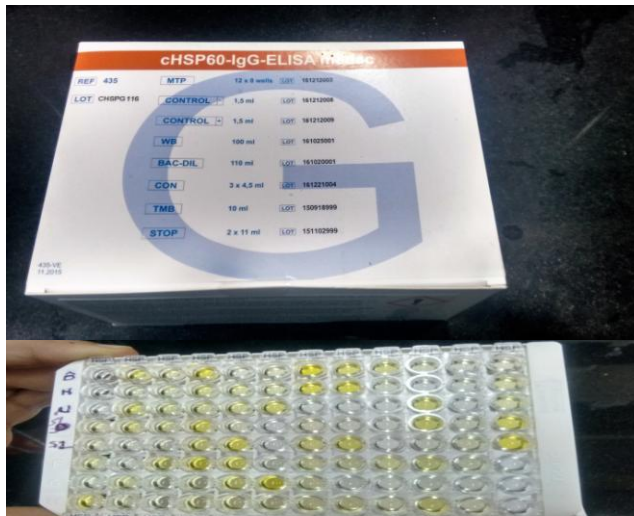


Figure 1: *Chlamydial* heat shock protein (cHSP60) ELISA Results

A. Enrolled women were grouped on the basis of their clinical history and presentation (**Table-1, Figure-1**).

Table 1: Defined group of subjects (n=100) enrolled for specimen collection

Clinical groups	Number of patients(N)	Percentage
Infertility(Primary(n=13)+ Secondary(n=6))	19	19.0
Bad Obstetric history (BOH)	6	6.0
Lower Genital Tract Infection (LGTI)	57	57.0
Ante Natal Care-ANC (Control group)	18	18.0
Total	100	100.0

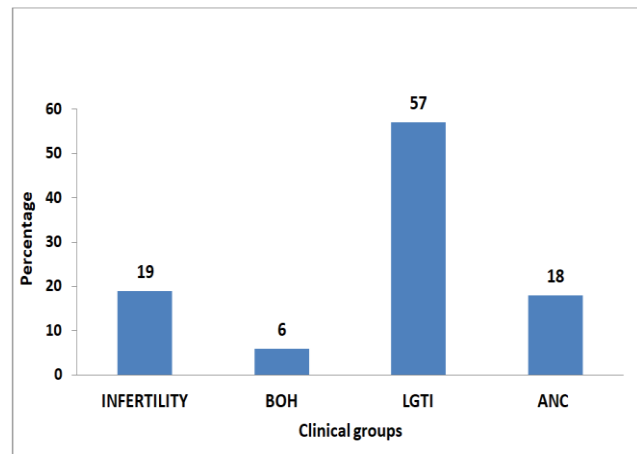


Figure 1: Defined group of subjects (number) enrolled for specimen collection

BOH-Bad Obstetric History, LGTI-Lower Genital Tract Infection, ANC-Ante Natal Care,

B. Seroprevalence of cHSP60 (*Chlamydia trachomatis* heat shock protein 60 ELISA) antibodies in the defined population

Of the 100 subjects 28% had antibodies to *Chlamydia trachomatis* Heat Shock Protein 60 (cHSP60) (**Table-2**).

Table 2: *Chlamydia trachomatis* serological markers in the defined population

Subjects	<i>C.trachomatis</i> Heat Shock Protein 60
N	cHSP60
100	28(28%)

cHSP60-*C.trachomatis* Heat Shock Protein 60

Chlamydial Heat Shock Protein 60 antibodies(cHSP60) was present in 42.1% of the Infertile patients while among the other clinical groups 24.6 % had cHSP60 antibodies ($P>0.05$)-(Table-3,4.Figure-2,3).

In the primary infertility group 39% of the patients had cHSP60 antibodies while 50% of the secondary infertility cases had cHSP60 antibodies ($p>0.05$) (Table-3).

