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Investigating the Effect of Drugs of Chloramphenicol and Gentamicin on Acanthamoeba genotype T3 Causing keratitis Isolated from Environmental samples in vitro

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Abstract:

This study is an investigation of the drugs effect on some pathogenic *Acanthamoeba* isolated from Iraqi waters, where the problem of environmental adaptation that characterizes this organism in addition to being a reservoir for many pathogenic microorganisms that take shelter in it to escape disinfectants and medicines is sometimes difficult to treat it with traditional treatments. Twenty water samples were collected from different water regions in Iraq, namely the Dokan Lake, Tigris River, Euphrates River and Najaf Sea, 5 samples from each source. *Acanthamoeba* was isolated from water samples on NNA and PYG media, using an inverted microscope with an electron microscope to determine their phenotypic features. PCR and Sequencing were also used to determine their genotype. The isolates were belonged to the T3 genotype that causes corneal infections. Then two types of drugs were used to treat it, which were Chloramphenicol and Gentamicin at three different concentrations, 0.1%, 0.3% and 0.6% and their inhibitory effect was compared with each other and with the positive control of the Chlorohexidine drug at a concentration of 0.2% which was recommended to treat *Acanthamoeba keratitis*. The results showed that both drugs have an inhibitory effect against *Acanthamoeba* growth, and that the chloramphenicol had more effect to inhibit compared with gentamicin and chlorhexidine, and it can be used as an alternative treatment instead of chlorohexidine for treating *Acanthamoeba keratitis*.

Keywords: *Acanthamoeba,* Drugs, Genotype T3, Keratitis, Waters.

Introduction:

Some of free-living amoeba, including *Acanthamoeba* in water and soil cause serious diseases to humans, such as *keratitis*, and can also infect the skin, lung, and central nervous system [1]. Genotypes of *Acanthameba* range from T1 to T20. The genotype T4 is the main cause of infection worldwide. By contrast, the genotypes T3 and T11 show lower prevalence than genotype T4, but they are the most common causative agents of amebic *keratitis* [1–4]. *Acanthamoeba keratitis* is especially common in swimmers and contact lens wearers, and sometimes in people who have immunity deficiencies.

During the two past decades, *Acanthamoeba* has been increasingly recognized as important medical microbes in the ecosystem, and has a role in the ecosystem being as vectors and reservoirs of prokaryotes such as bacteria [5]. In spite of the studies that reported it, it is still not enough.Many drugs eliminate *Acanthamoeba* active phase (trophozoite) *keratitis*, such as antibacterial, antifungal, etc., while there is no drug that eliminates its active and inactive phase (cyst) at one time. [6] used in their study the chlorohexidine to observe its effect on *Acanthamoeba* strains isolated from environment and from patients infected by *keratitis* in *vitro*. They also showed that the drug (chlorhexidine), at a concentration of 0.02% - 0.2% (0.2 mg/ ml – 2 mg/ ml), inhibited the growth of *Acanthamoeba*, because it works to undermine the cell wall of trophozoites [6 – 9]. Due to the effectiveness of this drug (chlorohexidine) and its low toxicity to the cornea, it is used as a medicine for the treatment of *Acanthamoeba keratitis* [10, 11].

Gentamicin is an antibiotic isolated from *Micromonospora purpureochromogenes*. It has broad activity against different types of gram-negative bacteria and some streptococcal strains,thus it is used to treat bacterial infections of the eye [12]. Chemical formula: $C_{21}H_{43}N_5O_7$**.** Chloramphenicol is an antibiotic that has broad activity against different types of Gram-negative, anaerobic and aerobic bacteria, therefore it is used to treat bacterial infections of the eye [13]. Chemical formula: $C_{11}H_{12}Cl_2N_2O_5$**.**

There are many studies in the world and neighboring countries have reported the presence of pathogenic *Acanthamoeba* and its treating in both *vitro* and vivo, but there is not any study about *Acanthamoeba* treating in Iraq. The aim of this research is finding suitable drugs, which may be an alternative from of chlorohexidine recommended for treating of *Acanthamoeba keratitis*.

