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Cytokine profile in Nigerians with tubal infertility

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Abstract

Introduction: *Immune response to genital Chlamydia trachomatis infection is involved in both immunity and pathology. The cytokine profile during infection has been implicated in the disease outcome, either resolution or severe sequelae. Serum cytokines of Chlamydia positive Nigerian women with tubal infertility were assessed to determine their possible relationship with tubal occlusion.*

Material and methods: *One hundred and fifty age-matched consenting women (100 fertile and 50 with tubal infertility) were recruited based on C. trachomatis antibody positivity and grouped into infertile Chlamydia positive (CTpos) women (n = 50), fertile Chlamydia positive women (n = 50) and fertile Chlamydia negative (CTneg) women as controls (n = 50). High vaginal swabs and endo-cervical swabs were collected for microscopy, culture and gram staining. Cytokines [transforming growth factor β1 (TGF-β1), interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin (IL)-4, IL-10 and IL-17A] were estimated by ELISA in sera. Data were analyzed using ANOVA, χ^2 and Spearman's correlation at p = 0.05.*

Results: *Lower IFN-γ levels were observed in infertile women compared to fertile women. Fertile CTneg women had significantly higher TNF-α, and TGF-β1 compared to fertile and infertile CTpos women, respectively. Lower IL-10 levels were seen in fertile CTpos women compared to the infertile CTpos group. Vaginal discharge was negatively correlated with TNF-α and IFN-γ and positively with IL-4 in Chlamydia positive women.*

Conclusions: *Chlamydia positive women with tubal infertility have higher IL-10 and lower IFN-γ levels than controls, which may contribute to their development of tubal pathology.*

Key words: *cytokines, infertility, Chlamydia trachomatis, tubal obstruction.*

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Introduction

Uncontrolled immune reactions in the upper genital tract following repeated *Chlamydia* infections are believed to contribute to the disease pathogenesis and development of late complications such as pelvic inflammatory disease and tubal occlusion associated with a genital *Chlamydia* infection [1]. A strong adaptive immune response is a key mechanism involved in controlling or eliminating the infection having a double-edged nature which can be both protective and tissue damaging [2]. The exact pathologic mechanism of *Chlamydia*-induced tissue damage has not been elucidated, however, chronic inflammatory processes have been implicated. *Chlamydia* infection of the upper genital tract induces the production of T-helper 1 (Th1) and 2 (Th2) cells with different functions and pattern of cytokine release. An association between cytokine profiles, duration of infection and subsequent outcome of genital *Chlamydia* infection, either resolution or tissue damage, has been demonstrated [3]. Th1 cytokines promote protection from and resolution of *Chlamydia* infection and disease whereas Th2 cytokine responses may be associated with susceptibility, persistence, tissue fibrosis and immunopathology. Interferon γ (IFN-γ) has been shown to have a major role in anti-chlamydial immune response but can also promote inflammatory damage and fibrosis [4]; tumor necrosis factor α (TNF-α) has been reported to participate in enhancement of anti-chlamydia effector mechanisms, it has also been implicated in excessive inflammation and tissue damage, whereas interleukin (IL)-4, IL-5, and IL-10 are believed to be involved in anti-inflammatory response and defense against extracellular pathogens [5]. Interleukin 17 has been shown to contribute not only to host defense against invading pathogens, but also to severe immunopa-

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thology. Transforming growth factor β1 (TGF-β1) is secreted by regulatory T cells to prevent tissue damage [6]. They reduce the amount of mucosa inflammation but may contribute to bacterial persistence and colonization of the genital tract and in peritoneal adhesions [4, 7].

Deficiencies or overstimulation in the production and activity of these cytokines may be associated with failure of protective immunity or harmful inflammation. Determining the specific responses that promote tissue damage and differentiating them from those that lead to benign resolution of infection may be important in predicting the disease outcome. This study therefore examines the serum cytokine profile of *Chlamydia* positive Nigerian women with tubal infertility to determine their possible relationship with tubal occlusion.

Material and methods

Study design

The study was a case control study conducted in the Gynaecology and Family Planning Clinics of the Department of Obstetrics and Gynaecology, University College Hospital and Adeoyo Maternity Hospital, Ibadan, Nigeria. The study protocol was approved by the University of Ibadan/University College Hospital Ethics Committee reference number UI/EC/08/0083. This study was conducted between April 2009 and January 2010. Informed consent was obtained from the subjects before recruitment into the study.