Table 3: Prevalence of cHSP60 antibodies in infertility group

Infertility Group	cHSP60 60		Fisher's Exact test	Significant at 5% level
	Positive (%)	Negative		
Primary(n=13)	5(38.5)	8	1.000	Not
Secondary(n=6)	3(50)	3		
Total(n=19)	8(42.1)	11		

$p>0.05$, cHSP60- *Chlamydial trachomatis* Heat Shock Protein 60

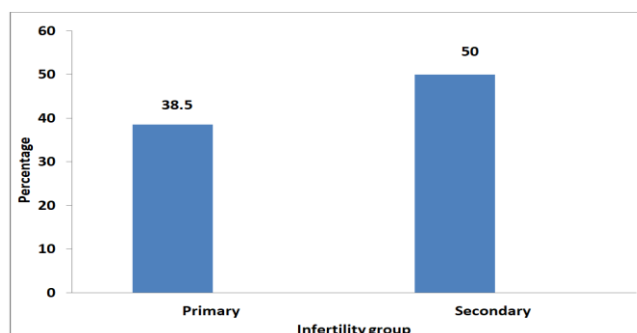


Figure 2: Prevalence of cHSP60 antibodies in infertility group

cHSP60 - *Chlamydial trachomatis* Heat Shock Protein 60

Amongst the other clinical groups cHSP60 antibodies was observed in 33% in ANC group, 19% in LGTI cases and 50% in BOH group, which was not statistically significant (**Table-4, Figure-3**).

Table 4: Prevalence of cHSP60 antibodies in other clinical groups

Other Clinical Groups	cHSP60		Chi square Test	P-value	Significant at 5% level
	Positive (%)	Negative			
ANC(n=18)	6(33)	12	3.681	0.159	Not
LGTI(n=57)	11(19)	46			
BOH(n=6)	3(50)	3			
Total(n=81)	20(24.6)	61			

$p > 0.05$, cHSP60 - *Chlamydial trachomatis* Heat Shock Protein 60

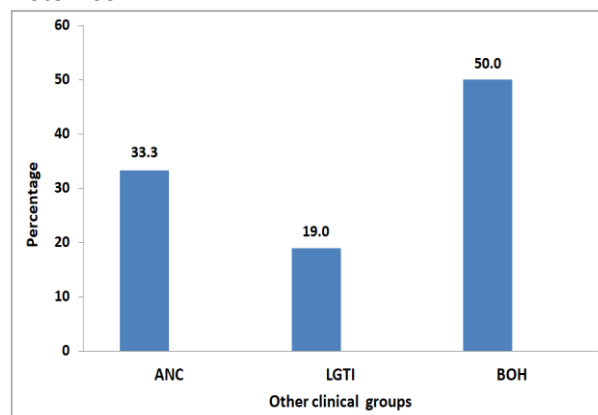


Figure 3: Prevalence of cHSP60 antibodies in other clinical groups

cHSP60 - *Chlamydial trachomatis* Heat Shock Protein 60

C. Infertility and anti *Chlamydial* antibodies

Use of cHSP 60 antibodies (cHSP60) as a marker for primary infertility had a sensitivity of 38.5% and a specificity of 73.5%, its sensitivity and specificity for diagnosis of secondary infertility was 50.0% and 73.4% respectively while as a marker for infertility in general it had a sensitivity of 42.1% and a specificity of 75.3% (**Tables-5-7**).

Table 5: cHSP60 Elisa as a marker for primary infertility

Clinical Groups		Primary Infertility		Total	95% Confidence Interval			
cHSP60		+ve	-ve		Sensitivity	Specificity	PPV	NPV
	+ve	5	23	28	38.46%	73.56%	17.86%	88.89%
	-ve	8	64	72	(12.02% to 64.91%)	(64.30% to 82.83%)	(3.67% to 32.04%)	(81.63% to 96.15%)
Total		13	87	100				

Table 6: cHSP60 Elisa as a marker for secondary infertility

Clinical Groups		Secondary Infertility		Total	95% Confidence Interval			
cHSP60		+ve	-ve		Sensitivity	Specificity	PPV	NPV
	+ve	3	25	28	50.0%	73.4%	10.71%	95.83%
	-ve	3	69	72	(9.99% to 90.01%)	(64.47% to 82.34%)	(0.00% to 22.17%)	(91.22% to 100.0%)
Total		6	94	100				

Table 7: cHSP60 Elisa as a marker for infertility

Clinical Groups		Infertility		Total	95% Confidence Interval			
cHSP60		+ve	-ve		Sensitivity	Specificity	PPV	NPV
	+ve	8	20	28	42.11%	75.31%	28.57%	84.72%
	-ve	11	61	72	(19.90% to 64.31%)	(65.92% to 84.70%)	(11.84% to 45.30%)	(76.41% to 93.03%)
Total		19	81	100				

Discussion

Serological study using the marker Chlamydial Heat Shock Protein 60 (cHSP60) was carried out in the defined population. There was no significant association between age groups and presence of *Chlamydia trachomatis*

cHSP60 antibodies. Anti Chlamydial Heat Shock Protein 60 antibodies (cHSP60) was detected in 42.1% of the infertile patients while among the other clinical groups 24.6% had anti-cHSP60 antibodies.