Material and Methods:

Site and period of samples collection

Twenty water samples were collected during October 2018 to April 2019 from different regions in Iraq, with five isolates from each site as follow: Dokan Lake, Tigris River, Euphrates River and Najaf Sea (Fig.1). The collected samples were stored in sterile 250 ml plastic bottles at 4 °C until they reached to the laboratory for examination.

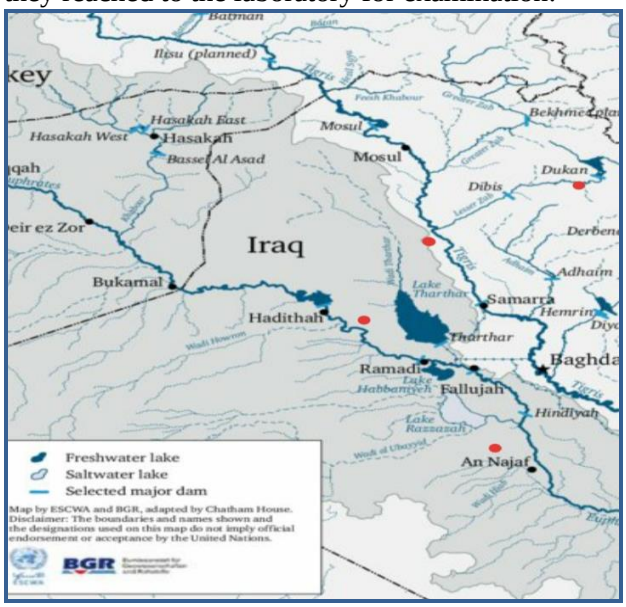

Figure 1. The map of water sampling sites, which are marked in red [www.google maps].

Filtration and cultivation on Non – Nutrient Agar (NNA) medium

The water samples were concentrated by filtering through a wattman filter papers (0.45µm) under vacuum, and placed by overturned form on a non – nutrient agar medium (15 g difco agar - 2.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 40 mM $CaCl_2$, and 20 mM $MgSO_4$), supplemented with 0.1 ml of heat-inactivated *E.coli*, then incubated at 30□ for two week. The positive growth of plates was re-cultured several times on fresh plates until to obtain pure isolates [14].

Cultivation in Peptone Yeast extract Glucose (PYG) medium

A suspension of *Acanthamoeba* was prepared by scraping with a sterile scalpel from the surface of non – nutrient agar (NNA) media and suspending it in 3 ml of buffer phosphate 0.1 M ((PH = 7.2). Then, 0.1 ml of the suspension was transferred to a peptone yeast extract glucose broth (20 g of peptone, 10 g of yeast extract and 10 g of glucose in 1 liter of distilled water),with antibiotics 0.5 ml of penicillin and 0.5 mg/ml streptomycin. Thence, incubated at 30 °C for a week [6]**.**

Microscopic examination

The growth cultures on NNA media were examined directly by inverting microscope at magnifications of 10x and 40x in order to distinguish the positive growth of *Acanthamoeba* from the negative. Negative growth cultures were excluded and left again in the incubator for a month to ensure that they are free of *Acanthamoeba*, whereas the positive were re-cultured in fresh NNA media for cloning, with three replications for each culture [14].

Examination using an electron microscope

One ml of suspended *Acanthamoeba* in buffer phosphate was centrifuged at 500 rpm for 5 minutes. Then, it was fixed with 2% of buffered Glutaraldehyde for two hours, followed by washing process with Buffer solution 0.1 M. Thence, it underwent post fixation with 1 % of Osmic acid for one hour. Then, the samples were subjected to the drying process (Critical drying point) by grading concentrations of alcohol starting from 30% - 50% - 70% - 90% - 95% - 100% for 10 minutes at each concentration (15). Later, it was plated with gold (thickness 30 nanometers) in sputter device, and then photographed with a scanning electron microscope (FEI Inspects 50) at the magnification of 1200x and 2500x in the Faculty of Science / University of Kufa.

