Identification and Characterization of *staphylococcus aureus* Phage Isolated from Sewage

A.M. Al-khafaji
Unive. of Babylon

Abstract

Phages are nonliving agents and require use of the host's metabolic processes to replicate itself. In this study, interest on phage that infect and lyses *staph. aurous* host cells. When phage are released from the ruptured host, distinct zones of clearing (plaques) form. the original *staph aureus* host cells for this experiment came from a sample of raw sewage. in order to obtain the bacteriophage, a procedure of enrichment, isolation, dilution and seeding was followed, the presence of distinct plaques indicated that lytic bacteriophage had been successfully amplified, separated and grown.This study included determination of phage titre, latent period , rise period and the burst size of the phage and effect of some factors on phage titre as (temperature, ether and chloroform) .For determination of phage titre used series of dilutions(10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},10^{-5} 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) the dilution factor gave the best countable number of plaques was(10^{3}). this dilution factor was then used for all other experiments, the latent period , rise period and the burst size of the phage are determined by countable number of plaques and phage titre(titer: plaque-forming unit(p.f.u)) during 10,20,30,40,50, and 60 minutes . it was ($28x10^{4}$, $36x10^{4}$ and $51x10^{4}$) during 10,20 and 30 minutes respectively in the latent period ,but it was ($58x10^{4}$,$67x10^{4}$,$77x10^{4}$)during 40,50,and 60 minutes respectively in the rise period .then the burst size of the phage is counted by the ratio of the phage titer after rise period to that during the latent period it was(1.422) this study also included effect of temperature on phage titre the statistical analysis revealed significantly increase $P<0.05$ in phage titre at the temperature37 Comparing with phage titre at the temperature 50 C° and phage titre at the temperature 65 C°. effects of ether and chloroform on number of plaques and phage titre during 5,10,15 ,20,25 ,30,35 and 40 minutes was(0.7x105 , 0.3x105 , 0 , 0 , 0 , 0, 0 and 0) respectively in ether sensitivity, but the phage titre in chloroform sensitivity was completely inactivated by chloroform treatment, the statistical analysis revealed high significant increase ($P<0.05$) in phage titre in normal saline comparing with phage titre in ether and chloroform sensitivity.

Introduction

Bacteriophage are small viruses that infect bacteria and kill them by multiplying and basically filling the bacterial cell to bursting[1] [2] [3]. They don't effect the infected host, because bacteriophage activity ceases once the bacterial cells are killed. bacteriophage, which exist in many varieties, do not attack bacteria indiscriminately, they each usually attack only one specific kind[3][4]. Most phages are 24—200 nm in length. head or capsid - every phage has a head structure, which can vary in size and shape. Some are icosahedral (20 sides), others are lilamentous. The head (or *capsid)* is composed of many copies of one or more type of protein, and it contains the phage's genetic material (i.e. nucleic acid). The genetic material can be ssRNA, dsRNA, ssDNA, or dsDNA between *5* and 500 kilo base pairs (kbp) long in either a circular or linear arrangement[5][6][7]. tail - many, but not all, phages have tails attached to the phage head, the tail is a hollow tube through which the nucleic acid passes during infection, and its size can vary considerably. in the more complex phages, like T4, the tail is surrounded by a contractile sheath which contracts during infection of the bacterium, at the end of the tail, some phages have a base plate and one or more tail fibers attached to it; these structures are involved in the attachment of the phage to the bacterial cell [8] to enter a host cell, bacteriophages attach to specific receptors on the surface of bacteria, including Iipopolysaccharides, teichoic acids, proteins or even flagella. this

specificity means that a bacteriophage can only enter bacteria that bear the certain types of receptors that they can bind to, and it is these portals of entry that determine the phage's "host range" [9][10]. phages may be released via cell lysis or by host cell secretion. in T4 phage, upwards of three hundred phages will be released via lysis in approximately twenty minutes after injection, host lysis is usually achieved through an enzyme called endolysin which attacks and breaks down the cell wall structure surrounding the bacterial cell which is composed of a sugar- amino acid co-polymer called peptidoglycan [11].Phage must attach to a receptor on the surface of a bacterial cell in order to initiate an infection (12). This interaction between the phage and receptor is very specific - a given phase type only will bind to a specific receptor molecule. thus, all phage are not alike[9][13]. .the study of bacteriophage lead to a number of observations that suggest reconsideration of phage therapy might provide a mechanism for circumventing the rising concerns about the growing spectrum of resistance to antibiotics by using a new approach[14][15].The aim of the present work was to characterization of S.aureus phage isolated from sewage, determine the phage titre and study the effect of some factors as temperature, ether and chloroform.

