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Introduction Prophylactic role of anti-fimbriae type 3 from biofilm formation by *Klebsiella pneumonia*.

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Abstract

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One hundred and thirty specimens of urine and sputum were collected for the period from September 2010 to February 2011 in sterilized containers from patients attending four hospitals in Baghdad including: Educational Al-yarmook Hospital, Educational Laboratories, Educational Baghdad Hospital, and Educational Ibn Al-Nafees Hospital.

All specimens were identified using biochemical tests and API 20 E system, the genus *Klebsiella* Formed 22.3% (29 isolates) in which 25 isolates 86.2 % were identified as *Klebsiella pneumoniae*.

The ability of *K. pneumoniae* isolates to produce biofilm were evaluated using crystal violet staining technique in polystyrene microtiter plates and then O.D. was determined at 540 nm. Isolate: K21 which isolated from urine produced the thickest biofilm (O.D = 1.987) , type 3 fimbriae expression by *K. pneumoniae* K21 isolate was detected by mannose-resistant hemagglutination (MRHA) of tanned human blood group (A) and Ox RBCs in the presence of 2% D-mannose, type 3 fimbriae was extracted by heating and mechanical shearing and partially purified by Ultracentrifugation (48,000xg for 3hrs), Anti-type 3 fimbriae antisera was prepared in rabbits.

The microtiter plate method was applied to estimate the role of the prepared anti-type 3 fimbriae antibodies in reducing the biofilm formation by *K. pneumoniae.* Prevention of bacterial adherence and subsequent biofilm formation to polystyrene microtiter plate was studied by employing different dilutions (1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640) of rabbit sera containing anti-type 3 fimbriae antibodies. The maximum inhibition of biofilm formation in terms of optical density (540 nm) and viable bacterial count was found in lowest dilution (1/10) (highest concentration of Abs) . However, the minimum inhibition was observed in highest dilution of rabbit antisera (1/640) (lowest concentration of Abs).

الخلاصه:

جمعت 130 عينة ادرار و قشع للمدة من ايلول 2010 الى شباط 2011 في اوعية معقمة من مرضى زائرين لاربع مستشفيات في مدينة بغداد وشملت مستشفى اليرموك التعليمي, المختبرات التعليمية ,مستشفى بغداد التعليمي و مستشفى ابن النفيس التعليمي لامراض القلب.

شخصت العينات باستخدام الفحوص البايوكيميائية ونظام Api 20 E تعود لجنس 22.3% (29 عزلة) , وجد ان *Klebsiella .K.pneumoniae*, منها 25 عزلة 19.23% شخصت

على انتاج الغشاء الحياتي باستعمال تقنية التصبيغ بالبنفسج البلوري في اطباق *K.pneumoniae* قدرت قابلية عزلات المعايرة الدقيقة وحددت قيمة الكثافة الضوئية عند الطول الموجي 540 نانوميتر والتي مثلت درجة سمك الغشاء الحياتي . وجد ان 52% من العزلات عالية الانتاجية في حين كانت 28% من العزلات ذات انتاجية جيدة و20% انتاجيتها ضعيفة . ومن الجدير بالذكر ان العزلة K211.987 المعزولة من الادرار اظهرت اعلى انتاجية للغشاء الحياتي

بوساطة اختبار التلازن الدموي *K.pneumoniae* K21 بكتريا تم التحري عن التعبير عن خمل النمط الثالث من قبل المقاوم لسكر المانوز لكريات الدم الحمرالمدبغة للأنسان و الثور بوجود سكر المانوز. اظهرت العزلة المنتخبة تلازن دموي مقاوم للمانوز قوي و مميز لخمل النمط الثالث عند الفحص تحت المجهر وباستخدام طريقة رج البلاطة وطريقة التسوية للتحري عن التلازن, وقد وجد ان التعبير عن الخمل يستحث باجراء زرع متكرر للعزلة المنتخبة 3-4 مرات كل 48 ساعة وحضن بدرجة 37 درجة مئوية في وسط مرق اللوريا في ظروف هوائية طبيعية وبدون حركة.

