Evaluation of *Aspergillus terreus* as potential biological control agent of the dengue mosquito (*Aedes aegypti*)

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Abstract

Vector control of dengue is widely used in preventing the disease transmission worldwide. The growing demand in reducing the use of chemical insecticides has provided imputes to explore safer alternatives. Biological control of dengue vector is the suitable approach to this problem. Myco-biocontrol using *Aspergillus terreus* was evaluated for its potential larvicidal activity against *Aedes aegypti* and safety to nature. Local strain of *A.terreus* was isolated from Jallo forest, Lahore, and its efficacy against the 3rd and 4th instar larvae of *A. aegypti* was assessed.*A.terreus* was mass cultured in potato dextrose agar (PDA) medium under controlled laboratory conditions. Bioassay of the prepared fungal suspension was applied against the larvae and the mortality rate was observed after 24, 48 and 72 hours. The experimental groups consisting of 10^7,10^6 and 10^5 conidia mL^{-1} showed the mortality rate of 90%, 65% and 50%, respectively. The LC_{50} values after 24, 48 and 72 hours was recorded as 8.1E+6, 4.27E+5 and 1.99E+4, respectively. Besides the efficacy of *A. terreus* against dengue vector, the current paper evaluatedits safety against plants, fish and indoor air for its consideration as safe to natural environment.*A.terreus* showed no potential side effects on fish, plants and indoor air proving it safe for the environment.

Keywords: *Aedes aegypti*, *Aspergillus terreus,* Biocontrol, Entomopathogenic fungi, *Solanum melongena*.

Introduction

Aedes aegypti is the vector of viral disease dengue (Gubler, 1998). According to a new estimate, 3.6 billion (55% of the world's population) are at risk of dengue. In the absence of a vaccine and effective drugs, vector control has been considered an important tool in the prevention and control of these diseases (Loujana *et al*., 1981). Dengue outbreaks have appeared in many areas of Pakistan causing great losses in terms of mortalities and morbidities in last two decades (Ali *et al*., 2016). A very devastating Dengue outbreak was observed in Pakistan in the year 2011, particularly in Punjab province with Lahore as the most affected city (Shakoor *et al*., 2012). The potential vector and supporting environmental conditions which promote the infectious agent to proliferate are playing important role in spreading disease in the area. Dengue patients are mostly asymptomatic and re-infection with different serotypes of the viruses may lead to hemorrhagic fever with increased chances of mortality (Morais *et al*., 2004). Symptoms of dengue fever includes headaches, pain and rash, and in sever case it may cause internal bleeding leading to mortality of the patient (Sarwar, 2015).

Viral diseases can be controlled by vaccination, In case of dengue CYD-TDV a chimeric tetravalent vaccine has been prepared by Sanofi Pasteur, but access to this vaccine is far long to be common (López-Gatell *et al*., 2016). As wait for dengue vaccine to be in common use is not a realistic approach the only way is to control the mosquito vector to avoid the spread of disease. Keeping in view the very limited access to vaccine for the treatment of dengue sickness and the most widely used methods for control of disease carrier *Aedes* mosquitoes are certain insecticides (David *et al*., 2010). Indeed, insecticides are often deliberately introduced into the mosquito habitat in the fight against many human diseases they transmit (Lounibo, 2002). As a consequence, mosquito control programs are now threatened by the selection of mosquito populations resistant to these chemical insecticides (Hemingway *et al.,* 2002*)*. Growing insecticide resistance in the primary mosquito vector, *Aedes aegypti*, limits the effectiveness of vector control, so alternative tools are urgently needed. The utility of biological control methods using bacterial pathogens is not considered for the containment of immature *Aedes aegypti* because of the high costs involved due to the frequent treatments that are necessary. Other biological control agents such as larvivorous fish could be used in wells, water storage containers and cisterns, but their release is unacceptable to the inhabitants and the fish also die due to chlorination (Santos *et al*., 1996).

To resolve these problems, entomopathogenic fungi have been used as larvicides which can provide an alternate to synthetic chemical insecticides (Howard *et al*., 2010). Mosquitoes show vulnerability towards entomopathogenic fungi. They have low toxicity to non-target organisms and using

entomopathogenic fungus as larvicides may be a promising approach for biological control of mosquitoes due to their selective toxicity and ready decomposability in the ecosystem (Soni *et al*., 2010). Unlike the dangers which are associated with the processing of synthetic insecticides, the process for the manufacture of microbial products is safe and less pollutant (Bukhari *et al*., 2011).