Inclusion criteria: consenting subjects with infertility of at least one year's duration, child birth of less than 2 years for the fertile controls. Exclusion criteria: subjects with previous history of uterine surgery, subjects undergoing any form of contraceptive therapy, those with malignancies on long-term medication, or chronic organ or systemic illness, and those that did not give consent. Infertility was defined in this study as the inability of a couple to conceive after a consecutive period of 12 months of unprotected sexual intercourse [8]. Evidence of fertility was taken as ability to have at least one child, with the last childbirth within the last 2 years. Evaluation of infertility was carried out using standard procedures according to the National Health Service evaluation criteria [9].

Socio-demographic characteristics of the study population – family history, social history, past medical history, medication and gynaecological history were obtained using a semi-structured questionnaire. Anthropometric indices – height, weight, hip and waist circumference were taken to calculate the body mass index and waist-to-hip ratio, respectively. The women were screened for the presence of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Staphylococcus aureus* and *Candida albicans* using standard methods [10], to rule out any current reproductive tract infection, which could be a confounder. These women were further screened for presence of *Chlamydia trachomatis* IgG antibodies (CTIgG). Bilateral tubal blockage was identified by hysterosalpingogram (HSG). A significant number of patients are unable to afford diagnostic laparoscopy in our resource-poor setting, therefore, HSG diagnosis was used for consistency. Other causes of tubal blockage were ruled out in those that had laparoscopy. Cases of unilateral tubal blockage were excluded, as sexually-transmitted, ascending *Chlamydial* infection is usually a bilateral disease. It was assumed that unilateral damage was due to other causes.

Selection of subjects

A total of 150 age-matched women of reproductive age (100 fertile and 50 with tubal infertility) without microbial antigens (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Staphylococcus aureus* and *Candida albicans*) were consecutively recruited into this prospective case control study. These women were sub-divided into 3 groups based on chlamydia antibody positivity into infertile *Chlamydia* positive women ($n = 50$), fertile *Chlamydia* positive women ($n = 50$) and corresponding fertile *Chlamydia* negative women as controls ($n = 50$). Recruitment was discontinued after the first 50 women in each group were identified. Further data were not collected or analyzed on subsequent recruited participants in the completed groups, while other participants in other groups were still being recruited.

Sample collection

Ten milliliters of venous blood samples were collected aseptically from each subject at recruitment. Samples were dispensed into 10 ml plain sample containers. After clot retraction, samples were centrifuged at 500 g for ten minutes after which sera were extracted and stored in small aliquots at –20°C until time of analysis. High vaginal swabs (HVS) and endocervical swabs (ECS) were collected from all subjects of study for isolation of such pathogens as *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Treponema pallidum*, *Staphylococcus aureus* and *Candida albicans* within one hour after sample collection. Cytokines (TGF-β1, IFN-γ, TNF-α, IL-4, IL-10 and IL-17A) were estimated in the sera of the study population.

Laboratory methods

Detection of *Chlamydia* antigens was carried out by the immunochromatographic method using prepared test kits (Diaspots Diagnostics, USA) [11]. Isolation of *Candida* spp. and bacterial vaginosis were done by Gram staining procedure; reagents procured from Oxoid chemicals (USA) [12]. Identification of *Trichomonas vaginalis* was done by microscopy [13]. Isolation of *Neisseria gonorrhoeae* was made by culture method using Thayer Martins culture media (Becton, Dickinson and Company, USA) [14]. Quantification of *Chlamydia* antibodies was carried out by the

enzyme immunoassay (EIA) method using a prepared test kit (Orgenics Ltd, Germany) [15]. Detection of *Treponema pallidum* antibodies was done by immunochromatographic methods with test kits (Acon diagnostics, USA) [16]. All cytokines: TGF-β1, IFN-γ, TNF-α, IL-4, IL-10 and IL-17A, were estimated by the enzyme linked immunosorbent assay (ELISA) (eBioscience, USA) [17-20].

Statistical analysis

Data were analysed using the statistical package for social sciences (SPSS version 20.0). Analysis of variance (ANOVA) was used to test significance of variations within and among group means. Fisher's least significant difference (LSD) test was used for comparison of multiple group means. χ^2 analysis was used for comparison of means for non-quantitative variables while Spearman's correlation was used to determine associations between non-parametric variables. A two-sided probability value $p < 0.05$ was considered statistically significant.