Molecular diagnostic DNA extraction

One ml of suspended *Acanthamoeba* in buffer phosphate was added to 1.5 ml Eppendorf tubes and centrifuged at 2000 g for 5 minutes to remove the supernatant. After that, the extraction procedure was completed according to the kit (DNA extraction - Promega USA) instructions.

Primers and polymerase chain reaction protocol

In order to molecularly confirm *Acanthamoeba* isolates, the JDP Primers (Macrogen, Korea) sequences were used to amplify as follow: (JDP Forward GGCCCAGATCGTTTACCGTGAA) and (JDP Reverse TCTCACAAGCTGCTAGGGAGTCA) [12], depending on the program (initial denaturation step at 95 ℃ for 5 minutes, then 30 cycles at 95 ℃ for 40 seconds, 56 ℃ for 45 seconds, and 72 ℃ for 55 seconds, followed by a final extension step at 72 ℃ for 7 minutes).

Electrophoresis were used to confirm the presence of PCR products by using 1% agarose with 100 Volt/ Milliampere at 75 min [14].

Standard Sequencing and analysis

The PCR product was sequenced by Macrogen Corporation (Korea), using Sanger sequencing (ABI3730XL, automated DNA sequencer). The results were analyzed using geneious software.

Calculation of *Acanthamoeba* density and vitality

The 0.1 ml of positive growth media of PYG which was incubated for week, and inoculated in 4.9 ml of fresh PYG media prepared for experiment purpose in this study. Then, incubated at 30 °C for 48 hours, after completing 48 hours of incubation, 0.2 ml was taken from last growth to determine the numerical density of *Acanthamoeba* growth. The density of *Acanthamoeba* was 7 x 10^5 cells /ml, with a vitality of 100% by using hemocytometer counting, and eosin stain 1% depending on its incapability to penetrate the living cell [16].

Drugs used in the study

Preparing drug solutions and measurement their ^{2}cytotoxicity on *Acanthamoeba* growth

The drug solutions were prepared under sterile conditions using Millipore filter for each of Chloramphenicol (50 mg / ml Drops) and Gentamicin (20 mg / 2ml ampoule) at a concentration of 0.1, 0.3 and 0.6 mg /ml. Then, 0.2 ml of each concentration added to sub-culture media incubated 48 hours with three reps of each concentration. In the same way, the drug Chlorohexidine used at concentration of 0.02% (0.2 mg /ml) as a positive control for comparison. On the other hand, the negative control group was left without treatment and kept all in the incubator at 30 °C.

The growth index was calculated to determine the Inhibition Concentration Fifty values every 24, 48, 72, 96 and 120 hours by using the hemocytometer and eosin stain according to the following equation [17]:

(Percentage of Growth index = Number of Live *Acanthamoeba* / Number of Total *Acanthamoeba* X 100) The counting process was repeat three times for each experiment and the mean was used.

Results and Discussion:

Microscopic examination

After 2-5 days of inoculation and examination of the cultures using invert microscope, trophozoites of *Acanthamoeba* were observed in the positive samples (Fig 2). Cysts of *Acanthamoeba* were observed in the positive cultured samples after two weeks of inoculation and examination by SEM microscope (Fig 3).