Entomopathogenic fungi, with its cosmopolitan existence and rich diversity, present a sustainable solution towards biological pest management programs. These entomopathogens, due to their eco-friendliness and bio-persistence, are preferred to kill insects at various stages of theirlife cycle. Several entomopathogens, when introduced into a variety of habitats, can provide effective long-term to short term control (Sandhu *et al*., 2012). The objective of the present study was to investigate the efficacy and safety of the local strain of *A. terreus* as biological control agents against *A. aegypti*.

Material and Methods

The soil samples were collected from Manga Mandi, Lahore. The isolation of *A. terreus* was done by insect bait method using larvae of *Galleria mellonella* (Meyling, 2007). The pure cultures of *A. terreus* were obtained after mass culturing on PDA and Sabouraud dextrose agar (SDA) media. The fungus was isolated and identified on the morphological and molecular basis. The study was conducted after approval of ethical committee and review board of the university.

Morphological and molecular identification

The cultures were identified based on their colony characteristics and microscopic examinations were done. Morphological identification was done based on the shape of colony as well as the spores, the size of spores, colony color, margins, colony reverse, elevations, nature of growth, and nature of pores. The growth parameters like radial growth, spore count and days taken to cover up the full plate were also observed.

The molecular identification was done via DNA extraction, quantification, PCR based amplification, Gel electrophoresis, Gel DNA extraction and DNA sequencing at the Centre of Excellence in Microbiology (CEMB).

Efficacy of *Aspergillus terreus* as biocontrol agent against *Aedes aegypti*

The prepared dilutions of *A. terreus* suspension were applied on *Aedes* larvae in separate cups under standard lab conditions (26 °C and 70% relative humidity) set at the Parasitology Department of University of Veterinary and Animal Sciences. The fungal efficacy was observed after 24, 48 and 72 hours to evaluate the larval mortality rate. The LC_{50} of *A. terreus* was obtained after prohibit analysis via MS Excel 2007.

Effect of *Aspergillus terreus* on fish

The test fish, inland silverside *(Menidia beryllina*) fingerlings, were obtained from fish farm near Lahore. During this acclimation period, water in the tanks was continuously aerated. Fingerlings were fed with commercial food twice a day. The test used 4 treatments, 0.01% tween 80 solution control and 3 densities of approximately 10^7, 10^6 and 10^5 conidia mL^{-1}. A volume of 10 mL from each was poured in the respective fish bowls. Fish bowls containing ten fingerlings were labeled accordingly. The test bowls were re-exposed with the same densities of fungal suspension dilutions and 0.01% tween 80 solution every third day during the span of 20 days experiment. Behavioral changes (feed intake, swimming) were followed closely. After the completion of 20 days experiment the fish under study was examined morphologically considering the aspects (mouth infection, cloudy eyes, eye pop/bulge, fin rot, fin hemorrhage, gill rot, gill inflammation and scale infection) and their survival percentage was observed.

Persistence of *A. terreus* in indoor air

A room was selected to carry out the experiment. There was no storage of food or any other eatables in the rooms. No one was allowed to enter in the rooms for three days until the setup was removed. Samples were taken with all the precautions followed. A bare (un-covered) Petri plate containing growth media was placed in Air Sampler and its reading was recorded for a period of three minutes. Then, the plate was covered and sealed by using tape and properly labeled. After this, a black cotton cloth was evenly sprayed by 20 mL prepared spray of fungal suspension containing 10^7 conidia mL^{-1} in oil. Two uncovered Petri plates were immediately placed in the air sampler for a time period of three minutes and readings were recorded for six minutes. The said plates were then sealed with tapes and labeled properly. For next two consecutive days, the procedure was repeated. Incubation of plates was done for seven days at 32 ± 2 ºC. Plates were then observed and assessed for the growth and fungal colonies were counted by using microscope after 5-7 days.