ذات الغشاء الحياتي الاسمك والتي اظهرت تلازن دموي قوي ومميز من *K.pneumoniae* (K21) تم اختيار العزلة بين جميع العزلات كمصدر لاستخلاص بروتين الخمل من النمط الثالث

تم استخلاص خمل النمط الثالث بالمعاملة الحرارية والقوة الميكانيكية وتمت تنقيته جزئيا باستخدام النبذ المركزي الفائق ثم لغرض التحري عن النقاوة تم تحضير المصل المضاد لخمل النمط الثالث في SDS ترحيله كهربائيا بوجود المادة الماسخة الحيوانات المختبرية (الارانب) وتم التحري عن خصوصية وعيارية اضداد خمل النمط الثالث بواسطة التلازن البكتيري حيث لازن المصل المضاد الخلايا البكتيرية عند التخفيف 80/1 .

استخدمت طريقة اطباق المعايرة الدقيقة للتحري عن دور اضداد خمل النمط الثالث المحضرة لاختزال قدرة بكتريا على تكوين الغشاء الحياتي . حيث تمت دراسة منع الالتصاق البكتيري وتكون الغشاء الحياتي التابع لذلك *K.pneumoniae* باستخدام عدة تخافيف من المصل المضاد للخمل (10/1, 20/1, 40/1, 80/1, 160/1, 320/1, 640/1) . وقد اظهرت نتائج استخدام عامل الكثافة الضوئية عند الطول الموجي 540 نانوميتر والعد الحي للخلايا ان اعلى تثبيط للغشاء الحياتي وجد عند اقل تخفيف من المصل المضاد 10/1 (اعلى تركيز لاضداد الخمل) في حين ظهراقل تثبيط للغشاء الحياتي عند التخفيف الاعلى للمصل 640/1 (اقل تركيز لاضداد الخمل).

Introduction

Most bacteria live as complex communities adhered to surfaces, rather than as planktonic isolated cells . These compact communities, referred to as biofilms, are commonly associated with many health problems.[1,2] It is estimated that biofilms contribute to more than 80% of human infections.[2] Biofilm-linked infections are particularly problematic, because biofilm associated bacteria can withstand host immune defenses, antibiotics and hydrodynamic shear forces far better than the corresponding planktonic bacteria. These characteristics made biofilm-associated infections particularly resistant toward treatment and it is a common and frustrating experience that after treatment surviving biofilm associated bacteria will carry on the infections. [3] The role of biofilm formation and development by bacteria has been suggested to be an important stage in the pathogenesis of different organisms such as *Klebsiella*.[2]

*K. pneumonia*e is widely distributed in the gastrointestinal, urinary, and respiratory tracts of healthy people and is one of the most important pathogens causing nosocomial infection.[4] It causes opportunistic infections, such as pneumonia , sepsis, inflammation of the urinary tract, and wound infection, in compromised patients.[5] Biofilm-associated *K. pneumoniae* have been shown to be associated with several human diseases [6], and to colonize a wide variety of medical devices.[7] Several studies have clearly demonstrated that the *K. pneumoniae* type 3 fimbriae (a hair-like appendage expressed on bacterial surfaces), play an important role in mediating the adhesion process, have a significant role in *K. pneumoniae* biofilm formation , they found that type 3 fimbriae, but not type 1 fimbriae, strongly promote biofilm formation in *K. pneumoniae*. [8,9]

Since type 3 fimbriae mediate biofilm formation, it is altogether plausible that type 3 fimbriae play a significant role in biofilm-associated infections, in particular the nosocomial infections with *K. pneumoniae*.[10] Blocking the early stages of biofilm formation, namely bacterial adherence to biotic and a biotic surfaces, may considered an effective strategy to prevent bacterial biofilm.[11] Preventing bacterial adhesion can be addressed in a variety of ways, such as blocking adhesion using adhesion-based vaccines which could be achieved either by active or passive means. [12] This mean, the anti -type 3 fimbriae antibodies could be used as a preventive strategy to block the onset of pathogenic biofilm growth on biotic and a biotic surfaces, hence we carried out this study to investigate the role of the prepared anti-type 3 fimbriae antibodies in blocking the adherence of type 3 fimbriae in *vitro* and find out it's affect on biofilm formation for *K. pneumonia*.

Materials and methods

1- Specimens collection, Isolation and identification of Klebsiella isolates:

From September 2010 to February 2011, One hundred and thirty specimens (urine and sputum) were collected in sterilized containers from patients in four hospitals including:

Educational Baghdad Hospital, Educational Al-yarmook Hospital, Ibn-Al Naffees Teaching Hospital and Educational Laboratories.