Results

The mean age, anthropometric indices [weight, height, body mass index (BMI), waist circumference (WC), hip circumference (HC), and waist–hip-ratio (WHR)] and cytokine profile of fertile women (CTneg and CTpos) and infertile *Chlamydia* positive women are shown in Table 1. Significant variations in the levels of IFN-γ were observed among the groups ($p < 0.05$). No significant variations were observed in the mean age, anthropometric indices and other indices among the groups ($p > 0.05$).

Comparison of gynecologic characteristics and symptoms of genital *Chlamydia* infection (GCI) in fertile women (CTneg and CTpos) and infertile *Chlamydia* positive women are shown in Table 2. Significant differences were observed in such characteristics as dysmenorrhea and symptoms as vaginal discharge, vaginal itching and lower abdominal pain among the groups ($p < 0.05$). No significant difference was observed in the women in relation to dyspareunia among the groups ($p > 0.05$).

Table 3 shows a comparison of serum cytokines (TGF-β1, IFN-γ, TNF-α, and IL-10) of fertile women (CTneg and CTpos) and infertile *Chlamydia* positive women using Fischer's LSD *post hoc* analysis. Fertile CTneg controls had significantly higher TNF-α as compared to fertile CTpos, and also higher IFN-γ and TGF-β1 compared to infertile CTpos women ($p < 0.05$). Fertile CTpos women had significantly elevated IFN-γ and lower IL-10 compared to the infertile CTpos group ($p < 0.05$).

Table 4 shows a correlation of vaginal discharge with IFN-γ, TNF-α and IL-4 in *Chlamydia* positive women (fertile and infertile). Vaginal discharge correlated negatively with IFN-γ ($r = –0.767$, $p = 0.005$), and TNF-α ($r = –0.430$, $p = 0.000$) and positively with IL-4 ($r = 0.253$, $p = 0.11$).

Table 1. Age, anthropometric indices and cytokines in fertile women (CTneg and CTpos) and infertile *Chlamydia* positive women (CTpos)

Index	Fertile CTneg *n* = 50	Fertile CTpos *n* = 50	Infertile CTpos *n* = 50	F	*p*
Age (years)	34.90 ±0.46	33.94 ±0.48	33.76 ±0.51	1.099	0.351
Weight (kg)	68.60 ±2.19	64.92 ±1.83	69.60 ±1.38	1.38	0.249
Height (cm)	159 ±7	150 ±1	160 ±1	0.855	0.465
BMI (kg/m^2)	26.90 ±0.78	25.87±0.65	27.01 ±0.49	0.709	0.548
WC (cm)	86.34 ±1.84	83.40 ±1.65	86.64 ±1.19	1.168	0.323
HC (cm)	103.94 ±1.72	102.76 ±1.71	104.32 ±1.18	0.202	0.895
WHR	0.83 ±0.07	0.81 ±0.07	0.83 ±0.06	1.937	0.125
Cytokines					
TGF-β1 (pg/ml)	545.52 ±108.90	386.62 ±68.16	348.58 ±72.20	1.81	0.147
IFN-γ (pg/ml)	128.58 ±9.36	126.12 ±9.66	72.20 ±0.20	16.67	0.000*
TNF-α (pg/ml)	134.61 ±25.12	79.94 ±2.10	92.77 ±19.30	1.68	0.172
IL-4 (pg/ml)	32.90 ±5.44	33.02 ±24.35	37.48 ±5.9	0.214	0.887
IL-10 (pg/ml)	23.62 ±2.97	16.93 ±0.64	28.99 ±6.67	0.792	0.449
Il-17A (pg/ml)	37.58 ±12.75	28.18 ±10.04	43.50 ±15.53	0.404	0.751

*TGF-β1 – transforming growth factor 1β; IFN-γ – interferon γ; TNF-α – tumor necrosis factor α; IL-4 – interleukin 4; IL-10 – interleukin 10; IL-17 A – interleukin 17A; * – significant at p < 0.05; p – significant level; F – F-ratio; BMI – body mass index; WC – waist circumference; HC – Hip circumference; WHR – waist-to-hip ratio; CTpos – Chlamydia positive; CTneg – Chlamydia negative*

Table 2. Comparison of gynecologic characteristics and symptoms of GCI in fertile women (CTneg and CTpos) and infertile *Chlamydia* positive women (CTpos)