Effect of *A. terreus* on plants

Out of 24 test plants of eggplants (*Solanum melongana*), 18 plants were assigned to experimental group and 6 plants were assigned to control group exposed with 0.01% tween 80 solution. Three experimental groups were created and exposed with prepared dilutions of 10^5, 10^6 and 10^7 conidia mL^{-1}. The base of plant was covered by aluminum foil or plastic cover to avoid fungal suspension runoff. From each suspension, 3 mL solution was taken in the sprayer and applied onto the leaves of plants. The plants were covered with plastic bags in order to maintain humidity. The exposed plants were

observed after 1, 4 and 7 days. Morphological changes and disease symptoms (rust, downy mildew, powdery mildew, gray mold, necrosis, leaf mold and late blight) in plants were observed. LC_{50} and LC_{90} were calculated by using Probit analysis (Robertson *et al.,* 2017: Finney, 1971)

Results and Discussion

Morphological and molecular identification

A. terreus was brownish in color when observed after 3rd day of incubation and gets black in color when observed after 7–8 days. It has conidial heads that were compact and densely columnar, reaching 500 × 30–50 µm in diameter. Conidiophores of *A. terreus* were smooth and up to 100–250 × 4–6 µm in diameter. The conidia of *A. terreus* were small, about 2 µm in diameter, globose-shaped and smooth-walled. It was distinguished from the other species of *Aspergillus* by its cinnamon-brown colony coloration. Nature of pore was powdery (Fig. 2).

For the sequencing the gene, sequencing was performed utilizing 18S rRNA gene of isolated *Aspergillus* species when subjected to BLAST search device in database of NCBI to distinguish the isolated *Aspergillus* species. The fungus was identified as *A. terreus* with Genbank Accession No. MK 371711

Efficacy of *A.terreus* as biocontrol agent against *A.aegypti*

The local strain of *A. terreus* demonstrated its entomopathogenic efficacy against *A. aegypti* larvae (Table 1). In 24 hours, the mean mortality of *A. aegypti* larvae was recorded as 50% with 1×10^7 conidia mL^{-1} dose treatment, 30% with 1×10^6 $conidiaml^{-1}$ dose treatment and 16.66% with 1×10^5 conidia mL^{-1} dose treatment. In next 24 hours, the mean mortality percentage was observed to be increased. The mean mortality of 86.66%, 63.33% and 36.66% were obtained with three different doses of 1×10^7, 1×10^6 and 1×10^5 conidia mL^{-1}, respectively. Maximum mortality was observed in 72 hours i.e. 100 with 1×10^7 conidia mL^{-1} dose treatment. After 72 hours of exposure 80 mortality percentage was observed with 1×10^6 conidia mL^{-1} dose treatment and 60 with 1×10^5 conidia mL^{-1} dose treatment. The mortality rate was observed to be proportional to the exposure time. Positive relation was shown between the conidia mL^{-1} concentrations of *A. terreus* with exposure time to cause mortality in *A. aegypti* larvae. The LC50 and LC90 values were calculated using Probit analysis. The LC_{50} value of *A. terreus* against *A. aegypti* larvae in 24 hours post treatment was 1.0×10^7 conidia mL^{-1} and LC_{90} was 4.20×10^9 conidia mL^{-1}. A 48 hours post reatment showed the LC_{50} and LC_{90} values as 2.99×10^5 and 1.63×10^7 conidia mL^{-1}, respectively.

Safety of *A. terreus* on fish

Eye pop and Scale Infection were the major morphological effects observed. The level of severity of effects is shown in Table 5. After 6 days of experiment, some behavioral changes were observed in the fish in bowls exposed with the dilutions of 10^7 conidia mL^{-1}.

Persistence of *A.terreus*in air

After five days of incubation, colonies in the Petri plates of three days were counted under microscope. Average of two plates of the same day was taken and this was repeated for the plates of next two days as well. The concentration of conidia for three consecutive days was counted to be 37098, 15486 and 3963 m^{-2}. This data is presented in Fig. 2.

Safety of *A. terreus* on plants

No effect of *A. terreus* was observed on egg plant. Pathogenic property of various fungal species has been utilized in reducing the mosquito population that helps in promoting biological control technologies (Scholte *et al.,* 2004). In the present study, natively isolated *A. terreus* was used to target the *A. aegypti* larvae to evaluate the entomo-pathogenic activity. For infection the immature stage of *A. aegypti* vector are reported to be most perfect stage by biological control (Yap, 1985; Conti *et al.,* 2010). According to many experiments, mortality of *Aedes* larvae has been observed when treated with the spores of *Aspergillus* fungus. Mycotoxins are produced by *Aspergillus*. The fungus can penetrate the mosquito larvae cuticle, cause expansion of hyphae internally (Seye *et al.,* 2009). One more research accomplished by Moraes *et al.* (2001) in which eleven different isolates of *Aspergillus* were treated against *Aedes* and *Culex* mosquitoes and testified 100% mortality in some cases within 24 hours.