Our findings correlated well with studies carried out by Dutta R et al (2008)^[9] from Delhi, Srivastava et al (2008)^[10] from Delhi, Dadamessi et al (2005)^[11] from France as well as Caroline J et al (2004)^[12] from Netherlands.

Limitation of our study might be the small number of subjects which affects the generalization of the results and the extent to which statistical interferences can be made.

This study shows that *C.trachomatis* infections are highly prevalent in India which could have major consequences on female fertility. Introduction of a serological test allowing specific diagnosis of infertility due to *C.trachomatis* into routine clinical practice would help to confirm clinical data, exclude other possible causes of infertility, and lead to prescription of appropriate antibiotic treatment, before an in vitro fertilization process is undertaken.(chsp60).

References

1. Gerbase AC, Rowley JT, Mertens TE. Global epidemiology of sexually transmitted diseases. Lancet 1998; 351 (Suppl. 3): S2-4.
2. Singh V, Salhan S, Das BC, Mittal A. Predominance of *Chlamydia trachomatis* serovars associated with urogenital infections in females in New Delhi, India. J Clin Microbiol 2003; 41:2700-2.
3. Schachter J. Chlamydial infections. N Engl J Med 1978; 298:428-35.
4. Golden M, Schillinger J, Markowitz L, St Louis M. Duration of untreated genital infections with *Chlamydia trachomatis*: a review of the literature. Sex Transm Dis 2000; 27:329-7.
5. Tiitinen A, Surcel HM, Halttunen M, Birkelund S, Bloigu A, Christiansen G, Koskela P, Morrison SG, Morrison RP, Paavonen J. Chlamydia trachomatis and chlamydial heat shock protein 60-specific antibody and cell-mediated responses predict tubal factor infertility. Hum Reprod. 2006;21(6):1533-8.
6. Raulston, J., Paul, T., Knight, S. et al. (1998b), " Localization of Chlamydia trachomatis heat shock proteins 60 and 70 during infection of a human endometrial epithelial cell line in vitro. " Infect. Immun. 66: 2323-2329.
7. Witkin, S, and Ledger, W. (1992), " Antibodies to Chlamydia trachomatis in sera of women with recurrent spontaneous abortions." Am. J. Obstet. Gynecol. 1-61:1 35-139.
8. Van Voorhis, W., Barrett, L., Cosgrove-Sweeney Y, . et al. (1996), " Analysis of lymphocyte phenotype and cytokine activity in the inflammatory infiltrates of the upper genital tract of female macaques infected with Chlamydia trachomatis". J. Infect. Dis. 174: 647-650
9. Dutta R1, Jha R, Salhan S, Mittal A. Chlamydia trachomatis-specific heat shock proteins 60 antibodies can serve as prognostic marker in secondary infertile women. Infection. 2008 Aug;36(4):374-8. doi: 10.1007/s15010-008-7129-9. Epub 2008 Jul 19.
10. Pragya Srivastava,1 Rajneesh Jha,1 Sylvette Bas,2 Sudha Salhan,3 and Aruna Mittal In infertile women, cells from Chlamydia trachomatis infected site release higher levels of interferon-gamma, interleukin-10 and tumor necrosis factor-alpha upon heat shock protein stimulation than fertile women. Reprod Biol Endocrinol. 2008; 6: 20.
11. Innocenti Dadamessi a, Francois Eb b, Fotini Betsou Combined detection of Chlamydia trachomatis-specific antibodies against the 10 and 60-kDa heat shock proteins as a diagnostic tool for tubal factor infertility: Results from a case-control study in Cameroon FEMS Immunology and Medical Microbiology 45 (2005) 31-35
12. Caroline J, Bax P J, Dörr Baptist B Trimbos Chlamydia trachomatis heat shock protein 60 (cHSP60) antibodies in women without and with tubal pathology using a new commercially available assay Sex Transm Infect 2004;80:415-421