Materials and Methods

The methods used were adapted from [16] Phage isolation Enrichment (amplification of phage) . Measure out 45 ml raw sewage into graduated cylinder and decant into Erlenmeyer flask and pipette 5 ml double sterile phosphate buffer saline and 5 ml *staph aureus* into the sewage flask then incubate flask at 37° C for 24 hours. Isolation of phage and seeding: distribution of sewage and *staph aureus* mixture into 8 centrifuge tubes, filling each up to within 1/2 inch from the top and then pipette the liquid into a membrane filter assembly and run vacuum until all the liquid is pulled into the container then transfer the final filtrate from the filtration assembly to a flask and liquefy four tubes (labeled l-4) of 5 mL soft nutrient agar and maintain a 50°C temperature then pour tubes quickly onto corresponding hard nutrient agar plates (labeled 1 - 4) before soft nutrient agar hardens finally incubate plates at 37°C for 24 hours. (if possible, check plate at 6 hour intervals. determination Phage titer : made dilutions of (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-4}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) of the sample and then plated 0.lml each of the dilution and the *staph aureus* culture onto NA plates and then incubated overnight, after which the phage titer could be determined by counting the number of plaque forming units (p.f.u.) for each dilution. For determination (dilution factor) to used for all other experiments: counting the number of plaque forming units (p.f.u.) for each dilution it was also determined at this stage which dilution factor gave the best countable number of plaques. this dilution factor was then used for all other experiments.determination of Latent period , Rise period and the Burst size of the phage the tube was mixed and placed in a 37C water bath. 0.lml of this culture was removed after 10,20,30,40,50, and 60 minutes had elapsed. Just before the culture was removed at each time interval, 0.lml of *staph aureus* culture was added to a sloppy agar tube to which the culture from the water bath was added. this was then plated immediately on NA plates and incubated overnight, the number of p.f.u. was then determined for each time A latent period, characterized by a steady low level of viruses a rise period, characterized by a sudden increase in the number of viruses the burst size of the virus or the number of viruses released by an individual bacterium is given by the ratio of the phage titer after the burst to that during the latent period.

Determination Temperature sensitivity: optimal phage dilution were prepared using saline and one placed in a 50C , the other in a 65 Cº water bath and 37Cº as a control . 0.l ml of phage dilution were removed after 10,20,30,40,50, and 60min had elapsed and added to sloppy agar previously inoculated with *staph aureus* culture then plated on NA and incubated

overnight at each temperature the number of p.f.u. were determined for each time interval and this was plotted versus time exposed to that temperature.

Determination Ether and chloroform sensitivity:

0.lml of the optimal phage dilution, diluted using saline, was added to 0.9ml ether. after 5,10,15,20,25,30,35,and 40 min had elapsed 0.lml was removed from this tube and added to sloppy agar, which had been previously inoculated with *staph aureus* culture. This was then plated on NA and incubated overnight. 0.lml of phage dilution was added to 0.9ml of chloroform and mixed after 5,10,15,20,25,30,35,and 40mm had elapsed 0.lml was removed from this tube and added to sloppy agar, which had been previously inoculated with *staph aureus* culture. this was then plated on NA and incubated overnight, the number of p.f.u. were determined for each time.

Statistical analysis : Statistical analysis were conducted to describe different variables and parameters in the research, and to describe relationship with each other as well, calculation of mean value and standard deviation (SD) were made for immunological parameters. the statistical significance of difference in mean of variable between more than two groups was assessed by ANOVA test .probability values of $P<0.05$ were considered statistically significant.

