RESEARCH ARTICLE
PREDICTION OF ACUTE TRICHOMONIASIS IN WOMEN COMPLAINING VAGINAL DISCHARGE BY DIFFERENT IMMUNOLOGICAL METHODS
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ABSTRACT

Trichomonas vaginalis is commonly causes vaginitis and perhaps cervicitis in women and urethritis in men and women. Neutrophils are important immune cells in response to Trichomonas vaginalis. In this study, we investigated whether human neutrophils could be used as predictive marker of acute infection by detection of their surface CD62L, however confirm their recruitment by estimation of IL-8 chemokine and predict their apoptosis by estimation of IL-10 in early infection by screening of serum protein profile and finally characterize the systemic immune response in infected women with trichomoniasis by detection of C-reactive protein (CRP) in serum. The results suggested that Trichomonas vaginalis stimulate human neutrophils significantly P<0.001 higher in early infected women than in late infected women, however IL-8 elevated significantly in early infected women than late infected one while, IL-10 decreased significantly in acute infected women in comparison to chronic infected women. Serum protein profile showed normal values of total protein in both disease activity (early and late) while other components revealed an unexpected results, as well as protein electrophoresis on agarose gel revealed normal bands in all patients women except some of late infected women showed abnormal bands at β and γ regions. Finally the results detected that vaginal infection with T. vaginalis during both disease activity was associated with a clear systemic inflammatory response exhibited by an increased serum CRP concentrations significantly P<0.001 in infected women in comparison to control.

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Performing serum protein electrophoresis is a valuable screening test since changes in certain bands are clearly associated with particular disorder (25). Several principle plasma proteins can be grouped as acute-phase reactant (ARP). The concentration of these proteins rises significantly during acute inflammation (26).

For these reasons this study aimed to predict the acute infection of *Trichomonas vaginalis* by detection of anti-human selectin(CD62L) antigen expressed on earlier inflammatory cells neutrophils in women blood, however confirm the recruitments of these cells (neutrophils) by evaluation of proinflammatory chemokine IL-8 and predict the apoptosis of neutrophils by estimation the level of anti-inflammatory cytokine IL-10, then differentiate between acute infection and late by screening of serum protein profile by electrophoresis technique, and confirm the systemic immune response in infected women by evaluating C- reactive protein in their sera.

**MATERIALS AND METHODS**

**Subject samples**

This study included 160 women within the reproductive age group attending the obstetrics and gynecology out-patients department of Al-Kadhimiya Teaching Hospital in Baghdad, Iraq during the period from October, 2011 till April 2013. Vaginal swabs, heparinized blood and serum samples were collected from both asymptomatic and symptomatic (itching and dysuria) women complaining vaginal discharge within different periods of illness.

**Culture**

Study participants had a *T. vaginalis* culture performed with each pelvic examination. The swabs were inserted intothe pooled vaginal secretions and also touched both fornices and the mid third wall of vagina. Each swab was used to inoculate the upper chamber of the In-Pouch TV bag (BIO-med Diagnostics,USA). The upper chamber of the In –pouch was immediately examined for the presence of motile *T. vaginalis* by using the 10X objective. In-pouch cultures which demonstrated motile trichomonads were considered positive and not further examined.

**Neutrophils phenotyping**

Heparinized blood was processed according to Boyum in 1968 (27) for granulocytes separation, two major steps were involved, each using specific conditions to separate cells based on their intrinsic density: Sedimentation in dextran at 1gm and differential sedimentation in discontinuous density gradient ofisopaque-ficoll. The aspirated granulocytes were washed three times with PBS and centrifuged at room temperature. At 300g for 5min. The cell pellet was resuspended in buffer. Cell number was adjusted to contain 1x10^6 cell/ml. 10µl of granulocytes suspension were placed per well of an immunofluorescent slide, allowed to air –dry at room temperature, then10µl aceton as a fixative was added, allowed to air-dry at room temperature, wrapped in foil and stored at -20ºC until used in immunostaining. The indirect method used for the determination of neutrophils phenotyping and (CD62L) is reactive with neutrophil antigens.

