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COMPATABILITY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AND *LAGENIDIUM GIGANTEUM* FOR MOSQUITO CONTROL. I. EFFECT OF TIME OF APPLICATION AND TOXIN CONCENTRATION

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ABSTRACT

In laboratory experiments, 3-day-old larvae of the mosquito *Culex quinquefasciatus* Say exposed to *Bacillus thuringiensis* subsp. *israeliensis (B.t.i.)* and to the pathogenic fungus *Lagenidium giganteum* showed no difference in susceptibility when *B.t.i.* was applied before or after the larvae were exposed to *Lagenidium* zoospores. However, the number of dead larvae infected with the fungus and the extent of mycelial growth within the larvae were less when *B.t.i.* was applied first and this diminishing pattern was also shown in relation to increasing *B.t.i.* concentration.

Zoosporogenesis from larval cadavers was accclerated when *B.t.i.* was used in combination with the fungus. *B.t.i.* delayed but did not prevented the production and maintenance of epizootics by *Lagenidium* under the conditions tested.

INTRODUCTION

Integrated control of vector species has been supported to reduce the present dependence on chemicals and because of the success achieved in the control of agricultural pests. Although in 1982 the WHO Expert Committee on Vector Biology and Control suggested "to consider the combined effects of various biological control agents in the reduction of vector populations," the main effort has been directed at testing *Bacillus thuringiensis* subsp. *israeliensis (B.t.i.)* with chemicals (Merriam and Axtell, 1983 b; Laird et al., 1985) and the effect of chemicals and some microbes on *Lagenidium giganteum* (Merriam and Axtell, 1983 a; Lord and Roberts, 1985), and little is known of the interaction of two biological control microorganisms in vector control.

Since 1977, when Goldberg and Margalit reported the discovery *of B.t.i.*, considerable attention has been directed to this pathogenic bacterium, as well as other species as promising biological control agents for vector of diseases; and extensive research has been done on *B.t.i.* and *Lagenidium giganteum*, as summarized by Davidson (1981), Federici (1981), Lacey (1985), and Lacey and Undeen (1986).

The main incentives for research and development of *B.t.i.* as a biological control agent are its efficacy, specificity, biodegradable nature (Lacey, 1985), and the fact that B.t.i. does not produce a broad spectrum heat-stable exotoxin, which is toxic to vertebrates (de Barjac, 1978).

B.t.i. delta-endotoxin is synthesized as a protoxin, and is active only when ingested by the mosquito larvae with subsequent activation in the alkaline gut environment (Lacey, 1985). These conditions are found in the gastric coeca and mid-to posterior part of the larval midgut (Dadd, 1975). The pathogenicity of this bacterium on the larval gut has been studied by Charles and de Barjac

(1981), and Lahkim-Tsror et al. (1983); the molecular mode of action has been studies by Thomas and Ellar (1983a,b), Ellar et al. (1986), and Nizeyimana et al. (1986).

Lagenidium giganteum, a water mold, is a facultative pathogen of mosquito larvae, that has been shown to be a promising biological control agent (Federici, 1981; Axtell, 1983a,b; Lacey and Undeen, 1986).

The life cycle, pathogenicity, and biochemistry of this fungus has been extensively studied by Brey (1985), Dean and Domnas (1983), Domnas (1981), and Domnas et al. (1974, 1982). Production and field ecology have been studied by Fetter-Lasko and Washino (1983), and Jaronski and Axtell (1982, 1984, b).

MATERIALS AND METHODS

The *Culex quinquefasciatus* larvae used for these experiments were obtained from laboratory colonies routinely maintained at North Carolina State University and originated from specimens collected in the vicinity of Raleigh, North Carolina. *Bacillus thuringiensis* subsp. *israelensis* was obtained from Abbott Laboratories, as Vectobac AS. Previous bioassays indicated that this commercial formulation at rates of 13.9 and 31.5 International Toxic Units (ITU) per 100 ml produces 50 and 90% of mortality in 3-day-old *Culex quinquefasciatus* larvae. The California isolate of *Lagenidium giganteum* used in these experiments was cultured on agar with sunflower seed extract (SFE) as described by Jaronski and Axtell (1984b) and Guzman and Axtell (1986). Previous bioassays indicated that a 20 mm^2 disc of fungal mycelia produces approximately 95% mortality of *Cx. quinquefasciatus* 3-day-old larvae.

