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Kinetics of Serum and Local Leukotriene B_4 Response in Experimental Intravaginal Trichomoniasis by *T. vaginalis* Isolates from Symptomatic and Asymptomatic Women

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Abstract

Trichomoniasis is most common sexually transmitted disease caused by *T. vaginalis*. The clinical manifestation varies from severe manifestation to asymptomatic condition. However, the exact virulence markers led to varied symptomatology was not well clarified. The role of leukotriene B_4 (LTB_4) in the pathogenesis of many parasitic diseases has been reported. The present study reports the leukotriene B_4 levels on different days post infection (3rd, 7th, 14th, 21st and 28th d.p.i.) in serum and vaginal washes (VWs) and vaginal tissues of mice infected intravaginally with *T. vaginalis* isolates from 10 symptomatic and 10 asymptomatic women. A significant increase in LTB_4 was observed on the 3rd to 28th d.p.i. in serum and VWs of mice infected with *T. vaginalis* isolates from asymptomatic as compared to symptomatic women. The present study also reports the histopathological changes of the posterior vaginal fornix's and upper portion of the vagina of mice infected intravaginally with *T. vaginalis* isolates from 10 symptomatic and 10 asymptomatic women. The results show that there are no significant differences between symptomatic and asymptomatic isolates regarding histopathological changes.

Keywords

Trichomoniasis, Clinical Isolates, Experimental Study, Pathogenesis, Immune Response, Leukotriene B_4

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1. Introduction

Trichomonas vaginalis is the protozoan flagellate responsible for most common worldwide sexually transmitted diseases (STDs) called trichomoniasis [1]. One-third of *T. vaginalis*-infected women are asymptomatic [2], while some clinical manifestations as cervicitis, vulvo-vaginitis and urethritis with complications as infertility [3] and Cancer cervix happen [4].

Multifactorial mechanisms explain this phenomenon. Many authors suggested the presence of two different strains with different virulence factors [5]-[7]. Others relate this phenomenon to host immune response [8]. Many other factors play important role in parasite virulence e.g. cytoadherence [9] and higher levels of D-lactosyl residues on the surface of pathogenic strains [5]. However, virulence factors responsible of wide variation of symptomatology have not been well-defined.

Leukotrienes (LTs) are lipid mediator that are generated *via* the 5-lipoxygenase (5-LO) pathway in the arachidonic acid metabolism. Leukotriene B_4 (LTB_4) is synthesized from combined action of 5-LO and LTA_4 hydrolase on LTA_4 [10]. Microorganisms interact with phagocytes *in vitro* and experimental infections *in vivo* leading to LTs production [11]. LTs play essential roles in both innate and adaptive immune responses [10] [11]. LTB_4 are involved in the defense against protozoan and helminthic infections through modulation both Th1 and Th2 immune responses [12]. Antimicrobial activity [13] antiparasitic activity [14] and antifungal activity of LTB_4 occur through nitric oxide (NO) and cytokine production during infection [15]. Any defect in LTB_4 biosynthesis pathways will decrease the phagocytic and antimicrobial activities against microorganisms including parasites [14] [16] bacteria [17] and fungi [15].

Many researchers studied the immune responses to *T. vaginalis* in both murine models and human hosts. Production of Th-1 cytokine such as IL-2 and IFN-γ, IL-4 [18] and IgM, IgA, and IgG subclass [19] responses has also been demonstrated. Others suggested that *T. vaginalis*-derived secretory lipid mediator LTB_4 induces IL-8 production in mast cells [20]. However, the role of LTB_4 in host defense during *T. vaginalis* infection has been given less attention. How LTB_4 contributes to host resistance in experimental trichomoniasis is not also well understood and very little information is available in this point. In experimentally infected rats, data showed that infections did not differentiate between symptomatic and asymptomatic human isolates regarding histopathological and immunohistochemical changes. They revealed that interaction between parasite and vaginal epithelium was species specific [21]. However, data about this point is scarce. This issue isn't clarified using experimentally infected mice.

The present study aims to assess serum and local LTB_4 level and pathological changes in the vagina of mice infected with *T. vaginalis* isolates from symptomatic (SYM) and asymptomatic (ASYM) women.

