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In vitro Antifungal Activities of Various Plant Crude Extracts and Fractions Against Citrus post-harvest Disease Agent *Penicillium digitatum*

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Abstract

The aim of the present study is to in vitro evaluate various plant extracts and liquid fractions against citrus post-harvest disease agent Penicillium digitatum. Crude extracts of seven plant materials (fenugreek seeds, harmal seeds, garlic cloves, cinnamon bark, sticky fleabane leaves, and nightshade leaves and fruits). In addition to their methanolic, hexane, and aqueous - fractions were assayed by agar well diffusion and amended agar methods. Regression analysis of results was carried out by using Microsoft Excel and SPSS program. Results indicated that crude extracts of nightshade fruits cinnamon bark have completely inhibited the growth of tested fungal isolates (IC_{50} = 57.5 µg ml^{-1}, 190-252.5 µg ml^{-1}) respectively. Methanolic (except fenugreek), hexane and aqueous fractions of all tested plants have resulted in complete inhibition of tested isolates. The methanolic fraction of cinnamon bark extract has shown the highest antifungal activity as compared with the same fraction from other plants (IC_{50} in the range of 5-23 µg ml^{-1}). Moreover, cinnamon bark hexane fraction was the most effective hexane fraction in all tested plants (IC_{50} range: 12.25-14.5 µg ml^{-1}). Concerning fractions of garlic extract, only methanolic fraction has resulted in complete inhibition of fungal growth (IC_{50} range: 3.75-18 µg ml^{-1}). However, the nightshade leaves aqueous fraction (IC_{50} range: 6.75-10.5 µg ml^{-1}) was the most effective over other fractions of the same plant.

الملخص

تهدف هده الدراسه الى تقييم فاعلية بعض المستخلصات النباتيه بالكحول وأجزائها العضويه و المائية للسيطره على فطر البنسيليوم الاخضر المهاجم لثمار الحمضيات. تم دراسة سبعة أنواع من المستخلصات النباتيه ومجزءات الميثانول، الهكسين والماء على سلالات الفطر في المختبر سواء باستخدام طريقة وضع المستخلص في حفر داخل الوسط الغذائي أو بتوزيع المستخلص على سطح الوسط الغذائي ثم زراعة الفطر. دلت النتائج أن مستخلص ثمار النبات nightshade أدت الى تثبيط نمو الفطر بشكل كامل حيث كان التركيز المثبط للنمو بنسبة 50% يساوي 57.5 ميكروجرام/مل. مستخلص لحاء القرفه أدى كذلك الى تثبيط كامل لنمو الفطر. مجزء أو مستخلص الميثانول لجميع النباتات المدروسه (باستثناء الحلبة) أدى الى تثبيط كامل لنمو سلالات الفطر. كذلك مستخلص الهكسين و المحلول المائي لجميع النباتات قيد الدراسه أدت الى تثبيط النمو في العزلات المستعملة. مستخلص الميثانول للحاء القرفه أظهر أعلى فاعليه مثبطة للفطر مقارنة مع نفس مستخلص من النباتات الاخرى. اضافه لذلك مستخلص الهكسين للحاء القرفه كان الأكثر فاعليه مقارنة مع نفس النوع من المستخلصات في النباتات الأخرى. مستخلص الميثانول من الثوم كان الوحيد من جميع مستخلصات الثوم القادرة على تثبيط نمو الفطر بشكل كامل بينما المستخلص المائي لنبات nightshade كان الاكثر فاعلية مقارنة مع بقية المستخلصات من نفس النوع من النبات.

Keywords: green mold, citrus fruits, post-harvest diseases.

1. Introduction

Post-harvest green mold caused by *Penicillium digitatum* [(Pers: Fr) Sacc.] is considered to be a universal disease that leads to the spoilage of almost all kinds of mature citrus fruits (Plaza *et al*., 2004). This disease is currently controlled through the massive use of chemical fungicides (Pramila and Dubey, 2004). However, consumer demands for fungicide-free products, development of resistant fungal strains as a result of continuous use of fungicides, and the effectiveness of applied fungicides necessitate the search for alternative control options (Obagwu and Korsten, 2003; Soylu *et al*., 2005). Plant's extracts are one of several non-chemical control options that have recently received attention. However, actual use of these extracts to control post-harvest pathogens of fruits and citrus pathogens in

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particular is still limited (Obagwu and Korsten, 2003). In this study, the antifungal activity of crude extract and fractions of various medicinal, commercial, and wild-type plants were investigated against *P. digitatum* isolates. The studied plant materials include fenugreek seeds (*Trigonella foenum-graecum* L.), harmal seeds (*Peganum harmala* L.), garlic cloves (*Allium sativum* L.), cinnamon bark (*Cinnamomum cassia* L. Presl), sticky fleabane leaves (*Inula viscose* L. Aiton), and nightshade leaves and fruits (*Solanum nigrum* L.). Fenugreek is an annual Mediterranean herb with aromatic seeds that contain a number of steroidal sapogenins, (saponin fenugrin B) as well as alkaloids (Zargar *et al*., 1992; Zhao *et al*., 2002). The crude extract of fenugreek has shown to be highly specific for the dermatophytes (Shtayeh and Abu Ghdeib, 1999). In addition, the methanolic extract was potent in inhibiting *Candida albicans* (Olli and Kirti, 2006). Extracts from the seeds, as well as the roots of harmal, were found to contain a mixture of active alkaloids such as harmine, harmaline, and tetrahydroharmine. Harmaline was the most active (Kartal *et al*., 2003; Telezhenetskaya and Dyakonov, 2004). The crude extract of freshly crushed garlic cloves has shown strong inhibitory activity on several microbial growth systems including bacteria, fungi, and viruses (Yoshida *et al*., 1999; Elsom, 2000; Sokmen, 2001). Cinnamon bark crude extract has consistently been reported to have antifungal activity. This activity was attributed mainly to the presence of cinnamaldehyde, as well as to the presence of eugenol (He *et al*., 2005). Sticky fleabane is a perennial wild plant that has a wide range of distribution in the Mediterranian region (Curadi *et al*., 2005). The leaf extract of *Inula viscose* proved to have a significant antifungal efficacy against dermatophytes, *Candida* spp, and downy mildew. This high activity may be attributed to the high concentration of sesquiterpene compounds presence (Cafarchia *et al*., 2002; Cohen *et al*., 2006). Nightshade is an herbaceous annual wild plant that has a globular and smooth skinned green fruits (turned black or red at maturity); and are borne in small clusters (Dafni and Yaniv, 1994, Dhellot *et al*, 2006). The plant, due to the presence of steroidal alkaloids, showed antifungal activity against eleven agronomically important fungi (Al-Fatimi *et al*., 2007). The objective of this study is to "*In vitro*" evaluate antifungal activity of crude extracts of seven plants and their liquid fractions against green mould rot of citrus, *P. digitatum*.

2. Materials and Methods

This study was conducted during the year 2007 in laboratories of biological sciences department at Mu'tah University, Jordan.

2.1. Penicillium Digitatum Tested Isolates

Conidiospores of four *P. digitatum* isolates (dg2, dg4, dg5, and dg6) were obtained from spoiled citrus orange (*Citrus sinensis* L.), and lemon (*Citrus limon* L.) fruits were collected from two Jordanian cities: Irbid and Al-Karak.

2.2. Media Used

The *Aspergillus nidulans* complete medium (CM) described previously by Cove (1966) was used (gave maximum zone of growth as compared to potato dextrose agar (PDA) media) with slight modification (i.e. pH 5.5, supplemented with 10 mM glutamic acid, and 10 gl^{-1} fructose as C- source).

2.3. Purification of Isolates

Conidiospores suspensions in a 5 ml solutions of normal saline/Tween 80 (0.05%) were made from each tested isolate at a concentration of approximately 1×10^8 spores per milliliter. Aliquot of 100 µl from a dilution of 10^{-6} or 10^{-7} were plated again on complete media to confirm the identity of each single pure colony as a source of pure culture (Zhang *et al*., 2004).

2.4. Optimal Growth Conditions of Tested Fungal Isolates

Nine replicates (for each tested condition) of conidiospores suspension (20 µl) from each tested isolate were inoculated into complete media, having different pH regimes (i.e. 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 9) for optimal pH (5.5). At pH 5.5, L-glutamic acid at a concentration of 10 mM was the best N-source used among the following materials: Urea; L-proline, L-lysine, L-arginine, L-adenine, L-glutamine, NH_4^+, NO_3^-, and L-histidine. In addition, inoculated plates of complete media adjusted to optimal pH of 5.5, and tehn supplemented with 10 mM glutamic acid and then were incubated at five temperature regimes (i.e. 10 °C, 20 °C, 25 °C, 30 °C, 37 °C) in order to determine the optimal temperature of growth (20 °C). Also, various C-sources (glucose, sucrose, sorbitol, fructose, and maltose) were tested at a final concentration of 10 gl^{-1} to determine best serving C-source (fructose). Each group of nine replicates were incubated for 5 days, at 20°C or at the tested temperature, and then the radius of each growing colony was measured in two directions at right angles to each other.

2.5. Plant Material

Six crude extracts, out of seventy plant species, have shown antifungal activity against tested isolates of *P. digitatum* and these are fenugreek seeds (*Trigonella foenum-graecum* L.), harmal seeds (*Peganum harmala* L.), garlic cloves (*Allium sativum* L.), cinnamon bark (*Cinnamomum cassia* L. Presl), sticky fleabane leaves (*Inula viscose* L. Aiton), and nightshade leaves and fruits (*Solanum nigrum* L.). The former four plant materials were brought from traditional medicine shops in Irbid city whereas the latter two species were collected from wild-type populations occupying orchard fields and road sides of Mu'tah and Al-Iraq towns within Al-Karak area. Plant species were named and classified with the help of the plant taxonomist Dr. Saleh AL- Quran, Mu'tah University, Jordan.

2.5.1. Extracts Preparation

The plant material was dried in the shade, and then it was ground by using liquid nitrogen and extracted (48 h) with absolute ethanol in a soxhlet apparatus (Ndukwe *et al*., 2006). The solvent was removed using rotary evaporator (Heidolph, VV2000) under reduced pressure at temperature below 50 ºC. The resulting crude extracts were stored at 20 ºC until assayed. Stock solutions and serial dilutions of extracts and fractions were prepared in dimethylsulphoxide (DMSO) (Ambrozin *et al*., 2004). Control experiments were performed by using DMSO with identical concentration used to test the extracts. Extracts

were dissolved in DMSO and evaluated for their ability to inhibit the growth of *P. digitatum* isolates.

2.5.2. Fractionation of Plant Crude Extracts

Each crude extract sample was fractionated with (1:1) ratio of water /dichloromethane (v/v). The resultant aqueous fraction was further extracted with dichloromethane, and then combined and concentrated to dryness using rotary evaporator and kept in sterile containers at 4°C until used. The dichloromethane fraction was concentrated to dryness using rotary evaporator, and then portioned with (1:1) n-hexane/90% methanol. The hexane and methanol fractions were concentrated to dryness using rotary evaporator and kept in sterile containers 4°C until used. Each fraction was dissolved in dimethylsulphoxide (Ambrozin *et al*., 2004).

2.6. Antifungal Assay by Agar Well Diffusion Method

Aliquot of 100 µl spores suspension ($1x10^8$ spores/ml) of each tested isolate was streaked in radial patterns on the surface of complete media plates. Wells of 6 mm in diameter were performed in the media, and then each was filled with certain concentration (0.65, 1.3, 13, 32, 65, and 97 µg) of each tested crude extract. DMSO was used as control for the ethanolic extracts. The cultured plates were incubated at 20ºC for 3-5 days. The radius for the zone of inhibition was measured in two directions at right angles to each other. Experiments were carried out with three replicates per treatment and each treatment was repeated at least twice (Ndukwe *et al*., 2006).

2.7. Antifungal Assay of Crude Extracts and Their Fractions by Amended Agar Method

Each crude extract was fractionated into aqueous, hexane, and methanolic fractions. Also the emulsion that may form between layers sometimes was tested. Stock solutions of each fraction were sterilized through a 4 µm Millipore filter (Soylu *et al*., 2005). Each fraction was used in this experiment with different concentrations, depending on its inhibitory activity. Each concentration (25, 50, 130, 260, 390, and 520 µg ml^{-1}) of crude extracts or their fractions (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100,120, and 150 µg ml^{-1}) from the included plant species was amended (streaked in radial patterns) on the surface of solidified complete media prepared as described above. To ensure time was adequate for the diffusion of the extract into media, the plates were incubated at room temperature for at least two hours. 20 µl of conidiospores suspension ($1x10^8$) from each of the isolates were pipetted and left as drops on the surface of the amended media, and then were kept at room temperature for at least one hour until it became completely absorbed. Three inocula per isolate/plate and three replicate Petri plates were used per treatment, where each treatment was repeated at least twice. A long with each treatment, 20 µl of dimethylsulfoxide (DMSO) was replicated as mentioned above and used as controls for the ethanolic extracts. The inoculated Petri dishes were incubated for 3-5 days at optimal temperature (20 °C). Colony diameter was determined by measuring the average radial growth of each isolate. The radius of the growing colonies was measured in two directions at right angles to each other. The MIC was defined as the lowest concentration of the extract inhibiting visible growth of each isolate (Obagwu and Korsten, 2003).

2.8. Determination of Plant Crude Extract or Fraction Sensitivity

The percentage of mycelial growth inhibition by each extract or fraction concentration was calculated from the mean colony diameter (mm) on medium without plant fraction (control), and from the mean colony diameter (mm) on each fraction amended plate (zone of growth). A linear regression of percent inhibition versus plant fraction concentration, estimated produce 50% (IC_{50}), was determined from the regression equation or by interpolation from the regression line. Percentage of inhibition of mycelial growth was determined by using the following formula (Nwachukwu and Umechuruba, 2001).

$$\% \text{ MGI} = \frac{\overline{x} - xi}{\overline{x}} \times 100\%$$

Where:

% MGI denotes for: % of mycelial growth inhibition.

$\overline{x}$: refers to diameter (mm) of control colony on non-amended medium.

xi: refers to diameter (mm) of tested colony replicates on a single crude extract or fraction amended plate (zone of growth).

2.9. Statistical Analysis

The concentration of plant crude extract or fraction, producing 50% growth inhibition (IC_{50}), was calculated by regression analysis for the relationship between the size of inhibition zone (mm) and the concentration (µg) of crude extract or fraction (Log value). Both Microsoft Excel 2003 and SPSS (version 10) were used in such analysis.

3. Results

3.1. Antifungal Activity of Crude Extracts by Agar Well Diffusion Method

Regression analysis of the relationship between size of inhibition zone (mm) and plant crude extract concentration (Log value) showed that there was a significant correlation between concentrations of tested plant extracts and the mean inhibition zone of *P. digitatum* isolates (Table 1). However, such correlation was not significant when crude extracts of garlic cloves, sticky fleabane leaves, and harmal seeds were tested against isolates: dg2; dg4 and dg6; dg5 and dg6, respectively. In addition, none of the extracts has completely inhibited the growth of the four isolates within a range of concentrations from 0.65 to 97 µg ml^{-1}. Moreover, sticky fleabane and harmal extracts have shown the least inhibitory activity against fungal isolates whereas fenugreek seeds, followed by nightshade fruits and leaves extracts, have shown the highest antifungal activity (Table 1).

3.2. Antifungal Activity of Crude Extracts by Amended Agar Method

A linear regression of inhibition percentage versus plant crude extract concentration, estimated to produce 50% (IC_{50}) inhibition, was determined by interpolation from the regression line (Table 3). As clearly seen in (Table 2), the nightshade fruits crude extract has completely inhibited the growth of the four tested *P. digitatum* isolates with an MIC equal to 130 µg ml^{-1} (IC_{50} = 57.5). In addition, cinnamon bark extract has completely inhibited the growth of isolates dg2 (IC_{50}=252.5), dg4

(IC_{50}= 222.5) and dg6 (IC_{50}= 190). Furthermore, fenugreek seeds extract has completely inhibited the growth of dg6 isolate at an MIC value of 390 µg ml^{-1} (IC_{50}= 142). Moreover, results indicated that the tested concentrations of nightshade leaves extract (in the range of 25-520 µg ml^{-1}) has reflected significant correlation in the percentage of inhibition of isolates: dg2 and dg4 (r= 0.999; *P*= 0.001); dg2 and dg5 (r= 0.976; *P*= 0.024); dg2 and dg6 (r= 0.960; *P*= 0.040); dg4 and dg5 (r= 0.967; *P*= 0.033); dg5 and dg6 (r= 0.983; *P*= 0.017). Concentrations of garlic cloves crude extract (50-520 µg ml^{-1}) have also reflected significant correlation in the percentage of inhibition of isolates: dg2 and dg4 (r= 0.992; *P*= 0.008); dg2 and dg5 (r= 0.996; *P*= 0.004); dg4 and dg5 (r= 0.984; *P*= 0.016). In addition, harmal seeds extract has reflected significant correlation between isolates: dg2 and dg4 (r= 0.995; *P*= 0.005); dg4 and dg6 (r= 0.993; *P*= 0.007) percentage of inhibition.

3.3. Antifungal Activity of Plants Extracts Fractions

Results presented in (Table 3) show the *In vitro* antifungal activities of methanolic, hexane, and aqueous fractions of plant materials. The methanolic fractions of all plants (with the exception of fenugreek) have completely inhibited the growth of all fungal isolates. Hexane and aqueous fractions of all plants (not including fenugreek and garlic) have resulted in complete inhibition of fungal growth in the four isolates. The methanolic fraction of cinnamon bark has shown the highest antifungal activity against four *P. digitatum* isolates - followed by methanolic fractions of garlic, nightshade fruits, sticky fleabane, harmal seeds, and nightshade leaves respectively (indicated by the IC_{50} values). The hexane fraction of cinnamon was the most effective fraction against all tested isolates followed by sticky fleabane, harmal, nightshade fruits and leaves, and hexane fraction respectively (Table 3). Moreover, the aqueous fraction of nightshade leaves was the most effective against all isolates, followed by the aqueous fraction of nightshade fruits and cinnamon. Concerning the efficacy of fractions in each particular plant, results indicated that cinnamon methanolic fraction was the most effective - followed by hexane and aqueous fraction of cinnamon extract respectively. However, the fenugreek fractions have not caused complete inhibition of fungal growth in all tested isolates. In garlic, the methanolic fraction was the only fraction that has caused complete inhibition of all tested fungal isolates (Table 3). Moreover, methanolic fractions of both sticky fleabane leaves and harmal seeds were more effective, followed by hexane fraction of sticky fleabane, in controlling the growth of tested isolates although complete inhibition of fungal growth was generated with both fractions (Table 3). Concerning the effect of fractions in nightshade fruits, methanolic fraction was the most effective, and followed by the aqueous then hexane fraction. In contrast, the aqueous fraction of nightshade leaves was the most effective fraction, then followed by hexane and finally methanolic fraction as the least effective in all nightshade leaves fractions.

4. Discussion

Results indicated that growth of *P. digitatum* isolates was completely inhibited by all fractions of cinnamon bark and most effectively with the methanolic fraction. However, garlic methanolic fraction was the only fraction of garlic extract that generates complete inhibition in all isolates. Furthermore, both methanolic and hexane fractions of sticky fleabane leaves, harmal seeds, and nightshade fruits have generated complete inhibition of fungal growth. The aqueous fraction of nightshade leaves was the most effective fraction. A comparative study between these findings and previously obtained results (Al-Najar, 2007) against blue mold *P. italicum* indicated that *P. digitatum* isolates were more susceptible to the same plant extracts, where complete inhibition or higher percentage of inhibition was obtained within the same range of concentrations. Similarly, when results on fractions of tested plants were compared with those obtained against *P. italicum* (Al-Najar, 2007); all fractions of cinnamon bark have completely inhibited the growth of blue mold isolates. However, such fractions were more effective in controlling the growth of *P. digitatum* isolates. In contrast to what it is obtained in this study, the aqueous fraction of garlic cloves has caused complete inhibition to the blue mold isolates (IC_{50} values in the range of 49.5-70 µg ml^{-1}). However, methanolic fraction has shown higher efficacy to *P. digitatum* isolates although complete inhibition to *P. italicum* was obtained (IC_{50} values in the range of 30.5-31.5 µg ml^{-1}) (Al-Najar, 2007). Furthermore, the sticky fleabane methanolic fraction has shown almost the same activity to isolates from both species. Quite the opposite, hexane fraction of the same plant was much less effective against *P. italicum* isolates, where no complete inhibition was achieved (Al-Najar, 2007). Naturally, none of the harmal seeds or the nightshade fruits and leaves fractions (except for nightshade leaves hexane fraction) has caused complete inhibition of *P. italicum* isolates, as compared to their high efficacy against *P. digitatum* isolates in the present study. This indicates that *P. digitatum* isolates are more susceptible to plant extracts than isolates of *P. italicum*. Moreover, when results of this study were compared with those obtained from the *In vivo* use of crude extracts of the same plant species against *P. digitatum* isolates infecting orange and lemon fruits (Kanan 2007, data not presented), crude extracts of the nightshade fruits, cinnamon, and garlic were the most effective especially to isolate dg6 (MIC values within the range of 130-390 µg ml^{-1}). When results were compared to the *In vivo* results of the same crude extracts tested against *P. italicum* isolates (Al-Najar, 2007), all extracts have shown complete inhibition of growth to all isolates infecting orange rather than lemon fruits. Based on above results, it is suggested that high efficacy of cinnamon extract or fractions may be related to cinnamaldehyde, eugenol, cinnamic acid, as well as to various organic acids that have consistently been reported by different workers to show antifungal activities (Inouye *et al*., 2000; Gill and Holly, 2004). Yet, this activity may be traced mainly to cinnamaldehyde, which acts as a specific inhibitor for enzymes such as ß-(1, 3)-glucansynthase that participate in the biosynthesis of chitin and ß-glucans cell wall components(Cowan,1999).

Table 1. In vitro activity of different concentrations of various plant crude extracts (conc. range 0.65-97 µg ml^{-1}) against P. digitatum isolates using agar well diffusion method.

Source of plant crude extracts	Fungal Isolate	Mean size zone of inhibition (mm) ± SD/ Range	Corr. Value(r)	Sig-value	*Regression Equation*
Nightshade fruits	dg2	0.0 - 27.33 ± 4.41	0.954**	0.001	*y=9.72x+6.17*
Nightshade fruits	dg4	0.0 - 27.33 ± 13.04	0.901**	0.006	*y=8.19X+5.55*
Nightshade fruits	dg5	0.0 – 30.67± 9.97	0.890**	0.007	*y=9.15x+6.59*
Nightshade fruits	dg6	0.0 – 27.5± 1.22	0.974**	0.000	*y=12.93x+1.96*
Nightshade leaves	dg2	0.0-31.33± 3.44	0.957**	0.001	*y=10.68x+5.76*
Nightshade leaves	dg4	0.0-22.5± 0.55	0.941**	0.002	*y=8.02x+5.75*
Nightshade leaves	dg5	0.0-22.8± 1.72	0.925**	0.003	*y=7.64x+5.97*
Nightshade leaves	dg6	0.0-25.17± 0.98	0.902**	0.006	*y=8.02x+6.97*
Cinnamon	dg2	0.0-24.67± 1.97	0.896**	0.006	*y=10.31x-1.73*
Cinnamon	dg4	0.0-19.83± 7.08	0.780*	0.039	*y=7.60x-2.16*
Cinnamon	dg5	0.0-24.67± 4.18	0.783*	0.037	*y=10.03x-2.77*
Cinnamon	dg6	0.0-23.67± 7.03	0.783*	0.037	*y=9.48x-2.68*
Garlic	dg2	0.0-11.5± 1.05	0.628	0.131	*y=3.56x-1.28*
Garlic	dg4	0.0-20.5± 0.55	0.783*	0.037	*y=8.73x-2.39*
Garlic	dg5	0.0-22.0± 2.13	0.781*	0.038	*y=9.85x-2.65*
Garlic	dg6	0.0-25.33± 0.52	0.784*	0.037	*y=10.47x-2.90*
Sticky fleabane	dg2	0.0-26.33± 1.03	0.900**	0.006	*y=11.53x-1.68*
Sticky fleabane	dg4	0.0-0.0			*Y=0.0*
Sticky fleabane	dg5	0.0-19.00± 1.27	0.781*	0.038	*y=7.48x-2.09*
Sticky fleabane	dg6	0.0-0.0			*Y=0.0*
Fenugreek	dg2	0.0-39.83± 1.97	0.987**	0.000	*y=17.94x+1.33*
Fenugreek	dg4	0.0-39.17± 3.66	0.864*	0.012	*y=17.29x-3.72*
Fenugreek	dg5	0.0-38.17± 2.22	0.989**	0.000	*y=17.69x+1.75*
Fenugreek	dg6	0.0-41.17± 1.47	0.987**	0.000	*y=18.39x+1.78*
Harmal	dg2	0.0-25.17± 0.75	0.775*	0.041	*y=9.34x-2.74*
Harmal	dg4	0.0-11.0± 1.26	0.782*	0.038	*y=4.82x-1.33*
Harmal	dg5	0.0-14.17± 0.75	0.629	0.130	*y=4.55x-1.62*
Harmal	*dg6*	*0.0-13± 0.0*	*0.626*	*0.133*	*y=3.88x-1.41*

Values are means ± SD of at least two independent experiments.

** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).

Table 2. *In vitro* antifungal activity of crude plant extracts against four *P. digitatum* isolates using amended agar method.

Source of plant crude extracts	Conc (Range) (µg ml-1)	Fungal Isolate	IC50	% of inhibition (range)	Corr. Value (r)	Sig-value	*Regression Equation*
Nightshade fruits	25-125 130-520	Dg2,dg4 dg5, dg6	57.5	21-98 100			*Y=100*
Nightshade leaves	25-520	dg2		11-63.11	0.915	0.085	*y=0.022x+52.68*
Nightshade leaves	25-520	dg4		9.5-62.70	0.931	0.069	*y=0.038x+44.57*
Nightshade leaves	25-520	dg5		6.5-54.71	0.809	0.191	*y= 0.05x+30.29*

Nightshade leaves	25-520	dg6		9.5-67.92	0.773	0.227	*y= 0.041x+49.74*
Cinnamon	50-390	dg2	252.5	14.5-74.33	0.993**	0.007	*y=0.166X+11.4*
	520			100			
Cinnamon	50-390	dg4	222.5	11.5-56.48	0.939	0.061	*y=0.165X+6.49*
	520			100			
Cinnamon	50-520	dg5		3.5-59.42	0.913	0.087	*y=0.113X+4.99*
Cinnamon	50-260	dg6	190	14-67.38	0.944	0.056	*y=0.176X+18.46*
	390-520			100			
Garlic	50-520	dg2		9.0-55.62	0.972*	0.028	*y=0.091X+10.97*
Garlic	50-520	dg4		7.5-54.41	0.956*	0.044	*y=0.096X+9.08*
Garlic	50-520	dg5		5.5-43.53	0.952*	0.048	*y=0.086X+2.06*
Garlic	50-520	dg6		10-56.15	0.991**	0.009	*y=0.081X+12.58*
Sticky fleabane	50-520	dg2		7.5-35.84	0.962*	0.038	*y=0.051X+11.77*
Sticky fleabane	50-520	dg4		17-50.78	0.962*	0.038	*y=0.027X+38.09*
Sticky fleabane	50-520	dg5		18-59.99	0.975*	0.025	*y=0.041X+37.94*
Sticky fleabane	50-520	dg6		16-50.81	0.772	0.228	*y=0.027X+39.58*
Fenugreek	50-520	dg2		12.5-42.26	0.922	0.078	*y=0.029X+24.87*
Fenugreek	50-520	dg4		8.5-48.19	0.959*	0.041	*y=0.065X+16.33*
Fenugreek	50-520	dg5		9.5-58.23	0.996**	0.004	*y=0.091X+10.59*
Fenugreek	50-260	dg6	142	20.5-54.55	0.920	0.080	*y=0.155X+25.41*
	390-520			100			
Harmal	130-520	dg2		0.0-22.47	0.878	0.122	*y= 0.051X-8.56*
Harmal	50-520	dg4		1.0-34.72	0.950	0.050	*y= 0.076X-1.81*
Harmal	25-520	dg5		2.5-30.59	0.995**	0.005	*y=0.052+4.12*
Harmal	25-520	dg6		4.0-25.68	0.905	0.095	*y=0.014X+18.99*
Benomyla	0.01-20	dg2	20.7	10.0-29.5	0.875**	0.000	*y=19.86x+34.10*
	25-520			100			
Benomyl	0.01-35	dg4	37	0.0-13.5	0.837**	0.000	*y=23.29x+17.30*
	40-520			100			
Benomyl	0.01-250	dg5	262	10.5-27.0	0.796**	0.000	*y=18.9x+16.01*
	300-520			100			
Benomyl	0.01-35	dg6	36	0.0-30.5	0.884**	0.000	*y=21.88x+23.8*
	40-520			100			
DMSOb	*5.0-520*	*dg2,dg4 dg5, dg6*		*0.0*			

Values are means ± SD of at least two independent experiments.
** Correlation is significant at the 0.01 level (2-tailed),
* Correlation is significant at the 0.05 level (2-tailed).
[a] :positive control fungicide, [b] :negative control.

The high antifungal activity noticed with the crude extract and fractions of nightshade leaves and fruits could be related to the presence of steroidal alkaloids including solamargine, solasonine, solanine and saponin (Zhou *et al*., 2006).

Table 3. *In vitro* activity of different concentrations of methanolic, hexane and aqueous fractions from various plant crude extracts against *P. digitatum* isolates.

Source of plant extract Fraction	Fungal isolate	Conc (Range) (µg)	% of inhibition (range)	IC50	Corr. Value(r)	Sig-value	*Regression Equation*
Night shade/fruits methanolic	dg2	5.0	33.33	8.75	0.559		
		20-100	100			0.093	*y=0.38x+72.71*
	dg4	5	24.59	10	0.559	0.093	*y=0.43x+69.14*
		20-100	100				
	dg5	5.0	29.03	9.5	0.559	0.093	*y=0.403x+70.95*
		20-100	100				
	dg6	5.0-70	21.67-65.0	24.75	0.944**	0.000	*y=0.799x+22.12*
		80-100	100				
Night shade/fruits hexane	dg2	5-80	8.36-57.38	56	0.896**		
		90-100	100			0.001	*y=0.80x+10.82*
	dg4	5-80	11.21-56.45	62	0.892**	0.001	*y=0.81x+10.21*
		90-100	100				
	dg5	5-80	13.64-85.0	36.5	0.969**	0.000	*y=0.77x+22.17*
		90-100	100				
	dg6	5-80	10.91-53.33	70	0.858**	0.003	*y=0.80x+8.11*
		90-100	100				
Night shade/fruits aqueous	dg2	5-20	22.31-67.21	14.8	0.548		
		30-100	100			0.127	*y=0.219x+83.24*
	dg4	5-20	19.64-66.13	15.25	0.548	0.127	*y=0.226x+82.69*
		30-100	100				
	dg5	5-30	16.73-60.0	21	0.730*	0.025	*y=0.544x+57.15*
		40-100	100				
	dg6	5-80	12.94-66.67	39.5	0.870**	0.002	*y=0.669x+24.09*
		90-100	100				
Nightshade /leaves methanolic	dg2	20-70	9.24-36.11	72	0.894	0.106	*y=2.11x-87.78*
		80-90	100				
	dg4	20-70	11.63-36.11	72	0.883	0.117	*y=2.04x-81.91*
		80-90	100				
	dg5	20-70	17.94-67.24	44	0.739	0.261	*y=1.20x-6.11*
		80-90	100				
	dg6	20-90	0.0-8.33		0.878	0.122	*y=0.21x-12.06*
Nightshade leaves hexane	dg2	20-50	4.64-13.89	58.5	0.878		*y=2.21x-82.06*
		70-90	100			0.122	
	dg4	20-50	6.32-16.67	58	0.878	0.122	*y=2.14x-76.19*
		70-90	100				
	dg5	20-80	18.94-60.35	55	0.850	0.150	*y=1.15x-16.40*
		90	100				
	dg6	20-80	7.31-27.78	83.75	0.767	0.233	*y=1.74x-83.65*
		90	100				

Nightshade	dg2	5.0	43.33	6.75	0.581		
leaves aqueous		20-90	100			0.131	*y=0.39x+74.24*
	dg4	5.0	35.0	8.5	0.581	0.131	*y=0.45x+70.45*
		20-90	100				
	dg5	5.0	33.33	8.75	0.581	0.131	*y=0.46x+69.69*
		20-90	100				
	dg6	5.0	21.67	10.5	0.581	0.131	*y=0.54x+64.39*
		20-90	100				
Cinnamon methanolic	dg2	5.0-10	49.18-59.02	5.0	0.819*	0.046	*y=1.123x+55.7*
		20-50	100				
	dg4	5.0-20	15.25-20.34	9.5	0.900*	0.015	*y=2.35x-2.20*
		30-50	100				
	dg5	5.0-20	10.0-30.0	23	0.920**	0.009	*y=2.44x-4.66*
		30-50	100				
	dg6	5.0-10	40-51.66	9.25	0.819*	0.046	*y=1.33x+47.71*
		20-50	100				
Cinnamon hexane	dg2	5-10	32.79-36.07	12.25	0.817*	0.047	*y=1.59x+37.16*
		20-50	100				
	dg4	5-10	27.11-28.81	13	0.815*	0.048	*y=1.74x+31.05*
		20-50	100				
	dg5	5-10	5.0-5.0	13	0.814*	0.049	*y=2.29x+9.16*
		20-50	100				
	dg6	5-10	16.66-26.66	14.5	0.819*	0.046	*y=1.91x+24.68*
		20-50	100				
Cinnamonaa queous	dg2	20	38.33	25.5	0.926	0.074	*y=1.32x+18.51*
		50-70	100				
	dg4	20	16.66	32.5	0.926	0.074	*y=1.79x-10.12*
		50-70	100				
	dg5	20	21.66	31	0.926	0.074	*y=1.68x-3.51*
		50-70	100				
	dg6	20-60	23.33-51.66	47.5	0.881	0.119	*y=1.30x-8.21*
		70	100				
Garlic methanolic	dg2	5-10	18.03-60.66	8.5	0.784	0.065	
		20-50	100				*y=1.53x+40.19*
	dg4	5-10	64.52-64.52	3.75	0.814*	0.049	*y=0.86x+66.07*
		20-50	100				
	dg5	5-10	63.33-71.67	3.75	0.818*	0.046	*y=0.79x+68.57*
		20-50	100				
	dg6	5-20	33.33-55.0	18	0.929**	0.007	*y=1.73x+26.48*
		30-50	100				
Garlic hexane	dg2	50-100	8.20-16.39		0.959**	0.010	*y=0.16x-0.47*
	dg4	50-100	1.61-12.90		0.938*	0.019	*y=0.233x-11.09*
	dg5	50-100	0.0-8.33		0.824	0.086	*y=0.164x-10.16*
	dg6	50-100	1.67-18.33		1.000**	0.000	*y=0.33x-15*
Garlic aqueous	dg2	50-100	6.56-14.75		0.959**	0.010	*y=0.157x-2.11*
	dg4	50-100	9.68-19.36		0.882*	0.048	*y=0.18x+2.68*
	dg5	50-100	13.33-20.0		0.885*	0.046	*y=0.13x+5.65*

	dg6	50-100	11.67-18.33		0.814	0.094	*y=0.126x+7.16*
Sticky fleabane methanolic	dg2	20	34.43	27.25	0.874	0.053	
		50-80	100				*y=1.114x+24.53*
	dg4	20	25.81	30	0.874	0.053	*y=1.26x+14.61*
		50-80	100				
	dg5	20	20	31.75	0.874	0.053	*y=1.36x+7.93*
		50-80	100				
	dg6	20	26.68	29.75	0.874	0.053	*y=1.25x+15.60*
		50-80	100				
Sticky fleabane hexane	dg2	20-80	22.63-81.97	38	0.926		
		90	100			0.074	*y=0.86x+16.96*
	dg4	20-80	27.94-72.58	38.5	0.825	0.175	*y=0.82x+16.27*
		90	100				
	dg5	20-80	31.76-65.0	36.5	0.683	0.317	*y=0.70x+23*
		90	100				
	dg6	20-80	19.46-66.67	48	0.864	0.136	*y=1.07x-8.10*
		90	100				
Fenugreek methanolic	dg2	20-90	0.0-13.88		0.969	0.160	*y=0.233x-5.43*
	dg4	20-90	6.52-25.42		0.955	0.192	*y=0.42x-6.72*
	dg5	20-90	0.0-23.33		1.000*	0.015	*y=0.50x-16.71*
	dg6	20-90	0.0-16.66		0.945	0.212	*y=0.496x-21.03*
Fenugreek hexane	dg2	20-90	0.0-13.89		0.899	0.101	*y=0.19x-2.14*
	dg4	20-90	0.0-13.89		0.841	0.159	*y=0.37x-18.5*
	dg5	20-90	0.0-6.90		0.775	0.225	*y=0.21x-11.72*
	dg6	20-90	0.0-6.67		0.990**	0.010	*y=0.233x-11.83*
Fenugreek aqueous	dg2	20-90	0.0-24.59		0.870	0.055	*y=0.33x-2.95*
	dg4	20-90	8.33-32.26		0.945*	0.015	*y=0.323x+1.29*
	dg5	20-90	0.0-30.0		0.877	0.051	*y=0.42x-11.83*
	dg6	20-90	0.0-18.33		0.866	0.058	*y=0.15x+5.83*
Harmal methanolic	dg2	20-80	0.0-59.02	66.5	0.811*	0.027	*y=0.821x-1.41*
		90-150	100				
	dg4	20-80	0.0-58.07	68.5	0.815*	0.026	*y=0.882x-8.91*
		90-150	100				
	dg5	20-90	0.0-61.67	85	0.876**	0.010	*y=0.809x-7.78*
		100-150	100				
	dg6	20-120	0.0-83.33	81.5	0.984**	0.000	*y=0.830x-19.57*
		150	100				
Harmal hexane	dg2	20-90	23.38-65.57	47	0.825	0.086	*y=0.79x+6.34*
		100	100				
	dg4	20-80	19.67-61.29	40.5	0.819	0.090	*y=0.93x+3.71*
		90-100	100				
	dg5	20-90	12.86-46.67	90.5	0.705	0.184	*y=0.93x-18.06*
		100	100				
	dg6	*20-100*	*9.74-51.67*		*0.953**	*0.012*	*y=0.244x+25.41*

This is in agreement with previous results (Al-Fatimi et al, 2007), which indicate that steroidal alkaloids have shown antifungal activity against eleven agronomically important fungi including Aspergillus spp, Rhizopus spp, Fusarium spp, Alternaria brassicicola. Garlic extracts have shown significant effect on the growth of P. digitatum isolates. This finding agrees with earlier reports that stated extracts of garlic can inhibit mould growth. And the effectiveness of this inhibition is related to the solvent used in the extraction (Irkin and Korukluoglu, 2007). The antifungal activity of garlic is related to allicin, which is the main biologically active component of garlic extract inhibiting essential enzymes for pathogen infection (Miron et al., 2000). Similarly, ajoene (allicin derivative) has shown also strong inhibitory activity against several fungal species including the black mold (Aspergillus niger), Candida albicans, and Paracoccidiodes brasiliensis (Naganawa et al., 1996). Ajoene was superior to allicin in the severity of inhibiting fungal growth by disrupting the cell wall (Yoshida et al., 1987). Moreover, leaf extracts of I. viscose proved to have a significant antifungal activity against dermatophytes and downy mildew. This may be attribuatble to high concentration of sesquiterpene as well as phenolic compounds present in the methanolic or aqueous fractions (Cafarchia et al., 2002; Cohen et al., 2006; Al-Najar, 2007). These findings confirm results obtained earlier (Shtayeh and Abu Gheleib, 1999; Cohen et al, 2002). Yet, results disagreed with the findings of Wang and his co-workers (2004), which indicate poor activity of I. viscosa water extract against plant diseases. The strong inhibitory activity of methanolic and hexane fractions of harmal may be related to the high content of alkaloids (harmine, harmaline and tetrahydroharmine), and the presence of phenolic compound. This is well-matched with earlier reports (Kartal et al., 2003; Telezhenetskaya and Dyakonov, 2004; Al-Najar, 2007). Although fenugreek fractions are rich in alkaloids, as well as phenolic compounds (Al-Najar, 2007), reduced activity was detected in this study against Penicillium isolates. And this may be linked to the fractionation process. However, these findings disagreed with that obtained by Olli and Kirti (2006), who stated that the methanolic extract of Fenugreek was highly specific for dermatophytes.

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The Effect of Crown Restorations on The Types and Counts of Cariogenic Bacteria

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Abstract

This study investigated the effect of crown restorations on the numbers and types of cariogenic bacteria. Plaque samples were collected from thirty eight individuals who had crown restoration (crown or fixed partial denture) and an adjacent normal tooth by using sterile curettes. The bacterial counts (Colony Forming Unit/ ml) were obtained by the cultivation of plaque samples on certain selective media that were used for the cultivation of *Streptococcus mutans*, *Lactobacillus* species, and *Actinomyces* species. The number of *Lactobacillus* species were higher in the samples obtained from crown restorations than the samples obtained from natural teeth (P=.02). Also, it was found that the metal-acrylic crown restorations have higher number of lactobacillus species compared to the metal-ceramic (P =.003) and the crowns with subgingival margin has the highest counts of Streptococcus (P=.001) and Actinomyces species (P= .032). Moreover, the number of the cariogenic bacteria was found to be significantly associated with the periodontal conditions of the person and the age of crown restorations. It can be concluded that high counts of cariogenic bacteria was found to be associated with; crowns, metal acrylic crowns, placing the crown margin subgingivally, as well as the age of the crown restoration.

الملخص

لقد تم دراسة تأثير التركيبات السنية (التيجان والجسور) على أعداد وأنواع البكتيريا المسببة للتسوس، الدراسة شملت 38 شخص، حيت أن كل شخص لديه تلبيسة سنية (تاج أو جسر) وسن طبيعي مجاور للتلبيسة، تم جمع عينات البلاك (الصفائح الجرثومية) بواسطة أداة المجرفة (curette) . وتم احتساب أعداد المستعمرات البكتيرية بزراعة عينات البلاك على الأوساط الغذائية الاختيارية المناسبة لنمو كل من الأنواع والأجناس البكتيرية التالية: الستريتوكوكس ميوتانس، اللاكتوباسيلاس ، والأكتينومايسيس. حيث وجد أن أعداد اللاكتوباسيلس كانت أعلى في العينات المأخوذة من أسطح التركيبات السنية (التلابيس السنية) منها من أسطح الأسنان الطبيعية المجاورة، وأعداد اللاكتوباسيلاس أيضا كانت أعلى في العينات المأخوذة من التلابيس السنية المصنوعة من الأكريل بالمقارنة مع أنواع التركيبات السنية الأخرى. ولقد تم ايجاد أن أعداد الستربتوكوكس و الأكتينومايسس كانت أعلى في التركيبات السنية ذات الحافة تحت اللثة. ولقد وجد أيضاً أن العدد الكلي للثلاثة أنواع من البكتيريا اللاهوائية الاختيارية له علاقة إرتباط معنوي (significant) مع وضع اللثة للشخص وأيضا مع العمر الزمني للتركيبة السنية.

Keywords: Streptococcus mutans, *Lactobacillus* species, *Actinomyces* species, Crown restorations, Cariogenic bacteria .

1. Introduction

The placement of crown restoration maintains the morphology and the function of the tooth for a long period of time. Crowns and fixed partial dentures (FPDs) can be made from various types of material combinations; all ceramic, full cast metal, metal- ceramic and metal- acrylic. However, certain studies have reported that the margin of dental restorations stimulates bacterial recolonization and the acid production from cariogenic bacteria could attack the tooth restoration margin interface (Savarino et al., 2002; Mjor, 1985).

The bacterial community of dental plaque is subjected to physiological and compositional shifts as a result of environmental stresses generated by the placement of dental restoration and this is could lead to serious complications that result in the failure of the restoration (Mjor, 1997). Furthermore, many studies have found that there is variation in the effect of the various types of the restorations on the growth of certain bacteria in dental plaque according to its material combinations (Beyth et al., 2007; Satou et al., 1988).

Inspite of the widespread use of crown and fixed partial denture (FPD) restorations made from different types of materials, there are no studies to evaluate their effect on host tissues. The indirect effect of different types of crown and fixed partial denture (FPD) restorations on host tissues can be determined by the detection of the changes of

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bacterial counts in dental plaque. Comparison of the bacterial counts between the control site (natural tooth) and the experimental site (crown or retainer), is critical in the determination of whether different material types of crown and fixed partial denture (FPD) restorations have an effect on the bacterial composition in dental plaque or not. Therefore, this study aimed to investigate the effect of the various types of crowns and fixed partial dentures (FPDs) on the counts of Streptococcus mutans, lactobacillus species, and Actinomyces species in dental plaque.

2. Material and Methods

2.1. Subjects and Plaque Sampling

The current study composed of 38 subjects (9 men {23.7%}, and 29 women {76.3%}) ranging in age from 19 to 76 years (a mean age: 42.26 years). Each subject had a crown or a fixed partial denture (FPD) restoration and a natural tooth in the closet proximity to the crown site. Four samples were obtained from each participant at two sites, test and control: supragingivally and subgingivally for each. Samples were taken from 14 crowns and 24 fixed partial dentures (FPDs), made from metal- ceramic (26 cases), metal- acrylic (9 cases), metal only (only 1 case), and all ceramic (2 cases). The sample included 3 cases with diabetes, 6 with hypertension, and only 1 case with diabetes and hypertension. The study protocol was approved by the "Committee of the Search on Human", Jordan University of Science and Technology. The subjects were patients of the Dental Teaching Center at Jordan University of Science and Technology, and all of them provided their informed consent. The pregnant cases were excluded (4 cases out of 64), individuals who were having scaling and or taking antibiotics one month before sampling were excluded (16 cases out of 64), in addition to these, 6 cases out of 64 were excluded because of their missing data. All the thirty eight volunteers were non-smokers except for four.

The subjects were sampled at two sites, including natural teeth and teeth with crown restorations. Dental plaque samples were collected from the buccal side by using sterile curettes (Gracey Curettes). For both the tooth with crown restoration and the tooth without a restoration (control), the plaque sample was taken first from supragingival then the subgingival. The plaque samples were suspended in 1 ml of sterile phosphate buffer saline (PBS) (0.12 M NaCl, 0.01 M Na_2HPO_4, 5mM KH_2PO_4 [pH 7.5]). The samples were transported on icebox to the laboratory until processed within 24 hours. There were 76 plaque samples obtained from crown sites (experimental sites), and 76 samples from natural sites (control sites). The number of the cariogenic bacteria was obtained per ml of the plaque (CFU/ml) by cultivation on the proper selective media for each cariogenic bacteria.

2.2. Isolation and Enumeration of Bacteria From Plaque Samples

Plaque samples were dispersed by vortexing for 30 seconds with glass beads (diameter 4 mm), and diluted into different decimal serial dilutions in phosphate buffer saline (pH 7.5). Colony forming unit per ml (CFU/ml) was determined for each plaque sample by plating the appropriate dilutions on the following selective media (all media were purchased from Himedia Laboratories Pvt. Limited, Bombay, India): Mitis salivarius agar (MSA) supplemented with 0.2 U/ml bacitracin (Fluka; BioChemika, Buchs, Switzerland) and 5% sucrose (MSBS), Rogosa SL agar, and Cadmium Flouride Acriflavine Tellurite (CFAT) medium supplemented with 5% human blood were used for the cultivation of Streptococcus mutans, Lactobacillus species, and Actinomyces species respectively. The plates were incubated at 37°C for three days in an anaerobic jar with CO_2 gas generating kit (Oxoid Ltd, Cambridge, UK). A total of 152 plaque samples obtained from 38 persons were cultivated on (MSBS), Rogosa, and (CFAT) media. The resulting colonies were repeatedly subcultured for further analysis and detection.

2.3. Characterization of Cariogenic Bacteria in Plaque Samples

Morphological characterizations of bacterial isolates on the three selective media were performed according to the color, size, colony characteristics (margin, form, and elevation) and gram staining was done as a basic microbiological test for the identification. The Streptococcus isolates where further characterized using the following biochemical tests (based on Bergey's Manual of Systematic Bacteriology) (Sneath et al., 1986): Catalase activity, Fermentation of sugars: mannitol, sorbitol, raffinose, and Voges-Proskauer (VP) test. For the identification of the bacterial isolates that were grown on both Rogosa and (CFAT) media, the RapID ANA II biochemical kit (Remel, Lenexa, KS) was used according to the manufacture's instructions.

2.4. Statistical Analysis

Statistical analysis of the data was conducted using SPSS software (Statistical Package for the Social Science, version 11.5; SPSS Inc., Chicago, IL). All the bacterial count distributions were noticeably positively skewed; therefore, the nonparametric tests were used for the analysis. Mann Whitney U test and Kruskal-Wallis tests were used to analyze the data of the bacterial counts. Chi square test χ^2 was used to study the association between the numbers of bacteria with the age of the crown and the periodontal inflammation around the tooth. The level of significance was considered at $\alpha = .05$.