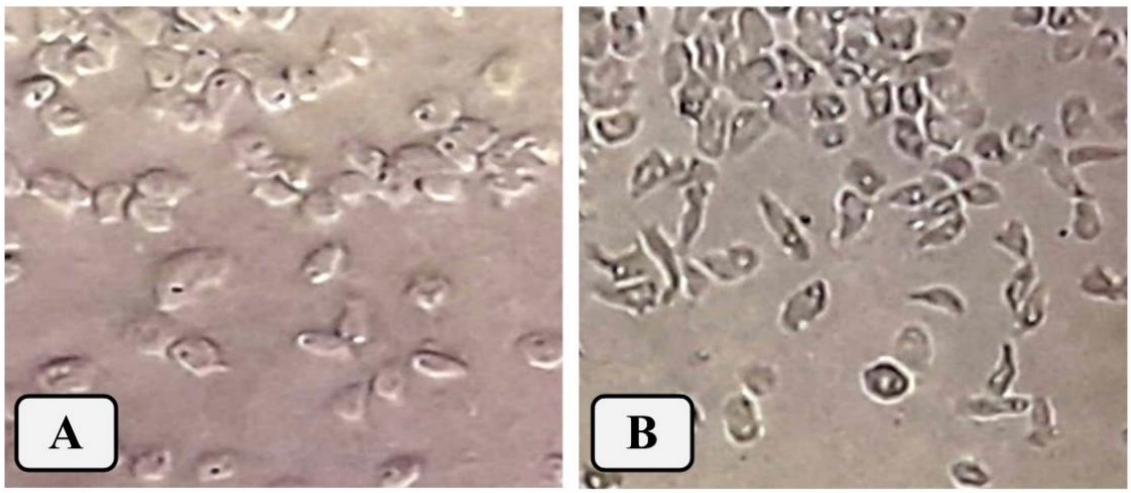

Figure 2. The trophozoites of *Acanthamoeba*, Photo graphed by inverted microscope with a magnification of 400 x. A: trophozoite of *Acanthamoeba* in NNA medium. B: trophozoite of *Acanthamoeba*in PYG medium.

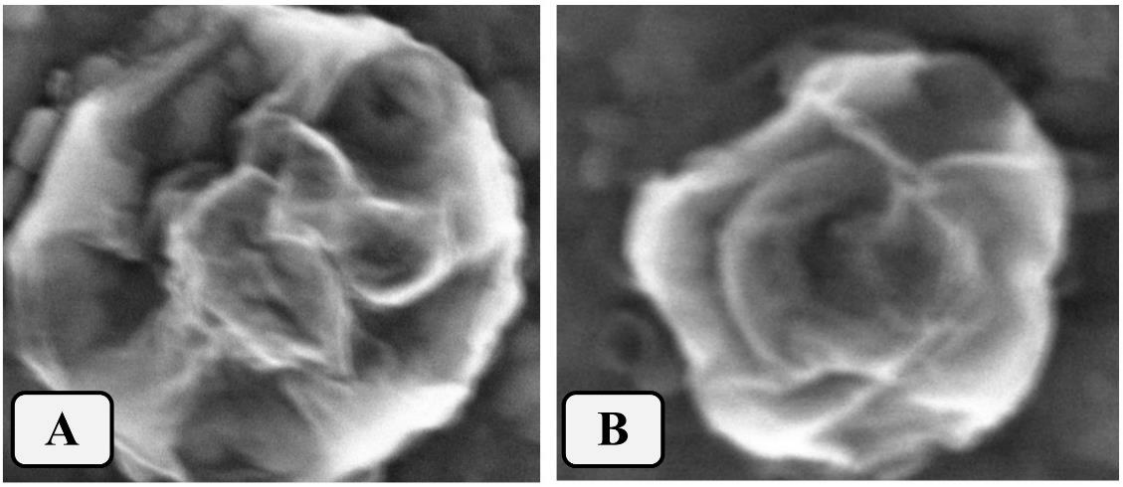

Figure 3. A and B: The cysts of *Acanthamoeba,* Photo graphed by SEM microscope with a magnification of 2500x.

Table 1. Microscopic examination of Acanthamoeba growth in NNA.

