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Serum Levels of Inflammatory Cytokines in Helminth Infested Pregnant Women and Cord Blood of their Babies in Relation to Pregnancy Outcome

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Summary: Pregnancy places a very high demand on physical, physiological and immunological responses of females, especially when aggravated by parasitic infestation. There is strong evidence that maternal infestations with helminth have profound effects on immunity to helminths and other pathogens. This case-control study involved 245 pregnant women aged 18-40 years (>30 weeks of gestation) recruited from three secondary level hospitals in Ibadan, Nigeria. Morning stool samples collected from pregnant women were examine for intestinal helminths using formol-ether concentration method. A total of 38 participants comprising 17 Helminth Positive (HP) and 21 Helminth Negative (HN) pregnant women were purposely selected for the study. Sera from these women (38) and their babies' cord (38) were analysed for immune factors [interleukins 6 and 8 (IL-6, IL-8), tumor necrosis factor-alpha (TNF-α) and immunoglobulin E (IgE)] were analyzed using ELISA. Anthropometric indices [weight and height in mothers and babies and Chest Circumference (CC) in babies] were measured using standard methods. Data were subjected to descriptive statistics and analysed using Student t-test and Pearson correlation at $\alpha_{0.05}$. Only *Ascaris lumbricoides* was found in the 17 (6.9%) infested pregnant women. The mean levels of IL-6 (57.8 ± 32.8 vs 52.8±39.6 pg/mL), IL-8 (24.3±3.5 vs 22.0±7.1 pg/mL) and IgE (333.3±96.6 vs 242.3±96.8 IU/mL) were similar in HP when compared with HN. In cord sera, IL-8 level was significantly higher in babies of HP (23.7±3.9 pg/mL) compared with babies of HN (20.1±5.9 pg/mL). The levels of IL-6, TNF-α and nutritional indices in HP had significant positive correlation with corresponding levels in babies of HP mothers. Only CC was significantly lowered in babies of HP compared with HN mothers. Other anthropometric indices were not significantly different. Therefore, this present study suggests that helminth infestation may lead to strong Th2 immune responses as is reflected by the cytokine levels of mothers and babies as well as anthropometric measurements of babies of infested mothers. The outcomes of this study provide basis to deworm pregnant women during pregnancy.

Keywords*:* Helminths, Pregnancy, Cytokines, Cord-Blood, Pregnancy-Outcomes, IgE

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INTRODUCTION

Pregnancy places a very high demand on physical, physiological and immunological responses of females (Tulman and Fawcett, 2003; Warning et al., 2011; Mpairwe et al, 2014). These changes are aggravated by parasite infection (Mpairwe et al, 2014). Intestinal parasitic infections in pregnancy have been associated with serious adverse outcomes both for the mother and the unborn baby. Exposure of the foetus to helminth antigens and maternal antibodies may modulate the infant's immunity against these infections at a later stage (Gwegweni and Ntombela, 2014).

T-helper (Th) 1 cytokines (interleukin (IL)-8, tumour necrosis factor (TNF) -α, TNF-β and interferon-γ) and Th2 cytokines (IL-4, IL-5, IL-6, IL-10) were detected at various levels in the sera of women at different stages of normal pregnancy (Makhseed *et al.*, 2000; Szarka *et al.*, 2010). Although, there is no systemic inflammation during pregnancy, circulating cytokines (such as IL-6, IL-8) are found to be elevated in maternal plasma (Vitoratos *et al.*, 2010). During pregnancy, the immune system is biased towards Th 2 cytokine immune response rather than towards Th 1 cytokine immune responses which is fundamental for fetal well-being (Saito *et al.*, 2006; Szarka et al, 2010; Saito *et al.*, 2010; Mor *et al.*, 2011). However, Szarka *et al.* (2010) noted that the third trimester of normal pregnancy seems to be a controlled state of systemic inflammation.