3. Results

The morphological and biochemical characterization revealed the presence of the following cariogenic bacterial species: Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis, Lactobacillus acidophilus, Actinomyces odontolyticus, Actinomyces meyeri, and Actinomyces israelii. The mean number of CFU per ml of plaque for each cariogenic bacteria showed considerable variability between teeth sites (natural tooth vs. crown restoration) and in relation to plaque position (supragingival plaque vs. subgingival). (Table 1)

Table 1: The mean values of bacterial counts (CFU/ml) for each type of bacteria in relation to plaque position and teeth site

Bacterial genera	Natural site		Crown site	
	supra[a]	sub[b]	supra[a]	sub[b]
Streptococcus	$(5.5\pm34)\times10^{12}$	$(7.0\pm37)\times10^{3}$	$(5.3\pm33)\times10^{12}$	$(1.5\pm9.1)\times10^{12}$
Lactobacillus	$(5.9\pm13)\times10^{3}$	$(1.1\pm4.2)\times10^{4}$	$(2.5\pm8.5)\times10^{4}$	$(2.3\pm13)\times10^{3}$
Actinomyces	$(2.7\pm9.3)\times10^{10}$	$(5.5\pm15)\times10^{8}$	$(7.3\pm18)\times10^{9}$	$(1.6\pm5.9)\times10^{10}$

[a]: supragingival plaque.

[b]: subgingival plaque.

Table 2: Frequency distribution of plaque samples with Lactobacillus species counts CFU/ml in terms of plaque positionand teeth site

T.site[b]	Plaque	CFU classes n (%)[a] 0.0	>0.0-10^3	>10^3-10^6	Total [c]
natural	supra[d]	34 (89.5)	3 (7.9)	1 (2.6)	38(100.0)
	sub[e]	33 (86.8)	3 (7.9)	2 (5.3)	38 (100.0)
crown	supra[d]	26 (68.4)	7 (18.4)	5 (13.2)	38 (100.0)
	sub[e]	32 (84.2)	3 (7.9)	3 (7.9)	38 (100.0)

[a] count of samples (percentage of samples).

[b]: tooth site.

[c]: Total: was estimated from all CFU classes within each group.

[d]: supragingival plaque.

[e] : subgingival plaque.

Analysis of the proportions of plaque samples in relation to CFU classes showed that Lactobacillus species have the largest frequencies of zero count with regard to plaque type and tooth site (Table 2). Table 3 shows that Actinomyces species were the predominance species being cultivated with the greatest proportions on the selective media. The total prevalence of Lactobacillus and Actinomyces in supragingival plaque was higher at crown sites than at natural sites (Tables 2, 3).

Table 3: Frequency distribution of plaque samples with Actinomyces species counts CFU/ml in terms of plaque position and teeth site

T. site	Plaque	CFU classes n (%) 0.0	>10^3-10^6	>10^6-10^9	>10^9-10^{12}	**Total**
natural	supra	19 (50.0)	14 (36.8)	0 (0.0)	5 (13.2)	38 (100)
	sub	25 (65.7)	5 (13.2)	3 (7.9)	5 (13.2)	38 (100)
crown	supra	11 (29.0)	14 (36.8)	5 (13.2)	8 (21.0)	38 (100)
	sub	19 (50.0)	3 (7.9)	13 (34.2)	3 (7.9)	38 (100)

All symbols and abbreviations are in Table 2.

While there were no large differences in the total prevalence of Streptococcus species in plaque samples between natural teeth and crowned teeth (Table 4).

The crown sites displayed significant increased in Lactobacillus counts in the supragingival plaque (P = .02). While there was no statistically significant difference between the natural tooth and crown tooth sites in the counts of *Streptococcus* and *Actinomyces* species (P > .05). Furthermore, the different types of crown material combinations revealed significant differences in Lactobacillus counts (Figure1), where metal-ceramic crowns have lower counts than the metal- acrylic (P= .003). Also the position of the crown margin displayed differences in bacterial counts (Figure2), where the subgingival margin has the highest counts of Streptococcus (P= .001) and Actinomyces species (P= .032). Significant differences in Lactobacillus counts were recorded according to the location of teeth in the oral cavity, where anterior teeth have lower counts than the posterior (P = .01). The count of the cariogenic bacteria was found to be significantly associated with the periodontal conditions (χ2 test, P=.002) and the age of the crown restoration (χ^2 test, P = .006).

Table 4: Frequency distribution of plaque samples with Streptococcus species counts CFU/ml in terms of plaque position and teeth site

T. site	Plaque	CFU classes n (%) 0.0	>0.0-10^3	>10^3-10^6	>10^6-10^9	>10^{12}	Total
natural	supra	25 (65.9)	6 (15.8)	6 (15.8)	0 (0.0)	1 (2.6)	38 (100)
	sub	27 (71.1)	6 (15.8)	5 (13.2)	0 (0.0)	0 (0.0)	38 (100)
Crown	supra	22 (57.9)	8 (21.1)	6 (15.8)	1 (2.6)	1 (2.6)	38 (100)
	sub	25 (65.8)	8 (21.1)	4 (10.5)	0 (0.0)	1 (2.6)	38 (100)

All symbols and abbreviations are in Table 2.

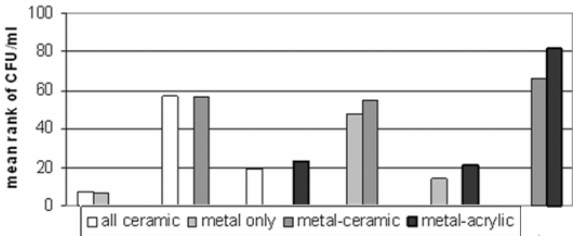

Figure1: Mean rank values of Lactobacillus species across 4 types of crown material combinations. According to Mann Whitney U test, significant differences lie only between metal ceramic crowns and metal acrylic crowns (P=.003).

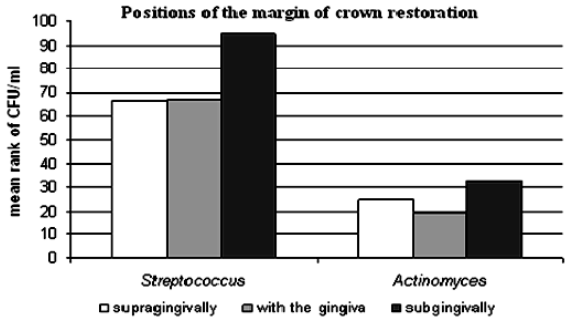

Figure 2 : Significant differences in bacterial counts (CFU/ml) of Streptococcus and Actinomyces species across 3 positions of crown restoration margin. Subgingival margin has highest counts of Streptococcus (P=.001) and Actinomyces species (P=.032) compared to supragingival margin and margin at gingival level.

4. Discussion

The present study aimed to evaluate the effect of crown restorations made from different combination of materials on the level of the most cariogenic bacteria compared to

the bacterial level on natural teeth sites. The number of CFU/ml of plaque was enumerated on each selective media for each patient and the proportions of each cariogenic bacteria were estimated. Low counts of Lactobacillus species in dental plaque were found in this study and this is expected since the Lactobacillus species are found in high numbers in samples taken from a caries lesion (Ahumada et al., 2003), which is not the case of the present study.

An interesting and significant result obtained is the difference in the lactobacillus counts in supragingival plaque on natural teeth compared to the crowns. There was no significant difference in the count of both Streptococcus and Actinomyces species between teeth sites. It is well known in most studies regarding the effect of dental restorations on oral bacteria that their surface roughness increases bacterial accumulation (Hannig, 1999; Weiman and Eames, 1975). Moreover, some of the elements that might be released from the restoration may have an influence in the bacterial adhesion and growth (Khalichi et al., 2004). In this study because there were various types of crown restorations involved, all the increased level of Lactobacillus could be explained to both the surface roughness and the physico-chemical properties of the restorations.

It was found also that the materials of crown restorations have a significant effect on the count of bacteria, where the differences lie between the metal-ceramic and metal-acrylic restorations (P=.003). The metal-ceramic had lower count than metal-acrylic. In spite of the fact that resin restoration is widely used in dental practice due to its low cost, it was reported in many studies that resin restoration promotes the accumulation of bacteria more than any other restoration, because their surface is highly rough (Weiman and Eames, 1975), and their released biodegradation by- products may stimulate bacterial growth. (Khalichi et al., 2004)

The current study showed that subgingival margin has the highest counts of Streptococcus and Actinomyces. The enamel close to margin restoration may be rapidly affected by secondary caries formation (Savarino, 2002; Mjor, 1985). The incomplete sealing between the enamel surface and the margin could lead to marginal leakage that allows the penetration and colonization of bacteria along the margin of the restoration and because the cleaning and removing of this accumulated bacteria become difficult when the restoration margin is below the gingival level.

Also this study revealed that the counts of Lactobacillus species from posterior teeth were significantly higher than from the anterior teeth. The accessibility of cleaning the anterior teeth may interfere with plaque accumulation and resulted in a reduction in bacterial counts compared to the posterior teeth. Differences in cariogenic bacterial counts between upper and lower teeth were not observed (P>.05).

The present study shows that persons with chronic periodontitis have the highest bacterial counts. The cariogenic bacteria have an indirect role in the periodontitis, by the interaction with bacterial species that cause periodontitis, Porphyromonas gingivalis (species that cause periodontitis) can co-aggregate with Streptococcus species (Cook et al., 1998). This study indicated that the age of the crown restoration had a significant effect on bacterial counts, the older the crown the higher the counts of cariogenic bacteria found. Previous studies showed that dental materials stimulate the accumulation of bacteria (Satou et al., 1988; Weiman and Eames, 1975; Khalichi et al., 2004), so it could be concluded that the older the restoration, the larger the accumulation of bacteria that could be found. This also could be due to the deterioration of the marginal seal between the restoration and the tooth margin, which subsequently enhance the conditions for the bacteria to accumulate and multiply.

Finally, it should be stated that in this study it was difficult to have a standard plaque sample size during the clinical sampling; therefore the quantitative comparison should be interpreted with caution. It's recommended for future research to standardize many parameters including; the plaque sample size, the condition of the periodontal tissue, and the level of oral hygiene of the patients.

5. Conclusions

Under the conditions of this study high counts of cariogenic bacteria was found to be associated with crown restoration vs. natural tooth, subgingival margin vs. supragingival and margin with the gingival level, metal acrylic crown vs. metal ceramic crown, as well as, the age of the crown and the periodontal inflammation.

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Hepatoprotective Activity of "*Orthosiphon stamineus*" on Liver Damage Caused by Paracetamol in Rats

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Abstract

The objective of this study was to investigate the hepatoprotective activity of Methanol extract of leaves of Orthosiphon stamineus against paracetamol induced hepatotoxicity. The material was dried in shade, they were powdered and Extracted with methanol. Preliminary phytochemical tests were done. Methanol extract showed presence of phenolic compound and flavanoids. The hepatoprotective activity of the methanol extract was assessed in paracetamol induced hepatotoxic Rats.Alteration in the levels of biochemical markers of hepatic damage like SGOT, SGPT, ALP and lipid peroxides were tested in both Paracetamol treated and untreaed groups. Paracetamol (2g/kg) has enhanced the SGOT, SGPT, ALP and the Lipid peroxides in liver. Treatment of methanolic extract of O.Stamineus leaves(200mg/kg)has brought back the altered levels of biochemical markers to the near normal levels in the dose dependent manner.Our findings suggested that O.Stamineus methanol leaf extract possessed hepatoprotective activity.

Keywords : Hepato protection, *Orthosiphon stamineus* ,Leaves, Methanol, paracetamol

1. Introduction

Liver diseases are the most serious ailment and are mainly caused by toxic chemicals (Excess consumption of alcohol, high doses of paracetamol, carbon tetrachloride, chemotherapeutic agents, peroxidised oil, etc). Inspite of the tremendous advances made in allopathic medicine, no effective hepatoprotective medicine is available. Plant drugs are known to play a vital role in the management of liver diseases. There are numerous plants and polyherbal formulations claimed to have hepatoprotective activities. In India, more than 87 medicinal plants are used in different combinations in the preparation of 33 patented herbal formulations (Handa SS et al.,1989; Hikino H, et al., 1988; Evans WC et al.,1996; Sharma A, et al., 1991).

Liver damage is associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels.In addition serum levels of many biochemical markers like SGOT, SGPT,triglycerides, cholesterol,bilirubin,alkaline phosphatase are elevated (Mascolo N et al 1998).

Orthosiphon stamineus benth (Lamiaceae) better known as poonai meesai by the locals is rich in flavanoids.Most flavanoids are bioactive compounds due to the presence of phenolic group in their molecule.Twenty phenolic compounds were isolated from this plant including nine lipophilic flavones,two flavonol glycosides, nine caffeic acid derivatives (Sumaryono W., et al 1991) and the new compound 5,6,7,8-tetra hydroxy-6-methoxy flavone was isolated from this plant (M Amzad Hossain et al.,2007). It is widely used in India for treatment of eruptivefever,urinarylithiasis, edema, hepatitis, jaundice,

Hypertension diabetes mellitus,Gout,Rheumatism, diuretic, anti-inflammatory and influenza. They exhibit excellentantibacterial Antifungal, antimicrobial, anti-tumer, and insect anti feed ant activities (Saravanan D., et al 2006; Hossain M.A et al 2001). OS have been reported to possess anti inflammatory (Masuda, T et al 1992) ,antihypertensive (Ohashi K et al 2000)Hypoglycemic activity (Mariam, A et al 1999) and Diuretic effect (Galyuteva, G.I., et al 1990; Dona DD et al 1992). In the present study we have evaluated the hepatoprotective activity of this plant against paracetamol overdose – induced hepatotoxicity in rats.

2. Materials and Methods

2.1. Drugs and Chemicals

Paracetamol (farmsons,Gujarat). All other chemicals were obtained from local sources and were of analytical grade.

2.2. Plant Materials

The leaves of *Orthosiphon stamineus* were collected from siddha research institute, Arumbakkam, Chennai. The plant was identified and voucher specimen was deposited in the herbarium of the department of biology (Specimen no;L-121),Annamalai University, chidambaram.The material was dried in shade and powdered leaves 1kg were extracted with methanol in a Soxhlet extractor for 36 hr. Extract was evaporated under low pressure by using Buchi type evaporator.

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2.3. Animals

Adult male wistar rats weighing 200-250g were obtained from Raja Muthiah Medical College, Annamalai University, Chidambaram, Tamil Nadu. They were maintained at standard housing conditions and fed with commercial diet and provided with water ad libitum during the experiment. The institutional animal ethical committee (Reg.no 160/1999/CPCSEA) permitted the study.

Table 1 Effect of *O.Stamineus* Extract on Biochemical Parameters in Rats Subjected to Paracetamol Induced Hepatotoxicity

Group		Lipid peroxides (nmole of MDA/mg protein)	ALP(IU/L)	SGOT (IU/L)	SGPT (IU/L)
I (Control)		46.5 ± 2.47	1.0817 ± 4.542	100.33 ±3.16	65.42 ± 2.16
II (Paracetamol)		128.17 ± 3.05	2.1067 ± 4.030	207± 4.05	180 ± 3.03
(Paracetamol & *O.Stamineus* extract)					
III	100 mg/kg	75.83 ± 2.04	1.6950 ± 2.918	174.5 ± 3.13	155.67 ± 3.84
IV	200 mg/kg	54.33 ± 3.16	1.2567± 2.171	115.17 ± 3.63	70.83 ± 3.32
One-way ANOVA	F	169.097	232.550	203.482	344.513
	d.f	23	23	23	23
	P	0.001	0.001	0.001	0.001

Values are mean ± SEM of 6 animals in each groups Group II compared with Group I (P<0.001), Group III and IV compared with Group II (P<0.001).

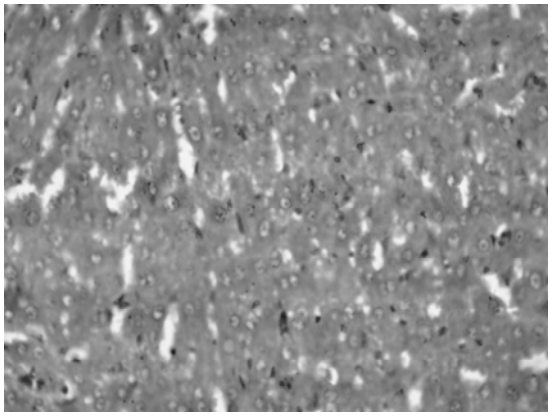

Figure 1. Liver tissue of control rats showing normal histology

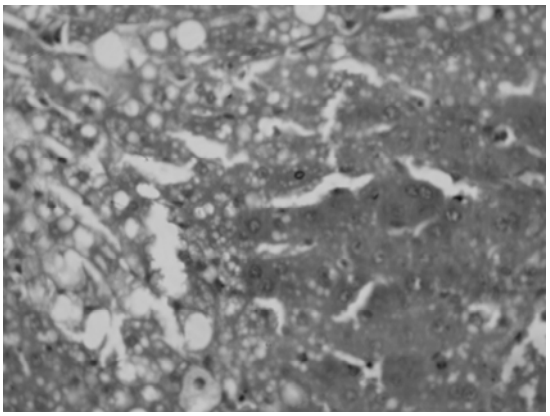

Figure 2. Liver tissue of paracetamol treated rats showing necrosis of the hepatic cells

2.4. Experimental Design

Four groups of six animals were used for the study.Control group received single daily dose of 5 % tween 80 (5 ml/Kg; po) for 4 days and a single dose of 40% sucrose solution (1 ml/rat; po) on day 3.

Paracetamol group received single daily dose of 5 % tween 80 (5ml/kg; po) for 4 days and a single dose of paracetamol suspension (2g/kg ,po)on day 3.

Test groups received daily doses (100mg/kg and 200mg/kg) of OS extract for 4 days and single dose of paracetamol suspension on day 3. Animals were sacrified under light ether anaesthesia, 48 hour after paracetamol administration.

2.5. Biochemical Study

Animals were sacrificed by cervical dislocation.The blood samples were cpllected by direct cardiac puncture . The blood samples were allowed to clot and serum were separated and the serum was used for the assay of maker enzymes viz.,Glutamate oxaloacetate transaminase(SGOT) ,Glutamate pyruvic transaminase,(SGPT) (Reitman S, et al1957) alkaline phosphatase (ALP)(Bessey OA et al 1964).

2.6. Estimation of Liver Lipid Peroxides

Estimation of liver lipid peroxides malondialdehyde (the product of lipid peroxidation) in the liver homogenate was measured as described(Ohkawa H et al 1979). Protein in the liver homogenate was measured according to the method of lowery et al (Lowry O et al 1951).

2.7. Histopathological Examination

Small pieces of liver tissue were collected in 10% formaldehyde solution for histopathological study. The pieces of liver were processed and embedded in paraffin wax sections were made about 4-6μm in thickness. They were stained with hematoxylin and eosin and photographed.

2.8. Statistical Analysis

The results were expressed as mean ± SEM of six animals from each group. The statistical analysis were carried out by one way analysis of variance (ANOVA)
P values < 0.05 were considered significant.

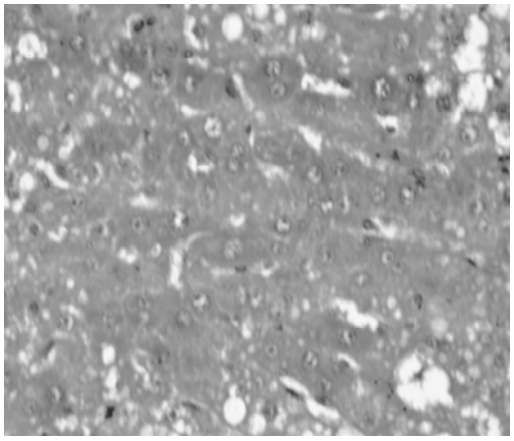

Figure 3. Liver tissue of paracetamol + OS extract (200 mg/kg) treated rats showing normal hepatic cells and central Vein

3. RESULTS

3.1. Paracetamol-Induced Hepatotoxicity

Preliminary phytochemical studies revealed the presence of phenolic compound and flavonoids were noticed in methanolic leaf extract. Table 1 shows that administration of paracetamol induced 48 hour after intoxication, a marked increased in serum SGOT, SGPT, alkaline phosphatase.

The toxic effect of paracetamol was controlled in the animals treated with methanol extracts (100mg/kg and 200 mg/kg) by way of restoration of the levels of the liver function.

At a dose of 100 mg/kg, the effect was only marginal whereas at higher dose (200mg/kg) the drug effectively prevented the paracetamol induced liver damage.

Paracetamol treatment group resulted in an increase in the lipid peroxide levels in liver homogenates. Administration of the methanol extract of O.stamineus leaves prevented the accumulation of lipid peroxides. At a lower dose (100mg/kg) there was a marginal effect in the lipid peroxide level where as at higher dose (200mg/kg) the drug effectively prevented paracetamol – induced elevation of lipid peroxides in liver (Table1)

3.2. Histopathology

Histological studies also confirmed the hepatoprotective effect of the methanol extract of O.stamineus. Paracetamol treated rat liver sections showed cloudy swelling and fatty degeneration of hepatocytes, necrosis of cells were also seen (Figure 1). The drug treatment (200mg/kg methanol extract) almost normalized these effects in the histoarchitecture of liver (Figure3).

4. DISCUSSION

Paracetamol is a known antipyretic and an analgesic which produces hepatic necrosis in high doses. Paracetamol is normally eliminated mainly as sulfate and glucuronide. Administration of toxic doses of paracetamol the sulfation and glucuronidation routes become saturated and hence, higher percentage of paracetamol molecules are oxidized to highly reactive N-acetyl-p-benzoquinemine by cytochrome-450 enzymes.Semiquinone radicals, obtained by one electron reduction of N-acetyl-p-benzoquineimine, can covalently binds to macromolecules of cellular membrane and increases the lipid peroxidation resulting in the tissue damage.Higher doses of paracetamol and N-acetyl-p-benzoquineimine can alkylate and oxidise intracellular GSH, which results in the depletion of liver GSH pool subsequently leads to increased lipid peroxidation and liver damage (Diadelis R et al 1995). In our experiments it is observed that the lipid peroxidation levels in the paracetamol group is increased.This clearly indicates that there is a significant hepatic damage due to paracetamol and this is further evident from the fact that there is elevation in the levels of various markers of hepati damage like SGOT, SGPT and ALP. Treatment with O.stamineus leaf extract has decreased the levels of lipid peroxidation and the elevated levels of above mentioned biochemical markers to the near normal levels. It may be concluded that the hepatoprotective effect of O.stamineus leaves is due to the prevention of the depletion in the tissue GSH levels. Literature review shows that the O.stamineus contains phenolic compound and flavanoids which are present in the methanol extract. Therefore there is a possibility that the O.stamineus leaf extract may possess hepatoprotective activity.

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Cytokine and Antibody Response to Immunization of BALB/C Mice with *E.Granulosus* Using Various Routes

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Abstract

Antibody and cytokine response were both studied in BALB/c mice immunized with *Echinococcus granulosus* crude sheep hydatid fluid (CSHF), antigen B (AgB) and protoscoleces homogenate (PSH) via subcutaneous (sc), intraperitoneal (ip) and intramuscular (im) routes. The present study aims at determining the most suitable antigen source and route of immunization, which stimulates the production of T helper type 1 response known to induce a protective immunity against secondary hydatidosis. Hydatid cyst fluid (HCF, a mixture of parasite and host proteins) has lead to the expansion of both IgG1 and IgG2a antibodies regardless of the immunization route. This was accompanied with the expression of moderate IFN-γ gene in spleen cells being the highest user of im route of immunization. Animals immunized with PSH using the ip route induced the highest IFN-γ and IL-4 gene expression, which is an indicator of a mixture of Th1 and Th2 responses. In this group of mice, the titer of antigen specific IgG3 was significantly higher than that for the other groups. Moreover, immunization of mice with AgB (a lipoprotein immunoregulatory component of HF) lead to high levels of IgG1 and IgG2a regardless of the route of immunization. However, using the ip route of immunization, AgB induced higher levels of IL-4, an indication of polarization towards the Th2 response. Immunoblotting profile showed the inability of IgG3 subclass in all groups to recognize the 16 kDa band of antigen B, whereas the reactivity to the 24 kDa band of antigen B was absent only in mice immunized ip with AgB. In conclusion, the im route of immunization of mice with CSHF lead to high Th1 response. While the ip immunization with PSH induced both Th1 and Th2 responses. AgB immunization with the same route lead to dominant Th2 response. Moreover, the IgG3 response in this model requires further investigation using cytokine gene knockout mice to elucidate the primary cytokine responsible for high IgG3 levels.