2. Subjects and Methods

2.1. Subjects

Vaginal swabs were collected from 133 women in the reproductive age group, attending the Obstetrics and Gynaecology Outpatient Department by specialist, Suez Canal University Hospitals and Port Said General Hospital. This study was conducted from July to December 2013. Isolates from women attending the clinic with complaints of vaginal discharge, itching, dysuria and dyspareunia were considered as symptomatic isolates and from women attending hospital for routine antenatal check-up, infertility and family planning advice without any symptoms were considered as asymptomatic isolates.

2.2. For *in Vivo* Study

Vaginal swabs samples were subjected to wet smear examination and culture in Diamond's medium supplemented by 10% horse serum for detection and isolation of parasites [22]. Axenization of *T. vaginalis* isolates was achieved by adding antibiotics, penicillin (1000 U/ml) and streptomycin (1000 µg/ml) in the first three to five subcultures. The parasites in log phase of growth were separated by chilling and centrifugation followed by washing of trophozoites in phosphate-buffered saline (PBS, pH 7.2) and sub-cultured in fresh media [23]. The isolates were used for inducing intravaginal infection in BALB/c mice.

Ten isolates from symptomatic and 10 from asymptomatic women were used to induce experimental infection in female BALB/c mice. Mice were divided into 2 groups (30 mice/each): GI and GII were inoculated by *T. vaginalis* isolates from symptomatic women (SYM) and asymptomatic women (ASYM) respectively. In addition,

5 uninfected mice (control group) were used. Each group was subdivided into Ga (10 mice) for histopathological assessment and Gb (20 mice) for assessment of LTB_4 level. Trichomoniasis was induced intravaginally by inoculating 10 µl of TYM medium (containing 10^6 T. *vaginalis* trophzoites/mL/mouse) in oestradiol-pretreated (0.1 ml of 500 µl estrogen on the day 1 and every 2 days throughout the study) mice [24]. To confirm the induction of infection, mice were checked daily for the presence of *T. vaginalis* by wet smear examination and culture technique. Mice of control group were inoculated by 15 µl of free TYM medium intra-vaginally. Mice in GIa and GIIa and two uninfected mice were sacrificed at 14 days post infection (PI). The posterior vaginal fornix and upper part of the vagina were fixed in 10% formalin and processed for paraffin sections and stained with hematoxyline & eosin. Histopathological changes were assessed according to Escario *et al.*, [25].

2.3. Assessment of LTB_4 Level

Serum, vaginal wash (VWs) (50 µL) and vaginal tissue were collected from G Ib (SYM), and G IIb (ASYM) on the following different post days of *T. vaginalis* infection diagnosis (3^{rd}, 7^{th}, 14^{th}, 21^{st}, and 28^{th}) and 3 uninfected mice on day 0 [18]. Vaginal tissues were homogenized in ethanol (5 mL/g). The homogenate was centrifuged for five minutes and Collected in a clean tube. Thirty µl of serum were dispensed in 1.5 ml plastic tubes. Samples were taken in duplicate. Samples were stored at −70˚C until further use. LTB_4 levels in vaginal tissue, VWs and serum were assessed by commercial ELISA kits according to manufactures protocol (Cayman Chemicals, Ann Arbour, MI). The detection limit of the assay was 13 pg/ml [26].

2.4. Statistical Analysis

For *in vivo* study, values were represented in means ± SD. Student's *t* -test and Chi square test were used to test statistical significance for categorical data. Significance levels were at $P < 0.05$ for Chi square test and at $P < 0.001$ for Student's *t*-test.

2.5. Ethical Considerations

Informed consents were taken from patients. Laboratory breed mice free from pathogen were purchased from the Faculty of Veterinary Medicine, Suez Canal University and they were housed (5/cage) in proper room temperature and offered drinking water and regular mouse feed ad libitum.

3. Results

The histopathological picture of vagina from mice of uninfected control mice showed normal appearance of posterior vaginal fornix and upper part of the vagina (**Figure 1(a)**). Different pathological changes were detected among mice infected by *T. vaginalis* isolates from SYM women. Moderate and severe pathological changes were in mice (30%) and 6 mice (60%) respectively. On the other hand, only one mouse (10%) showed mild pathological changes. No severe pathological changes in vagina of mice inoculated by ASYM isolates. Eight mice (80%) infected by *T. vaginalis* isolates from ASYM women showed no pathological changes, while mild and moderate pathological changes were observed in only one mouse per each. The differences were statistically insignificant (**Table 1**).