Location	No.	Positive		Negative	
		No.	%	No.	%
Dukan Lake	5	1	20	4	80
Tigris River	5	0	0	5	100
Euphrates River	5	3	60	2	40
Najaf Sea	5	1	20	4	80
Total	20	5	25	15	75

In Table No. 1 the results of microscopic examination of the *Acanthamoeba* cultivation show 5 positive growth cultures (25%) of 20 samples from the four regions, where Euphrates River shows the highest pollution percentage (3/5 or 60%), followed Dokan lake and Sea of Najaf show the same percentage of pollution (1/5 or 20%). While, no any pollution was observed in Tigris River.

Molecular identification (PCR & Sequence)

After PCR amplification of the samples and its electrophoresis, the isolates were belong to T3 genotype as *Acanthamoeba* genotype T3 Iraq (Accession Number MN462973) [14] (Fig 4).

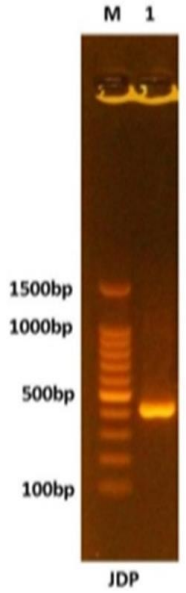

Figure 4. Electrophoresis of PCR product at 450 bp. (M) DNA Ladder marker 100 bp promega. (1) positive samples.

Effect of drugs on *Acanthamoeba* growth in *vitro*

The Gentamicin was added to each PYG media at a concentration of (1,3,6) mg /ml and their effects on survival at 24, 48, 72, 96 and 120 hours. (Table 2, Figs. 5 and 6)

Table 2. *Acanthamoeba* growth after Gentamicin addition.

Drugs Concentration% (mg / ml)	Growth Index % After :				
	24 hr	48 hr	72 hr	96 hr	120 hr
0.1 % (1 mg/ml)	97.1	92.8	85.7	78.5	68.5
0.3 % (3 mg/ml)	94.2	85.7	81.4	74.2	65.7
0.6 % (6 mg/ml)	87.1	82.8	71.4	61.4	51.4
Control positive	88.5	72.8	55.7	35.7	17.1
Control negative	100	100	100	100	100

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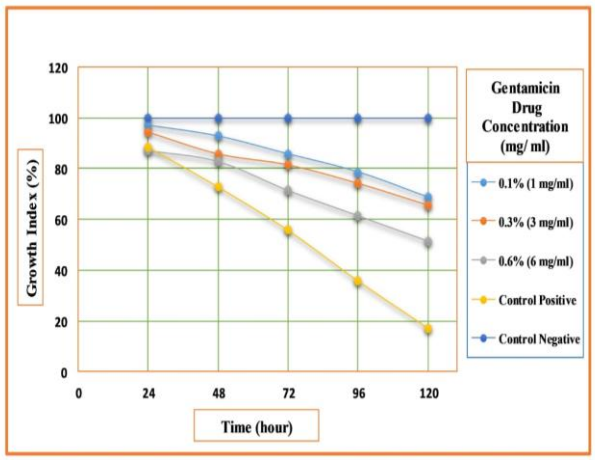

Figure 5. Curve shows growth Index of *Acanthamoeba* after Gentamicin addition.

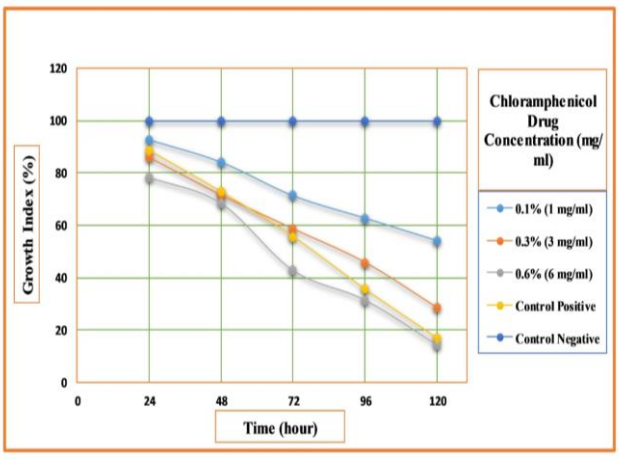

Figure 7. Curve shows growth Index of *Acanthamoeba* after Chloramphenicol addition.