الملخص

لقد تم دراسة الاستجابة المناعية ممثلة بالأجسام المضادة والسيتوكاينات المتكونة في عينات المصل المستخلصة من الفئران (سلالة BALB/c) نتيجة تحصينها بسائل الأكياس المائية الخام ، أوأنتيجين ب، أورؤوس اليرقات لطفيل إكينوكوكس جرانيولوسس *Echinococcus granulous* وذلك عن طريق حقن التجويف البطني، أو الحقن تحت الجلد، أوفي العضل. هدفت هذه الدراسة لتحديد أفضل الطرق وأفضل المحفزات المناعية اللازمة لتحفيز إنتاج استجابة مناعية للخلايا التائية T cells من النوع الأول بغرض تحقيق مناعة وقائية ضد تكون الأكياس المائية الثانوية. أدى حقن الفئران بسائل الأكياس المائية الخام (خليط من بروتينات الطفيل والعائل) لزيادة في الأجسام المضادة من نوع IgG1 و IgG2a بغض النظر عن طريقة الحقن. ترافق هذا مع تعبير (expression) معتدل لجين IFN-γ في خلايا الطحال والذي وصل أعلى مستوياته بالحقن العضلي.وقد أظهرت الفئران التي حقنت برؤوس اليرقات عن طريق التجويف البطني تعبيرا قويا لجينات IFN-γ و IL-4، كمؤشر لخليط من الاستجابات المناعية التائية من النوع الأول(Th 1) والثاني (Th 2). وقد كان تركيز الأجسام المضادة IgG3 المتخصصة في هذه المجموعة من الفئران أكبر مما كان عليه في المجموعات الأخرى. علاوة على ذلك، أدى التحصين المناعي للفئران باستخدام أنتيجين ب (بروتين دهني من المنظمات المناعية في محتوى الأكياس المائية) الى مستويات عالية من الأجسام المضادة IgG1 و IgG2a بغض النظر عن طريقة الحقن. ومع ذلك حفز أنتيجين ب باستخدام طريقة حقن التجويف البطني، تعبيراً جينياً لجين IL-4 وهذا مؤشرللانحراف باتجاه الاستجابة المناعية التائية من النوع الثاني. وأظهرت نتائج الرسم المناعي (Immunoblot) عدم قدرة الأجسم المضادة من ضرب IgG3 في أي من المجموعات على التعرف على الجزئ 16 كيلو دالتون من أنتيجين ب، بالمقابل لم يظهر أي تفاعل مع الجزئ 24 كيلو دالتون في الفئران المحصنة عن طريق حقن التجويف البطني بأنتيجين ب.

Keywords: Crude Sheep Hydatid Fluid (CSHF), Antigen B (AgB), Protoscolices homogenate (PSH), T helper Type I (Th1), Interferon gamma (IFN-γ), Real Time PCR (RT-PCR).

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1. Introduction

Cystic echinococcosis (CE) is a cosmopolitan zoonotic parasitic disease caused by the larval stage (metacestode stage) of the tapeworm *Echinococcus granulosus*, which cycles between canines, mainly dogs, as definitive hosts and various herbivores as intermediate hosts. The infection rates in dogs and herbivores, as well as the prevalence including seroprevalence, and incidence of the disease in humans have been extensively studied in Jordan. This is one of the endemic areas among Middle East countries (Abdel-Hafez *et al.,* 1997; Al-Qaoud *et al.,* 2003; Qaqish *et al.*, 2003).

Like many helminthic parasites, *E. granulosus* develops sophisticated mechanisms for avoiding the cytotoxic effects of the immune response. Larvae can develop into hydatid cysts in various host organs, particularly liver and lungs. The cyst wall consists of an inner nucleated germinal layer, where protoscoleces bud are, and an outer a cellular laminated layer surrounded by a host fibrous capsule (Schantz *et al.,* 1995). The coexistence of the chronic infection with detectable humoral and cellular responses against the parasite represents one strategy of host-parasite relationship. CE is associated with induction of Th2 response, which appears to be important for parasite survival (Torcal *et al*., 1996; Rigano *et al*., 2001). Furthermore, humoral immune response studies on hydatid disease patients showed high levels of IgE and IgG4, which are induced by Th2 lymphocytes (K6ing and Nutman, 1993; Shambesh *et al.,* 1997). Further analysis of antibodies reactivity showed that IgG4 was predominantly bound to the highly specific AgB subunits of hydatid fluid antigens (Wen and Craig, 1994).

Vaccine-based control of disease transmission is the main objective of several research projects. Generation of a protective immune response against parasitic infections is associated with induction of Th1 type immunity. Evaluation of different parasite antigen preparations and different immunization routes may form a solid base for vaccination trials. Therefore, this study aims at evaluating three *E. granulosus* antigenic preparations (CSHF, AgB and PSH) versus three immunization routes (im, ip and sc) in mice to find the best immunization strategy that activates immune response of the mouse, where secondary hydatidosis develops.

2. Materials and Methods

2.1. Preparation of Antigens:

Crude sheep hydatid fluid (CSHF) and human hydatid fluid were prepared by the aseptic withdrawal of the cyst fluid from liver or lung cysts according to Moosa and Abdel-Hafez (1994). The fluid was centrifuged at 3500×*g* rpm, and the supernatant was freeze-dried using an Edwards lyophilizer (UK). The proper concentration was prepared by dissolving lyophilized powder in PBS, then dialyzing against PBS. Finally, protein content was determined according to Bradford (1976). *E. granulosus* protoscoleces homogenate (PSH) was prepared from washed PSc by homogenization in sterile PBS using a Braun homogenizer (B. Braun, Germany). The homogenate was centrifuged at 15,000×*g* for 30 min at 4°C.

Thermostable lipoprotein antigen B (AgB)-enriched cyst fluid was prepared according to McVie et al. *(*1997*),* based on Oriol et al. *(*1971*).* Hydatid fluid obtained from fertile sheep liver or lung cysts was dialyzed against 5 mM acetate buffer (pH 5); and was centrifuged at 15,000×*g* for 30 min. The precipitate was dissolved in 0.2 M phosphate buffer (pH 8); and boiled for 15 min. After centrifugation, the protein concentration of the supernatant was determined. And purity of AgB was assessed, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

All antigens were stored at -20°C until analyzed. These antigens were diluted to the desired concentration for animal immunization, or used for cell culture stimulation in RPMI containing 5% fetal calf serum (FCS) after filtration through a 0.2 µm Millipore filter (Schleicher & Schuell, Germany).

2.2. Immunization and Infection of Mice

Groups of 8 female BALB/c mice aged 6-8 weeks, bred at animal facilities (Yarmouk University) were immunized with 120 ug/mouse/dose of CSHF, PSH or AgB using the ip, im, or sc routes for each antigen. Antigens were mixed with equal volumes of complete Fruend`s adjuvant as first injection, and followed by 3 boosters of antigens in incomplete Fruend`s adjuvant. Similar groups of mice were immunized with the same volumes using PBS mixed with the relevant adjuvant. The interval between first injection and subsequent boosters was 7 days. The experiment was terminated after 12 days of the last booster. Mice were killed by ether overdosing, and blood was collected for serum preparation while spleens were collected for mRNA preparation**.**

2.3. Determination of Specific Antibodies

E. granulosus-specific IgG1, IgG2a, and IgG3 were quantified by ELISA. Microtiter plate wells were coated with 200 ul of 5 µg/ml crude human hydatid fluid diluted in carbonate buffer (pH 9.6). After incubation with 1:100 dilutions of sera, plates were washed four times and then incubated with a predetermined dilution of goat anti-mouse antibodies including IgG1, IgG2a, and IgG3 (Sigma, USA). HRP anti-goat IgG was added after 1 hr incubation. After addition of substrate solution, *o*-phenylene diamine (OPD, Sigma), and H_2O_2 (C.B.H.: UK), optical density (OD) was read on 450- and 630-nm reference filters. Reactivity index (RI) was calculated to overcome the interplate variation. A pool of positive samples with high titer was used as reference values in each plate. And OD of each sample was divided by reference value to obtain RI for all antibodies. For each plate, six wells with pooled negative control sera from normal mice were included. The same was done to other two wells containing sera from pooled positive control, immunized and infected mice from other studies carried out in our lab.

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2.4. SDS-PAGE and Immunoblot

SDS-PAGE was performed along the lines of Lammeli (1970). Low molecular weight protein standards (Bio-Rad, CA, USA) ranging from 14.4 to 97.4 kDa were used after being processed equally to antigens. After electrophoresis of CSHF samples and protein marker, gel was fixed, stained, and destained. To be used in immunoblot, the gel was neither fixed, nor stained. Immunoblotting technique was performed similarly to Towbin et al (1979). At end of electrophoresis, separated proteins were transferred to a nitrocellulose sheet by using the transblot cell (Bio-Rad, USA). After the end of transfer, cellulose sheet was removed and cut into 2 mm wide strips; and blocked with 2% bovine serum albumin in PBS (BSA-PBS) incubated with mouse sera (diluted 1:50) after being washed for three times. HRP conjugated goat anti mouse IgG1, IgG2a, and IgG3 were added after being diluted at 1:1000 for each antibody. The strips were incubated for 1 hour at R.T. After washing steps, three ml of substrate solution was added for 15 minutes at R.T. until color develops.

2.5. Measurement of Cytokine Gene Expression

To analyze cytokine gene expression, RNA was extracted from spleens of immunized mice and reverse-transcribed into cDNA. Quantitative analysis was done using a Real time PCR machine. Approximately, one third of fresh spleen samples were prepared by homogenizing the sample in TRI-REAGENT^{TM} (SIGMA, USA) according to manufacturer protocol. The RNA pellet was dried, for 5-10 minutes, and reconstituted in the appropriate volume of DEPC-treated dH_2O. Concentration and purity of isolated RNA were determined spectrophotometrically at 260 and 280 nm. The first strand cDNA synthesis was performed in 20µl reactions at which 2-5 µg of RNA were added to 1 µl of oligo(dT); and continued with DEPC-treated dH_2O to a volume of 11µl. The reaction mixture was incubated at 70^{o}C for 5 min. Then, the contents were centrifuged quickly after being chilled on ice for 1 minute. The reaction mixture was supplemented with 5µl of 4X M-MLV buffer, 1µl (40U/µl) RNAase inhibitor, 1µl (200U) M-MLV-RT enzyme and 2µl dNTPs mix (10mM each). The mixture was incubated at 42^{o}C for 60 minutes after being vortexed gently. The synthesized cDNA was stored at -70^{o}C until usage. Amplification and detection of synthesized cDNA for each sample were accomplished - using the real time PCR machine (Rotor-Gene 3000A, Corbett Research, Australia). SYBR-green dye was used for the purpose of DNA quantification. The PCR- reaction mixture constituted of 1µl cDNA, 2.5µl 10x reaction buffer, 2 mM $MgCl_2$, 400 µM dNTP mix, 200 nM sense and anti-sense primers, platinum DNA-polymerase enzyme (2.5 U), SYBR-Green (0.5x final conc.) and finally, sterile distlled H_2O was added to 25 µl total reaction volume. For primer design see Al-Qaoud and Abdel-Hafez (2008). Primers were purchased from Promega (Madison, Wisconsin, USA). The sequence of primers was as follows: for β-actin (forward primer CCC CGG GCT GTA TTC CCC TCC A, reverse primer TCC CAG TTG GTA ACA ATG CCA); for IL-4 (forward primer CAC TTG AGA GAG ATC ATC GGC, reverse primer TGC GAA GCA CCT GGA AGC CC); and for IFN-γ (forward primer CCT GCA GAG CCA GAT TAT CTC T, reverse primer TCG CCT TGC TGT TGC TGA AGA A). The optimization of real time PCR reaction was performed according to the manufacturer's instructions, and as described by Bustin (2000). Amplification steps consisted of initial denaturation at 95^{o}C for 5 min; and followed by 40 cycles of denaturation at 95^{o}C for 30 sec, and annealing and extension were at 60^{o}C. Relative quantification of cytokine gene expression was calculated using comparative C^T method in relation to β-actin gene as internal control. After determination of the threshold cycle (C^T), the relative expression level (Expr.) of each cytokine was calculated in relation to the expression level of β-actin gene as follows:

Expr. of cytokine gene = $2 - \Delta\Delta CT$

$\Delta\Delta CT = \Delta CT$ sample - ΔCT no template control (NTC)

ΔCT sample = CT sample – CT positive control.

$$\text{Relative Expr. level of each cytokine gene} = \frac{\text{Expr. level of cytokine gene}}{\text{Expr. level of } \beta\text{-actin gene}} \times 100\%$$

The positive control is a plasmid construct (kind gift from Prof. Fleischer, Bernhard Nocht Institute, Hamburg, Germany) containing similar sequence for that of the target cytokine gene; and is amplified by the same primers, which were used to amplify the target. Gel electrophoresis, using 1.5% agarose, was performed for all samples to test real time PCR product for both target gene and β-actin.

2.6. Statistical Analysis

Statistical analysis for differences among groups was done using the non-parametric (Mann-Whitney) *t* test. Results were considered significant at $P < 0.05$.

3. Results

AgB induced both high IgG1 and IgG2a whereas PSH induced the highest levels of IgG3 Evaluating IgG antibody subclasses revealed that IgG1 is the predominant subclass in all immunized groups with the im route being comparatively the lowest (Figs. 1 a, b & c). The highest level of IgG1 was recorded in AgB immunized mice with no significant differences among routes. However, a significant difference (P=0.007) was noticed in the titer of IgG1 antibody in CSHF immunized mice between ip and sc routes. Furthermore, immunization with PSH indicated significantly lower IgG1 in im route when compared to the ip (P=0.001) and to sc routes (P= 0.003) (Figure 1).

The levels of IgG2a were highest in AgB immunized mice when compared with the other two antigens for all immunization routes (Figure 1b) with a significant difference between ip and sc routes (P=0.03). Moreover, immunization with PSH resulted in significant differences in IgG2a titers between the im and ip routes (P=0.002) and the im and the sc (P=0.001).

With respect to IgG3, the highest level was noticed in PSH immunized mouse group regardless of immunization route (Figure 1c). Moreover, a significant difference in IgG3 levels was noticed between the ip and im (P=0.006), also between the sc and im immunization routes of mice (P=0.024).

3.1. Immunization Route Affected The Type and Reactivity of Igg Subclasses to Parasite Components

Immunoblotting of sera against CSHF fractions showed reactivity of sera with AgB fractions (8, 16, and 24 kDa bands) in CSHF, and in AgB immunized mice regardless of the immunization route (Figure 2 I, II and III). Further bands of 45-48 and 66 kDa, in addition to high molecular weight bands, were also prominent. However, in these mouse groups, a remarkable observation was that the IgG3 subclass using all routes did not recognize the 16 kDa polymer of AgB.

3.2. Intraperitoneal Immunization with PSH Induced Both Th1 and Th2 Cytokine Genes

The highest expression levels for both IL-4 and IFN-γ genes were induced in PSH immunized mice, by using ip r oute (Figure 3). Th1 cytokine (IFN-γ) gene expression dominated im and sc immunized mice, by using CSHF as antigen source. But lower levels were expressed in ip immunized mice. In contrast, AgB induced a Th2 cytokine (IL-4) gene expression using the ip route only.

4. Discussion:

This study aimed at exploring the most suitable hydatid cyst antigenic sources and the most appropriate injection route that lead to the development of Th1 response in mice. Such response is advantageous to the host but critical to the metacestode stage of the parasite.

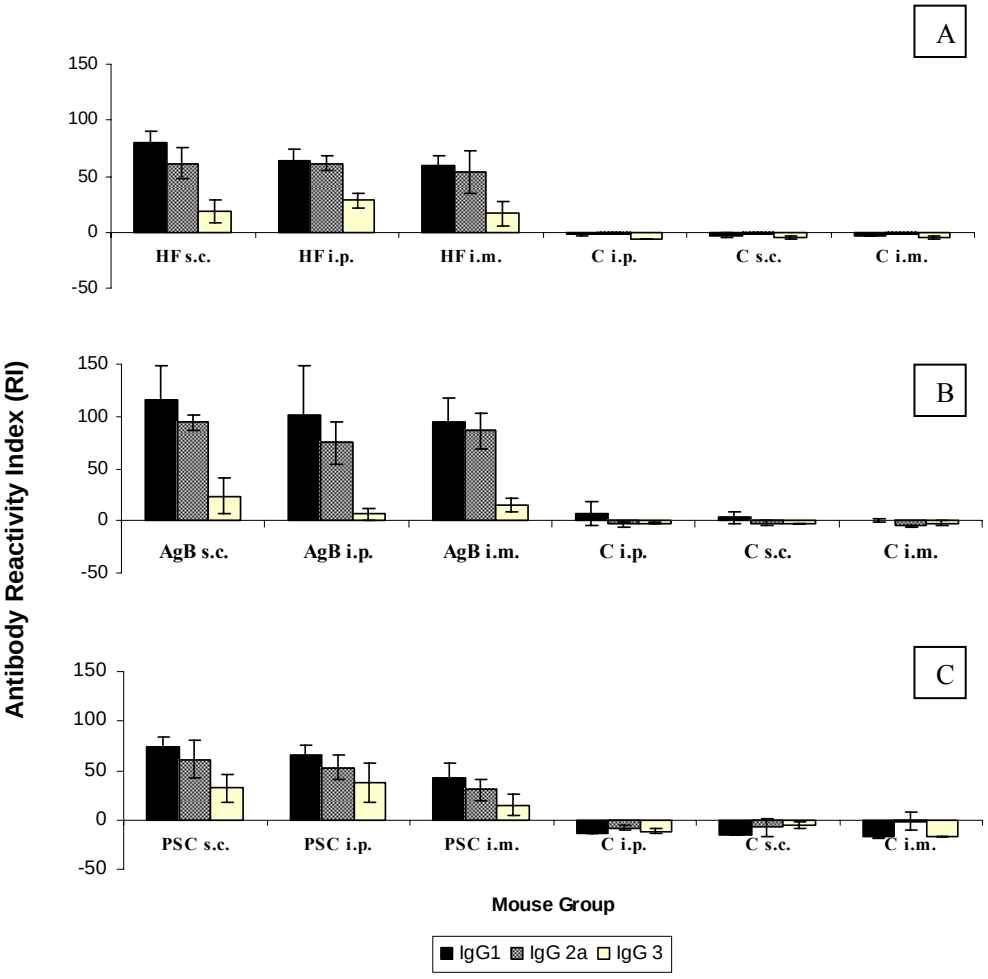

Figure 1 : Antibody isotype profile (IgG1, IgG2a and IgG3) of mice immunized with CSHF **(a)**, AgB **(b)**, and PSC antigens **(c)**, using sc, ip and im routes. Mice were killed on day 12 after final booster and anti-CSHF, anti-AgB and anti-PSC specific antibodies were measured using ELISA technique. Each block represents the mean value of reactivity index (R.I.) for 8 mice of each immunized group and 4 mice of control groups.

$$\text{R.I.(reactivity index)} = \frac{\text{Optical density of sample (O.D.) cut off point (COP)}}{\text{O.D. of positive control}} * 100\%$$

The COP was measured as mean O.D. +3 standard deviations of 6 wells containing pooled negative control sera.

Immunization of mice with protoscoleces using the ip route (Figure 3) induced both Th1 and Th2 typ responses. Rogan (1998) phased the two cell subsets in mice injected with protoscolices. It appears that, at the early stage, exposure to protoscoleces is accompanied by Th1 response, which is intended for the clearance of injected protoscoleces. Moreover, the death of considerable quantities of protoscoleces, which occurs in the first 3 weeks in secondary experimental hydatidosis may account for the development of Th1 (Rogan and Craig 1997). Consistently, in mice inoculated with live protoscoleces, the Th2 markers (high IL-4, IL-5 and IL-10 as well as IgG1) were found dominant during early days of infection, whereas inoculation with dead protoscoleces induced reversed type of response (Dematteis et al 1999, 2001).

Prominent levels of IgG3, which were induced by PSH immunization, agree with a (Al-Qaoud and Abdel-Hafez 2005). Induction of IgG3 secretion is mainly through T-independent carbohydrate antigens that are dominant on the surface of protoscolices (Ferragut and Nieto 1996; Dematteis et al. 2001), supported by high titres of IgG3 found in the peritoneal lavage of PSH-inoculated mice (Dematteis et al. 1999). Studies correlating the type of cytokine response with the IgG3 titers do conflict. While IFN-γ induced IgG3 production in response to T cell independent type 2 antigens IL-4 inhibited it (Snapper and Mond 1996). Absence of IFN-γ blocked IgG3 production completely (Snapper and Paul 1987). Data revealed that lack of IFN-γ receptor reduced IgG2a production, but not IgG3 (Huang et al 1993). The parallel expression of both IFN-γ and IL-4 cytokines genes in PSH immunized mice may explain why IgG3 may be induced by any or both of the opposing responses.

Upon immunization of mice with HF, all routes induced the expression of IFN-γ gene, and same groups demonstrated low levels of IL-4 gene expression, but still higher than that of control groups (Figure 3).

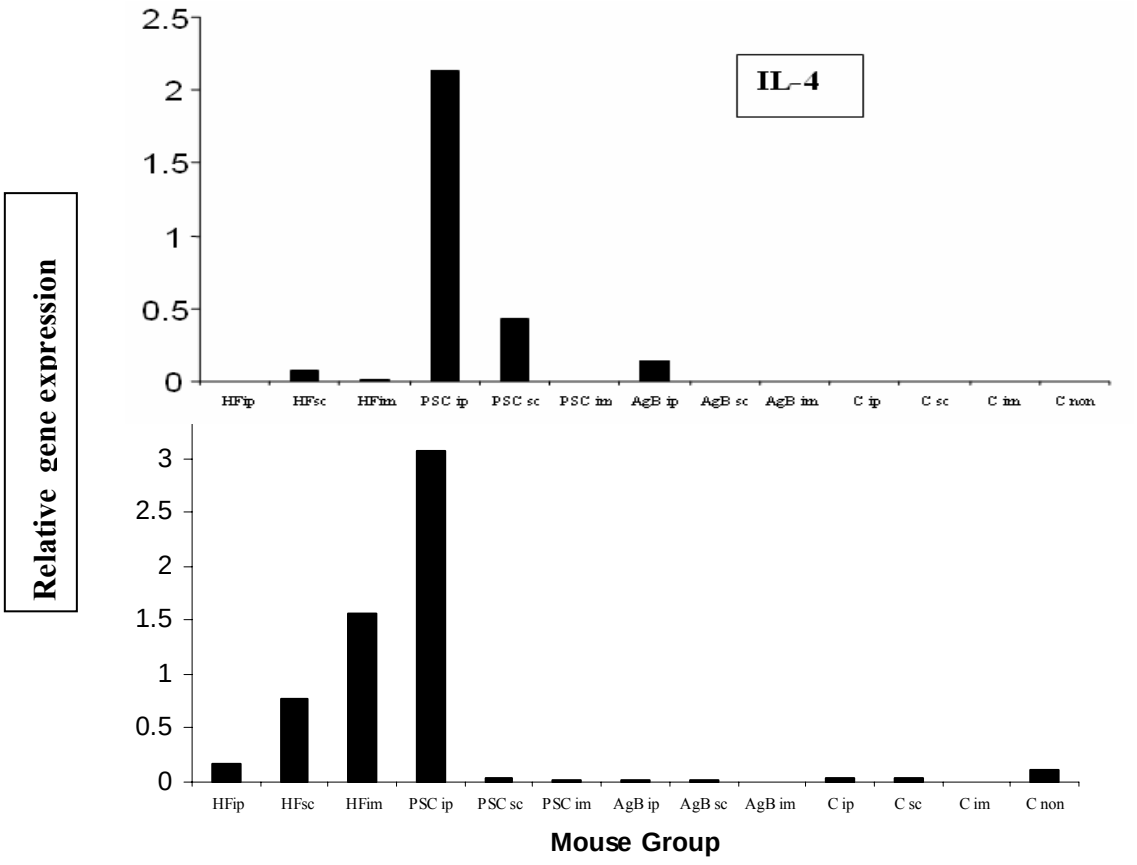

Figure 2 :Relative expression (Expr.) levels of IL-4 (a) and IFN-γ (b) genes determined in mice immunized with CSHF, PSH antigen and AgB, using sc, ip and im routes using the SYBR-Green I assay. Mice were killed on day 12 after final booster and the expression level of each cytokine gene was measured using RT-PCR. Each block represents the average of expression level for two mice from each group. Comparative C^T method ($\Delta\Delta C^T$) was used to calculate the expression level of each cytokine gene relative to β-actin gene as internal control as per text.