Results and discussion:

In this study, interest on phage that infect and lyses *staph aureus* host cells. when phage are released from the ruptured host, distinct zones of clearing (plaques) form, the original *staph aureus* host cells for this experiments came from a sample of raw sewage. in order to obtain the bacteriophage, a procedure of enrichment, isolation, dilution and seeding was followed, the presence of distinct plaques indicated that lytic bacteriophage had been successfully amplified, separated and grown. this study included determination of phage titre by using series of dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}) and counting the number of plaque forming units (p.f.u.) for each dilution it was (460×10^{1}, $520x10^{2}$, $570x10^{3}$,$310x10^{4}$, $190x10^{5}$ $110x10^{6}$, $30x10^{7}$, 0 and 0 respectively)this results means that the dilution factor gave the best countable number of plaques is(10^{-3}), this dilution factor was then used for all other experiments Table (1). the presence of these zones of clearing indicated that the first part of the hypothesis was proven, that is, amplifying bacteriophage from a raw sewage sample and inoculating the phage into *staph aureus* host was an effective way of isolating and visualizing phage [17].Amplify (increase the numbers) of phages in the sewage sample by allowing them to infect and reproduce within fresh *staph aureus,* The infection then "spread" as the viruses reproduced and cells lyses, eventually forming a visible plaque the titer of a phage suspension, therefore, is determined by counting the number of plaques that form from a given volume of suspension. Phage titer is expressed as plaque forming units (PFU) per milliliter (ml), it was also determined at this stage which dilution factor gave the best countable number of plaques [18]

Table(1):determination of *staph aureus* phage titer .

Plate NO.	Volume of phage plated (ml)	Dilution	Dilution Factor (DF)	Plaque per plate	Titer calculation = Plaque× DF / Volume of phage plated (ml)	Titer Plaque forming unit
1	0.1	10^{-1}	10^{1}	46	$46\times10^{1}/0.1$	460×10^{1}
2	0.1	10^{-1}	10^{2}	52	$52\times10^{2}/0.1$	520×10^{2}
3	0.1	10^{-1}	10^{3}	57	$57\times10^{3}/0.1$	570×10^{3}
4	0.1	10^{-1}	10^{4}	31	$31\times10^{4}/0.1$	310×10^{4}
5	0.1	10^{-1}	10^{5}	19	$19\times10^{5}/0.1$	190×10^{5}
6	0.1	10^{-1}	10^{6}	11	$11\times10^{6}/0.1$	110×10^{6}
7	0.1	10^{-1}	10^{7}	3	$3\times10^{7}/0.1$	3×10^{7}
8	0.1	10^{-1}	10^{8}	0	$0\times10^{8}/0.1$	0×10^{8}
9	0.1	10^{-1}	10^{9}	0	$0\times10^{9}/0.1$	0×10^{9}

The latent period , rise period and the burst size of the *staph aureus* phage are determined by countable number of plaques and phage titre(titer: plaque-forming unit(p.f.u)) during 10,20,30,40,50, and 60 minutes. it was($28x10^4$, $36x10^4$ and $51x10^4$)during 10,20and30minutes respectively in the latent period ,but it was($58x10^4$,$67x10^4$,$77x10^4$) during 40,50,and 60minutes respectively in the rise period .then the burst size of the phage is counted by the ratio of the phage titer after rise period to that during the latent period it was(l.422) Table (2).the statistical analysis was significant different $P<0.05$ in comparing between rise period and latent period.another study done by [19] on latent period, characterized by a steady low level of viruses 870 p.f.u/ml, was followed by a sudden increase in the number of viruses or a rise period, to a titre of 2500 p.f.u./ml. [20] • found the burst size of the virus or the number of viruses released by an individual bacterium is given by the ratio of the phage titre after the burst to that during the latent period. In this case it turned out to be 3 phages released per infected bacterium.

Table(2):determination of Latent period , Rise period and the Burst size of the *staph aureus* phage(the number of phage released by an individual bacterium) .

Time (min)	Volume of phage plated (ml)	Dilution Factor (DF)	Plaque per plate	Titer Plaque forming unit	Type of period	Means	Burst size
10	0.1	10^{3}	28	$28\ x10^{4}$	Latent period	47.3333	Phage Titer after /Rise period Phage Titer during Latent period= 1.422
20	0.1	10^{3}	63	$63\ x10^{4}$			
30	0.1	10^{3}	51	$51\ x10^{4}$			
40	0.1	10^{3}	58	$58\ x10^{4}$	Rise period	67.3333	
50	0.1	10^{3}	67	$67\ x10^{4}$			
60	0.1	10^{3}	77	$77\ x10^{4}$			