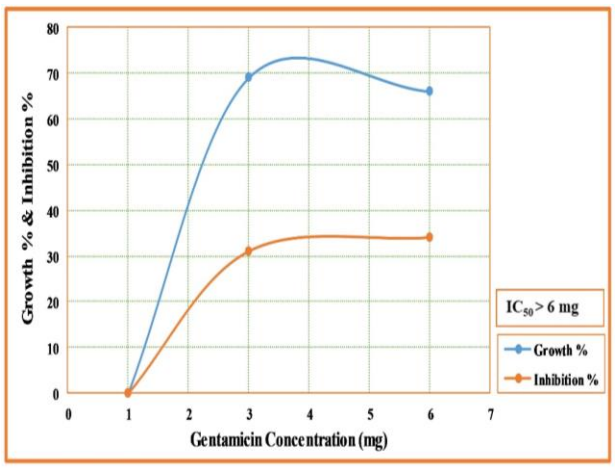

Figure 6. Inhibition of *Acanthamoeba* growth % by Gentamicin.

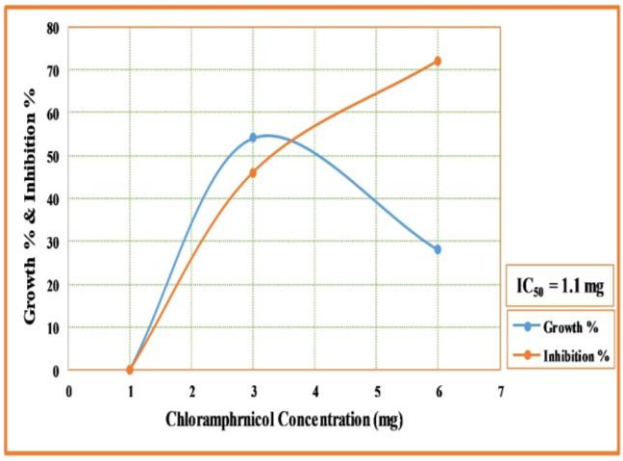

Figure 8. Inhibition of *Acanthamoeba* growth % by Chloramphenicol.

The Chloramphenicol was added to each PYG media at a concentration of (1,3,6) mg / ml and their effects on survival at 24, 48, 72, 96 and 120 hours. (Table 3, Figs. 7 and 8).

Table 3. Acanthamoeba growth after Chloramphenicol addition.

Drugs Concentration % (mg / ml)	Growth Index % After:				
	24 hr	48 hr	72 hr	96 hr	120 hr
0.1 % (1 mg/ml)	92.8	4.2	71.4	62.8	54.2
0.3 % (3 mg/ml)	85.7	1.4	58.5	45.7	28.5
0.6 % (6 mg/ml)	78.5	8.5	42.8	31.4	14.2
Control positive	88.5	2.8	55.7	35.7	17.1
Control negative	100	100	100	100	100

The drugs used in treating diseases caused by *Acanthamoeba* are very few, and their lack effect because of *Acanthamoeba* resistance to environmental conditions, in addition to being a reservoir for many pathogens that shelter for escaping from disinfectants and drugs, which is continuous obstacle for researchers [18]. In this study, the drug gentamicin showed an inhibitory effect on *Acanthamoeba* growth, and this is consistent with the results of researchers studies that continued for ten-years about the effect of antibiotics against bacterial keratitis in vitro [19]. In another study, the researchers showed that gentamicin had an ability to inhibit the cysts of *Acantamoeba* in vitro and can be used as a treatment for keratitis, they also added that the *Acanthamoeba* cysts isolated from environment were more resistant to gentamicin than the *Acanthamoeba* cysts that were clinically isolated [20], and this is consistent with what [21] also mentioned in their study, that showed the lack effect of gentamicin against *Acanthamoeba* isolated from environment in vitro [21]. As for the drug chloramphenicol, [19] mentioned in his study the