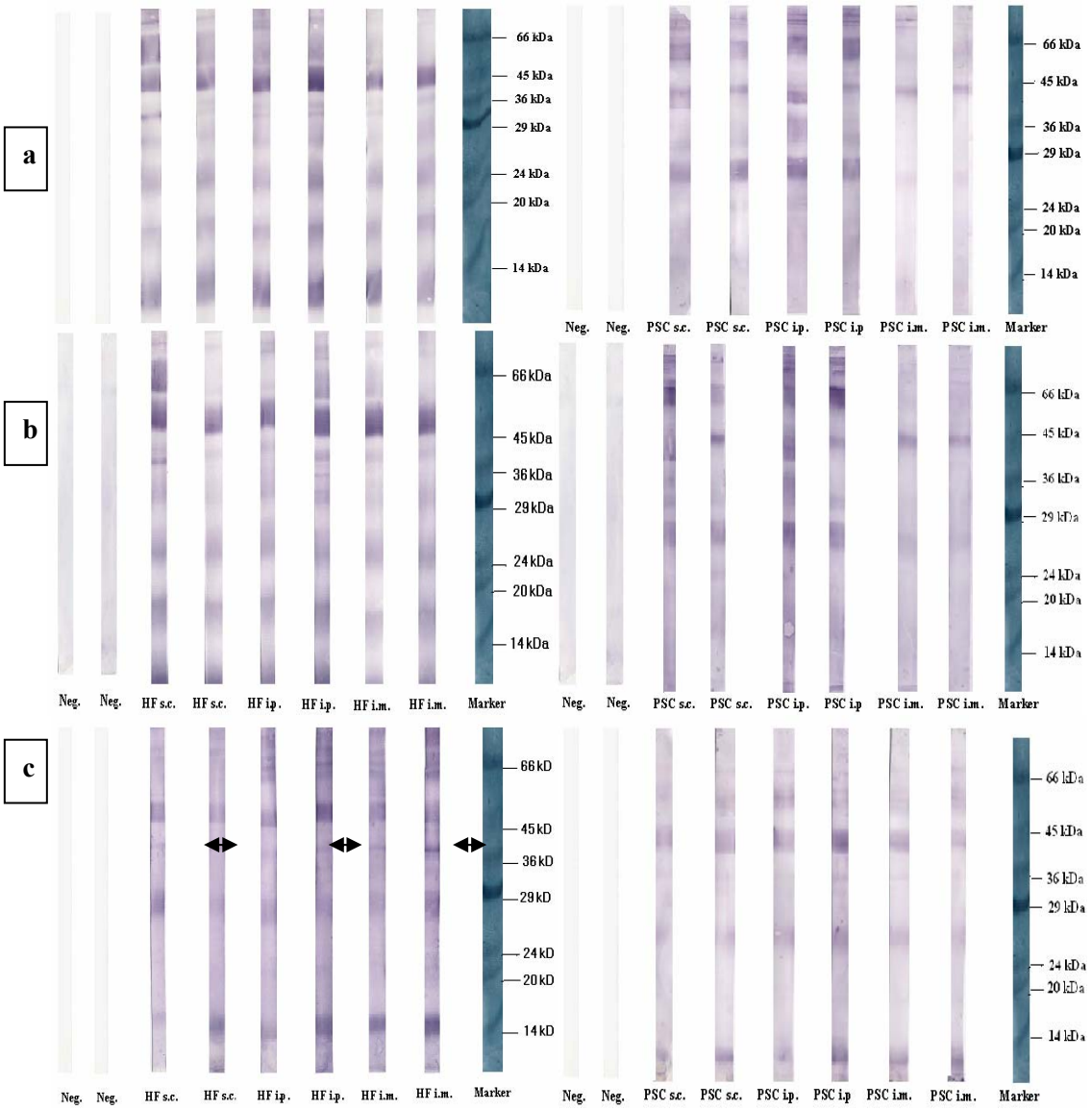

Figure 3 :Immunoblotting pattern of sera collected from mice immunized with CSHF (I), PSC homogenate antigens (II) and AgB (III) using sc, ip and im routes. The peroxidase conjugated secondary antibodies used were anti mouse IgG1(a), IgG2a (b) and IgG3 (c). The first 2 lines are blotting strips incubated with negative control sera and the final lane is a low molecular weight marker. CSHF, PSC homogenate and AgB were electrophoresed on 12.5% SDS-PAGE and blotted into nitrocellulose membrane.

Similar results were reported by Haralabidis *et al*. (1995), Rigano *et al*. (1999 and 2001), who attributed this dichotomy (Th1 and Th2) to the presence of a mixture of host and parasite antigen in the CSHF. Yet, using the same antigen in our laboratory resulted in variable responses and protection rates. This may be explained by using different antigen preparations from different antigen sources of variable purities. Promising data, in this study, stresses the importance of using either highly purified antigens or recombinant proteins in the immunization trials.

The absence of IgG3 reactivity to the 16 kDa subunit of AgB in all immunization routes was remarkable (Figure 2). It is well known that AgB is a polymeric lipoprotein that is made of an 8 kDa molecular weight building blocks. Until recently, it was thought that all AgB monomers are homologous. However, new studies indicated that AgB is encoded by a multigene family (EgAgB8/1, EgAgB8/2, EgAgB8/3, and EgAgB4) (Kamenetzky et al, 2005), each code for one subunit of 8 kDa. Recent data from our laboratory showed that a monoclonal antibody, which is raised against AgB8/2 subunit, did not react with all batches of parasite hydatid fluid (Al-Qaoud *et al.*). Moreover, polyclonal antibodies produced by mouse gene immunization recognized only one subunit of AgB. Since one batch of HF was used in this study, this phenomenon can be attributed to the heterogeneity of the AgB subunits.

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The Latency and Reactivation of Temperature- Sensitive Mutants of Mouse Cytomegalovirus in Different Organs of Mice.

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Abstract

Nine temperature sensitive (*ts*) mutants of mouse cytomegalovirus (MCMV) were compared with wild type (*wt*) for their ability to become latent and then be reactivated in different organs of Swiss mice. Three mutants (*tsm20*, *tsm23* and *tsm27*) failed to replicate in mice and were avirulent. Three other mutants (*tsm12*, *tsm21* and *tsm25*) were of similar virulence to the parental (*wt*) virus. All infected mice with *wt* died without prior infection after seven days, but the mice infected with *ts* mutants were still alive. Titer of virus in hearts, lungs and salivary glands homogenates from mice infected with *tsm15* continued to rise with time. After immunosuppressive treatment, infected mice with *tsm15* and *tsm25* had detectable virus in all tested organs; infected mice with *tsm29* had undetectable virus in spleens and kidneys; whereas, *tsm12* and *tsm21* had detectable virus in lungs and salivary glands. Mutant *tsm10* was unable to be reactivated. Virus recovered from salivary glands of mice infected with *tsm15*, *tsm25* or *tsm29* remained *ts*. These different mutants should prove useful for examining the viral and host factors involved in latency and reactivation.

الملخص

تم مقارنة تسع سلالات حساسة للحرارة من فيروس Cytomegalo مع نظيراتها من الأصل البري لقدرتها على الكمون ثم اعادة النشاط في الأعضاء المختلفة للفئران السويسرية. وقد تبيّن من الدراسة أن ثلاث سلالات(tsm20,tsm 23,tsm27) قد فقدت قدرتها غلى النمو والتكاثر ، وهناك ثلاث سلالات (tsm12,tsm21,tsm25) لها نفس خصائص الأصل البري. كما تبيّن أن جميع الفئران التي حقنت بسلالة الأصل البري قد نفقت خلال سبعة ايام بينما بقيت جميع الفئران التي حقنت بالسلالات الحساسة للحرارة على قيد الحياه. وقد بيّنت الدراسة أن الفيروس الحساس للحرارة tsm 15 يتكاثر مع الوقت أثر حقنه باالرئتين أو الغدد اللعابية أو القلب، كما ظهرمن عدد الفيروسات المتواجدة في مطحون هذه الأعضاء.كما أن حقن الفئران بفيروسات tsm15,tsm 25 بعد علاجها بمثبطات جهاز المناعة اظهر اعدادا من الفيروس يمكن قياسها في جميع الأعضاء التي تم فحصها. كما أن حقن الفئران بفيروس tsm29 تسبب بوجود اعدادا من الفيروس يمكن قياسها في الكلى والطحال. كما ان حقن الفئران بفيروس tsm 12 او tsm21 تسبب بظهور اعدادا من الفيروس يمكن قياسها في الرئتين والغدد اللعابية. كما بينت الدراسة أن الفيروس tsm 10 قد فشل في النمو بعد حقنه. كما أظهرت الدراسةأن الفيروسات tsm 15, tsm25,tsm29 المتحصّل عليها من الغدد اللعابية للفئران قد بقيت ذات طبيعة حساسة للحرارة. كما تظهر هذه الدراسه جدوى دراسة هذه الأنواع من الفيروسات .

Keywords: Cytomegalovirus; mouse; temperature sensitive; latency; reactivation.

1. Introduction

The establishment and maintenance of cytomegalovirus (CMV) latency in infected hosts has been subject of intense investigation for many decades (Tsutsui *et al.*, 2002). However, the mechanisms involved in the induction and maintenance of latency are not fully understood. Virus latency is achieved *in vivo* when infectious viral particles cannot be isolated from the host. *In vitro* induction of CMV latency has been accomplished through the use of viral inhibitors and temperature manipulation (Hummel *et al.*, 2001; Hummel and Abecassis, 2002).

Cytomegalovirus is a β-herpesvirus that is fairly ubiquitous in the human population and causes mild or subclinical disease in healthy individuals (Mocarski, 1996). Human cytomegalovirus (HCMV) has been shown to establish latency in cells of the monocyte/macrophage lineage including hematopoietic progenitor cells (Söderberg-Nauclèr *et al.*, 2001); and there is evidence of persistent HCMV infection in aortic endothelial cells (Fish *et al.*, 1995). The ability of CMV to reactivate from a latent state that is subsequently accompanied by asymptomatic viral shedding can periodically occur in healthy, seropositive individuals; however, the specific cell types from which recurrent virus comes are unknown. A significant amount of CMV morbidity can be attributed to reactivation events that are normally controlled by the immune system, based on the observation that immunosuppressed individuals often suffer from HCMV disease (Kercher and Mitchell, 2002).

Murine cytomegalovirus (CMV) has been used successfully to study parameters of latency in visceral organs such as salivary gland, lung and spleen (Balthesen *et al.*, 1994; Shinmura *et al.*, 1997; Pollock *et al.*, 1995).

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MCMV is similar to HCMV with respect to pathogenesis and the ability to establish or reactivated from latent infections.

Temperature-sensitive (*ts*) mutants, the first generation of conditional mutants in virology, have been helpful and often superior to null mutants in mapping and identifying viral functions (Roizman and Sears, 1996). Random procedures for the generation of *ts* mutants have been described for alphaherpesviruses (Schaffer *et al*., 1970). However, this method did not lead to major findings in betaherpesviruses, as their most prominent member human cytomegalovirus (HCMV), an important human pathogen. Propagation of conditional betaherpesvirus mutants by selection of *ts* alleles is technically difficult, and a specific gene has not been assigned for any of the reported *ts* mutants (Akel and Sweet, 1993). However, one *ts* allele was generated recently for the UL122 gene of HCMV by rational mutagenesis (Heider *et al.*, 2002).

Recently, Sweet *et al.* (1989 and 1993) had described the isolation and preliminary characterization of 25 *ts* mutants of MCMV derived by mutagenesis of virulent wild-type(*wt*) virus with N-methyl-N-nitro-N-nitrosoguanidine. These mutants varied in virulence from a virulent through 10-100 fold less virulent than *wt* virus (Akel and Sweet, 1993; Sandford and Burn, 1988).

Nine mutants out of 25 *ts* mutants were used to study the latency and reactivation of MCMV in various organs of mice.

2. Matrials And Methods

2.1. Mice

Out bred Swiss mice were obtained from Animal House of the University of Sciences and Technology, Irbid, Jordan. Foetuses of sixteen-day old pregnant mice were used to prepare mouse embryo fibroblast (MEF) cultures and for litters to be used for mouse passaged virus. Litters of Swiss mice were used to determine the lethality of mutants of MCMV. Infected and control mice were housed separately.

2.2. Virus

Wild type and mutant viruses were originally supplied by Dr Clive Sweet (Department of Bioscience, Birmingham University, Birmingham, United Kingdom) who had developed them from the Smith strain after many passages *in vivo* in mouse salivary glands (Mims and Gould, 1979). These viruses were cloned in mouse embryo fibroblast cells using terminal dilution as described by Sammons and Sweet (1989). Cloned stocks containing 1.4-3.2 x 10^5 pfu/ml was used for subsequent working.

2.3. Mouse Passaged Stocks of Virus

Groups of twenty of one-week-old Swiss mice were inoculated intraperitoneally (i.p.) with 50 µl of the seed stock of the virus, either undiluted or diluted up to 10 fold in growth medium. Twenty to 26 days later, mice were killed by anaesthetic overdose (Pentobarbiton sodium B. P.) and the salivary glands removed aseptically. These were homogenized in a small volume of growth medium using ultra-turrax T_8 IKA Labortechnik mixer (Germany) to give 10% suspensions, clarified by centrifugation, sonicated, filtered and stored at -70°C. These stocks were labeled mouse passage one (MP1). The procedure was repeated to produce mouse passage two (MP2) stocks using MP1-stock as inoculum.

Growth Curve of Ts Mutants at **37°C-**

Confluent monolayer containing 10^6 cells/well in 24-well multidishes were inoculated with 150 µl of the *ts* mutants in growth medium at multiplicity of infection (moi) of 1. After 60 min adsorption at 37°C cultures were washed with 0.5 ml of phosphate buffered saline (PBS) and overlaid with 0.5 ml of maintenance medium. The multidishes were incubated in a humid atmosphere of 5% CO_2/95% air, in CO_2 incubator at 37°C. At various times (0 hr, 1 hr, 4 hr, 10 hr, 24 hr, 48 hr, 96 hr and 120 hr) after infection, the viruses were harvested and stored at -70°C until yields were assayed at 37°C.

2.4. Determination of Lethality of Ts Mutants for Mice (LD_{50}):

The susceptibility of one-week old Swiss mice to infection with *ts* mutants virus determined by i.p. inoculation of groups of mice with 50 µl of serial 2 fold dilutions of virus in growth medium; groups of 20 mice were used per dilution. Initially the end-point was death of the animal allowing calculation of the 50% lethal dose (LD_{50}) by the method of Reed and Muench (1938). Later the feasibility of non-lethal end points was explored and this is described in the results.

2.5. Measurement of Ts Mutants Titers in Various Organs:

Twenty litters of Swiss mice were harvested on each of the day 2, 4, 7 and 11 post-infection (p.i.) with 300 pfu MCVM. The 300 pfu MCMV was lethal for 95% of Swiss mice, which died at ≤7 days p.i. . Spleens, livers, hearts, lungs, brains and salivary glands were removed aseptically, pooled from each litter and weighed. Thus, each litter of mice gave rise to one sample of each organ. Organs were homogenized and the levels of infectious MCMV were determined by a plaque assay in MEFs as described previously (Sammons and Sweet, 1989), modified to use 24-well trays seeded with 10^6 cells/ well. The results are expressed as the pfu of MCMV per g of tissue.

2.6. Activation of Latent Virus from Various Organs:

Four week-old Swiss mice were inoculated i.p. with 10^2 pfu of MCMV. Control mice were given normal rabbit serum and saline. Infected and control mice kept for one year. Infected mice then were given rabbit antilymphocyte serum (MA Biorpoducts) at a dosage of 0.3 ml twice week and cortisone acetate at a dosage of 125 mg/kg of body weight up to 21 days. Both agents were administered i.p. . At the end of three weeks, the mice were killed, and spleens, livers, hearts, lungs, brains and salivary glands were removed aseptically. Organs were homogenized and the levels of infectious MCMV were determined by a plaque assay in MEM, modified to use 24-weel trays seeded with 10^6 cells/well. The results are expressed as number of positive/ number of tested mice.

3. RESULTS

3.1. Titration of Stocks and Virulence of Mutants

The pfu/LD_{50} of the parental and various mutant viruses grown as stocks by passage in mouse (mouse passage 1 and 2) are shown in Table-1.

Table 1: Viral titration of tissue culture grown, mouse passage 1 and mouse passage 2 stocks, and virulence of *wt* and ts mutants, grown and assayed at 37°C.

issue culture grown stocks				Mouse grown stocks							
				Mouse passage 1			Mouse passage 2				
Virus titer (log_{10} pfu/ml)[a] after assaying at:				Virus titer (log_{10} pfu/ml)[b] after assaying at:			Virus titer (log_{10} pfu/ml)[c] after assaying at:			Virulence (pfu/LD_{50})	
	33°C	37°C	41°C	33°C	37°C	41°C	33°C	37°C	41°C		
wt	4.0	4.2	2.9	6.1	6.1	5.8	6.3	6.1	6.1	≥39	
tsm10	4.2	3.8	<1	2.1	1.0	<1	4.3	3.1	<1	≥205,000	
tsm12	5.0	5.1	1.4	4.3	4.1	<1	5.2	4.7	3.0	≥50,000	
tsm15	5.1	5.0	<1	2.1	3.7	<1	3.2	2.1	<1	≥125	
tsm20	4.2	3.4	1.5	1.1	<1	<1	<1	<1	<1	-	
tsm21	4.0	3.0	<1	3.2	<1	<1	4.2	3.1	1.5	≥12,562	
tsm23	4.2	3.2	<1	1.2	<1	<1	<1	<1	<1	-	
tsm25	4.1	4.6	2.2	3.3	3.0	<1	4.0	3.3	2.0	≥150	
tsm27	4.9	3.3	<1	2.3	<1	<1	3.6	<1	<1	-	
tsm29	4.0	3.3	1.2	2.8	<1	<1	3.5	2.6	<1	≥575	

[a, b and c] Results are expressed as the mean of three samples ± SEM.

Mouse stocks were prepared by i.p. inoculation of one-week-old Swiss mice with non-passaged (to produce mouse passage 1) or mouse-passaged (to produce mouse passage 2) stocks. The LD_{50} of *wt* virus for 2-week-old Swiss mice was 39 pfu in agreement with previously published data (Akel and Sweet, 1993). Three mutants (*tsm20, tsm23* and *tsm27*) were avirulent; their attempt to produce mouse-passaged virus were unsuccessful Table 1. Mutant (*tsm21*) did not replicate when inoculated into mice to produce mouse passage number 1; the other four mutants (*tsm10, tsm12, tsm21* and *tsm25*) showed ability to grow in mice.

Mutants that could be grown in mice showed considerable differences in virulence. Two mutants (*tsm15* and *tsm25*) were 86 and 111 fold less virulent than *wt* virus; one mutant (*tsm29*) was 536 fold less virulent and the remaining mutants (*tsm10, tsm12* and *tsm21*) were 2261 up to ~499961 less virulent than parental *wt* virus.

3.2. Growth curves of mutants

The growth curves at temperature 37°C of wild-type virus and of five mutants (*tsm12*, *tsm15*, *tsm21*, *tsm25* and *tsm29*) each inoculated at multiplicity of 1 are shown in Figure 1. Wild-type virus first produced infectious virus after 50 hrs of onset of infection and reached maximum

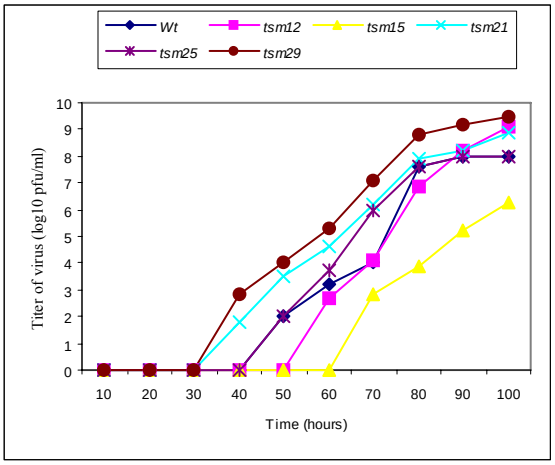

Figure 1. Growth curve of *wt* and ts mutants of MCMV grown and assayed at 37°C. Cells were inoculated at an input multiplicity of 1. Where, *tsm10* was not determined.

virus yields by 90 hrs p.i. Two mutants (*tsm21* and *tsm29*) released infectious virus particles at 40 hrs after infection and virus yields reached maximum yields by 100 hrs. The other mutants (*tsm12*, *tsm15* and *tsm25*) were a little slower and infectious particles did not appear until 60, 70 and 50 hrs i.p. respectively, although virus yields

reached maximum levels by 100 (*tsm12* and *tsm15*) and 90 hrs (*tsm25*) respectively.

3.3. Cumulative Mortality of Wild Type and Mutant Viruses

Table 2 shows the percent cumulative mortality induced by the *wt* virus and *ts* mutants with the lethal dose, 100% deaths generally occurred within 8 days of inoculation as seen with *tsm29*.

Table 2. Cumulative mortality of *wt* and ts mutants of MCMV for one-week old Swiss mice.

Virus	Dose (pfu/0.05 ml)	**Cumulative mortality (%)** Days post infection											
		1	2	3	4	5	6	7	8	9	10	11	12
wt	1,250	0	0	100	100	100	100	100	100	100	100	100	100
	625	0	0	0	70	90	100	100	100	100	100	100	100
	313	0	0	0	40	50	100	100	100	100	100	100	100
	156	0	0	0	0	0	70	90	100	100	100	100	100
	78	0	0	0	0	0	20	90	90	100	100	100	100
	39	0	0	0	0	0	0	0	0	20	52	70	100
	20	0	0	0	0	0	0	0	0	0	0	0	0
tsm10	20,000,000	0	100	100	100	100	100	100	100	100	100	100	100
	10,000,000	0	0	0	0	100	100	100	100	100	100	100	100
	50,000	0	0	0	0	0	0	0	80	80	100	100	100
	205,000	0	0	0	0	0	0	0	0	0	50	70	80
	102,500	0	0	0	0	0	0	0	0	0	0	0	0
tsm12	200,000	0	0	0	0	0	30	50	50	100	100	100	100
	100,000	0	0	0	0	0	0	10	90	100	100	100	100
	50,000	0	0	0	0	0	0	20	42	82	82	82	100
	25,000	0	0	0	0	0	0	0	0	0	0	0	0
tsm15	2,000	0	0	0	50	100	100	100	100	100	100	100	100
	1,000	0	0	0	0	15	100	100	100	100	100	100	100
	500	0	0	0	0	0	20	20	100	100	100	100	100
	250	0	0	0	0	0	0	0	0	80	80	100	100
	125	0	0	0	0	0	0	0	0	0	10	20	30
	63	0	0	0	0	0	0	0	0	0	0	0	0
tsm21	202,000	0	0	40	50	100	100	100	100	100	100	100	100
	101,000	0	0	0	50	80	80	100	100	100	100	100	100
	100,500	0	0	0	0	0	0	60	73	80	80	100	100
	50,125	0	0	0	0	0	0	40	40	40	40	50	70
	25,125	0	0	0	0	0	0	0	0	10	10	40	40
	12,562	0	0	0	0	0	0	0	0	0	5	35	35
	6,281	0	0	0	0	0	0	0	0	0	0	0	0
tsm25	8000	0	0	45	100	100	100	100	100	100	100	100	100
	4000	0	0	30	90	100	100	100	100	100	100	100	100
	2000	0	0	0	0	0	45	60	60	60	100	100	100
	1000	0	0	0	0	0	15	50	60	60	60	100	100
	500	0	0	0	0	0	0	30	43	43	70	80	100
	250	0	0	0	0	0	0	0	20	43	50	50	50
	150	0	0	0	0	0	0	0	0	0	5	35	40
	63	0	0	0	0	0	0	0	0	0	0	0	0
tsm29	5,200	0	0	0	60	100	100	100	100	100	100	100	100
	4,600	0	0	0	30	100	100	100	100	100	100	100	100
	2,300	0	0	0	0	0	60	90	100	100	100	100	100
	1,150	0	0	0	0	0	0	40	40	60	65	65	80
	575	0	0	0	0	0	0	0	30	45	45	45	45
	287	0	0	0	0	0	0	0	0	0	0	0	0

With some viruses, however, the lowest lethal dose took a little longer to kill, e.g. *tsm10, tsm15* and *tsm21* took 10-11 days while *wt* virus, *tsm12* and *tsm25* took 12 days to kill 100% of the animal at the lowest lethal dose.

It appears that there is very sharp dose response curve; generally, an increase in dose of as little as 2 fold can change lethality of the virus from 0% to 100% mortality such as *wt*, *tsm10* and *tsm12*. Even with the other mutants

only 4-6 fold difference in inoculum resulted in a 0-100% difference in mortality.

