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PREPARATION AND EVALUATION OF LOCAL MEDIUM FOR CULTURE AND GROWTH OF *Trichomonas vaginalis* ISOLATED FROM PATIENTS IN MOSUL, IRAQ

Nawfal Y. Al-Dabbagh
Department of Microbiology/
College of Medicine/
University of Mosul

Wahda A. Kharofa
Department of Anatomy/
College of Medicine/
University of Mosul

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ABSTRACT

This study was conducted to prepare a local culture medium from orange leaves for cultivation of *Trichomonas vaginalis.* Five culture media, namely: Oxide *Trichomonas* media (CM 161), CPLM, Barbarowski, Protose pepton and Pavlova, which are used for clinical diagnosis of vaginal trichomoniasis were compared *in vitro* to this new medium. Growth studies using fresh clinical isolates from different patients showed clear ability of *Trichomonas* to grow on this medium. The organisms inoculated initially (25×10^4 cm^{-3}) into this media reached a population of (78×10^4 cm^3) organism in 72 hours. Although (CM 161) and (CPLM) media allows more prolific growth over a shorter period of time (24 hours). It was obvious that the orange leaves media (OLM) was suitable for detecting *T. vaginalis* in patients with vaginitis.

Key words: *Trichomonas vaginalis,* culture, orange leaves.

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تحضير وتقييم وسط محلي لزرع ونمو طفيلي المشعرات المهبلية *Trichomonas vaginalis* المعزول من المرضى في الموصل / العراق

نوفل ياسين الدباغ
فـــرع الأحياء المجهريـــة
كلية الطب/جامعة الموصل

وحدة عبد الرزاق خروفة
فرع التشريح /كلية الطب
جـــــامعة المـــوصـــــل

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الخلاصة

أجريت هذه الدراسة لتحضير وسط زرعي محلي يعتمد على أوراق البرتقال (OLM) لغرض نمو طفيلي المشعرات المهبلية وأستعملت لهذه الدراسة خمسة أنواع من الأوســـاط الزرعيـــة وهـــي: Trichmonas Medium (CM 161) و Oxide و CPLM و Barbarwski و Protose Peptone و Palvova لغرض تشخيص هذا الطفيلي. تمت مقارنة هذه الأوساط الخمسة في الزجـــــاج (*In vitro*) مع الوسط الجديد (OLM) إذ أظهرت دراسة النمو لهذا الطفيلي والمأخوذة من عدة عينات مرضية أن طفيلي المشعرات المهبلية قد نما في الوسط الجديد. إن عدد الطفيليات الابتـــدائي (25×10^4 /سم^3) التي تم استخدامها لغرض الزرع في الوسط الجديد قد نمت الى (78×10^4 /سم^3) خلال 72 ساعة. على الرغم من أن الأوساط الآخرى مثل (CM161 وCPLM) قد أظهرت نمو الطفيلي بوقت أقـــصر خلال 24 ساعة, إلا أن هذه الدراسة أظهرت أيضاً ملائمة هذا الوسط الجديد لغرض الكشف عن الطفيلي في المرضى الذين يعانون من الالتهاب المهبلي.

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INTRODUCTION

T. vaginalis is a parasitic protozoan that is the cause of trichomoniasis, a sexually transmitted disease (STD) of worldwide importance (1). The disease shows a broad range of symptoms ranging from a state of severe inflammation and irritation with a frothy maladorous discharge to a relatively asymptomatic carrier state (2,4). Data have shown that the annual incidence of trichomoniasis is more than 170 million cases worldwide (5). This disease has important medical, social, and economical implications. It causes approximately one-third of all vaginal discharge complaints (6). Despite the fact that the disease was characterized and the protozoan was described since 1836 by Donne (7), its detection remains a problem today (6,8,9). Diagnosis of trichomoniasis has traditionally dependent on the microscopic observation of motile protozoa in vaginal or cervical secretions (7,8). *Trichomonas* can be differentiated on the basis of their characteristic motility. The sensitivity of this technique varies from low as 38% to as high as 82% (10,12). Although this method is certainly the most cost-effective diagnostic test, it is far from optimal in terms of its reliability because it has low sensitivity. This may be due to the loss of distinctive motility after the protozoan has been removed from body temperature (1).

The broth culture method is the **"gold standard"** for the diagnosis of trichomoniasis because it is simple to interpret and requires as few as 300 to 500 *Trichomonas* per ml of inoculum (8) to indicate growth in culture. Culture, with a sensitivity of (86 to 97%), is considered the best method for detection of *Trichomonas* (13). Evaluation of six commercial culture media for growth of *T. vaginalis* (9) indicated the superiority of modified diamond media among other media.