The collected specimens were streaked directly on MacConky agar, incubated at 37ºC for 24 hrs, the large, pink and mucoid colonies were selected and sub cultured on another MacConky agar to obtain isolated colonies, Morphological characteristics and biochemical tests were carried out depend on Bergey's Manual of Systematic Bacteriology, 2nd edition. (13)

2- Biofilm assay:

Method described by Maldonado *et al.* (14) was followed:

Bacterial isolates were cultured in lactose broth (LB) and incubated at 37ºC for 18 hrs, after that bacterial culture was diluted in LB medium with phosphate buffer saline (PBS) and adjusted in comparison to MacFarland tube 0.5, (200) µl of this bacterial culture were used to inoculate pre-sterilized 96-wells of polystyrene microtiter plates and later incubated for 24 hrs at 37ºC. After incubation, all wells were washed (2-3) times with PBS for elimination of unattached cells.

Afterward, (200) µl of 0.1% crystal violet was added to each well, shaking the plates three times to help the colorant to get the bottom of the well. After 10 minutes at room temperature, each well was washed with (200) µl sterile (PBS) to remove the planktonic cells and stain which not adhered to the well. Only the adhered bacteria forming the biofilm were kept on the surface of the well. The Crystal violet bound to the biofilm was extracted later with (200) µl of ethyl alcohol, and then absorbance was determined at 540 nm in an ELISA reader for determination of the degree of biofilm formation (this reading designated as O.D1). Controls were performed with Crystal Violet binding to the wells exposed only to the culture medium without bacteria. The control reading referred as to O.Dc. All assays were performed in triplicates. The biofilm degree was calculated as follows:

Biofilm O.D = O.D1 – O.Dc

The data obtained were used to classify the strains as high producers (O.D higher than 0.500), good producers (O.D between 0.500 and 0.100) or poor producers (O.D lower than 0.100) (14)

3- Mannose resistant hemagglutination activity(MRHA) test:

This test was done for *K. pneumoniae* isolates which considered as high biofilm producers in order to determine the presence of type 3 fimbriae.(10 and 15)

4- Extraction and partial purification of type 3 fimbriae:

This procedure was done according to. [15]

5- Antisera preparation:

Anti- type 3 fimbriae antisera were prepared according to.[16] Four wild type males rabbits (weight 2-2.5 Kg) were used, the rabbits were divided into two groups each included two rabbits:

- **Group1:** injected with PBS pH 7.2 (used as control).
- **Group2:** injected with partially purified type 3 fimbriae of *K. pneumoniae*.

Tow hundred µg of antigenic protein / Kg of body weight in 1ml of PBS pH 7.2 was emulsified with an equal volume of Freund's complete adjuvant (sigma). Each rabbit was injected with 2ml of suspension 0.5ml amounts into four sites, namely subscapularly, subcutaneous at two sites and intramuscularly into the two hind legs. After four weeks, a booster injection 200 µg protein / kg body weight with Freund's incomplete adjuvant (sigma) was given. One week after the second injection, the animals were bled by cardiac puncture, and the serum was obtained by centrifugation at 2000 xg for 10 min, heated at 56°C for 30 min to inactivate the complement , and stored at -20°C.

6- Role of anti-type 3 fimbriae antisera in the prevention of biofilm formation by *K. pneumoniae in vitro*

Serial dilutions of serum containing anti-type 3 fimbriae antibodies with PBS (1/10, 1/20 ,1/40, 1/80, 1/160, 1/320, 1/640) were investigated to evaluate their ability to prevent biofilm formation by *K. pneumoniae*. Same protocol previously was followed to produce a biofilm but with little modification , one hundred microliters of each dilution of serum were added to wells containing one hundred microliters of LB broth containing *K. pneumoniae* then the plates were incubated for 24 hours at 37^{o}C. The bacterial biofilm was evaluated by using Crystal Violet. The O.D reading designated as O.D1. *K. pneumoniae* biofilm with sterile PBS without serum was included as a positive control , its OD represented the reading before treatment. One hundred microliters of sterile LB broth plus 100 µl of sterile PBS without serum represented the negative control (its reading considered as ODc). All assays were performed in triplicates and the biofilm degree was calculated as follows

Biofilm O.D = O.D1 – O.Dc

A viable count was performed depending on the procedure described by [17] to determine the viability of bacterial cells within the biofilm, briefly:

- Nine test tubes filled with 5 ml of normal saline or phosphate buffer saline and the bottom of 9 petri plates with the following dilutions: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} were labeled.

