Humoral immunity response against *Gardnerella vaginalis* in rabbit

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**Abstract**

Three groups of rabbits were used to study the immunization with killed, live bacteria and cell free culture of *Gardnerella vaginalis* by using direct agglutination and passive haemagglutination. We found that these antigens stimulate local and systemic specific humoral immune response. Delayed type hypersensitivity was also studied and found it stimulate T cells.

**Introduction**

Bacterial vaginosis (BV) is the most prevalent vaginal disorder in women of reproductive age, affecting 10–20% of Caucasian women, and 30–50% of African American women, although estimates of its prevalence depend on the population studied (Eschenbach, 1993; Sobel, 2000; Ojiyi et al., 2012). The normal healthy vagina is populated mainly by hydrogenperoxide-producing lactobacilli that inhibit the growth of other vaginal flora (Eschenbach et al., 1989). BV is characterized by a loss of these protective lactobacilli, an increase in vaginal pH up to 4.5, and the proliferation of a variety of anaerobic species. Symptoms of BV can include a thin greyish-white vaginal discharge with a foul odour, and mild irritation. Of more concern is that BV is associated with serious disorders, such as pelvic inflammatory disease (Sobel, 2000; Larsson et al., 2005) and adverse pregnancy outcomes, such as preterm delivery, low birth weight and post-partum endometritis (DiGiulio et al., 2008). BV also increases the risk of male-to-female, and female-to-male, HIV transmission (Schmid et al., 2000).

*Gardnerella vaginalis* cells are gram-negative to gram-variabl, small, pleomorphic rods that are nonmotile and do not possess flagella, endospores, or typical capsules. In vaginal fluid smears the Gram reaction of G. vaginalis may vary from positive to negative (Catlin, 1992). It is present in up to 95% of cases of BV (Catlin, 1992; Marrazzo et al., 2008); however, with the advancement of molecular tools, it has been shown that the numbers and diversity of anaerobes associated with BV are high (Oakley et al., 2008). One study has found that pure cultures of *G. vaginalis* do not always cause BV, and that the organism can occur, albeit in low numbers, in healthy women (Gardner & Dukes, 1955; Fredricks et al., 2007). Together, these findings cast doubt on the role of *G. vaginalis* in the pathogenesis of BV. Recently, the term functional equivalent pathogen/pathogroup has been proposed for polymicrobial infections in which no one species alone is capable of causing disease, but rather the synergistic effects of the consortium give rise to the pathophysiology (Dowd et al., 2008). The aim of actual study to improve the ability of bacteria *G. vaginalis* to induce mucosal and systemic immune response.

**Materials & Methods**

**Bacterial isolate :**

Specimen taken from 350 pregnant and non pregnant women were attend to hospital of Babylon for labor and children, during a period from March 2011- April
An unlubricated sterile Cusco’s speculum was inserted and lateral and posterior vaginal fornices were swabbed with sterile cotton tipped applicators. Two vaginal samples were obtained from each pregnant women. One of the swabs was for culture to which one ml of sterile Stuart transport medium (Oxoid) was added and the other swab was for microscopic examination (Wet mount and Gram’s stain). After taking the swabs, vaginal pH was measured by a pH paper placed in contact with the secretions on the speculum after it had been withdrawn.

Amine test was performed by adding a drop of 10% KOH to the discharge on the used speculum and sniffed (Amsel et al., 1983). Wet mount preparation was done at the bed side within (5-10) minutes of collection of the specimen to detect the presence of *Trichomonas vaginalis*. Clue cells were diagnosed by Gram’s stained smears. Women were considered as a case of bacterial vaginosis when there was presence of at least three out of four clinical signs (homogenous vaginal discharge, pH greater than 4.5, positive amine test and clue cells which are epithelial cells covered with bacilli detected on Gram’s stained smears (Amsel et al., 1983; Jawetz et al., 1998). The isolation and identification of different microorganisms together with various laboratory tests was based on standard methods. Briefly, swabs were inoculated directly on to chocolate agar, Columbia blood agar incubated in candle jars with (5-10%) CO2 at 37°C for 48 hours.

**Rabbits:**

In this study we use twelve of female local rabbits (*Oryctologus cuniculus*) , age (8-9) months and weight from (1.5-2) kg 2.