Soil-transmitted helminths (STH) are a group of common parasites that infect more than a billion people worldwide (Mulu *et al.,* 2013). Immune responses in helminthiasis are generally associated with a Th2 response (Jackson *et al*., 2009). The triggering of the Th2 responses by helminths through activities of cytokines leads to a stereotyped cascade of effector mechanisms are typically associated with hyper-eosinophilia and considerable IgE production (Moreau and Chauvin, 2010). *In utero* exposure to pathogen derived antigens from helminths have been associated with prenatal immune priming that generates cytokine responses similar to adults and such sensitization is not skewed towards a dominant Th1 or Th2 profile (Polderman and Sobolay, 2008). Stimulation of B and T cell *in utero* by helminth infection during pregnancy is also a factor explaining IgE levels in neonates (King *et al.,* 1998).

There is strong evidence that maternal infestations with helminth have profound effects on immunity during pregnancy. Therefore, this present study suggests that helminth infestation may have effects on the anthropometric measurements of the babies, the cytokine levels in mothers and their babies.

MATERIALS AND METHODS

Study participants: The study was conducted among 245 pregnant women attending antenatal clinics at Our Lady of Apostle Catholic Hospital, Oluyoro, St Mary's Catholic Hospital, Eleta and Adeoyo Maternity Hospital, Yemetu in Ibadan, Oyo State. Nigeria. Maternal gestational age was based on calculated last menstrual period (LMP) and ultrasound assessment by Consultant Gynaecologist.

Ethical clearance: The ethical clearance for the human study was obtained from Institutional Health Research Ethics Committee of University of Ibadan/University College Hospital, Ibadan, Nigeria (UI/EC/14/0234) as well as the various collaborating hospitals and Oyo State Government, Nigeria (AD 13/479).

Inclusion criteria included pregnant women at third trimester who gave consents. Exclusion criteria included pregnant women who did not give consent or those with complicated pregnancies and infectious disease. More so, pregnant women who had diabetes mellitus, renal diseases and chronic hypertension predating pregnancy were also excluded.

Anthropometric measurement: The maternal height (m), weight (kg) and upper arm circumference (cm) were determined using portable stadiometer, digital weighing scale and tape rule respectively. At delivery, baby's birth outcomes such as weight and head to heel length as well as placental weight at delivery were recorded. Birth weight was determined using electronic weighing balance and recorded to the nearest 0.05kg while birth length was determined by measuring tape the nearest 0.1cm. Baby was considered underweight if less than 2.5kg (<2.kg) and preterm if less than 37weeks (<37weeks) gestational age.

Stool Specimen Collection and Processing: A sample of fresh stool specimen was collected from all the participants. Subjects were provided with a labeled leak-proof stool container (polypots), toilet paper, and applicator stick. Approximately 5gm of stool specimens was collected into polypots using applicator sticks. The stool specimens were examined microscopically within 24 hours of collection using the Kato-Katz concentration technique (Arinola *et al.,* 2003). The magnifications of ×10 and ×40 were used respectively to visualize and identify intestinal geohelminth ova.

Collection of blood samples: Five milliliter (5ml) of blood from the mother and cord blood of their babies was collected into plain vacutainer bottles for cytokine assay. Blood in plain bottles was spun at 2000g for 15 minutes to obtain serum which was frozen until analyses. Blood samples of 21 HN babies and their mothers were randomly collected for analysis.

Cytokines and IgE analyses: The serum levels of IL-6, IL-8, TNFα, and IgE were carried out based on kits (Assaypro, MO, USA) as previously carried out (Arinola *et al.,* 2012; Arinola *et al.,* 2014). Sample/standard was added to individual wells of microtiter plates provided by the manufacturer as part of the kit. The samples were run in duplicate. The mixture was incubated for at room temperature and washed four times with a plate washer (Tecan, Mannedorf, Switzerland). Specific biotin-conjugated secondary antibody was added to each well and incubated further at room temperature. Plates were washed four times and incubated for 30 minutes with streptavidin-HRP. Reaction was stopped by adding 50µl stop solution. The absorbance was read at 450 nm by using a microplate reader (Biotek , ELX 800, USA).