3.4. Effect of Mutant Infection on Body and Organ Weight

Individual control and infected mice were harvested and then weighed 11 days after inoculation. A minimum of 20 individual weights was used for each group. The mean weight of infected Swiss mice with *wt* was significantly lower than that of control Swiss mice Table 3 .

Table 3. Effect of *wt* and ts mutants of MCMV infection on body and organ weight[a].

	Control	Infected				
		wt	*tsm12*	*tsm15*	*tsm25*	*tsm29*
Mean body weight g±SEM[b]	11.60± 0.9	9.5± 0.44	11.3± 0.22	10.0± 0.1	7.85± 0.24	11.9± 0.2
Mean organ weight mg±SEM Spleen	48± 2	37± 2	45± 8	46± 4	43± 4	45± 3
Liver	250± 0	145± 8	208± 10	240± 10	185± 4	153± 6
Heart	66± 3	50± 4	62± 3	63± 4	55± 4	56± 3
Lungs	121± 5	75± 6	118± 3	122± 1	105± 2	100± 4
Brain	86± 3.0	71± 1	84± 6	84± 4	78± 4	80± 4
Organ weight as a % of mean body weight						
Spleen	0.41	0.39	0.40	0.46	0.55	0.38
Liver	2.16	1.53	1.84	2.40	2.36	1.28
Heart	0.57	0.53	0.55	0.63	0.70	0.47
Lungs	1.04	0.79	1.04	1.22	1.34	0.84
Brain	0.74	0.75	0.74	0.84	0.99	0.67

[a] Mice were inoculated i.p. with 6 pfu of MCMV or diluent alone on the day of 14 after birth and sacrificed for 11 days p.i.

[b] A minimum of 20 individual weights were used for each group.

tsm10 and *tsm21* – not determined.

Infected Swiss mice with *tsm15* and *tsm29* were slightly runted compared to control Swiss mice. The organs harvested from 20 controls and 20 infected suckling Swiss mice, 11 days after inoculation were weighted as a pool for each litter and the mean individual organ weight was calculated. The organ weights in infected mice were either significantly altered or were reduced compared to controls.

3.5. Latency of Mutant Viruses in Various Organs of Mice

Titer of virus in organs of mice infected with *wt* virus and the six *ts* mutants are presented in Table-4. Twenty mice infected with 300 pfu of tissue culture grown of *wt* virus or one of the six *ts* mutants or with no virus. Titer of virus in organs from mice infected with *wt* were at least >1000 fold higher than those for mice infected with *ts* mutants. After one week of infection, all mice infected with *wt* died without prior infection after seven days, whereas mice infected with mutants were still alive but mutants were below detectable level in brain. At seven days after infection, all tested mutants were recovered from spleen and liver. Titer of virus in hearts, lungs and salivary glands homogenates from mice infected with mutant (*tsm15)* continued to rise with time.

3.6. Activation of Mutant Viruses in Various Organs of Mice

To determine if *ts* mutants virus could become activated from a latent state, group of four-week-old mice kept for one year after infection and then subjected to an immunosuppressive therapy. This immunosuppressive regimen activated virus in all tested organs of nearly all twenty mice infected with *wt* virus Table 5, *tsm15* or *tsm25*. Infected mice with *tsm12* and *tsm21*, after immunosuppressive treatment, had detectable virus level in lungs (25% and 20% respectively) and salivary glands (10%). Virus titer in infected mice with *tsm29* subjected to immunosuppressive treatment was very low in spleen (5%), kidney (5%) and Brain (0%). No virus could be detected in almost all organs of any of twenty infected mice with *tsm10* that were subjected to immunosuppression. As seen in Table 6, virus recovered from salivary glands of mice infected with *tsm15*, *tsm25* or *tsm29* remained *ts*.

4. DISCUSSION

The results of our investigation showed various differences between nine mutants compared with *w*t or control (no virus infection): (i) a comparison of *wt* virus and *ts* mutants with form 86 fold up to >499961 fold differences in virulence may help to elucidate virus functions or virion components involved in latency, reactivation and immunosuppression. It remains to be useful in studies of any of the aspects of virulence; (ii) Organ weights of infected mice with *wt* and *ts* mutants were either significantly altered or were reduced compared to control; (iii) Mice inoculated i.p. with either virulent or attenuated MCMV develop latent infection, which we do not understand the latent state and those factors that maintain it or permit reactivation; and (iv) Activation of virus after immunosuppression indicated that all mice

appeared to develop latent infection with either *wt* virus or *ts* mutants of MCMV regardless of the mice strain, the dose of inoculation or the route of inoculation.

Table 4: Kinetics of *wt* and *ts* mutants of MCMV replication in organs after i.p. inoculation of newborn Swiss mice.

Days post* infection, Virus	Titer of virus (log_{10} pfu/gm of tissue±SEM)^					
	Spleen	Liver	Heart	Lung	Brain	Salivary glands
2						
wt	2.38	2.66	2.04	5.75	2	3.65
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	1.3	1.0	1.95	2.38	<1	2.00
tsm21	<1	<1	<1	<1	<1	<1
tsm25	1.1	1.1	1.0	1.2	<1	1.6
tsm29	<1	<1	<1	<1	<1	<1
4						
wt	4.08	4.43	3.76	3.61	3.39	6.78
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	1.35	1.6	2.00	2.20	<1	3.53
tsm21	<1	<1	<1	<1	<1	<1
tsm25	1.0	1.1	1.2	2.20	<1	3.20
tsm29	<1	<1	<1	<1	<1	<1
7						
wt	ND	ND	ND	ND	ND	ND
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	<1	<1	2.15	2.25	<1	3.28
tsm21	<1	<1	<1	<1	<1	<1
tsm25	<1	<1	<1	<1	<1	<1
tsm29	<1	<1	<1	<1	<1	<1
11						
wt	ND	ND	ND	ND	ND	ND
tsm10	<1	<1	<1	<1	<1	<1
tsm12	<1	<1	<1	<1	<1	<1
tsm15	<1	<1	3.0	2.86	<1	3.9
tsm21	<1	<1	<1	<1	<1	<1
tsm25	<1	<1	<1	<1	<1	1.1
tsm29	<1	<1	<1	<1	<1	<1

^ Results are expressed as the mean of the three samples ± SEM.

* Swiss mice were inoculated i.p. with 300 pfu of virus; organs homogenates from twenty mice killed at each times indicated were pooled.

* ND= Not determined.

Table 5. Activation of latent murine cytomegalovirus and dissemination to various organs of mice after immunosuppression with antilymphocyte serum and cortisone.

Virus			Activation[a]						
Strain	Type	Dose (pfu), route of inoculation	Spleen	Liver	Heart	Lung	Brain	Kidney	Salivary gland
wt	Virulent	10^2, i.p.	18/20	19/20	17/20	20/20	20/20	19/20	20/20
tsm10	Attenuated	10^2, i.p.	0/20	0/20	0/20	0/20	0/20	0/20	0/20
tsm12	Attenuated	10^2, i.p.	0/20	0/20	0/20	5/20	0/20	0/20	2/20
tsm15	Attenuated	10^2, i.p.	9/20	11/20	9/20	6/20	2/20	2/20	10/20
tsm21	Attenuated	10^2, i.p.	0/20	0/20	0/20	4/20	0/20	0/20	2/20
tsm25	Attenuated	10^2, i.p.	4/20	5/20	4/20	8/20	2/20	6/20	9/20
tsm29	Attenuated	10^2, i.p.	1/20	3/20	5/20	9/20	0/20	1/20	9/20

[a]Data are no. positive/ no. tested.

The virulence of mouse grown wild-type MCMV for one-week-old mice was ≥39 pfu/LD_{50} which was in agreement with previously published data (Shellam and Flexman, 1986; Sammons and Sweet, 1989; Akel and Sweet, 1993). Three mutants (*tsm20, tsm23* and *tsm27*) were avirulent for mice in that they failed to replicate in salivary glands of these mice. Similar results were obtained with *ts6* described by Sandford and Burns (1988), *ts21* described by Sammons and Sweet (1989) and *tsm5* by Morley *et al*. (2002). Clearly the genus defective in the viruses play a crucial role in their replication *in vivo* and may be involved in cell attachment or entry or more likely in early replication events. This was confirmed by the work of Gill *et al.* (2000) who used reverse transcription polymerase chain reaction (RT-PCR) to detect viral transcripts during latency by using 4 gene markers (IE 1, E 1, gB and GH); Their results indicated that replication of *tsm13* was blocked at a late phase, and *tsm22* was blocked at the immediate early phase. Whereas, *tsm9* and *tsm30* were blocked at a maturation step, probably of capsid formation, as gene transcription of all 4-marker genes occurred at 39°C and 40°C. In contrast, three mutants (*tsm12, tsm21* and *tsm25*) were able to grow in salivary glands of inoculated mice and were attenuated but the level of attenuation could not be determined since the highest dose used did not kill mice.

A major difference between *ts* mutants and parent virus infections was the replication pattern in heart, liver, lung, brain and salivary glands. One-week-old mice were inoculated i.p. with 300 pfu of mouse passaged virus. The *wt* virus was lethal at this dose and animals died within 5-7 days of inoculation; the virus become generalized, infecting hearts, lung, liver, spleen, kidney and salivary glands that was in agreement with previously published data (Furrarah and Sweet, 1994). In contrast, mutant viruses were not lethal at this dose but showed variability in replication in tested organs. For viruses (*tsm10, tsm12, tsm21* and *tsm29*) failed to replicate in any tissue while mutants (*tsm15* and *tsm25*) showed poor viral replication in heart (<3 log10 pfu/gm), lungs (<2.86 log10 pfu/gm) and salivary glands (<3.9 log10 pfu/gm).

Infected mice received an immunosuppressive regimen, after one year of inoculation, known to reactivate latent MCMV. The *wt* virus, as previously showed (Furrarah and Sweet, 1994), was most easily reactivated in that ≥85% of animals exhibited virus from hearts, spleens, livers, kidneys, lungs, brains and salivary glands. Mutants (*tsm12* and *tsm21*) could be reactivated but from fewer animals (lungs [25% and 20%] and salivary glands [10%]). Mutant (*tsm10*) could not be reactivated as an infectious virus after immunosuppression.

Table 6: Temperature sensitivity of the reactivated isolates.

Reactivated virus*	Titer (log10 pfu/ml) of virus at		
	33oC	37oC	40oC
wt	4.8	4.5	3.2
tsm 15	4.9	2.1	<1
tsm 25	3.8	1.2	<1
tsm29	4.0	3.2	1.1

*Salivary gland homogenates from (1) *wt*, nineteen mice; (2) *tsm 15*, eleven mice; (3) *tsm 25*, nine mice; and (4) *tsm29*, nine mice; that reactivated following immunosuppression therapy was pooled for this assay.

While, the mutant (*tsm29*) was able to reactivated but in very low in spleen and kidney (5%). Similar results were seen in six mutants (*tsm1, tsm2, tsm3, tsm4, tsm5* and tsm6) tested by Furrarah and Sweet (1994). All virus isolates from salivary glands of *tsm15, tsm25* and *tsm29* retained the *ts* phenotype. Clearly the *ts* lesion in *tsm15, tsm25* and *tsm29* was not in a gene whose product is important in initiating and maintaining latency.

Identification of all MCMV *ts* mutant genes and their ordering on the genetic map should eventually lead to a complete understanding of the structural and functional organization of the genome. This type of information is essential in order to understand the pathogenicity of the virus.

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Effect of *Allium sativum* and *Myrtus communis* on the elimination of antibiotic resistance and swarming of *Proteus mirabilis*.

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Abstract

Proteus mirabilis isolated from clinical sources (urine, wound and burn) in Erbil city hospitals. These isolates were characterized culturally, morphologically, and biochemically. The API20E system was used to support their identification. All isolates were tested for their resistance to twelve different antibiotics with the resistance of six isolates to all of the tested antibiotics (antibiotype A6). However, A1 antibiotype isolates were more sensitive and were resistant to seven of the tested antibiotics. Transformation experiment revealed that the resistant genes of (Chl, Dox, Ery, Gm, Kaf, Lin, and Pen) in P7 isolate and the resistant genes of all tested antibiotics from P23 isolate are not chromosomally coded. Sub-MIC of watery extract of A. sativum eliminated 50 and76% Kaf, and Gm resistance genes in P23 isolate, and reduced Ery resistance genes only 16.6% for P7 isolate, while M. communis eliminated Pen 3.3%, and curing of plasmid confirmed by determining the loss of resistance markers in the cured derivative culture. The Sub-MIC of crude extract of A. sativum habited the swarming of P7 at the concentration of 200 and 250 µg/ml when Amp, Am, Ceh, Chl, Lin, and Pan-c were applied to the nutrient agar plates, and M. communis at the concentration of 200 µg/ml inhibited the swarming when Chl and Pan-c antibiotics are applied. Neither A. sativum nor M. communis extracts were inhibited swarming at any concentrations in P32 isolate.

الملخص

عزلت بكتيريا *Proteus mirabilis* من مصادر طبية مختلفة في مستشفيات مدينة اربيل اعتمادا على الصفات المزرعية، المظهرية و البايوكيميائية والتي ضمنت اختبارAPI20E. اختبرت مقاومة جميع العزلاته تجاه عشر مضادات حيوية. اضهرت نتائج عملية النقل الجيني بان الجينات المسؤلة عن مقاومة المضادات Dox, Ery, Lin, Chl, Pen للعزلة P7 و جميع المضادات المختبرة للعزلة P32 تقع على DNA البلازميدي. عند استخدام Sub-MIC للمستخلص المائي لنبات الثوم اختزلت مقاومة الجينات Gen و Kaf بنسبة50 -76% للعزلة P23 و كذلك Ery جين فقط بنسبة 16و6%، في حين مستخلص نبات المورت (M. communis وان تحييد البلازميدات وجدت من خلال فقد المقاومة للمضاد في المزارع. ثبط المستخلص الخام لكلا النباتين عملية swarming للعزلة P7 بتركيز 200و250 ميكروغرام/ سم^3 عند تواجد المضادات Amp, Amx, Cep, Chl, Lin, Pan-c في الوسط الزرعي، في حين200 مايكرو غرام/سم^3 ثبط عملية swarming للعزلة P7 عند تواجد المضاد الحيوي Chl, Pan-c.

Keywords: Allium sativum, antibiotic resistance, Murtus communis plant extract, Proteus mirabilis.

1. Introduction

It has been well established that the most genes that are responsible for antibiotic resistance are borne on a plasmid DNA in most strains of *P. mirabilis* (Khder, 2006 and Alski, 2002). It found that frequency of loss resulted after growth of some strains at elevated temperature (Al-Safawi, 2001) or after exposure to compound which interfere with deoxyribonucleic acid (DNA) replication Such as acridin dyes(Staner et al, 1984 & Khder, 2002), and ethidium bromide (Villar et al, 1981), SDS (Ahmad, 2002) or by medicinal plant extract (Mawlud, 2006 & Khder, 2006).

Swarming which are multinucleated, nonseptated cells 20-80 mm in length containing even 500 fold more flagella, this phenomena is important in the pathogenicity of bacteria, and is important in ascending urinary tract infection (*UTI*) which are more common in Proteus strains (Alski, 2002).

The aims of this study are studding the effect of A. *sativum* and *M. communis* extracts on elimination of antibiotic resistance and swarming of *P. mirabilis* isolated from different clinical origins in Erbil city hospitals.

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2. Materials and Methodes

Thirty isolates of *p. mirabilis* were isolated from various clinical specimen at Komary, Rizgary and Emergency hospitals in the city of Erbil, Iraq. The reference bacteria *Escherichia. coli* K12JM83 was kindly provided by Dr.Khaled Daham Ahmed- University of Mosul-Iraq. All isolates identified by cultural, morphological and biochemical tests. Moreover the API 20E (Bio Merieux, Marcyl, Etoile, France) system and oxidase test were performed. All isolates were biotype on the basis of susceptibility testing. The isolates and reference bacteria were maintained as frozen stocks at -70 ^{o}C in presence of 80% glycerol and cultured in nutrient broth for 18 hr at 37 ^{o}C, for later analysis.

2.1. Antibiotic Susceptibility Test

Susceptibility to different antibiotics was tested using Muller-Hinton agar medium (Al-Najjar, 1976). Variable commercially and widely used antibiotics were used {Ampicillin (Amp), Amikacin (Am), Cepholothin (Ceh), Chloramphenicol (Chl), Doxicillin (Dox), Erythromycin (Ery), Gentamycin (Gm), Kanamycin (Kan), Kafalexin (Kfa), Lincomycin (Lin), Nalidixic acid (NA) and Penicillin (Pen). These antibiotics were used at final concentrations or plant extracts were added to medium after sterilization and cooling to 50 °C, the medium were mixed and poured into Petri-dishes, and inoculated with isolated bacteria using streaking method. Susceptibility or resistances of Proteus isolates to these antibiotics were recorded after incubation for 24 hr at 37^{o}C.

2.2. Genetic Site Determination of Antibiotic Resistant Genes.

Plasmid DNA from proteus isolates was extracted by following the method that described by (Birnboim and Doly, 1979). Transformation process was performed to determine the location of antibiotic resistance genes of tested isolates using the method of (Mandel and higa, 1970). The isolated plasmid DNA has been transformed to *E. coli* K12JM83 strain which has the following genotype {ara, Δ(lac pro A,B), rpsl, Ø80, lacZ ? M15, JM83 r^{+k} m^{+k} piR}. Competant cells were prepared using the method described by (Mandel and Higa 1970). A culture of *E. coli* K12Jm83 was prepared by inoculation of single colony in a test tube containing 5 ml of nutrient broth, then incubated with shacking (100rpm) for 18-24 hr at 37^{o}C, After growth, 1 ml of this bacterial culture was added to a flask containing 50 ml nutrient broth, then incubated under the above condition until the culture reach to active logarithmic phase with optical density of 0.5 at 600nm. The cells were harvested by centrifugation at 8000 rpm, and then resuspended in 1 ml of cooled transformation buffer (TE buffer pH 8, 50 mM $CaCl_2$ and 10mM Tris-HCl pH8), and the volum was completed to 40 ml using the same buffer. Resuspended cells were left in ice for one hour, and then centrifuged for 15 minutes at the same speed. Finally the pellet was resuspended in 1 ml of cooled transformation buffer.

To increase the efficiency of genetic transformation, the competent cells were kept at 4ºC for 24 hours before adding the plasmid. Transformation of plasmid DNA was performed by following the method of (Lederberg and Cohn, 1974). One hundred µl of prepared plasmid DNA was added to a tube containing 0.2 ml of competent cells, the mixture was placed in ice for 30 minutes, exposed to heat shock at 42ºC for 6 min (Hoekstra et al, 1980), then 1 ml of fresh nutrient broth was added to transformation mixture and then incubated at 37ºC for 60 minutes.

Aliquots of 0.1 ml from the transformation mixture were spread on the surface of 5 nutrient agar plates containing the appropriate antibiotic. Control plates were prepared by spreading of 0.1 ml of competent cells on the surface of nutrient agar containing the same antibiotics used as control. All plates were incubated at 37ºC for 48 hours. Number of transformant colonies were scored and purified several time on plates containing the different tested antibiotics used. The genetic transformation frequency was calculated according to (Puhler and Timmis, 1984).

2.3. Preparation of Plant Extracts

Extraction of *Allium sativum* and *Myrtus communis* was performed using Steam distillation technique as described by (Adler and Irobi, 1993). Twenty gram of plant powder was extracted using 300 ml deionized distilled water for 2-3 hours, with a distillation rate of 150 ml/minute (El- Astal et al, 2004; Rassol, 2004). The extract was dried for 2-3 hr using rotary evaporation (Bibby Re200 U.K.) (Rose et al, 1987), and then the extract was sterilized by membrane filtration using 0.22µ pore size membrane filters (Sharref, 1998).

2.4. Determination of MIC of Plant Extract.

MIC (minimum inhibition concentration) of plant extracts or chemical material (menthol) were performed through addition of different concentrations (25 µg to 750 µg) of the sterilized plant extracts to a sterilized nutrient broth, after incubation time for 24 hr at 37^{o}C with shaking, the turbidity was recorded using spectrophotometer at 600nm (Atlas et al, 1995).

2.5. Curing of Plasmid DNA

Plasmid DNA was cured by using pure menthol or water extracted from the tested plants as described by (Tomoeda et al, 1974). MIC of menthol and plant extracts was determined against the tested bacteria. Nutrient broth containing a range of concentrations (50-800 µg/ml) was prepared and 0.1 ml of fresh grown culture was inoculated in each test tube, then 0.1 ml was distributed on nutrient agar plates, then the plates were incubated at 37 ^{o}C for 24 hr then colonies were counted. The lowest concentration showing no significant viable count compared to the control plates (untreated plates) when plated on nutrient agar mediums was considered as the MIC of that curing agent/or plant extract. A range of sub-MIC concentrations were selected to treat the culture. A freshly grown culture of 0.1 ml was inoculated in each tube containing different concentrations of the curing agent. Control tubes were prepared without addition of curing agents. Tubes were incubated at 37ºC for 18 h. Cell broth was diluted by using a sterile normal saline and then spread on the surface of nutrient agar plates. Plates were incubated at 37ºC over night. Isolate colonies were replica-plated on to nutrient agar plates containing antibiotics to which the test bacterium was resistant. A plate without antibiotic was simultaneously also inoculated as control. Percent of cured colonies were determined by taking (the mean count of the colonies from antibiotic agar plates that did not grow of total mean colonies tested) X100.

3. Results

Clinical isolates of *Proteus mirabilis* isolated from different patients in three hospitals in the city of Erbil-Iraq were identified culturally, morphologically and biochemically. The API20E system was also used to support the identification process. All isolates were tested for their resistance profiles to twelve antibiotics representing different groups. The resistance pattern is shown in table (1), with the resistance of six isolates (P1, P23, P24, P26, P15, and P21) to all of the tested antibiotics (antibiotype A6). Isolates (P7, P11, P12, P29, and P30) of A1 antibiotype were more sensitive and were resisting to seven of the tested antibiotics.

To determine if the antibiotic resistance is encoded by plsmid or chromosomal DNA transformation process was performed to the most sensitive or resistant isolates of *Proteus mirabilis* and *E. coli*K12JM83 strain Table (2). Extracted plsmid DNA from P7 and P23 transferred successfully to JM83 strain, this derives us to conclude that the (Ch, Dox, Ery,Gm, Kaf, Lin, and Pen) in P7 and the resistant genes of all tested antibiotics in P23 are not chromosomally coded.

Table 1: Antibiogram groups, and numbers for *P. mirabilis* isolates.

Antibiogram	Amp	Amx	Ceh	Chl	Dox	Ery	Kan	Lin	Nal	Pen	Isolate No.	No.of antibiic resist
A1	-	-	-	+	+	+	-	+	-	+	7, 11, 12, 29, 30	5
A2	+	+	-	+	+	+	-	+	-	+	6, 13, 14	7
A3	+	+	+	+	+	+	-	+	-	+	8, 9	8
A4	+	+	-	+	+	+	+	+	-	+	3, 5,	8
A5	+	+	-	+	+	+	+	+	+	+	16, 17, 18, 19, 20, 22	9
A6	+	+	+	+	+	+	+	+	+	+	1, 23, 24, 26, 15, 21	10

Table 2: Number of transformation colonies and transformation frequency for P7 & P23 isolates of P. mirabilis.

Isolat	Am	Amx	Cep	Chl	Dox	Ery	Kan	Lin	Nal	Pen	Transformation frequency
P7 A1	S*	S	S	30	30	30	S	30	S	6	
P23 A6	18	20	30	30	30	30	30	30	21	20	

*: The bacteria are sensitive

Table 3: Curing % of *P. mirabilis* P23 andP7 isolates after treating with sMIC of *A. sativum* and *M. comunis* extract.

P23	sMIC µg/ml	Am	Amx	Cep	Chl	Dox	Ery	Gm	Kan	Kaf	Lin	Nal	Pen	Curing frequency
A. sativim	700	0.0	0.0	0.0	0.0	0.0	0.0	76.6	0.0	50	0.0	0.0	0.0	
M. comunis	700	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.3	0.23
P7	sMIC µg/ml	Chl	Dox	Ery	Kan	Lin	Nal	Pen	-	-	-	-	-	
A. sativum	700	0.0	0.0	0.0	0.0	0.0	16.6	0.0	-	-	-	-	-	1.15
M. comunis	700	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-	-	-	0.0
Menthol														
For P23	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
For P7	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	-	-	-	0.0

Table 4: Swarming of *P. mirabilis* after treating with *A. sativum* and *M.communis* extracts.