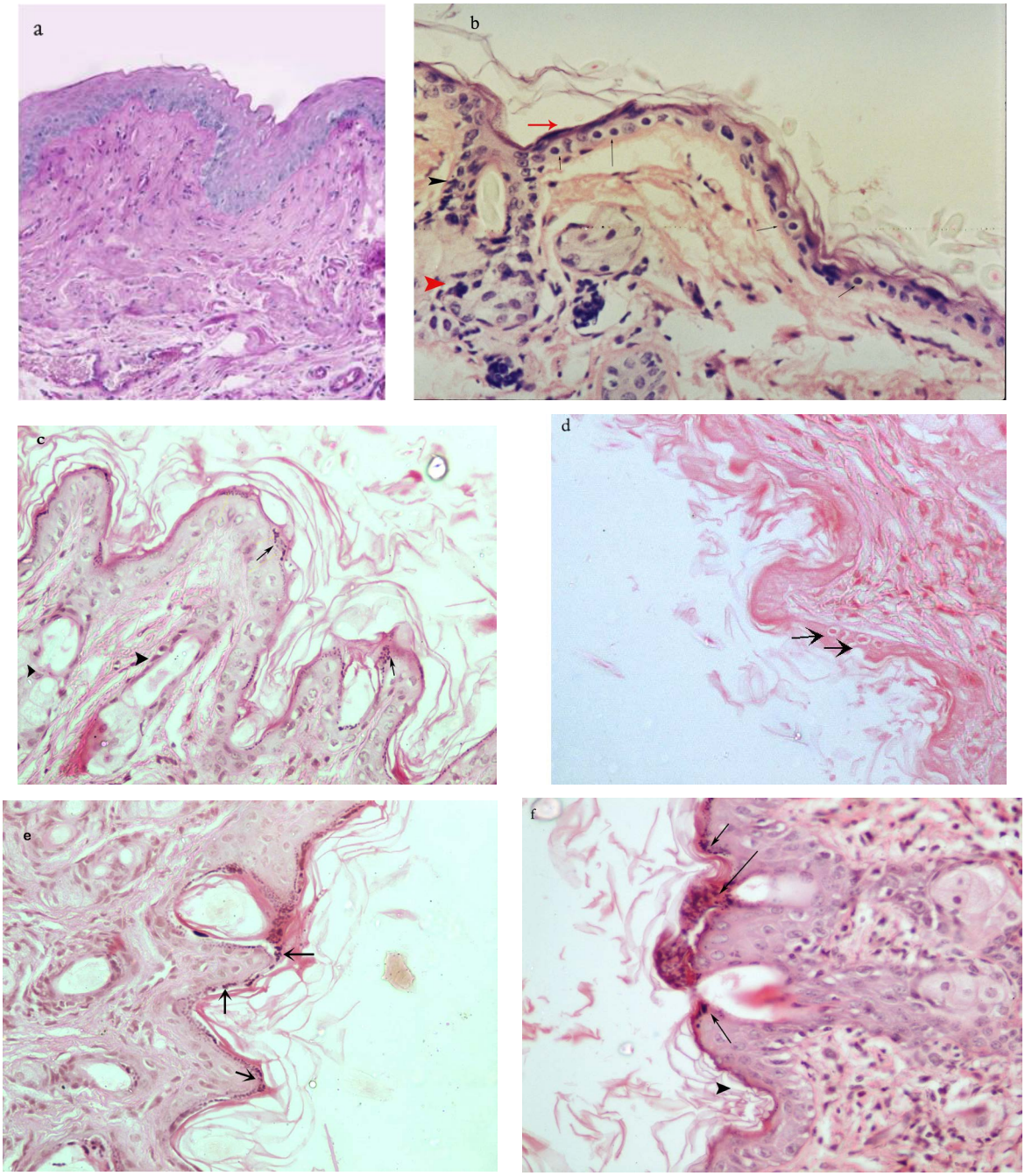
Mice vagina of group inoculated by SYM *T. vaginalis* isolates showed destruction of keratin layer and invaded the superficial layer of epithelium with desquamation and sloughing of epithelium (**Figure 1(b)**), dilated, ruptured vessels with interstitial hemorrhage in subepithelial lamina propria (**Figure 1(h)**). Mice vagina of group inoculated by SYM *T. vaginalis* isolates showed also marked increase in number of leukocytic infiltrates and cytoplasmic vacuoles with hydropic degeneration extend all over the fall thickness of stratified squamous epithelium in severe cases (**Figures 1(e)-(g)**). *T. vaginalis* parasite could be demonstrated as a round or flattened with a nucleus surrounded by a hallo (**Figure 1(b)**, **Figure 1(d)**).