chloramphenicol was used against Plankton organisms. In another study, the researchers [22] recommended the use of chloramphenicol or neomycin (an antibiotic belonging to the aminoglycoside group and its properties are near to gentamicin) for treatment of *Acanthamoeba keratitis* in secondary bacterial infection [22]. This is in consistant with the results of the current study. There are no abundant laboratory studies to evaluate the efficacy of gentamycin and chloramphenicol against *Acanthamiba*, so detailed comparisons cannot be made with the results of the current study.

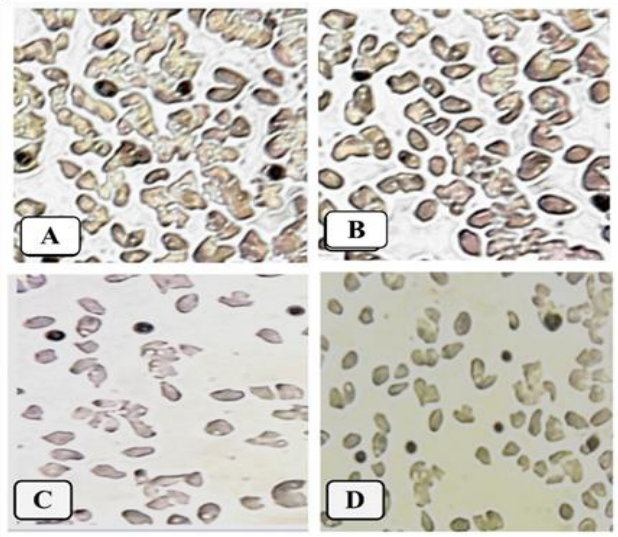

Figure 9. Represents the density of *Acanthamoeba* at 72 hours after Chloramphenicol addition. A) The highest density for growth at 0.1% con. B) growth at 0.3% con. C) growth at 0.6%. D) Positive control

The results of this study show the effect of gentamicin and chloramphenicol against the growth of *Acanthamoeba* genotype T3 isolated from Iraqi waters. Table No. 2 shows an inverse relationship between different concentrations of gentamicin with *Acanthamoeba* growth during different time periods, as observed when using concentrations 0.1 and 0.3% A slight decrease in the percentage growth within 24 hours, which amounted to 97.1% and 94.2%, respectively, then this decline gradually increased to 68.5% and 65.7% with the passage of 120 hours. But, in concentration 0.6%, it had a clear inhibitory effect on growth compared to the negative control sample, as the growth factor decreased from 87.1% to 61.4% within 96 hours and then decreased to 51.4% during the 120-hour period, equivalent to approximately 50% (IC50). This means that time also had a clear adverse effect on the growth rate of *Acanthamiba* in addition to the effect of different drug concentrations.

In Table No. 3 the results showed that the drug chloramphenicol had a higher effect against *Acanthamoeba* growth compared with gentamicin and positive control (Fig 9), where it was noticed that the growth index decreased when using concentration 0.1% from 92.8% to 62.8% during 96 hours period time of incubation, then continued to decline until it reached 54.2% within 120 hours, which is roughly equivalent to the value (IC50) of gentamicin concentration 0.6 % during the same time. The growth index when using the two concentration 0.3 % and 0.6% decreased from 85.7% to 28.5% and from 78.5% to 14.2% respectively, during the periods 24, 48, 72, 96 and 120 hours, meaning decrease was observed when using concentration 0.3 % to 45.7 at 96 hours. similarly, concentration 0.6 %, where growth decreased within 72 hours to 42.8%, and this result is less than the IC50 values, which are supposed to be within the limits of approximately 50% compared to the positive control which reached at the same period to about 55.7%. However, no inhibition was observed in the negative control group, as shown in Tables 2 and 3.