important characteristics of the viral reproductive process such as the number of bacteria released per infected cell and the time it takes to produce new viruses can readily be determined by plotting a single step growth curve. These important characteristics vary between viruses but are often similar for related ones. Thus, they can serve as taxonomic criteria, mostly for high-level taxonomy. The number of released phages or burst size, and the time it takes to make them or latent period, are mainly determined by the complexity and size of the virion so it is expected that these criteria are conserved between phages with similar morphology[21]. This study also included effect of some of characterization on phage

titre such as (temperature, ether and chloroform). ur results about temperature sensitivity effect on number of plaques and phage titre during 10,20,30,40,50, and 60 minutes was(28 $x10^4$,36 $x10^4$,51 $x10^4$,58 $x10^4$,67 $x10^4$ and 77 $x10^4$) respectively in the temperature37 C°. and phage titre was(43 $x10^{-5}$, 38 $x10^4$, 26 $x10^4$ '14 $x10^4$' 1 $x10^4$ and 0 $x10^4$respectively in the temperature5o C° . The phage titre was(3$x10^4$,0, 0, 0, 0 and 0) respectively in the temperature65 C° Table (3). The statistical analysis was significant increase P<0.05 in phage titre in temperature 37C° comparing with phage titre in temperature50 C° and phage titre in temperature 65 C° . most are susceptible to temperatures above 60°C and inactivated immediately at 100°C[22] The temperature at which a phage looses viability is called thermal inactivation temperature. This loss of viability has been shown to result from disruption of head proteins and the subsequent release of DNA, and the alteration of phage binding receptors (adsorption sites) [23]

Table(3):determination sensitivity of *staph aurous* phage titer in relation to temperature(37 C°, 50C° and 65C°).

Time (min)	Volume of phage plated (ml)	Dilution Factor (DF)	Titer: Plaque forming unit		
			37 C° (as control group)	50 C°	65 C°
10	0.1	10^3	28 $x10^4$	43 $x10^4$	3 $x10^4$
20	0.1	10^3	36 $x10^4$	38 $x10^4$	0
30	0.1	10^3	51 $x10^4$	26 $x10^4$	0
40	0.1	10^3	58 $x10^4$	14 $x10^4$	0
50	0.1	10^3	67 $x10^4$	1 $x10^4$	0
60	0.1	10^3	77 $x10^4$	0 $x10^4$	0
Means			52.83 $x10^4$	20.333 $x10^4$	0.5$x10^4$
Std. Deviation			2.0124 $x10^4$	9.6445 $x10^4$	0.3615 $x10^4$
Std. Error			0.8216 $x10^4$	0.7571 $x10^4$	0.1476 $x10^4$
Minimum			28 $x10^4$	0 $x10^4$	0.00
Maximum			77 $x10^4$	43 $x10^4$	3 $x10^4$

Table (4) showed effect ether and chloroform on number of plaques and phage titre during 5,10,15 ,20,25 ,30,35 and 40 minutes it was(5 $x10^4$' 2 $x10^4$' 0'0' 0'0' 0 and 0) respectively in ether sensitivity, but the phage titre was (0' 0' 0 '0' 0' 0' 0 and 0) respectively in chloroform sensitivity that means the phage was completely - inactivated by chloroform treatment, and the phage titre was (10 $x10^4$,32 $x10^4$ 47 $x10^4$ ' 51 $x10^4$ ' 58 $x10^4$ ' 66 $x10^4$' 74 $x10^4$' and 76 $x10^4$) respectively in normal saline as a control group . The statical analysis was high significant different P<0.05 in phage titre in normal saline comparing with phage titre in ether sensitivity and phage titre in chloroform sensitivity. [24]• found some phage contain lipids as a structure components of their virions and so detection of any lipids in a virus restricts its possible identity to only a few families . [25] showed these lipid are essential for maintaining the virus ability to infect new host. Any disruption of the lipid components will lead to a loss of viability of the virus, lipids are soluble in nonpolar solvents such as ether and chloroform, which are capable of extracting and disrupting the lipid components of the phages by interfering with the hydrophobic interactions between lipid molecules. Non-polar solvents are capable of denaturing proteins by disrupting the hydrophobic interaction between proteins, which can also lead to a loss of viability[26].

Table (4): determination of Ether, Chloroform and Saline sensitivity of *staph aureus* phage titers.