Isolate No.	Plant extract µg/ml	Antibiotics used at final concenterations										
		Amp	Amx	Ceh	Chl	Dox	Kan	Lin	Pen	Pan-c	St	Tri
P7 P23	Garlic 200	No* Sw**	No Sw	No Sw	No Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	No Sw	Sw Sw	Sw Sw
P7 P23	Garlic 250	No Sw	No Sw	No Sw	No Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	No Sw	Sw Sw	Sw Sw

P7 P23	Murts 200	No Sw	No Sw	No Sw	No Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	No Sw	Sw Sw	Sw Sw
P7 P23	Murts 250	Sw Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	Sw Sw	Sw Sw	Sw Sw	No Sw	Sw Sw	Sw Sw

*No: No swarming,**Sw: Swarming.

In table (2) MIC of watery extract for A. sativum and M. communis were determined (750µg/ml) and sub MIC (700µg/ml) was used as curing agent against multidrug resistance (was resist to all tested antibiotics) P23 and more sensitive isolate P7 (was resist to seven antibiotics). Antimicrobial activity was exhibited when plant extract used irrespective to drug resistance pattern of tested bacteria table (3). A. satevum eliminated 50 and 76% Kaf and Gm resistance genes in P23 respectively, and reduce Ery resistance genes only 16.6% for P7 isolate, while *M. communis* 3.3% eliminated Pen gene.

The effect of SMIC of both plant extracts on swarming of P7 & P23 isolates was determined, and the results were combined table (4). Swarming was inhibited in P7 only when 200 and 250 µg/ml of A. sativum or 200 µg/ml M. communis was applied when Amp, Amx, Ceh, Chl, Lin and Pan-c was applied to culture media, while no swarming was inhibited when 250 µg/ml M. communis was applied for P7, except with present of Chl and Pan-c swarming was inhibited. Swarming was not eliminated either using 200 or 250 µg/ml A. sativum extract or 200 and 250 µg/ml M. communis extract when applied for P23 isolate.

4. Discussion

Emergence of multidrug resistance in pathogenic bacteria has created immense clinical problem in the treatment of infectious disease. Elimination of plasmid DNA mediated antibiotic resistance in pathogenic bacteria is great practical significance both in chemotherapy of bacteria and in microbial genetics. Elimination of plasmid DNA can be performed using different chemical materials including plant extracts (Reuter and Sendel, 1994). Sulfur amino acids that is present in large quantity in garlic clove in which they named alliin, which was found to be the stable percussive that is converted to allicin by the active of enzyme termed allinase, which is also present in cloves of A. sativum (Mawlud, 2006). Allin is considered as a broad spectrum antibacterial for G^+ & G^- bacteria (Lawson, 1996; Sharref, 1998 and Feldberg et al, 1988). The main antimicrobial effects of Allicin is due to its interaction with important thiol containing enzymes, also it is believed to exert its primary antimicrobial effect through the inhibition of RNA synthesis via inhibition of RNA polymerase which seen in E. coli, and protein synthesis are also inhibited by allicin in S. typhimarium, and suggested that RNA polymerase be target for allicin (Feldberg et al, 1988; Ozolin and Kaxazolova, 1990 and Sharref, 1998), and other inhibited enzyme thiol-containing enzyme, alcohol dehydrogenase, and thioredoxin reductase (Ankir et al, 1997). Ajoen is antimicrobial which present in garlic cloves, and antimicrobial of ajoen is through additional microbe-specific enzymes may also target for allicin and wide spectrum antimicrobial effects of allicin and ajoene are due to the multiple inhibition effects they may have on vireos thiol-depending enzymatic.

The inhibition effect of M.communis towards proteus isolates may refer to the phenolic and polyphenolic they contain. These compounds were found to denature proteins and block enzymes and subsequently the bacteria losses its activity (Al- Asady, 1988). the phenolic activity of M. communis is refered to the hydroxyl phenol group (phenolic OH) that form a in hydrogen bond with the active sit of enzymes (Black, 1985). More over Myrtucomlone A&B are considered as two new acylphloraglucinols identified in the leaves and fruits of M. communis and shown a significant antimicrobial activity against. The inhibition activity may be regard to present of Tannin via producing hydrogen bonds with proteins, which converted its structure and lead to block the protein synthesis , and tannins considered as a phenolic compounds of plants which have ant oxidative effects (Makoto et al, 1995).

It is clear from this study that SMIC of tested plant extracts eliminated the resistance of Proteus isolates to certain antibiotics when applied with final concentrations, however the swarming of Proteus bacteria that is important in the pathogenesis of bacteria is reduced when these plant extracts are applied with some antibiotics, and the later being particularly important in ascending urinary tract infections, which are more common on proteus strains, one of the virulence factors and properties of proteus sp. mediating infectious process are swarming phenomenon.

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Optimization and Scale up of Cellulase free Endo xylanase Production by Solid State Fermentation on Corn cob and by Immobilized Cells of a Thermotolerant Bacterial Isolate

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Abstract

Different agro-residues were evaluated as substrates in solid state fermentation for xylanase production by a thermotolerent Bacillus isolate. Various fermentation parameters were optimized for enhanced endoxylanase production under solid state fermentation (SSF). Maximum enzyme production of 74.96±5.2 U/gds took place at 45^0C with corn cob (CC) and mineral salt solution (MSS) after 72 h, at pH 6.0 and particle size of 500 µm. A ratio of substrate: moistening agent of 1:2.5 was found to be optimum for CC, a solid substrate rarely utilized for bacterial xylanase in SSF. Continuous xylanase production by recycling immobilized cells could be achieved till 10 cycles with maximum enhancement of 156.05% and 219.21% after 5 and 4 cycles in static and submerged states respectively. Scale up in large trays under SSF yielded 157.12 ± 8.7U/gds in static state and 111.47± 8.1U/gds at 110 rpm.

Keywords: Cellulase free endoxylanase, Bacteria, Optimization, Solid state fermentation, Scale-up, immobilized cells.

1. Introduction

Xylanases (E.C.3.2.1.8) are key enzymes, which play an important role in the breakdown of xylan. Corn cob is a rich source of xylan (28%) and xylose (23%). Therefore it is an attractive substrate for production of xylanase enzyme. Xylan, a major component of hemicellulose, is a heterogeneous polysaccharides consisting of ß-1,4 linked to D-xylosyl residues on the backbone, but also containing arabinose, glucuronic acid, and arabino glucuronic acid linked to D-xylose backbone (Wong et al.,1988). Enzymatic hydrolysis of xylan is catalysed by different xylanolytic enzymes such as endo-1,4-ß-xylanase, ß-xylosidase, ά-glucuronidase, ά-arabinofuranosidase, and esterase. Among these endo-1,4-β-xylanase(E.C. 3.2.1.8) and ß-xylosidase are the most important enzymes where the first attacks the main internal chain linkages, and the second releases xylosyl residues by endwise attack of xylo-oligosaccharides (Bakir et al., 2001).

A variety of microorganisms including bacteria (Archana and Satanarayan, 1997; Poorna and Prema, 2006), fungi (Kheng and Omar, 2004), actinomycetes and yeasts have been reported to produce xylanase under SSF. Solid state fermentation is the growth of micro-organism on moist substrates in the absence of free flowing water. SSF offers distinct advantages over submerged fermentation including economy of space, simplicity of media, no complex machinary, greater product yields, and reduced energy demand (Sanghi et al., 2008). Although xylanase production in SSF from fungi and actinomycetes have been reported, only few reports using bacteria showing low enzyme yields are available (Archana and Satyanarayana 1997; Gessesse and Memo 1999; Heck et al. 2005; Battan et al. 2006; Sindhu et al. 2006).

Corn cob was also used for xylanase production in SmF by the *B. licheniformis* isolate under different conditions. However, xylanase yield was lower compared to the yield under SSF, which is being reported here. Although corn cob is a rich source of xylan and xylose, and is abundantly available in India, it has rarely been utilized for bacterial xylanase production under SSF. This could be due to low yield on corn cob as compared to other solid substrate like wheat bran. The objective of this work was to optimize various fermentation parameters for xylanase production

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by a thermophilic bacterial isolate on corn cob under SSF, and production enhancement by scaling up the solid state system. Since xylanase production was higher under SSF, and corn cob is an insoluble substrate suitable as solid state support, SSF was applied in the study. Improvement in xylanase yield by recycling immobilized cells for prolonged period are also being reported.

2. Materials and Methods

2.1. Materials:

Oat spelt xylan (Himedia Labroratories Pvt. Ltd., India) was used for enzyme assay. Corn cob was prepared by stripping corn of all kernels, drying, grinding, and sieving (500 mm. particle size). Dried corn cob was added to mineral salt medium; and autoclaved as mentioned in 2.4. Other than autoclaving, no other pretreatment was necessary. All other reagents were of analytical grade.

2.2. Microbial Strain:

Bacterial strain used in this study was isolated from decayed woody materials like xylan rich-wood materials, which are potentially good sources of xylanase producing micro-organism (Oliveira et al., 2006). This strain was identified by Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology, Chandigarh, India. And then was deposited as *Bacillus licheniformis* MTCC 9415. The optimum growth temperature of this strain is 45°C and it grows well in the range of 25^0C - 52^0C. The culture was grown and maintained on agar slants of 4% corn cob and mineral salts mentioned in 2.4.

2.3. Inoculum Prepararion:

Culture was maintained on agar slants of 4% corn cob and mineral salt mentioned in 2.4., then stored at 4^0C. And was subcultured routinely after every three four weeks. Inoculum was prepared by transferring one loopful of bacterial cells from a 48h old slant culture into 2 ml of fermentation medium; and incubated at 45^0C for 48h. This was used to inoculate 20 ml of fermentation medium.

2.4. Medium composition and growth condition:

Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 10 g of corn cob and 20 ml of mineral salt solution (MSS g/l : $MgCl_2.6H_2O$, 6.6g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5g; $(NH_4)_2S0_4$, 2.0g (Sanghi et al. 2007). The pH of medium was 6.7, and the medium was sterilized at 121^0C for 20 min. at 15 p.s.i., and was cooled and was inoculated with 10% (v/v) of inoculum (48h old) And was incubated at 45°C. At the desired intervals, the flasks were removed, and the contents extracted with 50 ml of 0.02 M phosphate buffer (pH 7.0).

2.5. Enzyme Extraction:

Enzyme was extracted with 50 ml of 0.02M phosphate buffer (pH 7.0), and squeezed through a wet muslin cloth. The extracted enzyme was centrifuged at 3000 rpm for 10 min. The clear supernatant was used in the enzyme assay.

2.6. Analytical Procedures:

Endoxylanase activity was measured by incubating 0.5ml of 0.4% (w/v) oat spelt xylan in 0.02 M phosphate buffer (pH 7.0). And 0.5 ml of suitably diluted enzyme extract at 45°C for 30 min. The release of reducing sugar was measured as xylose by dinitro salicylic acid method (Miller, 1959). One unit (U) of xylanase is defined as the amount of enzyme that releases 1 µmol xylose/ml/min under the assay conditions. Endoxylanase production in SSF was expressed as U/g dry fermented substrate (gds).

Cellulase activity was not detected in the culture supernatant. Cellulase was assayed according to Mandels et al., 1974 using sodium nitrate buffer (0.1M, pH 7.0) at 45^0C. One unit of cellulose is defined as the amount of enzyme that liberates 1µmol reducing sugar as glucose ml^{-1} min^{-1} under assay conditions.

2.7. Data Analysis:

All the experiments were carried out in triplicates. The analyses were done in duplicates. The mean values are shown in the figures and tables.

2.8. Statiscal Analysis:

Data were expressed as mean ± standard deviation for all experiments and statistical significance was calculated according to student two-tailed t test. Values corresponding to $p<0.001$ were considered statistically significant.

2.9. Endoxylanase Production Using Agro-Industrial Residues:

Bacterial strain was cultivated on different substrates such as wheat bran, corn cob, sugarcane baggase, rice bran, wheat straw, and rice straw. All growth conditions, mentioned in 2.4., were followed in all cases. Only agro-residues (10g in each case) were varied.

2.10. Effect of Incubation Period, Media Ph, And Temperature on Enzyme Production:

Effect of incubation period on endoxylanase production was determined by assays of enzyme after 24h, 48h, 72h, and 96h at 45^0C. Effect of media pH on endoxylanase production was estimated by culturing the strain in media of different pH-3, 4, 5, 6, 7, 8, and10. Effect of temperature on xylanase production was studied by incubating the strain at 30^0C, 35^0C, 40^0C, 45^0C, and 50^0C.

2.11. Effect of particle size:

The effect of particle size of corn cob on enzyme production was evaluated by culturing the organism on corn cob of different particle sizes (350µm, 500µm, 600µm, 710µm and 1000µm).

2.12. Effect of Moisture Contents:

The effect of moisture level on enzyme production was evaluated by varying the ratio of corn cob to mineral salt solution (66.6%-80%).

2.13. Scale up of Xylanase Production in Solid State:

The bacterial strain was cultivated in aluminum tray ($20\times8\times5cm^3$)containing 80g of corn cob moistened with MSS (ratio of 1:2.5), and other conditions as optimized in 250 ml Erlenmeyer flasks as mentioned earlier. The trays were covered with aluminum foil and sterilized at 121^0C for 20min., And then cooled and inoculated with 10% of 48h old inoculum. The trays were incubated at 45^0 for 96h. Samples were withdrawn at desired intervals, and xylanase was assayed as described in sections 2.4 and 2.5.

2.14. Immobilized Cell System:

Poly urethane foam (PUF) $1cm^2$ cubes, and scotch brite, SB,$1cm^2$ cubes were used for whole cell immobilization of bacterial strain. Cubes of PUF and SB were washed with distilled water and dried overnight at 60^0C in an oven, and then placed in a 250 ml flask

containing 50 ml of 1% yeast extract, peptone (YEP) medium supplemented with 1% xylose. After sterilization at 121^{0}C for 30 min, 10% (v/v) inoculum was added to each flask and was incubated at 45^{0}C. After 48h, PUF cubes with immobilized cells were carefully drained and washed with sterile water for eliminating all non adhering bacteria; also the broth was replaced with fresh medium for the next cycle. The process was carried out over 10 cycles.

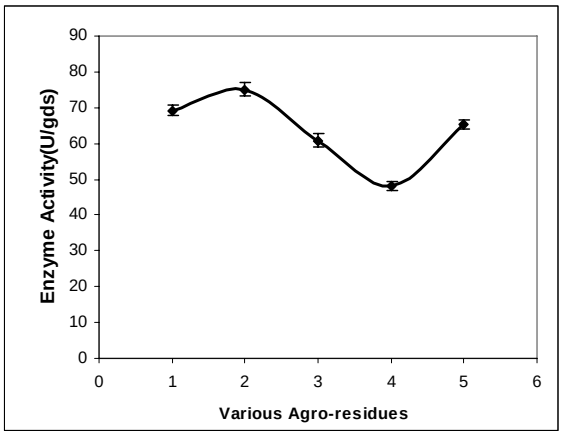

Figure 1 : Production of endoxylanase enzyme by *Bacillus* isolate under SSF on various agro-residues at 72h of incubation. Temperature 45^{0}C; pH 6.7. 1-WB; 2-CC; 3-WS; 4-RS; 5-SB.

3. Results and Discussion:

3.1. Effect of Different Agro Residues:

The effect of various substrates for xylanase production was examined with 10 g of each wheat bran, corn cob, wheat straw, rice straw, and sugarcane baggase in 250 ml Erlenmayer flasks with 20 ml of mineral salt solution. Cultivation was carried out at 450C for 96 h. As indicated in fig.1, corn cob (74.96±5.2 U/gds) was found to be the best substrate for endoxylanase production in the present study. The high level of xylanase production on xylan containg substrate (corn cob) suggest that xylan is necessary for effective induction of xylanase. Xylan may not be the direct inducer since it can not enter cells directly but its initial hydrolysis products like xylobiose, xylotriose, etc. are genrerated by constitutive xylanase action, which may act as inducers. Corn cob contained xylose (23%) and xylan (28 may fulfill the role of inducers, and use of corn cob for xylanase production under SSF by bacteria is very limited. Ninawe and Kuhad (2005) also reported wheat bran and corn cob as an enhancer for xylanase production by *Streptomyces cyancus* SN32. Consequently, corn cob was selected as the substrate for xylanase production in the present work.

3.2. Effect of Incubation Period, Media Ph, and Temperature:

Xylanase production by bacterial isolate under SSF showed that a low level of xylanase activity was detected in earlier stages of incubation and enzyme activity steadily reached a maximum level (74.96±5.2 U/gds) by 72h of incubation (Figure 2) there was a decrease in enzyme activity (64.54±4.8 U/gds) with further increase in incubation period. Similar findings have been reported with *B. lichenifoermis* where enzyme production reached a maximum level by 72 h in wheat bran medium (Archana and Satyanarayan, 1997). The reduction in xylanase yield after optimum period was probably due to the depletion of nutrient available to microorganism or due to proteolysis (Flores at. el., 1997).

effect of media pH on enzyme production is shown in Fig.3. The optimum pH for xylanase production was found to be 6.0. Each microorganism holds a pH range for its growth and activity with optimum value between around this range. Initial pH influences many enzymatic systems and the transport of several species of enzymes across the cell membrane (Poorna and Prema, 2006). The results of influence of temperature on xylanase production are shown in Figure 4, showing that the optimum temperature for xylanase production was 45^{o}C at 72h of incubation. Enzyme activity at 50^{0}C was also significant and comparable to that at 40^0 C till 72 h.

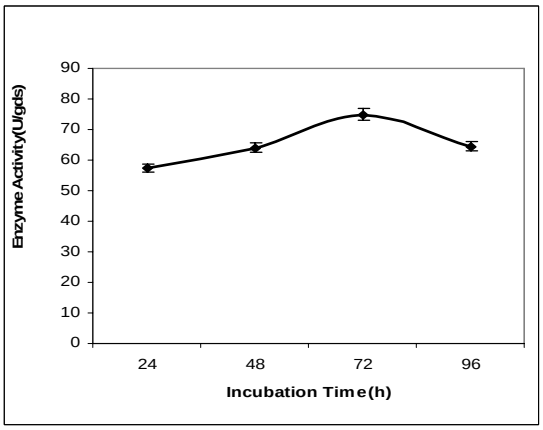

Figure 2. Effect of incubation time on endoxylanase and protein production by *Bacillus* isolate on corn cob as substrate in SSF. Temperature 45^{0}C; pH 6.7.

3.3. Effect of Particle Size:

Effect of particle size of corn cob on enzyme production is shown in Fig.5. Particle size of 500µm was found best for maximum xylanase production (74.96±5.2 U/gds). The results agree with Poorna and Prema, where 500 µm particle size gives maximum xylanase production with wheat bran.

3.4. Effect of Moisture Contents:

The moisture content in SSF is an important factor that determines success of the process. A moisture content higher than optimum moisture level causes decreased porosity of substrate, alternation in particle size, gummy texture, and lower oxygen transfer (RaimBault and Alazard, 1980 and Feniksova et al., 1960). A lower moisture level leads into a reduction in solubility of nutrients of solid substrate. And it leads to a lower degree of swelling and to a higher water tension (Ikasari and Mitchell, 1994). As indicated in fig.6, xylanase production was optimum (84.37±5.9 U/gds) when corn cob and moistening agent was 1:2.5.

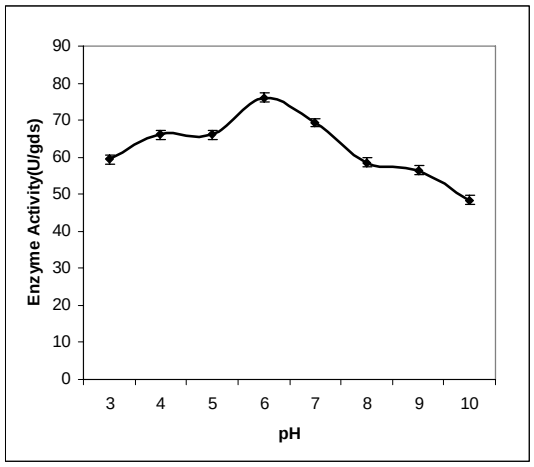

Figure-3. Effect of media pH on endoxylanase production by *Bacillus* isolate on corn cob as substrate in SSF.Temperature 45^0C.

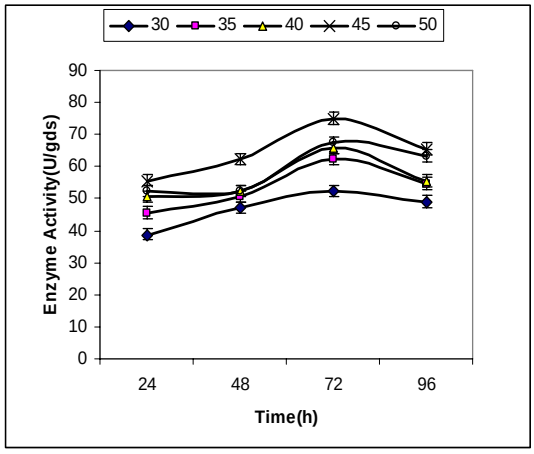

Figure-4. Effect of temperature on endoxylanase production by *Bacillus* isolate on corn cob as substrate in SSF at 72h of incubation. pH 6.7.

3.5. Scale-Up of Xylanase Production Under SSF:

Xyalnase production was enhanced by scaling up the solid state system by using enamel trays containg 80g corn cob. When SSF was performed in trays for xylanase production using bulk quantities of corn cob (80 g), xylanase production was higher if compared with Erlenmayer flasks. Cultivation in large enamel trays yielded 157.12 ±8.7 U/gds in static state and 111.47 ±8.1U/gds at 110 rpm when compared to the value obtained in 250 ml flasks (74.96 ±5.2 U/gds). The improvement of xylanase production in trays, more than flasks, may be due to efficient aeration, better mass, and heat transfer. A slight decrease (12.14%) in enzyme production by scaling up has been reported in *Bacillus megaterium* (Sindhu et al.,2006), while in *Bacillus licheniformis* (Archana and Satyanarayan, 1997), scaling up stimulate xylanase production. It may be possible to further improve the enzyme yield with higher quantities of the substrate - thus making corn cob a potential solid substrate for xylanase production.

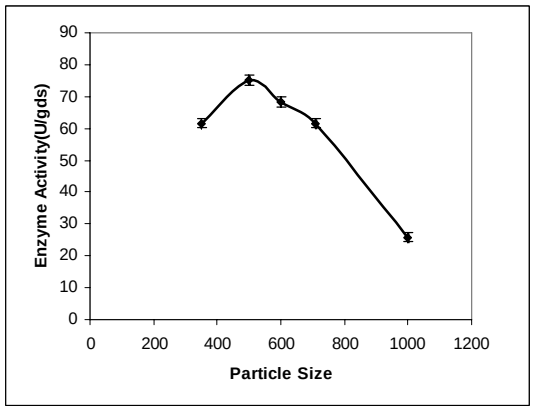

Figure-5. Effect of particle size on endoxylanase production by Bacillus isolate on corn cob as substrate in SSF at 45^0C. pH 6.7.

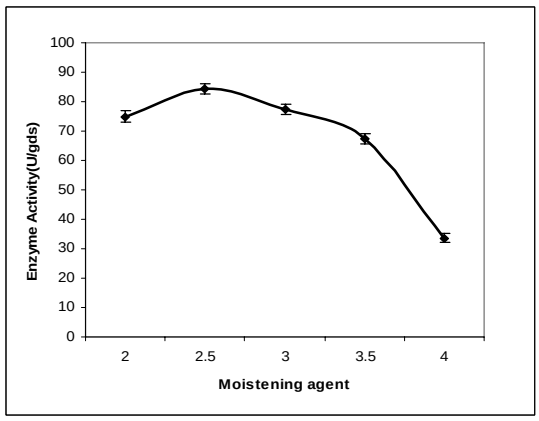

Figure-6. Effect of initial moisture level on endoxylanase production by *Bacillus* isolate on corn cob. Temperature 45^0C.

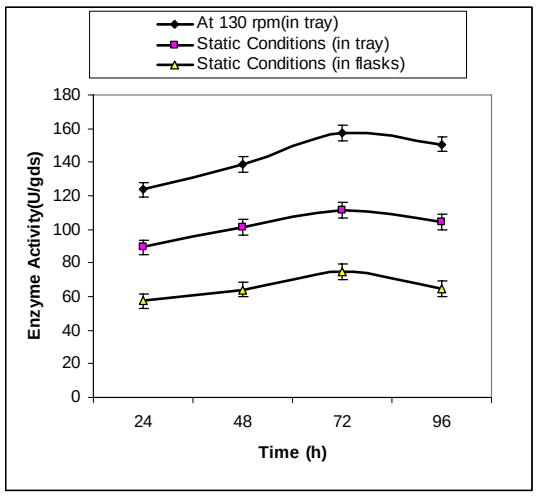

Figure-7. Scale up of xylanase production from bacterial isolate in large trays. Temperature 45^0C; pH 6.7.