Selection of third and fourth instar larval stage of *A. aegypti* in the present study is supported by different research studies. *A. aegypti* is the most commonly utilized in insecticide screening process because it is commonly low capable and it is easy to colonize at laboratory level. In the late third and early fourth instar larvae were utilized since the response of newly hatched larvae is not fully developed and they do not move during the first few hours (Essam *et al.,* 2005). *A. terreus* revealed higher mortality of *Aedes* larvae within 72 hours as entomopathogenic fungi do not show fast acting activity like chemical neurotoxins. Fungal pathogens do not cause fast mortality but take a number of days as the fungal hyphae has to penetrate the insect cuticle and then proliferate within the hemocoel (Thomas and Read, 2007). Our study also revealed that increasing level of mortality of *Aedes* larvae was observed with respect to increasing level of conidial concentration and duration of treatment in case of *A. terreus*.

The present research discovered that the larval body became crumbled and mummified when treated with *A. terreus*. Gopal *et al.* (2000) reported that the insect after the infection being to be slow, loss of desire food was observed and did not act toward the incentives. Due to the fungal parasitism death occurs and their body dried up, mummified and fragile. Popeye is scientifically known as exophthalmia, is the swelling of the eye, with or without cloudiness, which is caused by the pressure of fluid building up inside the eye itself. The first sign noticed was that both eyes were starting to bulge. The test bowls exposed with higher concentration of *A. terreus* conidial suspension has large number of test fish bearing eye-pop than the one exposed with lower concentration. Pop eye was recognized as one of the fungal infection in many studies. The study conducted by Hrajis (1991) concluded that endophthalmos (sunken eyes), exophthalmos (pop-eyes) are among the ocular diseases affecting wild and cultured fishes. These pathological conditions may be caused by nutritional deficiencies, environmental intoxication and other environmental stresses including other aspects of poor water quality, crowding, aggressive behavior and invasion of fungal organisms.

Clear dark red and black spots were seen on the scales of test fish. This effect was only found in the test bowls with high concentration of *Aspergillus terreus* conidial suspension. The entry of fungus in test fish body cause serious internal damage and leads to internal bleeding which appears as red spots on the body of the fish. In many studies this kind of infection has been related with fungus. According to Lester (2000) deep ulcerative lesions are the hallmark of the chronic stage of an infection. After 7 days of exposure fish started staying at the bottoms and constantly swim in sideways. The motion of fish slows down gradually after passing days and food intake was also slightly abnormal. Whenever the fish was caught to change the water of test bowls the fish behave aggressively. Same results were found in the study conducted by (Yildirim *et al.,* 2006) and considered as abnormal behavior.

There are concerns that the application of fungal bio pesticides may cause their spores to enter and increase the persistence of the spores in the air that can trigger the allergic response in the environment. Therefore, an experiment was performed in the present study to check if *A. terreus* can persist in air or not. When the spray of fungal solution formulated in oil was applied on the black cotton cloth, the spores of *A. terreus* showed high persistence immediately after the spray on the black cotton cloth. Studies by Alves *et al.* (2002) have shown that oil formulations enhance the endurance of conidia against abiotic factors. The reason why the concentration of spores fell so drastically after 24 and 48 hours can be supported by the findings of Thomas *et al.* (2007) who suggested that the drastic decline of spore concentration is an indicator that any no. of spores in the air, are not frequently freed, rather they rapidly dispose off.

Conclusion

The present research study lead to the conclusion that dengue vector should be controlled biologically by using *A. terreus* which showed no potential side effects on fish, plants and indoor air proving it safe for the environment.