Statistical analysis: Statistical analysis was performed using SPSS 17.0 analysis package. The mean (±Standard Deviation) was determined; Student t- test was used to determine the level of significance. Pearson's analysis was used to determine correlations between the data of mothers and neonates. Result was considered significant at $p<0.05$.

RESULTS

Sampling collection

Two hundred and forty five (245) consecutive pregnant women who submitted stool samples and in their third trimesters (30-41 weeks) of pregnancy, attending antenatal clinics namely: Adeoyo Maternity Hospital, Yemetu, Ibadan, St Marys Catholic Hospital, Eleta, Ibadan, and Our Lady of Apostle Catholic

Hospital, Oluyoro, Ibadan were recruited for the study. They were followed to childbirth when cord blood samples were collected from their babies immediately after delivery. Seventeen (6.9%) pregnant mothers were found to have ova of helminths (*Ascaris lumbricoides*) in their stool after examination. No other helminth was detected. See Table 1.

Antropometric measurement

The anthropometric indices of pregnant women with helminthiasis (HP) were compared with non-infected women (HN) in Table 2. The mean value for Weight, Height and Upper Arm Circumference were 1.56 ± 0.084 m, 59.33 ± 17.46kg, 15.8 ± 6.8cm and 1.55 ± 0.22m, 63.25 ± 17.93kg, 13.8 ± 3.5cm for helminth infested and non-infested pregnant women

Table 1 Prevalence of Helminth infestation (%) among pregnant women

	Ascaris lumbricoides	Others	Total
HP	17 (6.9%)	None (0%)	17 (6.9%)
H N	228 (93.1%)	None (0%)	228 (93.1%)
Total	245 (100%)	0	0

HP = Helminth Infested Pregnant women.
HN =Helminth Non-infested Pregnant women.

Table 2: Mean Height, Weight and Upper Arm Circumference (±SD) in helminth infested mothers compared with non-infested mothers at the third trimester.

	HP (n=17)	HN (n=21)	p
Height (m)	1.56 ± 0.084	1.55 ± 0.22	0.787
Weight (kg)	59.33 ± 17.46	63.25 ± 17.93	0.567
UAC (cm)	15.84 ± 6.84	13.78 ± 3.52	0.464

UAC = Upper Arm Circumference

Table 3: Mean Anthropometric parameters between babies from helminth infested mothers compared with babies from non-infested mother.

	Babies of HP (n=17)	Babies of HN (n=21)	p
BW (kg)	2. 3± 0.3	3.04 ± 0.4	0.8
PW (kg)	0.4 ±0.4	0.86 ± 0.2	0.0
CHL(cm)	42.9 ± 11.0	47. 94 ± 2.9	0.1
HC (cm)	32.89 ± 7.37	34.65 ± 3.4	0.4
CC(cm)	15.00 ± 1.00	33.33 ± 1.2	*0.01
GA(<37wks)	11 (73%)	14(68%)	0.2

*Significant at p=0.05
BW = Birth Weight; PW = Placental Weight; CHL =Crown to Heel Length; HC = Head Circumference; CC = Chest Circumference; GA =Gestation Age.

Table 4: Mean Cytokines and IgE levels (±SD) of Helminth infested (HP) mother compared with non-infested (HN) mothers

	HP (n=17)	HN (n=21)	p
IL-6 (pg/ml)	57.8 ± 32.8	52.8 ± 39.6	0. 67
IL-8 (pg/ml)	24.3 ± 3.5	22.0 ± 7.1	0.19
TNFα (ng/ml)	0.09± 0. 09	0.05 ± 0.07	0.25
IgE (IU/ml)	333.3 ± 96.6	242.3 ± 96.8	0.25

*Significant at p =0.05 (2-tailed).

respectively. There was no significant difference between the two groups (p>0.05). There was a significant increase in chest circumference of babies born to helminth non-infested mothers compared with babies born to helminth infested mothers (p<0.05). Seventy-three percent (73%) of babies born to HP mothers were below 37 weeks of gestation compared with 68% of babies born by HN mothers. Also, babies of HP mothers have lower mean birth weight (2.3±0.3kg) compared with weight of babies born by HN mothers (3.04±0.4kg) (Table 3).