Significant increase in mean of LTB_4 levels was observed on the 3^{rd} to 28^{th} PI in serum, VWs and vaginal tissue of mice infected with SYM (GIb) in comparison to mice infected with ASYM (GIIb) human isolates ($P < 0.001$) (**Figure 2**) and control. Mean LTB_4 concentration in mice infected by SYM and ASYM human isolates increased on the 3^{rd} PI, reached a peak on the 14^{th} PI followed by a decrease on the 21^{st} and 28^{th} PI. Vaginal tissue showed higher level of LTB_4 as compared with its level in serum and vaginal washes. Mean LTB_4 concen-

Table 1. The pathological degrees among mice infected with *T. vaginalis* isolates from symptomatic (SYM) and asymptomatic (ASYM) women.

Study group	Pathological degrees					Statistical analysis
	No	Mild	Moderate	Severe	Total	
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	
GIa	-	1 (10)	3 (30)	6 (60)	10 (100)	$X^2 = 9.364$ $P = 0.053^*$
GIIa	8 (80)	1 (10)	1 (10)	-	10 (100)	

GIa: Infected mice with symptomatic isolates (SYM). GIIa: Infected mice with asymptomatic isolates (ASYM). *Not significant.



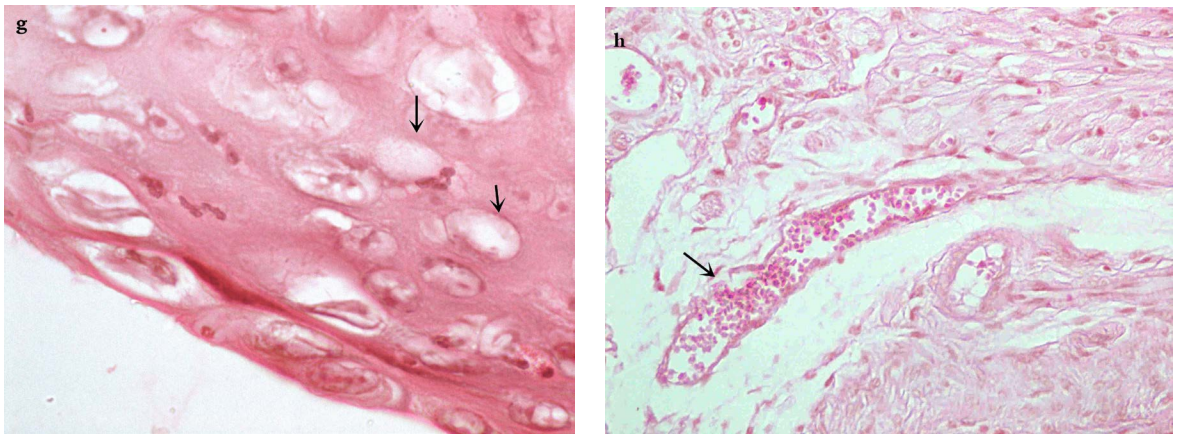


Figure 1. (a) Mice vagina of control group, showing normal histological structure. H & E. × 400; (b) Mice vagina of group inoculated by SYM isolates, showing desquamation of epithelium (red arrow), destruction of keratin layer and abundant lymphocytic infiltrate close to squamous epithelium (arrowhead) with severe invasion of the parasite to the epith. cells (black arrow) H & E. × 400; (c) Mice vagina of group inoculated by ASYM showing an moderate increase of leukocytic infiltrate in suprfecial layer of stratified squamous epithelium and lamina propria (arrow) with thick hyalinized blood vessels (arrowhead) (×400 H & E); (d) Mice vagina of group inoculated by ASYM showing mild increase in number of leukocytic infiltrate in the stratified squamous epithelium and mild invasion of parasite (×400 H & E); (e) Mice vagina of group inoculated by SYM isolates showing severe leukocytic infiltrate in the superficial layer of stratified squamous epithelium (×400 H & E); (f) Mice vagina of group inoculated by SYM isolates showing marked increase in number of leukocytic infiltrate in the superficial layer of stratified squamous epithelium (arrow) with desquamation of epithelium(arrowhead) (×400 H & E); (g) Mice vagina of group inoculated by SYM isolates showing cytoplasmic vacuoles with hydropic degeneration extend all over the superfacial layer of stratified squamous epithelium. (×400 H & E); (h) Mice vagina of group inoculated by SYM isolates showing dilated, ruptured vessels with interstatial hemorrhage in the subepithelial lamina propria (×400 H & E).

tration in mice (control group) was 13.3 ± 0.5 pg/ml and 13.6 ± 0.6 pg/ml and 14 ± 1 pg/ml in serum, VWs and vaginal tissue respectively.