In Mosul (Iraq), six medium were tested for cultivation of *T. vaginalis* (14). Although no significant differences were found between these culture media, it was shown that CM161 medium gave the best growth and multiplication. However, there are inherent limitations to culture diagnosis (7). An incubation period of 2 to 7 days is usually necessary to identify *T. vaginalis* in cultures, during which time infected patients may continue to transmit the infection (15). Also, no culture systems are widely available to clinicians. To improve the acceptability of diagnosis by culture, a new local culture media prepared from orange leaves was tested for culture and growth of *T. vaginalis* and compared with other known culture media used for cultivation of this protozoan. Since trichomoniasis is quite prevalent in many parts of Iraq (14,16, 18), so it was intended to prepare and test the growth characteristics of a rather cheap and efficient culture medium to be tested and used for the detection of this disease.

As far as we know, this is the first study to evaluate a new local medium for cultivation of *T. vaginalis* in Iraq.

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MATERIALS AND METHODS

Collection of *T. vaginalis* Isolates:

Fresh clinical isolates of *T. vaginalis* were obtained from patients attending the obstetrics and Gynecology clinic at Al-Batol Teaching hospital in Mosul, suffering from vaginal and uterine secretions. Vaginal swabs were taken using sterile speculum for direct microscopical examination. All isolates were obtained from different patients. Motile *Trichomonas* in wet-mount preparations of vaginal secretions obtained on cotton swabs premoistened with sterile saline were inoculated into culture media to be tested. This was done after an estimation of the number of organisms by dilution counting using haemocytometer. These culture media are Oxide Trichomonas Media (CM161) (19), (CPLM) (20), Barbarowski, Proteose pepton and Pavlova (21).

The pH of the culture media was adjusted with hydrochloric acid (HC1) or sodium hydroxide (NaOH) to the proper level. The number of trophozoites in culture was estimated by haemocytometer. The reading was done in wet smears *in vitro*. The recognition of *Trichomonas* is based on the motility of the protozoa and the mobility of the undulating membrane. The presence of non-motile organisms was not considered as positive results.

Preparation of Culture Medium (Orange Leaves Medium "OLM")

Orange leaves have been chosen for the preparation of this new medium. The leaves can be obtained from orange trees throughout the year. It is cheap with no economical value. 50 grams of orange leaves were kept at 50°C for 24 hours to get them dry, then leaves were crushed until the dry weight became 17.5 grams. To this 500 ml of tap water was added, then the mixture was boiled for 5 minutes, filtered, and the volume brought to 1-liter (22). Then the following ingredients were added:

Sodium chloride (6.5 grams)
Agar (1.0 gram)
Dextrose (5.0 grams)

The volume sterilized using autoclave at 121°C for 15 minutes. The pH was adjusted to 6.4. (14). The media cooled to 56°C and 80 ml per one liter. Chloramphenicol (0.125 gram) was added to inhibit bacterial growth. In this new modified medium the orange leaves were prepared and used instead of liver digest which is used normally with (CM 161) oxide Trichomonas medium. This new medium is designated Orange Leaves Medium "OLM". The chemical analysis of the orange leaves has been reported. The concentrations (mg/ml) of total protein, total carbohydrate (sugars), sodium (Na), calcium (Ca) and potassium (K), were analysed by some investigators and found to be 4.15, 1.15, 0.009, 0.12 and 0.003 respectively (22).

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"OLM" media were stored at 4°C to 8°C and brought to room temperature before use. (9).

Generation Time Determination

The calculation of generation time (doubling time) of *T. vaginalis* can be expressed as equations (23), using the following formula:

Let N_0 =The initial population number.
N_t = The population at time t
n = The number of generation in time t

$$\mathbf{n} = \frac{\log N_t - \log N_0}{\log 2}$$

So, generation time = $\frac{t}{n}$

Where t = time elapsed in hour.

RESULTS

The effect of (OLM) medium on the growth of *T. vaginalis* in comparison to other culture media is presented in Table (1). All media used supported the growth and multiplication of organisms but at different rates. However, (CM161) and (CPLM) media gave the greatest growth till 96 hours of inoculation. At 72 hours the growth rate by (OLM) media was comparable to (CPLM), Barbarowski and Proteose peptone media. All culture media used for the isolation of *T. vaginalis* gave an equivalent increase in the number of positives detected over those recorded by direct smears.

The generation time (G) for (OLM) medium and other culture media is shown in Table (2). Where (G) for (OLM) media was comparable to (CPLM) and Barbarowski media at 72 hours. At 48 hours, the generation time of (OLM) was comparable to (CM 161) and (CPLM) media. Regarding the number of generation, OLM medium showed similarity at 72 hours with Barbarowski, Proteose peptone and (CPLM) media. *T. vaginalis* has been maintained in this medium for many sub-cultures without any significant change in growth rates on final density becoming apparent.

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Table (1): The effect of orange leaves medium (OLM) on the growth of *T. vaginalis* in comparison to other culture media.