Table 5: Cytokine levels (±SD) in cord blood from babies of helminth infested compared with cord blood of babies from helminth non-infested mother.

	HP (n=17)	HN (n=21)	p - values
IL-6(pg/ml)	59.4 ± 3.4	49.9 ±3.9	0.42
IL-8(pg/ml)	23.7 ± 3.9	20.1 ± 5.9	*0.03
TNFα(ng/ml)	0.8 ±0.1	1.0 ± 0.2	0.68
IgE(IU/ml)	281 ± 224.9	263 ± 223.8	0.81

*Significant at p=0.05.

Table 6: Correlation of Cytokines and IgE levels of helminth infected mother- baby pairs.

Mothers' Samples / Babies' Samples	IL-6 (pg/ml)	IL-8 (pg/ml)	TNFα (ng/ml)	IgE (IU/ml)
IL-6 (pg/ml) p	0.580 0.015			
IL-8 (pg/ml) p		0.412 0.101		
TNFα (pg/ml) p			0.911 0.001	
IgE (IUml) p	`			0.084 0.758

*Correlation is significant at p<0.05.

Table 7: Correlation of Cytokine and IgE levels of helminth non-infested (HN) mother -baby pair.

Mothers' Samples / Babies' Samples	IL-6 (pg/ml)	IL-8 (pg/ml)	TNFα ng/ml)	IgE (IU/ml)
IL-6 (pg/ml) p	0.123 0.596			
IL-8 (pg/ml) p		0.378 0.091		
TNFα (pg/ml) p			0.402 0.109	
IgE (IUml) p				-0.004 0.987

*Significant at 0.05.

Inflammatory Cytokine and IgE

As shown in Table 4, HP pregnant women had slightly higher IL8 (24.3 ±3.5pg/ml), TNFα (0.09 ±0.0.09ng/ml) and IgE (333.3 ±196.6IU/ml) when compared with HN pregnant women. Significantly higher mean value of IL-8 (23.7 ± 3.9pg/ml) was observed in babies of HP when compared with babies born by HN mothers (20.1 ± 5.9, p=0.05) while the mean concentration of IgE and IL-6 were higher in babies born to HP mothers compared with babies born to HN mothers (Table 5). The serum levels of IL-6 and TNFα in baby born to HP mothers showed significant positive correlations with the serum levels of IL-6 and TNFα in their HP mothers. Such correlation was not observed in uninfested (HN) mother-baby pairs (Table 6 and 7 respectively).

DISCUSSION

Helminth infections during pregnancy cause significant morbidity in endemic areas through their effects on nutrition, growth and cognition (Crompton *et al.*, 2002; Bethony *et al.*, 2006; Cooper *et al.*, 2011) because intestinal parasites causes reduction in food intake, malabsorption, endogenous nutrient loss and anemia (Katona and Katona-Apte, 2008). Since mal-nutrition has been reported to cause immunodeficiency, it is hypothesized by this study that helminth infestation will modulate immune responses during pregnancy, thus affecting pregnancy outcomes.

This study reported 6.9% prevalence rate of *Ascaris lumbricoides* among pregnant women in their third trimester. This is contrary to the previous findings of Alli *et al*. (2011) and Omorhodion *et al*. (2012) who found prevalence rate among pregnant women to be 43.4% in Ibadan, and 23.7% in Warri. Obiezue *et al.* (2013) reported that helminth infection were found to be reduced during the third trimester while Aderoba *et al*. (2015) found 8.4% of pregnant women in the third trimester infested with Ascariasis in Benin City. *Ascaris lumbricoides* is transmitted faeco-orally and pregnant and pregnant women traditionally eat vegetables as source of micronutrients. This might explain the presence of Ascaris in the pregnant women.