**Chemokine and cytokine estimation**

In order to quantify specific chemokine IL-8 and cytokine IL-10 in women patients sera, ELISA Kit (Human IL-8, and IL-10Mabtch AB, Sweden) used. All assays were performed in accordance with manufactures specification.

**Serum protein profile**

The serum of each positive patient underwent to electrophoresis analysis by (Hellobio kit for protein electrophoresis, Greece). This test gives clear bands of major protein fractions with quantitative levels.

**C- reactive protein**

C- reactive protein concentration was quantified by a latex agglutination slide test for the qualitative and semi-quantitative determination in non-diluted serum (Cromatest, linear Chemicals, Spain).

**Statistical Analysis**

The Statistical Analysis System- SAS (2012) was used to effect of different factors in study parameters. Least significant difference –LSD test was used to significant compare between meansin this study.

**RESULTS**

Out of a total 160 women enrolled in this study, 35(21.87%) revealed the presence of *T. vaginalis* parasite by In-pouch TV system culture. Those infected women classified into two groups according to the symptoms and duration of illness. The first group included 18 (11.25%) asymptomatic early infected women (less than 2 weeks) and with discharge (frothy-yellowish green and mucopurulent), while the second group17(10.62%) symptomatic (pruritus and dyspunria) late infected women with duration of illness (more than 2 weeks) and discharge (frothy-yellowish green and mucopurulent).Table(1).All negative samples 125(78.125%) considered as a control group.

**Table 1** Subject samples included in the current study

<table>
<thead>
<tr>
<th>Total</th>
<th>Positive trichomoniasis women by in pouch TV</th>
<th>Negative trichomoniasis women by in pouch TV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>culture</td>
<td>culture</td>
</tr>
<tr>
<td>160 women with discharge (frothy-yellowish green and mucopurulent).</td>
<td>35(21.87%) Early infection 18(11.25)</td>
<td>125 (78.125%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17(10.62%) Late infection</td>
</tr>
</tbody>
</table>

| Immunostaining of neutrophils in peripheral blood |

The percentage of neutrophils (CD62L) positive cells was significantly higher (p 0.001) in early infected women with *T. vaginalis* (87.2±1.98) in comparison to control group(50.00±0.56) as well as ,early infected women showed.
significant increase percentage P 0.001 of neutrophils in comparison to late infected women (64.10±1.44 ).Table(2).

Table 2 The meanpercentage of neutrophil phenotype CD-62L in women infected with T. vaginalis

<table>
<thead>
<tr>
<th>Subject(group)</th>
<th>Mean ± SE of CD-62L (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Infection</td>
<td>87.21 ± 1.98 a</td>
</tr>
<tr>
<td>Late infection</td>
<td>64.10 ± 1.44 b</td>
</tr>
<tr>
<td>Control</td>
<td>50.00 ± 0.56 c</td>
</tr>
<tr>
<td>LSD value</td>
<td>4.212</td>
</tr>
<tr>
<td>P-value</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Means having different small later in columns are significant difference.

IL-8 and IL-10 cytokines estimation

The present study showed that the mean concentration of IL-18 in early infected women with trichomoniasis (139.90±2.71pg/ml) was significantly higher (p 0.001) than late infected women (113.00±1.07pg/ml) and both showed significantly increased concentration (p 0.001) in comparison to control group (70.00±0.77pg/ml).Table(3).

Table 3 Mean concentration of IL-8 chemokine in trichomoniasis infected women

<table>
<thead>
<tr>
<th>Subject(group)</th>
<th>Mean ± SE of IL-8 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early infection</td>
<td>139.90 ± 2.71 a</td>
</tr>
<tr>
<td>Late infection</td>
<td>113.00 ± 1.07 b</td>
</tr>
<tr>
<td>Control</td>
<td>70.00 ± 0.77 c</td>
</tr>
<tr>
<td>LSD value</td>
<td>5.047</td>
</tr>
<tr>
<td>P-value</td>
<td>0.001</td>
</tr>
</tbody>
</table>

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While the mean concentration of IL-10 in early infected women (65.00±1.01 pg./ml) was significantly lower than its concentration in late infected women (109.70±2.30pg/ml) and both disease activity were with higher significantly (p 0.001) levels than a control group (2.00 ± 0.39 pg./ml).Table(4).