The mode of gentamicin drug action is aminoglycoside causes codon misread by binding to the ribosomal subunit 30S, stops the peptidyl-tRNA translocation from the recipient to donor area and interrupts protein synthesis [15]. In chloramphenicol, the binding to the 50S ribosome unit of the microbial cell prevents the amino acids from transferring to the growth peptide chains, which also leads to inhibition of protein synthesis in the cell [16].

Conclusions & Recommendations

In this study, we reach an effect of gentamicin and chloramphenicol against the growth of pathogenic *Acanthamoeba*, but this effect is diverse at the different concentration during different times. Also, chloramphenicol has a more effective against *Acanthamoeba* growth compared to gentamicin and chlorhexidine as a positive control and it can be used as an alternative treatment instead of chlorohexidine for treating *Acanthamoeba keratitis*. In spite of these conclusions about the effect of the two drugs in the treatment of *Acanthamoeba* genotype T3, it is in vitro and not clinically, we need more researches to be conducted to identify other genotypes and treat them in *vitro* and in *vivo*.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the

Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.

- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Tikrit.

Authors' contributions statement:

Hassan H. F. and Mohammed S. A. they were contributed as supervisors to accomplish the current research and monitoring the acquisition of the results in each steps of the research.

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دراسة تأثير ادوية الكلورامفينيكول والجنتاميسين على الأكانثاميبا ذو النمط الوراثي T3 المسببة لإلتهاب القرنية المعزولة من العينات البيئية في المختبر

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[3]قسم علوم الحياة، اختصاص الطفيليات، كلية التربية للعلوم الصرفة - جامعة تكريت، العراق.

الخلاصة:

هذه الدراسة هي تحري عن مدى تاثير بعض الادوية على بعض انماط الاكانثاميبا الممرضة المعزولة من المياه العراقية ، وذلك لان مشكلة التكيف البيئي الذي يمتاز بها هذا الكائن اضافة الى كونه مستودعا لكثير من الاحياء المجهرية الممرضة التي تحتمي في داخله هربا من المطهرات والادوية يصعب احيانا علاجها بالعلاجات التقليدية . اذ تم جمع نماذج المياه من مناطق مائية مختلفة في العراق وهي بحيرة دوكان ونهر دجلة ونهر الفرات وبحر النجف بواقع 5 نموذج من كل مصدر . وتم عزل الاكانثاميبا من نماذج المياه على اوساط NNA و PYG . استخدم المجهر المقلوب والمجهر الالكتروني لغرض تحديد معالمها المظهرية ، كما استخدم تقنية PCR وSequencing لتحديد نمطها الوراثي ، انتمت العزلات الى النمط الوراثي T3 المتسببة بالالتهابات القرنية . بعد ذلك ، تم علاجها في المختبر باستخدام نوعان من الادوية وهما الـ Chloramphenicol و الـ Gentamicin بثلاث تراكيز مختلفة وهي 0.1 % ، 0.3 % و 0.6 % وقورنت كفائتهما التثبيطية مع بعضهما و مع السيطرة الموجبة المتمثلة بعقار الكلورهيكسيدين بتركيز 0.2 % الموصى به في علاج التهاب القرنية الشوكميبي ، حيث أظهرت النتائج ان كلا الدواءان لهما تأثير تثبيطي في نمو الاكانثاميبا. ، وان دواء الكلورامفينيكول له تاثير اقوى في تثبيط نمو الاكانثاميبا مقارنة بدواء الجنتاميسين وعقار الكلورهيكسيدين ، و انه من الممكن عند استخدامه بتركيز معين ان يكون دواءا بديلا في علاج التهاب القرنية الشوكميبي .

الكلمات المفتاحية: الأكانثاميبا، أدوية، نمط الوراثي T3، التهاب القرنية، مياه.