Index	Fertile CTneg *n* = 50	Fertile CTpos *n* = 50	Infertile CTpos *n* = 50	χ^2	*p*
Vaginal discharge					
yes	10 (13.7%)	18 (24.7%)	45 (61.6%)	53.85	0.000*
no	40 (51.9%)	32 (41.6%)	5 (6.5%)		
Vaginal itching					
yes	7 (9.6%)	16 (21.9%)	50 (68.5%)	19.27	0.000*
no	43 (55.8%)	34 (44.2%)	0 (0.0%)		
Lower Abd. Pain					
yes	3 (13.0%)	6 (26.1%)	14 (60.9%)	9.96	0.007*
no	47 (37.0%)	34 (34.6%)	36 (28.3%)		
Dysmenorrhea					
yes	6 (26.1%)	4 (17.4%)	13 (56.5%)	6.88	0.032*
no	44 (34.6%)	46 (36.2%)	37 (29.1%)		
Dyspareunia					
yes	5 (38.5%)	5 (38.5%)	3 (23.0%)	0.674	0.714
no	45 (32.8%)	45 (32.8%)	47 (34.3%)		

*Values are given as the number of subjects with a percentage in parenthesis; CTpos – Chlamydia positive; CTneg – Chlamydia negative; * – significant at p < 0.05; GCI – genital Chlamydia infection*

Table 3. Comparison of cytokines in fertile women (CTneg and CTpos) and infertile *Chlamydia* positive women (CTpos) using LSD *post-hoc* analysis

Parameter	Groups		Mean diff.	STD error	*p*
	Fertile CTneg	Fertile CTpos			
TNF-α (pg/ml)	134.61 ±25.12	79.94 ±2.10	54.672*	25.91	0.037*
	Fertile CTneg	Infertile CTpos			
TGF-β1 (pg/ml)	545.52 ±108.90	348.58 ±72.20	294.260*	136.38	0.033*
IFN-γ (pg/ml)	128.58 ±9.36	72.20 ±0.20	55.936*	9.56	0.000*
	Fertile CTpos	Infertile CTpos			
IL-10 (pg/ml)	16.93 ±0.64	28.99 ±6.67	–12.060*	5.98	0.046*
IFN-γ (pg/ml)	126.12 ±9.66	72.20 ±0.20	53.910*	9.55	0.000*

*TGF-β1– transforming growth factor β1; IFN-γ – interferon γ; TNF-α – tumor necrosis factor α; * – significant at p < 0.05; p – significant level; F – F-ratio; CTpos – Chlamydia positive; CTneg – Chlamydia negative*

Table 4. Correlation of cytokines with vaginal discharge in *Chlamydia* positive women (*n* = 100)

Indices		*r*	*p*
Vaginal discharge Vs	IFN-γ	–0.767	0.005*
	TNF-α	–0.430	0.000*
	IL-4	0.253	0.011*

*p – significant level; TNF-α – tumor necrosis factor α; IFN-γ – interferon γ; r – Spearman's correlation coefficient; IL-4 – interleukin 4; GCI – genital Chlamydia infection; * – significant at p < 0.05*

Discussion

The cytokine milieu in genital *Chlamydia* infection has been implicated in the disease outcome; either in resolution or severe sequelae such as tubal occlusion. In this present study, fertile women (CTneg and CTpos) had significantly higher IFN-γ levels compared to *Chlamydia* positive women with tubal infertility ($p < 0.05$). This is consistent with findings of Agrawal *et al.* [21] who reported higher IFN-γ levels in *Chlamydia* negative and positive fertile women compared to those with fertility disorders. Women with *Chlamydia* infection without pathological damage have been shown to secrete higher amounts of IFN-γ than women who developed sequelae to *Chlamydial* infection (tubal damage) suggesting that IFN-γ is down-regulated in women with damaging sequelae [22]. Cervical cell production of IFN-γ in response to stimulation with *Chlamydia trachomatis* elementary bodies (EBs) has been positively correlated with fertility in *C. trachomatis*-seropositive individuals [23, 24]. It appears that higher levels of IFN-γ seen in fertile women with or without GCI may be responsible for their protection against tubal pathology. These observations suggest that reduced IFN-γ may be involved in the development of *Chlamydia*-induced tubal occlusion [7]. Some of the mechanisms employed by

IFN-γ to control *Chlamydia* replication includes promoting the engulfment and destruction of extracellular EBs [25], up-regulation of inducible nitric oxide synthase [26], induction of indoleamine-2,3-dioxygenase (IDO) activity and down-regulation of the transferrin receptor thereby depleting the intracellular stores of iron available to the organism [27].