Table 4 Mean concentration of IL-10 cytokine in trichomoniasis infected women

<table>
<thead>
<tr>
<th>Subject(group)</th>
<th>Mean ± SE of IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early Infection</td>
<td>65.00 ± 1.01 b</td>
</tr>
<tr>
<td>Late infection</td>
<td>109.70 ± 2.30 a</td>
</tr>
<tr>
<td>Control</td>
<td>2.00 ± 0.39 c</td>
</tr>
<tr>
<td>LSD value</td>
<td>4.260</td>
</tr>
<tr>
<td>P-value</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Means having different small later in columns are significant difference.

Serum protein profile

To investigate the effect of trichomoniasis caused by Trichomonas vaginalis on serum protein profile, common monitoring technique (protein electrophoresis) was done.

Table 5 Mean concentrations of serum protein components in women infected with vaginal trichomoniasis

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total protein/dl</th>
<th>Albuming/ dl</th>
<th>Α1-globuling/dl</th>
<th>Α2-globuling/dl</th>
<th>Β- globuling/dl</th>
<th>γ- globuling/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early infection</td>
<td>6.10 ± 0.79 a</td>
<td>3.19 ± 0.18 b</td>
<td>0.359 ± 0.02 b</td>
<td>1.19 ± 0.12 a</td>
<td>1.33 ± 0.06 b</td>
<td>1.43 ± 0.07 b</td>
</tr>
<tr>
<td>Late infection</td>
<td>5.42 ± 0.09 a</td>
<td>3.10 ± 0.04 b</td>
<td>0.316 ± 0.01 c</td>
<td>1.43 ± 0.07 a</td>
<td>2.01 ± 0.04 a</td>
<td>1.99 ± 0.02 a</td>
</tr>
<tr>
<td>Control</td>
<td>5.86 ± 0.04 a</td>
<td>3.81 ± 0.04 a</td>
<td>0.400 ± 0.01 a</td>
<td>0.720 ± 0.05 b</td>
<td>1.11 ± 0.06 c</td>
<td>0.943 ± 0.05 c</td>
</tr>
<tr>
<td>LSD value</td>
<td>1.343</td>
<td>0.317</td>
<td>0.037</td>
<td>0.250</td>
<td>0.157</td>
<td>0.158</td>
</tr>
<tr>
<td>P-value</td>
<td>0.580</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Means having different small later in columns are significant difference.

The result revealed that all 18 samples of earlyinfected women and 17 late infected one within the normal values of total protein concentration, (6.10± 0.79 g/dL) and (5.42± 0.09 g/dL) respectively in comparison to control group (5.86 ± 0.04 g/dL).

C-reactive protein

The result revealed a significant increase (p 0.001) of CRP level (450.5± 12.52 mg/ml) in early infected women in comparison to late infected one (315.7± 17.06 mg/ml) and both
disease activities showed significant increase (p < 0.001) in comparison to control group (4.5 ±0.66 mg/ml).Table (6).

Table (6) Mean concentration of C-reactive protein in early and late infected women with T. vaginalis

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Early infected women</th>
<th>Late infected women</th>
<th>Control group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive Protein levels/ml</td>
<td>450.5±12.52a</td>
<td>315.7±17.06b</td>
<td>4.5±0.66c</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Means having different small later in columns are significant difference.

**DISCUSSION**

Trichomonad is one of the most commonest sexually transmitted diseases capable of causing considerable morbidity in infected patients (28). Infection with T. vaginalis may show diversities with respect to socio-cultural properties of the communities changing from a country to another from society to another (29).