3.6. Whole Cell Immobilization:

As indicated by fig.8, PUF was a superior support for immobilization if compared to SB. PUF was selected for further immobilization studies. The porous structure of foam allowed growth of the cells inside the pores, and a non-diffusion limited environment for substrate and

product. As indicated by Fig.9, it was observed that after immobilization of bacterial strain on PUF cubes, endoxylanase production was increased 156.05% in static state and 219.21% in submerged state compared to control value (free cells). Xylanase production by immobilized cells attained maximum level at V^{th} cycle in static state and at IV^{th} cycle in submerged state, After this xylanase production declined slowly up to ten cycle. Similar observations were made by Beg *et al.* (2000) in *Streptomyces Sp. QG-11-3* species, where immobilization on polyurethane foam (PUF) enhanced xylanase production by 2.5 fold. These results also indicated that this type of fiber materials has a significant role in providing a favorable environment for enzyme production. Increase in xylanase production, observed after immobilization, may be due to adherence of bacterial cells to the surface of PUF, as well as into the pores, thus increasing the residence time of cells in the medium.

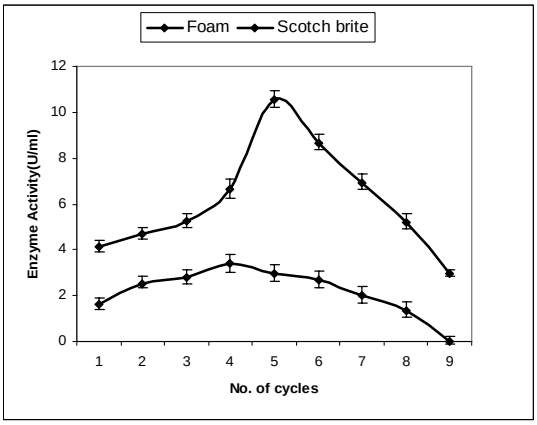

Figure-8. Xylanase production by the bacterial strain immobilized on polyurethane foam and scotch brite. Temperature 45^0C; Medium YEP+Xylose.

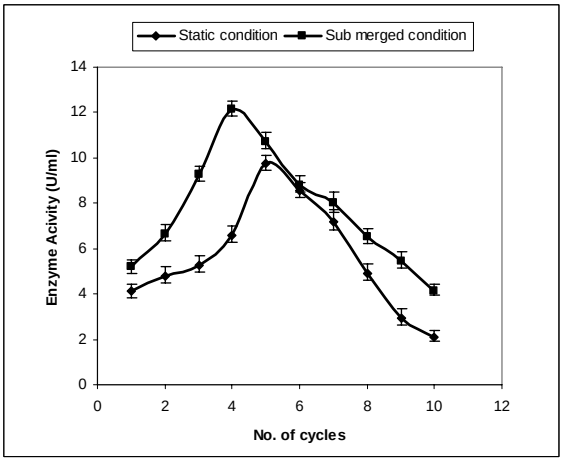

Figure-9. Xylanase production by immobilized cells under liquid surface and submerged conditions.

4. CONCLUSIONS:

Corn cob, an abundantly available agro-residue in India was successfully used as a solid state support by the *B.licheniformis* isolate at 45^0C for xylanase production, and it has not been reported so far.The results presented here show that optimization of process parameters and immobilization of the bacterial cells on PUF resulted in higher production of xylanase and could be continued by recycling the cells till 10 cycles. Scaling up under SSF also resulted in significant improvement in xylanase production.

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A Fast and Sensitive Molecular Detection of *Streptococcus mutans* and *Actinomyces viscosus* from Dental Plaques

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Abstract

Polymerase chain reaction was used in this study in comparison with conventional method for the detection of cariogenic bacteria (Streptococcus mutans and Actinomyces viscosus) in dental plaque samples taken from two different teeth sites, a crowned tooth (tooth with crown restoration) and a natural tooth. Nested Polymerase chain reaction (N- PCR) was performed on genomic DNA which was isolated directly from plaque samples and it revealed the presence of Streptococcus bacteria and Streptococcus mutans species using two sets of 16s rDNA specific primers. Actinomyces viscosus isolates were detected also by using conventional PCR. The plaque samples were recorded negative for the presence of cariogenic bacteria, depending on conventional microbiological methods. But the same samples were recorded positive for the presence of those bacteria depending on molecular approach. This finding demonstrates that the sensitivity and specifity of the PCR techniques in the detection of the cariogenic bacteria in plaque samples are higher than the conventional culture method.

الملخص

تمت في هذه الدراسة مقارنة تفاعل السلسلة المبلمرة مع الطرق التقليدية في زراعة البكتيريا في الكشف عن وجود البكتيريا المسببة للتسوس: الستربتوكوكس ميوتانس (Streptococcus mutans) والأكتينومايسس فيزكوسس Actinomyces viscosus. حيث تم الكشف عن هذه البكتيريا في عينات البلاك (الصفائح الجرثومية) المأخوذة من أسطح مختلفة من الأسنان: من على سطح تركيبة سنية (تاج) ومن سطح سن طبيعي. تم استخدام طريقة تفاعل السلسلة المبلمرة المتشابكة (N-PCR) للمادة الوراثية المعزولة مباشرة من عينات البلاك وأظهر التفاعل وجود بكتيريا الستربتوكوكس (Streptococcus) و الستربتوكوكس ميوتانس (Streptococcus mutans). باستخدام زوجين من البوادئ (primers) ، الزوج الأول مطابق للسلسلة النيوكلوتيدية لجين الحمض الرايبوسومي (rDNA) للجنس الستربتوكوكس والثاني مطابق لجين الحمض الرايبوسومي (rDNA) للنوع ستربتوكوكس ميوتانس (Streptococcus mutans). لقد تم الكشف أيضا عن بكتيريا الأكتينومايسس فيزكوكس Actinomyces viscosus باستخدام تفاعل السلسلة المبلمرة الإعتيادية. أظهرت الطرق الميكروبيولوجية التقليدية في زراعة البكتيريا عدم وجود البكتيريا بينما أظهرت نفس العينات وجود البكتيريا باستخدام الطرق الجزيئية (PCR). تبين هذه النتائج مدى حساسية و خصوصية وسرعة طرائق تفاعلات السلسة المبلمرة في الكشف عن وجود البكتيريا المسببة لتسوس الأسنان في عينات البلاك مقارنة بالطرق التقليدية.

Keywords: Streptococcus mutans. Actinomyces viscosus. Cariogenic bacteria. Nested PCR.

1. Introduction

The most cariogenic bacteria in dental plaque are *Streptococcus mutans* (Samaranayake et al., 1996; Rosan and Lamont, 2000; Hoshino et al., 2003). These bacteria have many properties which enable them to be potentially cariogenic (Melville and Russell, 1960). *Actinomyces* species were found to be associated with root caries, especially *Actinomyces viscosus* and *Actinomyces naslundii* (Bowden, 1989; Nyvad and Kilian, 1990).

Common methods of detection and characterization of pathogenic bacteria from the oral cavity are conventional especially culture methods, which are used to identify bacterial pathogens in dental plaque samples. Culture techniques are limited in their sensitivity and specificity, and they consume much time. In addition, it was found that 50% of oral micro-flora does not grow on culture media in the laboratory (Paster et al., 2001; Munson et al., 2004).

Molecular biology methods have been developed to overcome culture problems. Polymerase chain reaction (PCR) is now used in bacterial identification in environmental and clinical specimens. PCR methods are more sensitive and specific, and faster than conventional methods in bacterial determination. They allow the detection of viable and nonviable microorganisms, and consume less time and effort than conventional methods. A developed PCR method, which is called "Nested PCR' allows more sensitive detection of pathogenic bacteria. This method consists of first-step amplification with universal primers. The amplified products are used as

template in second-step amplification, where species specific primers are used (Sato et al., 2003).

This study is the first to be done in Jordan using molecular methods for the detection of the most cariogenic bacteria in the oral cavity obtained from dental plaque from different teeth sites.

The aim of this study is to detect the most cariogenic bacteria in plaque samples taken from different teeth sites by using polymerase chain reaction (PCR) method. PCR was performed for specific detection *Actinomyces viscosus*. Nested PCR was used to specifically detect *Streptococcus mutans species.* The approache of this study can be used in future research for detecting the effect of certain dental restorations, or materials, on the bacterial composition of dental plaque.

2. Materials and Methods

2.1. Subjects and Plaque Sampling

The study consisted of four plaque samples taken from one person who had a metal ceramic crown restoration and a natural tooth in the closet proximity to the crown site. The study protocol was approved by the Committee of Search on Human at Jordan University of Science and Technology. The subject was a patient of Dental Teaching Center.

Dental plaque samples were collected by using sterile curettes (Gracey Curettes). Supragingival plaque was taken first from the crown site, and then was taken from the subgingival plaque. The same procedure was applied to the plaque samples obtained from the natural tooth. The plaque samples were suspended in 1 ml of sterile Phosphate buffer saline PBS (0.12 M NaCl, 0.01 M Na_2HPO_4, 5mM KH_2PO_4 [pH 7.5]). The samples were transported on icebox to the laboratory.

2.2. Detection of Cariogenic Bacteria by Cultivation Methods

Plaque samples were dispersed by vortexing for 30s with glass beads (diameter 4 mm), and samples were diluted into different decimal serial dilutions in phosphate buffer saline (pH 7.5). The appropriate dilutions of each sample was plated on the following selective media: Mitis salivarius agar (MSA) supplemented with 0.2 U/ml bacitracin and 5% sucrose and Cadmium Flouride Acriflavine Tellurite (CFAT) medium supplemented with 5% human blood for the culture of *Streptococcus mutans* and *Actinomyces species* respectively. The plates were incubated at 37 °C for three days in an anaerobic jar with CO_2 gas generating kit. To confirm the presence of *Streptococcus mutan*s, the bacterial isolates grown on MSBS were subjected to certain biochemical tests (Sneath et al., 1986). For the identification of the bacterial isolates, which were grown on CFAT media, the RapID ANA II kit (Remel Compny. USA) biochemical kit was used according to the manufacture's instructions.

2.3. Extraction of The Genomic DNA from Streptococcus Isolates

Extraction of genomic DNA from bacterial colonies (local isolates in our laboratory), grown on MSA supplemented with bacitracin and sucrose was done as following: one colony from each pure bacterial isolate was inoculated into 10 ml of Trypticase soya broth and incubated for 24 hours at 37°C. 1 ml from each of the trypticase broth of the 24 hours broth was transferred to a new sterile eppendorf tube and the bacterial cells were harvested by centrifugation at 8000 xg for 15 minutes. The resulted pellet was digested by addition of 500 µl of lysis buffer (50 mM tris buffer, 1 mM EDTA, 0.5% Tween 20, and proteinase K (200µg/ml). pH 8) and then incubation at 55°C for 2 hours. Heating at 90 °C for 5 minutes was done for the inactivation of proteinase K. The DNA was precipitated by addition of an equal volume of cold isopropanol and incubated in freezer for 20 minutes. The pellet was washed with 70% ethanol and then rehydrated by addition of 35-50µl TE buffer (10 mM Tris- HCl, and 1 mM EDTA) (pH 8). This DNA was subjected to N-PCR reaction for amplification of 16s rDNA specific to the genus Streptococcus and species Streptococcus mutans.

2.4. Extraction of Total Genomic DNA Directly from Plaque Samples.

The total genomic DNA was isolated from plaque samples according to Paster *et. al.* (2001) with few modifications as follows: 100 µl of plaque sample was transferred to a new sterile eppendorf tube where 100 µl of the lysis buffer was added (50 mM tris buffer, 1 mM EDTA, 0.5% Tween 20, and proteinase K (200µg/ml). pH 8), then incubation at 55-60 ºC was done for 1.5 hour. Inactivation of proteinase K was done at 90 ºC for 5 minutes. After that, samples were cooled on ice for few minutes and the DNA was precipitated by the addition of an equal volume of ice-cold isopropanol and incubated at refrigerator overnight. The DNA pellet was washed with 70% ethanol, and was then rehydrated in 20- 30 µl TE buffer (10 mM Tris- HCl, and 1 mM EDTA) (pH 8).

2.5. Polymerase Chain Reaction (PCR)

Genomic DNA of 4 plaque samples that belong to one person was subjected to PCR reactions in order to detect the presence of *Actinomyces viscosus*. A Nested PCR (N-PCR) reaction was performed to detect the presence of mutans Streptococci. The first (N- PCR) was conducted to detect genus *Streptococci*, and the second (N- PCR) detected *Streptococcus mutans*.

For each PCR reaction, a negative control reaction was performed where no DNA template was added. All PCR reactions were performed in a Perkin Elmer DNA thermal cycler (Perkin Elmer 480). All PCR products were kept at 4 ºC until analyzed.

2.6. Detection of The Presence of Streptococcus Mutans Using Nested PCR Polymerase Chain Reaction, using primer pair specific to 16S rDNA specific to Streptococcus, was performed according to Sato et al. (2003) to detect Streptococcus. The target sequence of 16S rDNA was amplified by using PCR mixture (total volume 25 µl) containing 3mM $MgCl_2$, 0.4 mM dNTPs, 5U of Taq DNA polymerase, 1µl of each primer (5uM) (Table 1), 2.5 µl of 10x PCR buffer, and 1 µl of template DNA (20 ng). The PCR program consisted of initial denaturation at 95 ºC for 15 min, and 35 cycles involving denaturation at 94 ºC for 1 min, and annealing at 55 ºC for 1 min, and

extension at 72 ºC for 1.5 min followed by a final extension at 72 ºC for 10 min. All reaction mixtures were held at 4°C.

Table 1: Primers that were used for the amplification of oral bacteria

Primer set		Sequence 5'→3'	bp[a]	Reference
Streptococci species	8UA (F)	AGA GTT TGA TCM TGG CTC AG	1505	Sato et al. 2003
	1492 (R)	TAC GGY TAC CTT GTT ACG ACT T		
Streptococcus mutans	Sm1 (F)	GGTCAGGAAAG TCTGGAGTAAA AGGCT A	282	
	Sm2 (R)	GCG GTA GCT CCG GCA CTA		
Actinomyces viscosuss	A.vis (F)	ATG TGG GTC TGA CCT GCT	96	Suzuki et al. 2004
	A.vis (R)	CAA AGT CGA TCA CGC TCC G		

[a]: Size of the amplified product bp

The second nested PCR reaction was done for the detection of *Streptococcus mutans* by using species specific primers based on the 16S rDNA, Sato. et al. (2003). Briefly: The target sequence of 16S rDNA was amplified by using PCR mixture (total volume 25 µl) containing: 3mM $MgCl_2$, 0.4 mM dNTPs, 5U of Taq DNA polymerase, 1µl of each primer (5µM) (Table 1), 2.5 µl of 10x PCR buffer, and 1 µl of template DNA (20 ng). The PCR program is isentical to the one used in the detection of *Streptococcus* genus in the first reaction.

2.7. PCR Detection of The Presence of Actinomyces Viscosus by Using Primer Pair Specific to The Species

Polymerase Chain Reaction for *Actinomyces viscosus* using species specific primers to each species was performed according to Suzuki et al. (2004). The target sequence of 16S rDNA was amplified using PCR mixture (total volume 25 µl) containing: 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 5U of Taq DNA polymerase, and each of the primer added at a concentration of 0.2µM (Table 1), 2.5 µl of 10x PCR buffer, and 1 µl of template DNA (20 ng). The PCR program had initial denaturation at 95 ºC for 15 min, and 35 cycles involving denaturation at 94 ºC for 1 min, and annealing at 55 ºC for 1 min, with extension at 72 ºC for 1.5 min followed by a final extension at 72 ºC for 10 min. All PCR products were held at 4°C.

2.8. Gel Electrophoresis and Photography

The PCR amplified products were separated in ultra-pure agarose gels dissolved in 1X TBE buffer (0.89 M Tris Base, 0.89 M Boric Acid, 20 mM EDTA) (pH 8.3). 1.5 % w/v gel was used for separation of amplified PCR products for *Streptococcus* detection. And 3% w/v gel was used for the separation of PCR bands of *Actinomyces* amplification products. 5 µl of PCR product was mixed with 2 µl of 6x loading dye and then loaded into the well of the gel. 1 Kilo base pair (Kbp) and 100 base pair (bp) markers were included in the gel. PCR products were separated through the gel at an electric current of 90 V for 1 hour by using horizontal gel electrophoresis apparatus (Sigma Chemicals Co. USA). Gels were stained with ethidium bromide (0.5µg/ml) and visualized on a UV transilluminator by using BioDocAnalyze (Biometra, Germany).

3. Results

3.1. Detection of Cariogenic Bacteria by Cultivation Methods

All cultivated plaque samples were recorded 'negative' (no bacterial growth) regarding the detection of Streptococcus mutans and Actinomyces species.

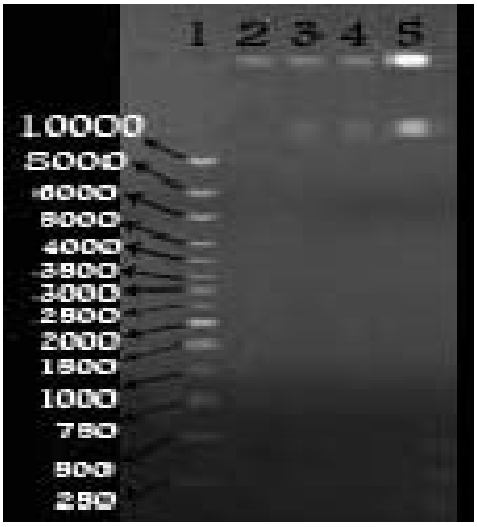

Figure 1: Agarose gel electrophoresis of the total genomic DNA isolated from four plaque samples (Lane 2 to Lane 5). Lane 1; molecular weight marker (1K bp ladder). Lane 2; the supragingival plaque of natural site, Lane 3; the supgingival plaque of the natural site, Lane 4; the supragingival plaque from crown site, Lane 5; the subgingival plaque from the crown site.

3.2. Total Genomic DNA Extraction Directly from Plaque Samples

The total Genomic DNA was isolated as described previously in materials and methods from four plaque samples (Figure 1), and revealed good quality and quantity of genomic DNA.

3.3. Detection of the presence of Streptococci mutans using Nested PCR

Total genomic DNA, which was isolated directly from plaque samples, was subjected to N-PCR reactions. The detection of the genus *Streptococci* was performed by using primer pair specific to 16s rDNA of the genus *Streptococci*. The positive amplified PCR product (1505 bp size), representing *Streptococcus* genus, was detected in all the 4 samples (Fig. 2). In the second reaction, the detection of the *Streptococci mutans* was done by using 16s rDNA primer pair specific to the species *Streptococcus mutans*. The species *Streptococcus mutans* was detected in all, four, samples. (Fig. 3) represents the presence of the amplified PCR product (282 bp).

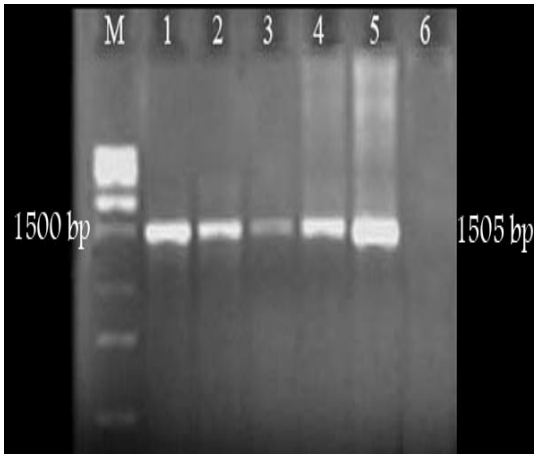

Figure 2: Agarose gel electrophoresis of PCR amplified products for 16s rDNA specific to Streptococcus genus for four plaque samples. The amplified band is 1505 bp. Lane M; molecular weight marker (1K bp ladder). Lane 1: positive control (PCR amplified product from genomic DNA isolated from the bacterial species Streptococcus mutans), (Lanes 2 and 3: represents plaques from natural tooth, (Lanes 4 and 5): represents plaques from crown tooth. (Lanes 2 and 4): represents the supragingival plaque, (Lanes 3 and 5): represents the subgingival plaque. Lane 6: negative control.

3.4. PCR detection of the presence of Actinomyces viscosus by using primer pair specific to these species

PCR detection of the presence of species *Actinomyces viscosus* using primer pair specific for this species (Table 1) resulted in an amplified product of 96 bp which was detected in all four samples (Fig. 4). All these samples were recorded negative of *Actinomyces* species by using the cultivation on CFAT medium.

4. Discussion

In this study four plaque samples, which belong to one person, were included in the study. The plaque samples, which were obtained from crown site, have zero count of *Streptococcus mutans* and of *Actinomyces viscosus*. However, PCR methodology was able to positively detect *Streptococcus mutans* and *Actinomyces viscosus* in all these samples. Wade (2002) indicated that many bacteria escaped the conventional culture techniques for detection either because they are unproductive, or because there are not distinguishable from similar species by observable phenotypic characteristics. In this study, *Streptococcus mutans* and *Actinomyces viscosus* were not detected in previous samples based on the conventional culture method. This could be attributable to their presence in low proportions in the samples, and/or the culture medium was not sensitive enough for the detection of the low levels of those bacterial species. This confirms and proves that PCR is more sensitive than conventional culture method.

The results of this study confirm the disadvantages of conventional method such as poor specificity and sensitivity; detection of only viable culturable bacteria - and that they are time consuming and laborious.

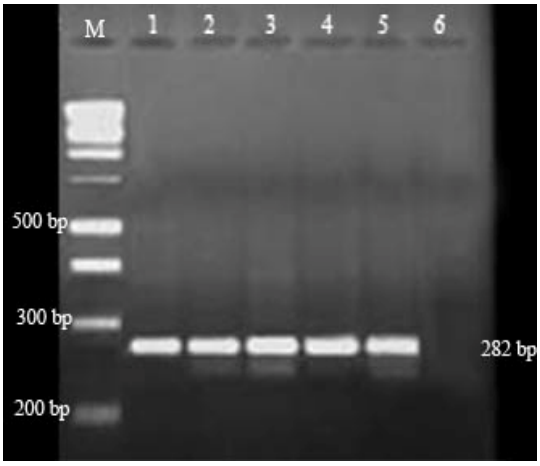

Figure 3: PCR detection of the species *Streptococcus mutans* in four plaque samples. Lane M; molecular weight marker (100 bp ladder), Lane 1: positive control, (Lanes 2 and 3): represents plaque samples from natural tooth, (Lanes 4 and 5): represents plaque samples from crown tooth, (Lanes 2 and 4): represents supragingival plaque samples, (Lanes 3 and 5): represents subgingival plaque samples. The size of amplified product is 282 bp. Lane 6: negative control.

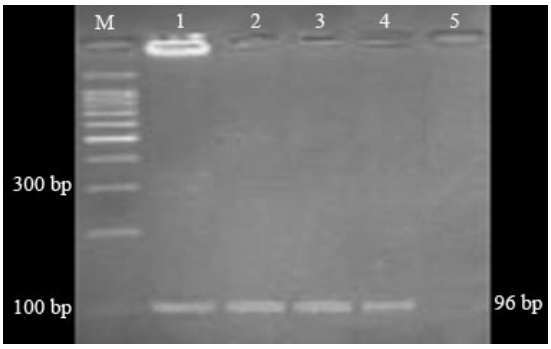

Figure 4: PCR detection of the species *Actinomyces viscosus* in four plaque samples. Lane M; molecular weight marker (100 bp ladder), (Lanes 1 and 2): represents plaque samples from natural tooth, (Lanes 3 and 4): represents plaque samples from crown tooth, (Lanes 1 and 3): represents supragingival plaque samples, (Lanes 2 and 4): represents subgingival plaque samples. The size of amplified product is 282 bp. Lane 5: negative control.

On the other hand, PCR methodology provides a more sensitive mean of detection of putative bacterial species even non-culturable bacteria if compared with conventional culture techniques. Also it is able to detect low numbers of bacterial species, being quick and relatively simple to perform. Moreover, a PCR assay has been found to be suitable for the specific detection and identification of human cariogenic bacteria like *Streptococcus mutans* (Sato et al., 2003). This study detected both *Streptococcus mutans* and *Actinomyces* species to be the most serious human cariogenic bacteria.

This research provides protocols that are potentially considered a cornerstone of the oral microbiological research that can be conducted in order to investigate the composition of dental plaque with many stressful factors, for instance, presence of dental materials.

5. Conclusion

With the methods of this study, the following conclusions could be drawn: PCR molecular approach was very sensitive, and it was specific and rapid in detecting and identifying the presences of cariogenic bacteria if compared to conventional culture approach. So it is recommended, for further research, to utilize PCR technique as a sensitive and specific method for the detection and identification of the human cariogenic bacteria.

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