Culture media	[0]No of parasites (× 10^4) per culture tube (mean ±SD)* after:			
	24h	48h	72h	96h
Orange leaves medium (OLM)	36±1	66±1	78±1	5±0
Oxide *Trichomonas* medium (CM 161)	45±2	74.33±2.08	93.4±1.52	111±1
(CPLM)	63.166±3.88	74.4±2.08	75.5±0.57	82.83±1.89
Barabarowski	34±3.6	64±3.6	73.7±3.21	60.1±1.25
Proteos peptone	53.4±1.52	64.7±2.51	74±1	79.7±0.57
Pavolva	35.7±0.57	44.7±1.52	60.4±1.52	79.7±0.57

[0] zero time=25.33±0.57
* all means based on (3) independent determinations.
h= hour

Table (2): The generation time (G) for orange leaves medium (OLM) and various culture media.

Culture media	Number of generation Time/h.				Generation time Time/h.			
	24	48	72	96	24	48	72	96
Orange leaves medium (OLM)	0.55	1.42	1.5	0	43.63	29.57	48	0
Oxide *Trichomonas* medium (CM 161)	0.87	1.598	1.92	2.183	27.58	30.03	37.5	43.9
CPLM	1.36	1.599	1.62	1.79	17.63	30.01	44.44	54.54
Barabarowski	0.74	1.382	1.58	1.296	51.06	34.73	45.56	74.0
Proteos peptone	1.12	1.398	1.59	1.704	21.4	34.5	42.25	56.33
Pavolva	0.54	0.83	1.26	1.70	44.4	57.8	57.14	56.47

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DISCUSSION

Further clarification of the growth requirements in culture may improve our understanding of parasite biology and is critical for development of a defined culture media. Our observation that orange leaves promotes growth of *T. vaginalis in vitro* has important implications including the possibility of cultivating the organism in (OLM) media is economically advantageous and may prove to be as good as other media. The purpose of this study was to examine the growth characteristics of freshly isolated samples of *T. vaginalis* in this new culture medium on order to be approved for clinical identification of trichomonads. Local culture medium used orange leaves were found suitable for cultivation of selected types of bacteria like *Lactobacillus salivarius* and *Streptococcus faecalis* (22). Where these two microorganisms showed clear ability to grow on this medium. Also the growing pattern microorganisms in this medium was as good as other important media like nutrient broth (oxide) for the same purpose. Furthermore, the (OLM) can be used for preparation of selective and enrichment media by adding some stains, blood or serum in order to become suitable for isolation and diagnosis of various microorganisms (22). One factor that would diminish sensitivity of culture medium is the adequacy of the patient samples. However, even with a poorly obtained patient sample with few organisms or a sample obtained from a patient in whom there were only rare trichomonads, the (OLM) medium could be relied upon to give an accurate results like those results obtained after using other trichomonads media for the cultivation of *T. vaginalis***.** A pH 6.4 was used to adjust (OLM) and other culture media, it was found that pH (6 - 6.4) gave the best growth and multiplication of *T. vaginalis* in various culture media (14). Control of PH during the course of growth of *T. vaginalis* seems to be of less importance than with certain other protozoa such as *Trypanosoma brucei* (24). *T. vaginalis* in culture does not reach such high final cell densities as these organisms reducing the acidification of the medium, and the organism is also tolerant of low medium pH. The effect of (OLM) medium on the growth of *T. vaginalis* showed comparable results with other culture media. Although (CM161) medium was the best among the media used for the growth and multiplication till 96 hours after inoculation, however, (OLM) medium proved to be more efficient than other media like (CPLM), Barbarowski and Proteose Peptone at 72 hours. On the other hand, a comparable results between (OLM), Barbarowski and Pavlova media were indicated at 24 hours of inoculation. The rapid decline of growth by (OLM) at 96 hours in comparison to other media is hard to explain and needs further clarification. Nevertheless it does not diminish the effectiveness of (OLM) medium shown in the present study. The efficient performance of (CM 161) medium was also recorded by others (14,25). Our data regarding the generation time and the number of generation after various times also support the effectiveness of (OLM) medium. Generation

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times vary markedly with the species of microorganism and environmental conditions. They range from less than 15 minutes to several hours or days (25, 26). Generation time in nature are usually much longer than in culture. Knowledge of microbial growth rates during the exponential phase is indispensable to microbiologists. Growth rate studies contribute to basic physiological and ecological research and the solution of applied problem in industry. During the exponential phase each microorganism is dividing at constant intervals. Thus the population will double in number during a specific length of time making the so-called generation or doubling time.

The data presented here are the first close approach to prepare a new modified medium for cultivation of a trichomonad flagellate. The ease with vaginal secretion may be cultured under ordinary laboratory conditions renders the culture method eminently desirable for widespread use. Cultivation is a more sensitive diagnostic method than either immediate microscopy of a wet mount or of the centrifuge deposit (27). *T. vaginalis* infection should not be diagnosed unless the characteristic morphology is clearly recognized.

In conclusion, various growth experiments indicated that (OLM) medium has got the ability to support the growth of *T. vaginalis* in vitro. It is hoped that the medium described here will be of use in the study of the nutritional requirements of *T. vaginalis* and its physiology under more controlled conditions than are possible in complex medium.

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