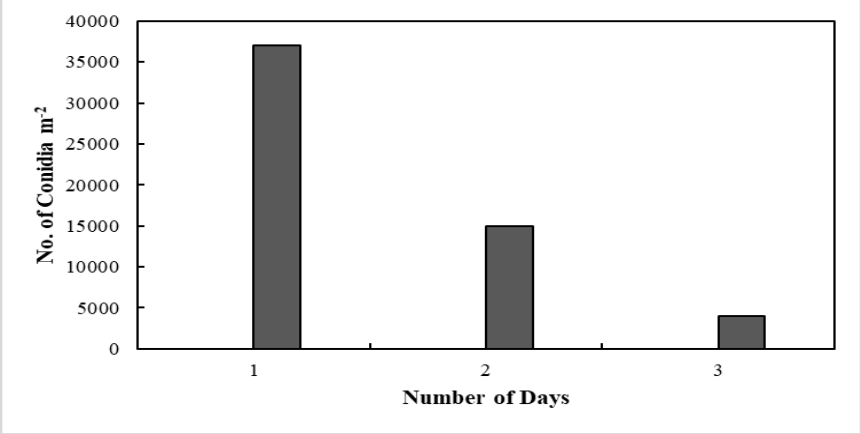

Fig. 1: The persistence of *Aspergillus terreus* spores in the air checked for 3 days after putting Petri plates containing growth media in the air sampler. There was a gradual decrease in the number of conidia after 3 days.