**Antigen preparation**

We use three antigen in this study:

1. Killed antigen by benza quinum chloride according to (McCoy & Kennedy, 1960) modified by (Al Thahab, 2006). by using pure culture of bacteria and formal saline solution , and serial steps of centrifugation, aid equal size of supernant and benza quinum chloride.

2. Cell free culture antigen according to (Shnawa & Thewaini, 2002).
   a. bacteria was cultured into tube containing 5 ml brain heart infusion broth and supplements.
   b. The tube was incubated anaerobic at (37)°C for (48)hrs .
   c. At the end of incubation period the tube was centrifuged at 2500rpm for 5mins.
   d. The culture then was filtrated by using 0.22μm (Millipore filter) and then was stored until use.

3. Live bacteria according to (Gibbs et al., 1987) by culture bacteria into tube containing 5 ml brain heart infusion broth and supplements and after incubation period the tube compare with macfarland tube.

**Immunization**

Three rabbits for each group were inoculated by each antigen with equal ratio 1:1 with complete Freund’s adjuvant (Difco) was injected as schedule according to (Al Thahab , 2006). and three rabbits as control for each group inoculated with normal saline. In the fifth week blood taken from animal by heart puncture. Blood samples were collected to study systemic immunity and autopsies were collected (appendex) as local study and secretory immunoglobulins were extracted according (Mancini, 1965).
Tube agglutination test
This test is done according to (Garvey et al., 1977).
1. Eleven clean agglutination test tubes were used and (0.9) ml normal saline was added to first tube and (0.5) ml was added to the rest tubes for sera while for antigens 0.2 ml of normal saline was added to other eleven tubes.
2. (0.1) ml of serum was added to first tube and mixed gently by pasteur pipette and then (0.5) ml was transferred to the second tube until 10th tube. Tube number eleven was considered as a control tube containing (0.5) ml normal saline, while 0.2 ml of antigens was added to tubes until 10th tubes.
3. (0.5) ml of antigen was added to each tube of serum.
4. The content was incubated at (37)°C for (24) hrs.
5. The titer was recorded.

Passive haemoagglutination test
According to (Boyden, 1951) Sheep blood was collected and preserved with Al sever's solution in a proportion volume: volume mixture was left at 4°C for at least 3 days and micro titration plate was used to dilute the serum and VIgs. 50 ml of normal saline was added to each well of plates including ten well and eleventh well was considered as control tube for each plates then (50) μl of VIgs was added to the first well and then mixed gently and pipette by micropipette to second well until ten well, while serum was diluted by adding 0.1 ml of serum to 0.9 ml of saline and from this tube then transferred 50 μl to first well of plate and then mixed and pipette to second well until ten well , and (50) μl of solution was added to each well of the eleven wells for each plates and incubated at (37)°C for 45 mins and then titer was recorded.

Skin test
Done according to (Tompkins et al., 1973) 0.25 ml from killed and cell free culture antigens were injected under skin of rabbit while animal control were injected with normal saline to see the result of this test.

Results
The result of this study show both killed antigen and live bacteria stimulate humoral, systemic and local immunity by tubes agglutination test with blood serum and with secretory immunoglobulins separated from appendix, results came as in table (1) animals immunized with killed antigen of bacteria G. vaginalis gave systemic antigenimia with average 1280 and the same animals gave local antigenimia with average 128, while animals immunized with live bacteria gave systemic antigenimia with average 640 and the same animals gave local antigenimia with average 64.

From result found in the table (1) show differences as comparison with control animals which gave systemic antigenimia with average 10 and the same animals gave local antigenimia with average 1.
Table(1) Specific antibody titers against *G. vaginalis* antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Average antibody titer</th>
<th>Systemic (serum)</th>
<th>Local (appendix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Killed bacteria</td>
<td></td>
<td>1280</td>
<td>128</td>
</tr>
<tr>
<td>Live bacteria</td>
<td></td>
<td>640</td>
<td>64</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

Numbers represent average of three variations*

Table(2) Specific antibody titers against *G. vaginalis* cell free culture

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Average antibody titer</th>
<th>Systemic (serum)</th>
<th>Local (appendix)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free culture</td>
<td></td>
<td>2560</td>
<td>32</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

Numbers represent average of three variations*

Also killed antigen and cell free culture of bacteria *G. vaginalis* induce delayed type hypersensitivity in rabbit which describe by erythema, induration diameter (cm) and necrosis as seen in table 3.