Prevalence rate of T. vaginalis obtained in this study was 21.87%. This percentage showed that the incidence of T. vaginalis infection agreed with previous results by (30, 31) that found the incidence rate in Iraq was 19.5% and 19.6% respectively. While other study showed an increased percentages 38.5%(32). The differences between results may related to time of the incidence, type of safe and effective method of prevention (33), potential side effects and clinical failures associated with the therapy drug of choice, metronidazole(32) and type of strain itself(34).

Surface CD62L receptor was used as a marker of neutrophil presence. Human neutrophils are the primary effectors cells in acute inflammation and have been implicated in the pathogenesis of a large variety of diseases. They are rapidly recruited from the blood stream in large numbers through the vascular endothelium to the inflamed site (35).

CD62L (L-selectin) is an adhesion molecule expressed on most leukocytes that has been shown to mediate rolling of neutrophils on the endothelium of post capillary venules (36). The expression of CD62L is at its highest level when the cells are released from the bone marrow(37) and then decreases as the cells age In vitro and In vivo(38). Matsuba and co-workers have shown that older circulating neutrophils with low CD62L expression are prone to undergo apoptosis in the circulation, which could mark them for removal by macrophages in the liver and spleen (39).

The current result was agreed with previous result by Beinertet al.(35) that showed the expression of the leukocyte selectin CD62L was maintained in non-apoptotic neutrophils, whereas apoptotic neutrophils expressed very low levels of CD62L.

Apoptosis represents a physiological clearance mechanism in the circulation and in the tissue to maintain homeostasis of leukocytes numbers in healthy humans (40).

Dransfieldet al. (41) reported that apoptotic neutrophils show reduced expression of CD62L. The altered adhesive potential of the apoptotic neutrophil may serve to limit release of its histotoxic contents and reduce inappropriate tissue injury (42). Moreover, apoptosis of human neutrophils is associated with loss of their surface CD16 (41, 43) and reduced expression of complement regulatory molecules (44).

Our finding of reduced of CD62L from the surface of neutrophil in late infected women with trichomoniasis was consistent with several studies uniformly describing low levels of CD62L expression on apoptotic neutrophils (39, 41, 45). So this phenomenon in our result may serve as predictive evaluation of acute and late trichomoniasis.

IL-8 is a major chemokine responsible for neutrophil recruitment to sites of tissue insult and inflammation (46, 47). Shaoiet al.(48, 49) reported the presence of IL-8 in the vaginal discharge from patients with symptomatic trichomoniasis, providing evidence for involvement of IL-8 by monocytes and neutrophils stimulated by live trophozoite(50). Here the result reports the presence of systemic IL-8 in sera of a symptomatic and symptomatic patient, characterized by elevation of this cytokine in both disease activities.

The difference among the mean concentration of IL-8 in early and late infected women in comparison to control group may result from different conditions of the parasite itself. Song (50) showed variously conditioned trophozoite, such as live, damage, or attenuated parasites, and secretory molecules from T. vaginalis could have an effect on immune response around the site of inflammation. Trichomonads caused neutrophils to produce low level of IL-8 compared to live T. vaginalis, and IL-8 may contribute to apoptotic changes of neutrophils (51).

During epithelial cell infection T. vaginalis stimulates cytokine production and evoke inflammatory responses (8, 52) that recruit a range of neutrophils across the epithelial barrier (50, 53). It also releases neutrophil activating factor (54). Accordingly, it is suggested that a balance of viable trophozoites, lyase and excretory-secretory products ESP from T. vaginalis could may contribute to progression of inflammatory reactions in trichomoniasis via the control of neutrophil apoptosis (17) and it has been hypothesized that the production of IL-8 upon T. vaginalis infection may be secondary to cytopathic effects and the release of the early response proinflammatory cytokine TNF-α and IL-1β by damage epithelial cells (55).

Clearance of apoptotic cells has an important role in tissue remodeling and resolving inflammation, as well as in protecting tissue from exposure to inflammatory and immunogenic contents of dying cells (56, 57).