Time (min)	Volume of phage plated (ml)	Dilution Factor (DF)	Titer: Plaque forming unit		
			Ether	Chloroform	Saline (as control group)
5	0.1	10^3	5×10^4	0	10×10^4
10	0.1	10^3	2×10^4	0	32×10^4
15	0.1	10^3	0	0	47×10^4
20	0.1	10^3	0	0	51×10^4
25	0.1	10^3	0	0	58×10^4
30	0.1	10^3	0	0	66×10^4
35	0.1	10^3	0	0	74×10^4
40	0.1	10^3	0	0	76×10^4
Means			0.875×10^4	0	53.500×10^4
Std. Deviation			0.2550×10^4	0	20.466×10^4
Std. Error			0.014×10^4	0	0.7236×10^4
Minimum			0	0	10×10^4
Maximum			5×10^4	0	32×10^4

References

1.Edberg, S.C., Allen, M.J., Smith, D.B.and Kriz, N.J.,(1990). Enumeration of total coli forms and *Escherichia coli* from source water by the defined substrate technology.Appi.Environ.Microbi al.56,366-369.

2.Alisky, J., K. Iczkowski, A. Rapoport, and N. Troitsky.(1998). Bacteriophage show promise as antimicrobial agents. J. Infect. 36:5-15.

3.Krukowska, A., and S. Slopek.(1987). Immunogenic effect of bacteriophage in patients subjected to phage therapy. Arch. Immunol. Ther. Exp. 5:553-561.

4.Perepanova, T. 5., 0. S. Darbeeva, G. A. Kotliarova, E. M. Kondrat'eva,L. M. Maiskala, V. F. Malysheva, F. A. Baiguzina, and N. V. Grishkova.(1995). The efficacy of bacteriophage preparations in treating inflammatory urologic diseases.Urol.Nefrol.5:14-17.

5.Eiserling, F. A.(1979). Bacteriophage structure, in Comprehensive Virology.. Vol .13: 543, Fraenkel-Conrat, H. and Wagner, R. R., Eds., Plenum Press, New York.

6.Hershey AD, and Chase M. (1952). Independent functions of viral protein and nucleic acid in growth of bacteriophage. J Gen Physiol 36:39-56

7.Ptashne M. (1967) .Isolation of lambda phage repressor. Proc NatI Acad Sci USA.57:306-13

8.Ginoza, W.(1967). The effects of ionizing radiation on nucleic acids of bacteriophages and bacterial cell. Annu. Rev.Microbial.21,325.

9.Rizvi, S., and Mora,p.D.(1963). Bacteriophage plaque-count assay and confluent lysis on plates without bottom agar layer. Nature (London) 200:1324.

10. Young, R., and Blasi,U.(1995). Functions in bacteriophage lysis. FEMS Microbial.Rev.17:191-205.

11. Summers, W.C. (2001). Bacteriophage therapy. Ann. Rev. Microbial. 55,437-451.
12. Havelaar, A. H. and Hogeboom,W.H.(1983). Factors affecting the enumeration of coli phages in sewage and sewage-polluted waters. Antonie Leeuwenhoek. 49:387-397.
13. Bell, R. G.(1976). The limitation of the ratio of fecal coli forms to total coli phages as a faecal pollution index. Water Res. 10:743-748.
14. Cislo, M., M. Dabrowski, B. Weber-Dabrowska, and A. Woyton. 1987. Bacteriophage treatment of suppurative skin infections. Arch. Immunol. Ther. Exp. 2:175-183.
15. Grabow, W.O. K.; Very, A.;Uys, M., de Villiers, J. C.(1998). Evaluation of the Application of Pathogens as indicators of Water Quality, Report to the Water Research Commission by the Department of Medical Virology, Faculty of Medicine, University of Pretoria, WRC Report No. *540/1/98:* 1-7.
16. Jaime M. and Tiffany F.(2003). Isolation of E coli Bacteriophage. Phage method.2,266-276.
17. Steele, P. R. M.(1971). Morphological manifestations of freezing and thawing injury in bacteriophage T4 Bo.J.Hyg. 77, 119.
18. Yoon, S.-S.; Kim, J.W.; Breidt, F., Fleming, H.P.(2001). Characterization of a lytic *Lactobacillus plantarum* bacteriophage and molecular cloning of a lysine gene in *Escherichia coli.* mt. J. Food Microbial. 65, 63—74.
19. Jarvis, A.W.(1989). Bacteriophage of *Escherichia coli* bacteria. S. Dairy Sci.72,3406-3428.
20. Soloff, B. L.; Rado, T. A.; Henry, B. E., and Bates, J. H.(1978). Biochemical and morphological characteristics of mycobacteriophage .Ri. J. Virol. 25, 253.
21. Kaiser, G.(2000). Temperance of Phages. California State University Northridge.Molecular Microbiology 30:295-304.
22. Bohannan, B..J.M.; Lenski, R.E.(1997). The effect of resource enrichment on a ehemostat community of bacteria and phage. Ecology 78,2303-2315.
23. Slopek, S., B. Weber-Dabrowska, M. Dabrowski, and Kucharewicz Krukowska,. (1987). Results of bacteriophage treatment of suppurative bacterial infections in the years 1981-1986. Arch Immunol Ther Exp Warsz) 35:569-83
24. Bertani, L. E. and Bertani, G.(1986). Preparation and characterization of temperate non-inducible bacteriophage P2. J. Gen.Virol. 2, 357.
25. Sands, J.A. and Lowlieht, R.A. (1976).Temporal origin of viral phospholipids of enveloped bacteriophage qi. Can. S. Microbial. 22, 154
26. Camerini- Otera, R. D. and Franklin, R. M.(1972). Structure and synthesis of a lipid- containing bacteriophage. XII. The fatty acids and lipid content of bacteriophage PM2. Virology. 49, 385.