The significant reduction in infestation rate between previous studies in Nigeria (Alli *et al*., 2011 and Omorhodion *et al*., 2012) compared with present study might be due to compulsory government policy on environmental sanitation. Obiezue *et al*, (2013) attributed reduction of helminth infestation in pregnant women observed during third trimester to the administration of anti-helminths drugs to pregnant women. Enforced regular sanitation exercise which complement monthly sanitation coupled with public enlightenment reduces unhygienic practices habit and indiscriminate waste disposal. Oranusi *et al*. (2013) suggested that consumption of contaminated fruits and vegetable causes parasitic infestation. Also, Ziegelbauer *et al.* (2012) reported that improved sanitation will reduce exposure to faecal contamination of soil-transmitted intestinal helminth infection.

Ascaris lumbricoides is a parasitic nematode that excretes a variety of molecules to evade immune attack of the host (Dzik, 2006), and also induces production of Th2-associated cytokines and IgE (Sykes *et al*, 2012). Based on this, raised levels of IgE in helminth infested pregnant women is expected as reported in this study. In this study, we reported that elevated IL-6 form part of the primary host response to helminth infestation and is frequently evident in altered cytokine profiles characteristic of unexplained adverse pregnancy outcomes that includes infertility, recurrent miscarriage, preeclampsia and preterm delivery (Prins *et al.*, 2012). Buonocore *et al* (1995) also associated increased IL-6 to fetal distress. In the present study, IL-6 was not significantly different in Ascaris infested mothers compared with Ascaris-free mothers. This might explain why mean of preterm birth and low birth weight were not statistically different in babies of helminth infested mother during pregnancy compared with babies of helminth free pregnant mothers. However, Sajjadian *et al.* (2011) reported maximum positive correlation between chest circumference and birth weight while Goto (2015) in his meta analysis of various studies reported that chest circumference is a better predictor of birth-weight less than 2500g. This study reported a significantly reduced chest circumference in babies born to helminth infested mothers.

During helminth infection, the Th2 response is suggested to have been moderated by parasite-expressed molecules (Fitzsimmons *et al.*, 2014). The significantly increased IL-8 observed in babies born to Ascaris infested mothers when compared with babies born to non-infested mothers signifies *in utero* IL-8 production induced by helminth antigen/molecules. Gebreegziabiher *et al (*2014) hypothesized that prenatal exposures to maternal helminth derived antigens elevate the level of total IgE in cord blood. Also, neonatal B cells have been shown to be intrinsically capable of IgE production (King *et al*, 1998; Soboslay *et al.*, 1999; Seydel *et al.*, 2012). Slight increase in the mean IgE levels in cord blood of babies born to helminth infested mothers compared to babies born to non-infested mothers in this study is supported by different studies conducted in helminth endemic areas in Africa (Seydel *et al.*, 2012) and could be attributed to the fact that the babies might have been sensitized *in utero* to produce Ascaris-specific B cells. Moreover, Terhell *et al.* (2002) also reported a positive correlation of IgE in sera of HP mothers and their babies as an indication of *in utero* sensitization to parasite antigens. The present study observed

significant positive correlations between IL-6 and TNF in cord sera of babies from Ascaris infested mothers and sera from their mothers during pregnancy. Such correlation was not observed in the cord sera of babies from helminth mothers compared with the sera of their mothers during. This is might be as a result of *in utero* sensitization by Ascaris molecules released into circulation of mothers during pregnancy.

The study concluded that the prevalence rate of helminth infestation among pregnant women in 3rd trimester is low and that IL-6, IL- 8, TNFα and and IgE were slightly raised in Ascaris infested pregnant mothers compared with un-infested pregnant women. Significantly, reduced chest circumference is the only negative neonatal anthropometric index observed in babies of Ascaris infected pregnant mothers.

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