Tumor necrosis factor α was significantly reduced in fertile CTpos women compared to their corresponding fertile CTneg controls. Consistently with our findings, some studies have shown that cells from *Chlamydia trachomatis* infected sites release small amounts of TNF-α [22, 28]. Tumor necrosis factor α has been reported to confer protection against GCI. Higher levels of TNF-α in synergy with elevated IFN-γ in fertile women without genital *Chlamydia* infection may be responsible for their strong resistance to GCI. Tumor necrosis factor α has been reported to participate in enhancement of anti-*Chlamydia* effector mechanisms through nutrient starvation; by synergistically enhancing induction of indoleamine-2,3-dioxygenase (IDO) activity and IDO mRNA expression. Indoleamine-2,3-dioxygenase degrades tryptophan, thereby resulting in reduced levels of intracellular tryptophan. Tryptophan is essential for *in vivo* and *in vitro Chlamydia* replication [29, 30]. Addition of TNF-α *in vitro* to *Chlamydia* cell culture has been found to disrupt the *Chlamydia* life cycle while its removal results in proliferation of reticulate bodies with their subsequent differentiation into infectious bodies and cell lysis. This *in vitro* observation may be analogous to *in vivo* scenario [31]. However, higher levels of TNF-α were detected in the infertile group compared to fertile controls after stimulation with a chlamydia inclusion membrane protein by Srivastava *et al.* [22]. Levels of TNF-α were also found to be significantly higher in *Chlamydia* inclusion bodies stimulated cervical cells and peripheral blood mononuclear cells (PBMCs) from *Chlamydia* positive women with or without fertility-related disorders compared to fertile controls [23, 28].

Lower TGF-β1 was observed in infertile CTpos women and also in fertile CTpos women (though not statistically significant), compared to fertile CT negative controls. This suggests that reduction in TGF-β1 may be associated with genital *Chlamydia* infection. Contrary to this finding, a significantly elevated expression of TGF-β1 and its receptors, TGF-β1R1 and TGF-β1R2, was observed in occluded fallopian tubal tissues of infertile women compared with normal specimens [32]. Song *et al.* [33] also observed that the staining intensity of TGF-β1 in the glandular epithelium and stromal cell of women with hydrosalpinges were significantly higher than those in fertile women. Transforming growth factor β1 interacts with the fibrinolytic system and many other cellular mediators involved in the process of adhesion formation in chronic inflammation and therefore have been implicated in scarring and occlusion of the uterus and fallopian tubes and infertility [34].

Fertile *Chlamydia* positive women had lower IL-10 compared to infertile *Chlamydia* positive women. The level of the Th2 cytokine IL-10 was found to be up-regulated in both cervical cells and PBMCs stimulated with the *Chlamydia* major outer membrane protein (MOMP) antigen in *Chlamydia* positive women. Interleukin 10 is known to selectively suppress the production of inflammatory cytokines IFN-γ, TNF-α and IL-1 [35], whose effects are needed to eradicate *Chlamydia trachomatis* infection. Interleukin 10 has also been found to be associated with susceptibility and typical pathological changes caused by the GCI such as granuloma formation and fibrosis [36]. Thus, higher IL-10 seen in CTpos women with tubal pathology may be responsible for their failure to eradicate GCI and the associated pathology. However, studies have demonstrated decreased production of IL-10 by decidual T cells of women with impaired infertility when compared with decidual cells of fertile women [37].

Interferon γ and TNF-α were negatively correlated with vaginal discharge in *Chlamydia* positive women (fertile and infertile). The anti-*Chlamydial* properties of IFN-γ and TNF-α have been established [4]. High doses of IFN-γ have been shown to confer protection against *Chlamydia* infection, albeit low doses of IFN-γ appear to rather promote persistent infection by increasing production of aberrant reticulate bodies (RBs) [1]. Thus, we can therefore deduce that the higher the IFN-γ levels, the faster the clearance and resolution of *Chlamydia* infection and associated symptoms and *vice versa*. A positive association was observed between IL-4 and vaginal discharge in *Chlamydia* positive women. Interleukin 4 has been implicated in immunopathological responses in GCI, accelerated tissue fibrosis, granuloma reaction and disease persistence [38] and perhaps persisting symptoms. The inability to completely eliminate *Chlamydia* organisms from hosts can lead to tissue destruction, tubal occlusion and tubal infertility.

The findings of this present study have shown that higher levels of IFN-γ, TNF-α and TGF-β1 seen in fertile *Chlamydia*-negative women may be responsible for their protection against *Chlamydia* infection. Higher levels of IL-10 and lower IFN-γ may be implicated in *Chlamydia*-induced tubal occlusion.

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The authors declare no conflict of interest.

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