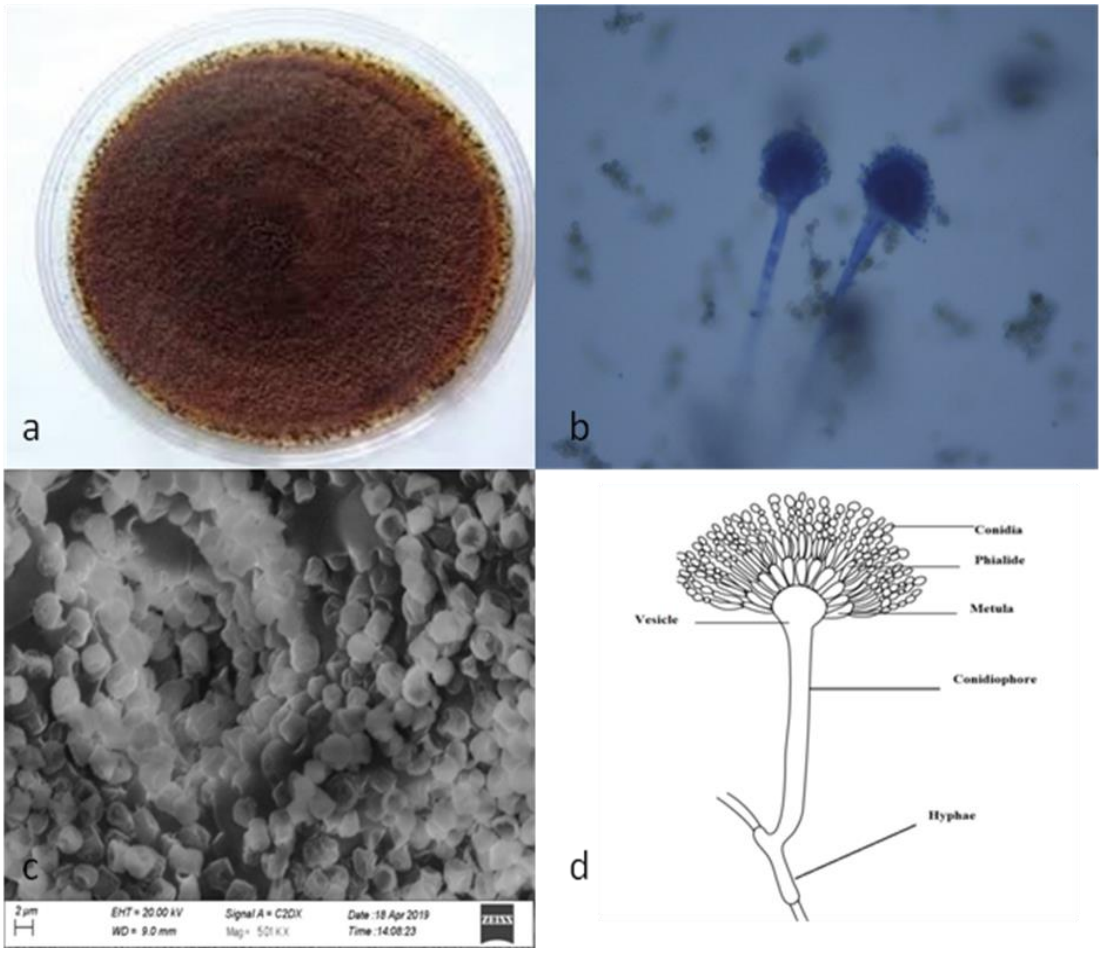

Fig. 2: *Aspergillus terreus* (MK 371711) **a.** Macroscopic features of colony; **b.** Microscopic features by LM showing Vesicle, conidia, Conidiophore, Phalides, Metula and Hyphae; **c.** SEM micrograph of conidia highlighting their shape, ornamentation and size: **d.** Schematic drawing.

Table 1: Percentage mortality rate of Larvae of *Aedes Aegypti* treated with *Aspergillus terreus* after 24, 48 and 72 hours.

S. No.	Conc.	24 h Dead larvae	24 h Mortality (%)	24 h Avg Mortality (%)	48 h Dead larvae	48 h Mort-ality (%)	48 h Avg Morta-lity (%)	72 h Dead larvae	72 h Mortality (%)	72 h Avg Morta-lity (%)
1	10^7	3	30	30	5	50	55	8	80	85
	Conidia mL^{-1}	3	30		6	60		9	90	
2	10^6	2	20	15	4	40	45	7	70	65
	Conidia mL^{-1}	1	10		5	50		6	60	
3	10^6	1	10	10	4	40	35	5	50	50
	Conidia mL^{-1}	1	10		3	30		5	50	
5	Control Positive	100	100	100						

Table 2: Lethal concentration of mortality rate of larvae of *Aedes aegypti* of *Aspergillus terreus* for 24 hours.

Conc.	Mean±SD	LC_{50}	LC_{90}	Lower CI	Upper CI	SS	SLOPE	DF
10^7 Conidia mL^{-1}	5±1							
10^6 Conidia mL^{-1}	3.5±0.5	8.10E+06	2.10E+09	-	-	1.4098	0.53	5
10^5 Conidia mL^{-1}	1.5±0.5							
Control Positive								

SD: Standard deviation, LC_{50}: Lethal Concentration at which 50% population is killed LC_{90}: Lethal Concentration at which 90% population is killed, CI: Confidence interval, SS: Sum of squares, DF: Degrees of freedom.

Table 3: Lethal concentration of mortality rate of larvae of *Aedes aegypti* of *Aspergillus terreus* for 48 hours.

Conc.	Mean±SD	LC_{50}	LC_{90}	Lower CI	Upper CI	SS	SLOPE	df
10^7 Conidia mL^{-1}	8.5±0.5							
10^6 Conidia mL^{-1}	5.5±0.5	4.27E+05	2.50E+07	-	-	2.3127	0.725	5
10^5 Conidia mL^{-1}	3.5±0.5							
Control Positive								

SD: Standard deviation, LC_{50}: Lethal concentration at which 50% population is killed, LC_{90}: Lethal concentration at which 90% population is killed, CI: Confidence interval, SS: Sum of squares, df: Degrees of freedom.

Table 4: Lethal concentration of mortality rate of larvae of *Aedes aegypti* of *Aspergillus terreus* for 72 hours.

Conc.	Mean±SD	LC50	LC90	Lower CI	Upper CI	SS	SLOPE	df
10^7 Conidia mL^{-1}	9±0							
10^6 Conidia mL^{-1}	8.5±0.5	1.99E+04	6.46E+06	-	-	1.3845	0.51	5
10^5 Conidia mL^{-1}	6±1							
Control Positive								

SD: Standard deviation, LC_{50}: Lethal concentration at which 50% population is killed, LC_{90}: Lethal concentration at which 90% population is killed, CI: Confidence interval, SS: Sum of squares, df: Degrees of freedom.

Table 5: Morphological effects of *Aspergillus terreuson* fish.

S. No.	Treatments	Dose/ ml	No. of Fish		% of Effected Fish	Morphological Aspects
			Observed	Effected		
1	Control	10	10	0	0	----
2	Dilution 10^5	10	10	2	20	Eye Pop/Bulge
3	Dilution 10^6	10	10	2	20	Eye Pop/Bulge
4	Dilution 10^7	10	10	2	40	Eye Pop/Bulge and Scale Infection

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