- By using aseptic technique50 µl from normal saline or PBS from each tube was transferred and throws away.

- The initial dilution was made by transferring 50 µl of liquid sample (BHIb+Bacteria) from the well of 96 polystyrene microtiter plate, to a 4.95 ml sterile saline blank . This was 1/100 or 10^{-2} dilution, this tube was capped.

- The 10^{-2} blank tube was then shaken vigorously 25 times by placing one's elbow on the bench and moving the forearm rapidly in an arc from the bench surface and back. This serves to distribute the bacteria and break up any clumps of bacteria that may be present.

- Immediately after the 10^{-2} blank has been shaken, the tube was uncapped and aseptically 50 µl was transferred to a second 4.95 ml saline blank. Since this is a 10^{-2} dilution, this second blank was represented a 10^{-3} dilution of the original sample.

- The 10^{-3} blank tube was shaked vigorously 25 times and 50 µl was transferred to the third 4.95 ml blank. This third blank represents a 10^{-4} dilution of the original sample. This tube was also capped and this process was repeated until reaching to tube with a 10^{-9} dilution.

- After that, 10^{-9} blank was shacked again and aseptically 50 µl ml was transferred and throws away.

- From each tube which contain different dilution 100 µl was transferred to each MacConky agar medium for the exact dilution and spreaded.

- All plates were cultured, inverted and incubated at $37C^{o}$ for 24 hrs .

- After incubation period the number of bacteria (CFU) per milliliter was calculated by dividing the number of colonies by the dilution factor.

Results and discussion:

From a total of one hundred and thirty specimens (urine and sputum) collected from patients, the genus *Klebsiella* formed 22.3% (29 isolates) in which (25 isolates) 86.2% were identified as *K. pneumonia* , Gram stain showed that they all were red single, double or short chain rods and also it showed a distinct capsule when they examined under oil immersion after capsule staining. Colonies reacted negatively with gram stain, cultured on MacConkey agar showed large, round, pink, positive and biochemical test table (1). mucoid and lactose fermenting colonies. all isolates were oxidase negative and catalase

Table (1): Biochemical test results for *K. pneumoniae*

Id	Biochemical tests	Results
1	Gram stain	–
2	Capsule stain	+
3	Citrate utilization	+
4	Catalase production	+
5	Growth at 10 C^{o}	–
6	Lactose fermentation	+
7	Indole production	–
8	VogasProskauer test	+
9	Methyl red	–
10	Motility	–
11	Oxidase production	–
12	String test	(+ approximately7cm)
13	Kliglar iron agar (KIA)	A/A, with gas , No H_2S
14	Urease production	+

Biofilm assay:

All *K. pneumoniae* isolates assayed for the production of biofilm, and the results obtained are presented in figure (1) and table (2). the results indicated that each isolate showed a different potential to form biofilm under the same conditions of experimentation. (52%) of the tested isolates were high producers while (28%) of isolates were good producers and (20%) were poor producers, moreover *K. pneumoniae* isolate (K21) which isolated from urine produced the thickest biofilm with O.D (1.987).

The differences in biofilm thickness resulted from different reasons such as differences in isolates capacity to form biofilm Perhaps the primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (quorum sensing signaling molecules) that produced from each isolate play an essential as well as important role. [18]

A local study done by (19) *K. pneumoniae* biofilm was evaluated using the same method , this study found that (39.29%) of isolates were considered as high biofilm producers while (60.71%) of isolates were good producers and none of the tested isolates were poor producers and the highest biofilm thickness was (1.698) . Another local study(7) mentioned that all *K. pneumonia* isolates were producing biofilm but with differences in thickness degree and absobance values ranged between (0.111- 2.899).

investigated the capability of different *Klebsiella* strains from clinical source to produce biofilm, found that each strain showed a different potential to form biofilm under the same conditions of experimentation and the highest biofilm strains were *K. pneumoniae subsp pneumoniae* isolated from an urinary catheter (20) . The strains able to form a good biofilm were considered those that produced O.D above 0.500 , being five different strains belonging to different species: *Klebsiella planticola* (one strain) and *K. pneumoniae subsp pneumoniae* (four strains). The strains able to produced a medium degree of biofilm were nine strains of *Klebsiella pneumoniae* and two *Klebsiella oxytoca*, and those classified as poor forming biofilm were three *Klebsiella pneumoniae* strains.