Removing cells before they undergo lysis, it is proposed that the ingestion of apoptotic cells results in potent anti-inflammatory and immune suppressive effects through the production of anti-inflammatory cytokines (22, 58).

The production of anti-inflammatory cytokine IL-10 and the suppression of pro-inflammatory mediators such as IL-8 in late infected women in this study may reflect the apoptosis process of neutrophils at this stage of disease.

In contrast to apoptotic neutrophils, pro-inflammatory cytokines induced by lysed neutrophils may happened by liberated protease (59). Meanwhile, fresh neutrophils caused human macrophage to induce increased proinflammatory cytokine and decreased IL-10 production (24).
In this study, it is noteworthy that only \textit{T. vaginalis} induced apoptotic neutrophils induced significantly increased IL-10 production among the apoptotic neutrophils induced by different methods. This result aligns with previous reports stating that apoptotic lymphocytes or neutrophils induce IL-10 or TGF-β production by monocytes or macrophages (22, 58, 60, 61).

So the current results suggested that high production of anti-inflammatory cytokine IL-10 in late infected women with trichomoniasis may lead to resolution of vaginal inflammation and apoptotic of early inflammatory cells.

According to serum protein component the decrease of albumin in the current study was within the normal values of albumin concentration in human but may happened due to vaginal inflammation or kidney disorder that accompanied with trichomoniasis insult or may some of studied women were pregnant these reasons account for low albumin level (62).

On one hand \(\alpha_1\) globulins are increased in inflammatory diseases (63) this may reflect sever inflammation in early infected women with trichomoniasis than in late infected women. \(\alpha_2\) component is increased as an acute–phase reactant (62). One of the most useful aspects of acute phase protein measurements is the sensitivity to small amounts of inflammation. (64). This increase in both disease activity may reflect that those patients under nephrotic disorder due to the infection with \textit{T. vaginalis} (63). Beta fraction has two peaks labeled \(\beta_1\) and \(\beta_2\), \(\beta_1\) is composed mostly of transferrin, and \(\beta_2\) contains beta–lipoprotein. IgA, IgM and sometimes IgG are along with complement proteins. So the increased in late infected women in comparison to other groups may explained by exposure of those women to iron deficiency anemia or carcinoma sometimes or may at third trimester pregnancy (65).

On the other hand much of clinical interest is focused on the gamma region of the serum protein spectrum because immunoglobulins migrate to this region. Gamma globulin levels are increased in acute and chronic infections (63). This fact agreed with the current result in a clear elevation of γ-globulins in both disease activities in comparison to control group.

The explanation of serumelectrophoresis results cleared that no obvious changes occurred in serum protein patterns of trichomoniasis women because all significant differences in serum protein levels that scored in the current result within the normal values or near from the normal values except in those seven women with late infection .The presence of abnormal band at β- region may reflect either those women pregnant at third –trimester or may had nephritic disease due to the effect of \textit{T. vaginalis} on the urinary tract or may had carcinoma, especially when there is evidence about a relative risk of developing invasive cervical cancer associated with \textit{T. vaginalis}(29).

While, those five women with abnormal bands at γ region may represent the sever chronic inflammation due to trichomoniasis (62)More researches and more numbers of patients with more broad clinical history will need to confirm such result.

Little work done characterizing the systemic immune response to sexually transmitted infections has been published, Hedegaet \textit{et al.} (66) examined the systemic immune response in non-pregnant women with gonococcal(GC) infection. They did not find a significant systemic immune response in non-pregnant women with GC alone, but found elevated concentrations of inflammatory cytokines when women were concomitantly infected with other sexually transmitted pathogens. Interestingly, they found that in women coinfected with GC and TV, the systemic immune response was intensified after treatment.

C-reactive protein (CRP) is a marker of systemic inflammation that is frequently elevated in response to infection. Is associated with overall increased risks of preterm birth, infection, and inflammation (67,68). The current study showed significant increase in its level in both disease activity which predict the acute (early) infection with trichomoniasis clearly.

References


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