4. Discussion

The pathogenesis of trichomoniasis is not clearly understood. Multiple virulence factors are involved in occurrence of symptoms among women. The parasite factors which may be involved in symptomatic infections of trichomoniasis are not clarified. The interaction between the host and *T. vaginalis* is complex. The existing literature shows little data on the host and parasite factors responsible for symptomatic or asymptomatic infection *in* women. The existence of two types of *T. vaginalis* strains, each differing in its morphological features and intrinsic virulence factors has been reported [5] [6]. Previous studies classify *T. vaginalis* isolates according to isoenzyme patterns [27], restriction fragment length polymorphism analysis [28] and random amplified polymorphic DNA (RAPD) analysis [29]. However, they show contrasting data.

The murine models had been proven to be valuable for investigating the pathogenesis and treatment of trichomoniasis [30] and can be applied for characterization of this variability [31].

The present study showed no significant difference in the pathological changes found in the vagina of mice infected with *T. vaginalis* isolates from symptomatic women as compared with asymptomatic women. Dissimilar result observed by Malla *et al.* [18] who showed that the mean parasite load significantly increase in mice inoculated with *T. vaginalis* isolates from symptomatic women versus those inoculated with parasites from asymptomatic women. The presence of two types of *T. vaginalis* strains with different intrinsic virulence factors has been suggested [32]. High pathogenic index between symptomatic isolates has been observed [33]. Others proved that cytoadherence to vaginal epithelium was higher in isolates from SYM women than ASYM one [34]. *T. vaginalis* isolated from symptomatic women was able to stimulate strong chemotactic response towards polymorphonuclear leukocytes (PMN) as compared to those isolated from asymptomatic one [35]. In experimental study, a higher inflammatory response also observed in mice infected with symptomatic isolates as compared to those infected with asymptomatic isolates [36]. The presence of chronic non-specific inflammation with sub epithelial infiltration by lymphocytes and neutrophils has been observed. Furthermore, with severe inflammation, deeper layers of the epithelium showed neutrophils [37]. Abundant PMN in vaginal smears with *T. vaginalis* in