Table (2): Absorbance O.D for *K. pneumoniae* biofilm at 540 nm and statistical analysis

Isolate number	Absorbance ± SD*
K1	1.240 ± 0.194
K2	0.088 ± 0.024
K3	0.083 ± 0.021
K4	0.263 ± 0.074
K5	0.072 ± 0.008
K6	0.879 ± 0.206
K7	0.950 ± 0.316
K8	0.088 ± 0.024
K9	0.155 ± 0.053
K10	1.697 ± 0.144
K11	1.272 ± 0.241
K12	0.246 ± 0.063
K13	0.222 ± 0.071
K14	0.197 ± 0.029
K15	1.863 ± 0.027
K16	0.691 ± 0.064
K17	0.130 ± 0.021
K18	0.976 ± 0.355
K19	0.078 ± 0.012
K20	0.128 ± 0.027
K21	1.987 ± 0.052
K22	0.602 ± 0.133
K23	1.303 ± 0.190
K24	1.513 ± 0.206
K25	1.490 ± 0.188

Each datum is a mean of triplicate.

SD* = standard deviation.

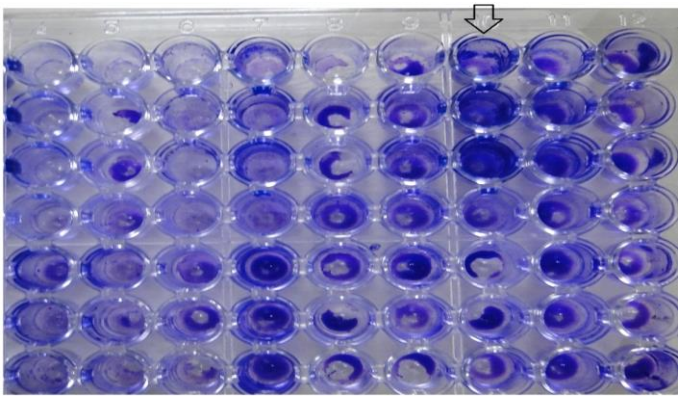

Figure (1) : Biofilm formation in microtiter plate by *K. pneumoniae* isolates (*K* 21 isolate pointed with an arrow).

The results showed that all the tested isolates (isolates with high capacity to produce biofilm) agglutinated the tannic acid treated ox RBCs and human group (A) RBCs in mannose resistant manner but with slight differences in the hemagglutination power ,the K21 isolate gave a strong MRHA activity which observed under microscope (40x) and in Rocked porcelain tiles (Rocked tile method) as shown in figure (2), the agglutination in the rocked tile method requires a strong enough adhesiveness of the bacteria for the red cells to withstand the shearing movements of the continuous mixing [20].

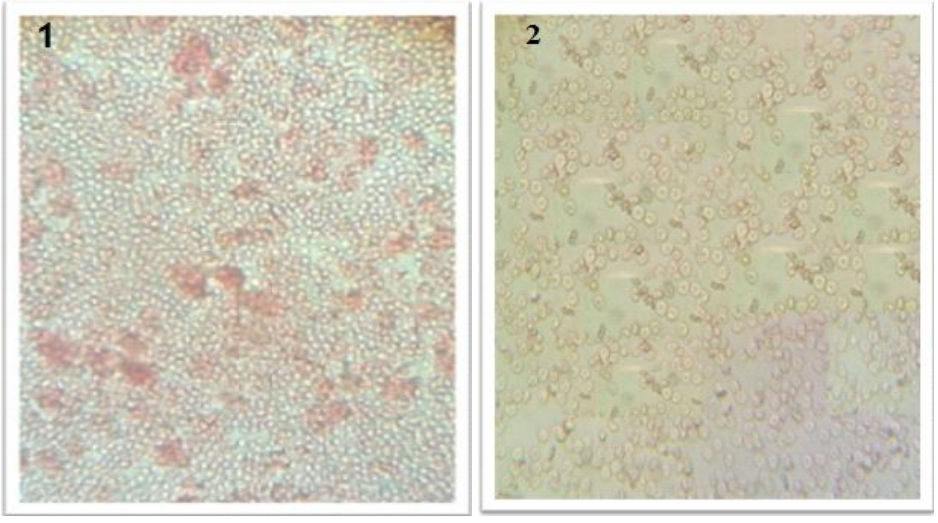

Figure (2) : Hemagglutination under microscope (40 x) to detect type 3 fimbriae expression by *K. pneumoniae* (K21) (1) : positive (2) : negative result.