**delayed type hypersensitivity test in immunized rabbits Result of Table(3) with different time and antigens**

<table>
<thead>
<tr>
<th>Antigens</th>
<th>4 h.</th>
<th>24 h.</th>
<th>48 h.</th>
<th>72 h.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E ID</td>
<td>N</td>
<td>E ID</td>
<td>N</td>
</tr>
<tr>
<td>Killed bacteria</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Live bacteria</td>
<td></td>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td>Cell free culture</td>
<td></td>
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<tr>
<td>Control</td>
<td></td>
<td></td>
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<tr>
<td>E :Erythema</td>
<td></td>
<td></td>
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</tbody>
</table>

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Discussion

Actual study referred to ability of *G. vaginalis* antigens to stimulate local and systemic specific humoral immune response and this agree with others studies, like Cohen and his workers refered to secretory immunoglobulin A, which may be present in vaginal secretions could affect the progress of bacterial vaginosis by coating *G. vaginalis* and blocking adherence to epithelial cells (Cohen et al., 1984).

While the study of (Heisterberg et al., 1987) determined the titers of agglutinating antibodies against *G. vaginalis* in sera from patients who had undergone first-trimester abortion and who harbored *G. vaginalis* in the cervix. A titer of 2560 was found in serum from one woman who experienced pelvic inflammatory disease, whereas the titers were not higher than 320 in 29 women with uncomplicated postabortal courses. Kristiansen et al. (1987) studied three patients with clinical signs of endometritis who underwent; in each case, *G. vaginalis* was isolated in pure culture from the fundus of the uterine cavity. Titers of serum antibodies that reacted with *G. vaginalis* were 64, 256, and 512 as determined by an immunofluorescence assay method in which a titer of 216 was regarded as positive. Sera from 28 women with clinical signs of "vaginitis" but with no evidence of extravaginal infection were studied by Kristiansen et al. (1987). Isolates from blood and urine specimens from a male patient with *G. vaginalis* septicemia were used by Wilson and Barratt (1986) as antigens in indirect immunofluorescence tests. Serum taken during the acute and convalescent phases showed an increase of antibody titer from <8 to >128 against both the blood and urinary isolates. When considering the design of serological tests, it is important to recognize that *G. vaginalis* strains isolated from different patients exhibit antigenic differences (Boustouiller et al., 1986). Elevated serum antibody titers can be detected in some infections if tests are performed with *G. vaginalis* antigens prepared from each patient's infecting strain. Reports of the lack of humoral response to *G. vaginalis* in patients with extravaginal infections (Gibbs et al., 1987) are difficult to evaluate when results were obtained with test antigens from *G. vaginalis* strains different from, and possibly unrepresentative of, the patients' strains. Zvirbliene et al. (2010) developed a panel of monoclonal antibodies (MAbs) against VLY and demonstrated the ability of some MAbs to prevent the lysis of human erythrocytes in vitro.

The results in table (3) presented bacteria antigen killed &cell free induce delayed type hypersensitivity in immunized rabbits this agree with (Berche et al., 1987) who found the role of exotoxin in the process of T cell activation was studied in vivo during the course of an experimental infection in the mouse. By using highly purified listeriolysin O, it was found that infection with viable, replicative bacteria induced in vivo the emergence of T cells specifically reacting against this exotoxin, as demonstrated by eliciting the expression of delayed-type hypersensitivity to listeriolysin O in *Listeria*-immune mice. So *G. vaginalis* also has vaaginolysin the type of exotoxin and resemble listeriolysin O.

REFERENCES


الخلاصة

استخدمت ثلاثة مجاميع من الأرانب لدراسة التثبيت بمستضدات بكتريا Gardnerella vaginalis (البكتريا المقوولة , البكتريا الحية والراشح الحر للخلايا ) باستخدام طريقة التلالون المباشر والتلالون الدموي المنفعل ووجد ان هذه المستضدات تعمل على حث الاستجابة المناعية الخلطية الموضعية والجهائية ودرس تأثيرها على فرط الحساسية المناخية ووجد انها تحف الخلايا للنية.