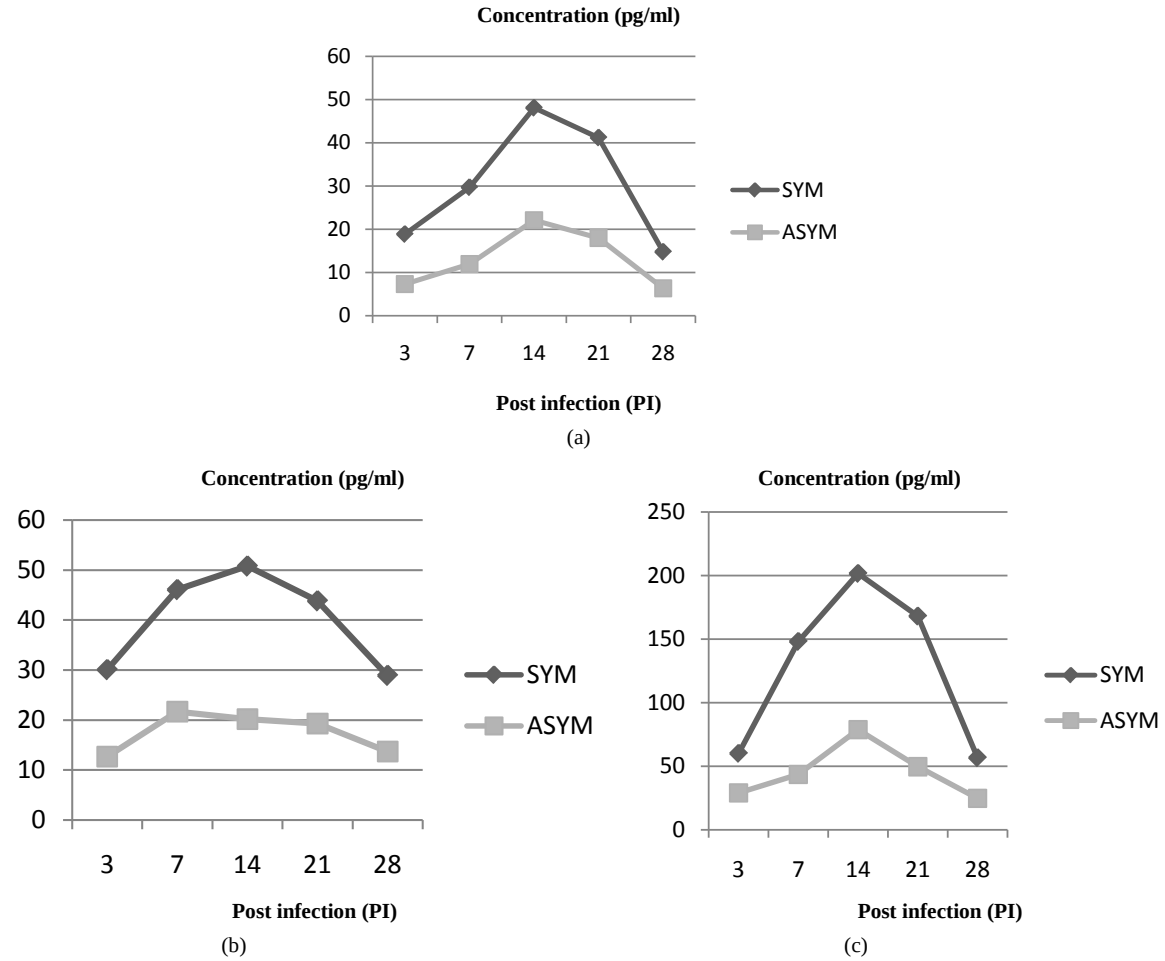


Figure 2. Mean Leukotriene B_4 levels in serum (a) and vaginal washes (b) and vaginal tissue (c) of BALB/c mice infected with *T. vaginalis* isolates from symptomatic (SYM) and asymptomatic (ASYM) patients. Results are expressed as mean and SD. NO = 20 per group (P <0.001 in 3rd, 7 th, 14th, 21st and 28th PI).

fected women were observed in another clinical study [38]. On the other hand, Helmy *et al.* [21] showed no difference among rats inoculated with *T. vaginalis* isolates from symptomatic and asymptomatic women regarding histopathological and immunhistochemical changes. Others failed to find correlation between clinical picture of host and pathogenicity of *T. vaginalis* in mice [39]. These contradictory findings may be explained by the fact that the effects of virulence factors are genetically initiated and controlled [31]. The diversity of host's immune response may also affect pathogenicity of *T. vaginalis* [40].

Local LTB_4 have been reported in women in previous study. A significant increase in the local LTB_4 was detected in vaginal washes of symptomatic, *T. vaginalis*-infected women when compared to asymptomatic patients [41]. However, the circulating LTB_4 level in symptomatic and asymptomatic women and local LTB_4 level in vaginal tissue in experimental mice hasn't been studied. In the present study, higher levels of LTB_4 were detected in the vaginal tissue than in the vaginal wash and serum of the all groups. Other work reported that reactive nitrogen intermediates is higher in vaginal tissue than vaginal washes and blood of infected mice [42]. This can be explained by the fact that vaginal washes and plasma may not be the ideal source of samples. Those samples may fail to identify the level of LTB_4 in the infected individuals exactly.

In the present study, LTB_4 level was highest on 14th p.i.d. and a significant difference was observed in LTB_4 levels in serum, vaginal washes and vaginal tissue of mice infected with *T. vaginalis* isolated from symptomatic patients as compared with asymptomatic subjects. A significant difference was also observed in LTB_4 in serum and vaginal washes of mice infected with symptomatic isolates as compared to asymptomatic isolates and uninfected mice. These results correlated well with the earlier studies conducted in clinical patients and in *vivo* stu-