In the present study, the microtiter plate method was applied to estimate the role of anti-type 3 fimbriae of *K. pneumonia* in reducing the biofilm formation of *K. pneumonia* as this method is easily to handle and available in our laboratories. Moreover, this method has been used by a lot of investigator to estimate either the ability of different bacteria to adhere and biofilm formation or in detecting the ability of antibodies to reduce the biofilm formations of different bacteria [7, 19, 21, 22].

Prevention of bacterial adherence and subsequent biofilm formation to polystyrene microtiter plate was studied by employing different dilutions of rabbit sera containing anti-type 3 fimbriae antibodies. The maximum inhibition of biofilm formation in terms of O.D (540 nm) and viable bacterial count was found in lowest dilution (1/10) (highest concentration of Abs). However, the minimum inhibition was observed in highest dilution of rabbit antisera (1/640) (lowest concentration of Abs) . Here the inhibition was in dose dependent manner as the biofilm formation increased dramatically with increasing in antisera dilution ,the results also showed that there is significant differences ($P<0.05$) in O.D. and viable count between data treated with sera and data without sera, also between serial dilutions in O.D. and viable count as shown in (table 3).

Here, the antisera (anti-type 3 fimbriae) blocked the bacterial fimbriae and that interfere with the ability of the bacteria to adhere to surfaces . This phenomenon reduced the ability of fimbrial protein to attach on the polystyrene , ultimately the reduction of bacterial ability to attach was yielded. Basically the adhesion is the first step of biofilm formation thus the reduction in adhesion will result the reduction in biofilm formation and this what was observed in the present study.

The current study in line with other studies which used the anti-fimbriae to reduced the ability of different bacteria to adhere on different kind of biotic and a biotic surfaces [23,24,25]. A local study[21] isolated and purified the p-fimbriae with pap GII adhesion from pyelonephritic, *Escherichia coli* and used to immunize rabbits for production of antisera and hence this was used in prevention of pyelonephritic *E. coli* adhesion to human uroepithelial cells , he found that anti-p-fimbriae antibodies have the same ability as the anti-pap GII adhesion antibodies in the prevention of pyelonephritic *E. coli* adhesion to human uroepithelial cells.

In another local study[26] Uroepithelial Cell Adhesion (UCA) fimbriae expressed by *P. mirabilis* were isolated and anti-UCA fimbriae antiserum was prepared in rabbits, this antiserum at 1:50, 1:100 and 1:200 dilutions inhibited adhesion of *P. mirabilis* to human uroepithelial cells. he also studied immunization and protection of female mice from urinary tract infection using UCA-fimbriae and anti-UCA fimbriae , he considered that UCA-fimbriae has a good ability to prevent the infection in bladder of immunized mice and decreased the number of bacteria in kidney of immunized mice compared with unimmunized mice .

Table (3): Viable count (CFU/ml) O.D (540 nm) for *K. pneumonia* (K21) biofilm before and after employing serial dilutions of type 3 fimbriae antisera and statistical analysis.

Parameter	Serial dilution	Without serum	With serum
O.D	1/10	1.987 ± 0.052 a	0.170 ± 0.024 e
	1/20		0.183 ± 0.035 e
	1/40		0.305 ± 0.106 d
	1/80		0.689 ± 0.184 c
	1/160		0.760 ± 0.241 c
	1/320		1.402 ± 0.426 b
	1/640		1.441 ± 0.517 b
P value		2.39 E-04	LSD 0.129
VC	1/10	9.92×10^{12} ± 2365.6 a	N.G b
	1/20		N.G b
	1/40		N.G b
	1/80		8.62×10^{12} ± 1975.2 a
	1/160		8.85×10^{12} ± 2047.4 a
	1/320		9.66×10^{12} ± 2262.3 a
	1/640		9.78×10^{12} ± 2912.3 a
P value		0.0144	LSD 974.37

- O.D= optical density, VC= viable count, NG= no growth, LSD = least significant difference, Each datum is the mean of triplicate.
- Different letters in the same column refer to significant differences.
- Similar letters in the same column refer to insignificant differences

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