تحديد وتوصيف عاثي بكتريا المكورات العنقودية المعزولة من المياه الثقيلة

علي مالك سعد الخفاجي
جامعة بابل

الخلاصة

العاثيات عوامل غير حية تحتاج إلى العمليات الايضية لخلية المضيف كي تضاعف نفسها . هذه الدراسة تمت على العاثي المحلل لخلايا جرثومة العنقوديات الذهبية , ذ تترك العاثيات الناتجة من تحلل خلايا المضيف مناطق واضحة تعرف بالباحات . تم الحصول على خلايا جرثومة العنقوديات الذهبية لهذه التجارب من عينات مياه المجاري الخام وللحصول على العاثيات الجرثومية تم إتباع طرق الاغناء أو التكثير والعزل والتخفيف ثم الزرع حيث إن وجود باحات واضحة مؤشر على أن التحلل بفعل العاثيات البكتيرية والتكثير والعزل والتخفيف تم بنجاح . وقد تضمنت هذه الدراسة تحديد عيار العاثي وفترة الكمون (المستتر) وفترة الارتفاع (الازدياد) وحجم الانفجار أو التحلل وتاثير بعض العوامل على عيار العاثي مثل (درجة الحرارة ,الايثر,الكلوروفورم) .وقد تم تحديد عيار العاثي باستخدام سلسلة من التخافيف(10,10,10,10,10,10,10,10,10,10^) وحساب عدد الباحات كما أن عامل التخفيف 1000 قد أعطى أفضل عدد محسوب من الباحات وقد تم استخدامه في التجارب اللاحقة . فترة الكمون (المستتر) وفترة الارتفاع (الازدياد) وحجم الانفجار أو التحلل تمت دراسته وتحديده خلال الفترات (10 و20و30و40و50و60) دقيقة أما حجم الانفجار والتحلل فقد كان 1.422 وهو يمثل نسبة فترة الارتفاع إلى فترة الكمون . كما تضمن هذا البحث دراسة تأثير بعض العوامل على عيار العاثي كالحساسية لدرجة الحرارة والايثر والكلوروفورم وقد بينت نتائج التحليل الإحصائي زيادة معنوية في عيار العاثي عند درجة حرارة 50م و 60م . وقد بينت دراسة تأثير حساسية العاثي للايثر وللفترات(5و10و15و20و25و30و35و40) دقيقة انخفاض واضح في عيار العاثي كلما طالت الفترة الزمنية . كما بينت دراسة تأثر العاثي بالكلوروفورم ولنفس الفترات الزمنية عدم وجود أي فعالية للعاثي . وقد بينت نتائج التحليل الإحصائي ارتفاع معنوي في عيار العاثي عند استخدام محلول الملح الفسلجي مقارنة مع الايثر والكلوروفورم .