dies in trichomoniasis. *T. vaginalis* releases neutrophil-activating factor known as LTB_4 [43]. Vaginal discharge from symptomatic patients with trichomoniasis showed high concentration of LTB_4 when compared to asymptomatic patients. Authors concluded that LTB_4 is involved in the inflammation and symptoms of trichomoniasis [41]. Whereas, others reported that this factor was extracted from both symptomatic and asymptomatic group of *T. vaginalis* isolates [44]. In *viro* studies showed that the supernatant of viable *T. vaginalis* induced LTB_4 production in neutrophils in an IgG- and complement-(C5-) dependent manner. This effect was decreased by LTB_4 antagonist treatment [45]. *T. vaginalis* has the ability to communicate directly with immune cells *via* interaction between LTB_4 and host cell BLT receptors resulting in modulation of the host's immune responses [20] and Neutrophil activation [46]. Interleukin IL8 production in mast cell and human neutrophils occur *via* BLT-dependent activation of transcription factors NF-κB and CREB [47]. On other hand, LTB_4 down-regulate IL-12, and up-regulate IL-10 by dendritic cells leading to immune tolerance [48].

These results also correlated well with the earlier reports conducted in experimental animals whereby Increase in LTB_4 in intestine has been demonstrated in other nematode-infected animals [49] [50] and other protozoan parasite as *Entamoeba histolytica* [51]. Experimental studies conducted on *Strongyloides venezuelensis* indicated greater LTB_4 release in the lungs, duodena and serum than that seen in the tissue of uninfected animals. Impairment of LTB_4 synthesis, either genetic or pharmacologic, is associated with increased numbers of worms and eggs in infected animals. Altered resistance to parasite in the absence of LTB_4 may explain this increase. LTB_4 levels during *S. venezuelensis* infection have main role in regulating IL-5 release, IgG and IgE [14] and production of IL-8 [52]. LTB_4 may effectively have a role in the rapid expulsion of *T. spiralis and* other helminthes by increase the contractility of smooth muscle cells, the permeability of epithelial cells and the production of mucus [53]. In experimental leishmaniasis, treatment with exogenous LTB_4 increase parasite killing by macrophages in BALB/c mice [54]. LTB_4 reduces the parasitic load of macrophages infected with *Leishmania amazonensis* through production of reactive oxygen species (ROS) and interleukin-1β (IL-1β) [55]. In addition, LTB_4, play a role in immune response to *Leishmania* infection by promoting leishmanicidal activity and modulation of the immune system in synergism with genetic factors [56]. Genetic variation in LTB_4 synthesis has been suggested and might influence resistance and susceptibility patterns to *L. amazonensis* infection [54]. In *Leishmania infantum* in *vivo*, the macrophage response is characterized by C-type lectin receptors (CLRs) signature. CLRs allow parasite resilience through inhibition of the LTB_4-IL-1β axis. So, CLRs might be a potential targets for treatment and prevention of visceral leishmaniasis [57]. Pharmacologic or genetic inhibition of 5-LO biosynthesis, with subsequent lack of LTs, lead to impaired clearance of bacteria, fungi and parasites with increased mortality. LTs play a role in the control of helminthes and protozoan infections by modulating the immune system and/or through direct cytotoxicity to parasites. In addition, the decreased production of LTB_4 in immunocompromised individuals might have a role in modulating the pathophysiology of helminthic and protozoan infections [12]. In contrast, LTB_4 overproduction in experimental cerebral malaria model lead to increased parasitemia and mortality rate of infected mice [58]. However, some protozoan parasite as *T. gondii* may inhibit LTB_4 production whereas exogenous LTB_4 promoted intracellular killing of ingested *T. gondii* in human monocytes [59]. By using of LTB_4 receptor antagonist and genetic approaches (5-LO-deficient mice), researchers have demonstrated increased parasitemia in mice infected with *T. cruzi* [60] [61]. In Chagas disease, LTB_4 control parasite differentiation and proliferation in the infected host cells allowing the infection to progress to a chronic state. So, LTB_4 initially ensure host survival but later on may cause cardiovascular damage [62]. In acute phase of infection, endogenous LTB_4 are important regulators of NO activity in the heart and therefore, affect cardiac parasite load without a direct action on IL-6 release [61]. Therefore, LTB_4 might represent a new series of targets for therapy with certain precautions [62].

5. Conclusion

The present study demonstrates that *T. vaginalis* infection induces production of LTB_4, which is essential for a protective immunity against parasites. Further study is needed to clarify correlation between pathological changes and LTB_4 in trichomoniasis.

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Author Contribution

AM Eida provided the original idea of the research and planned the study design. She shared in the experimental studies, assessment of LTB_4 level, results analysis and wrote the manuscript. OM Eida shared in the study design, the experimental studies, assessment of LTB_4 level and manuscript writing. AS SALEM did the histopathological assessment.

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