General bioassay techniques

Two types of bioassays were conducted. The first used 20 or 200 mm^2 agar discs of mycelia cultured on agar-SFE. Zoosporogenesis in these type of bioassays took place 5–8 h after the discs were immersed in assay containers. The second group used zoospores that were produced before mosquito larvae were exposed. *B.t.i.* was applied using the 10,000 fold dilution from the stock Vectobac AS.

The overall larval mortality in the bioassays was determined at 24 and 48 h after treatment with the pathogens. The presence of *Lagenidium* infection and the spread of the mycosis in the larvae was determined 48 h after exposure to the microbial agents. Dead larvae from each treatment was observed microscopically. The presence or absence of mycelial growth was recorded as well as its location within the larval cadaver.

All the bioassays were conducted at room temperature (25 ± 2°C). Data collected from the experiments were analyzed using SAS procedures (SAS Institute Inc.). Data were subjected to analysis of variance and differences between means were compared using Tukey's test. Interactions of the pathogens were evaluated using the controls with larvae exposed to each agent alone. The *B.t.i.* concentration used was 11.4 ITU/100 ml. The formula $0_\alpha + 0_\beta (1 - 0_\alpha)$ was used to calculate the expected mortality, where 0_α was the observed percentage of mortality for the bacterium alone, and 0_β was the observed percentage of mortality for the fungus alone (McVay et al., 1977; Fuxa, 1979; Richter and Fuxa, 1984). Chi square statistics were used for comparison of expected mortality with observed mortality. The same procedure was used to evaluate the bacterial fungal interactions for the variables, infectivity and growth rate.

Bacterial fungal interactions

1. *Lagenidium* applied as mycelial discs. The bioassay was conducted in plastic cups containing 100 ml of deionized water and twenty 3-day-old mosquito larvae. One 20 mm^2 agar-SFE disc with mycelia was placed in each cup, and *B.t.i.* doses were applied at rates of 45.6, 34.2, 22.8, 11.4, and 5.7 ITU/100 ml.

2. *Lagenidium* applied as zoospores. Six hundred 3-day-old mosquito larvae were exposed to the zoospores for 1 h, then washed with deionized water and in groups of 90 exposed to several *B.t.i.* concentrations for 1 h. The larvae were then removed and washed in groups of 20, placed in plastic cups containing 100 ml of deionized water.

To determine the zoospore production time from the larval cadavers in the bioassay cups, twelve water samples were taken from each replicate 24 and 48 h after exposure to the pathogens, and applied to newly hatched mosquito larvae contained in tissue culture plates. Data were collected 48 h after exposure to the 1 ml water samples.

3. Effect of *B.t.i.* on *Lagenidium* persistence. A single dose of *B.t.i.* was evaluated with two different bacterial application schedules. *B.t.i.* was applied to simulate a single and a slow release application. For each treatment, three experimental buckets were prepared containing 5 mm of sand in the bottom and 1 liter of deionized water. Twenty 1, 3, and 5-day-old larvae were added, as well as the fungal innoculum. Natural oviposition was simulated by daily addition of 20 newly hatched larvae for a period of 15 days. Every day 15.4 ITU/100 ml of *B.t.i.* were added to the buckets that were simulating a slow release application, and a single application of 15.4 ITU/100 ml was added the first day to the buckets that were simulating a single application. After 15 days the buckets were drained, allowing larval cadavers to remain on the sand and dry at room temperature. Buckets were kept dry for 15 days, then reflooded with deionized water. Newly hatched larvae were added every day; no pathogens were added at this point. The activity of the bacterial and fungal pathogens was measured indirectly by removing and counting all the pupae on a daily basis.

RESULTS

Lagenidium applied as mycelial discs. Analysis of variance was conducted to test the effects of time, *B.t.i.* concentration and presence or absence of *Lagenidium* on overall mortality on 3-day-old *Cx. quinquefasciatus* larvae. Interactions among treatments were also evaluated. The F test for time with 4 degrees of freedom was highly significant (P = 0.0001). The F test for all other treatments and interactions (188 df in the error term) were highly significant as well (P = 0.0001). This analysis showed that the effects of the pathogens on larval mortality changed with increasing concentration of *B.t.i.*, fungal presence, and time of exposure to the pathogens. Larval mortality 24 h after exposure, in the combined treatments, was significantly higher than the mortality in the *Lagenidium* control, except for the treatment that contained 5.7 ITU/100 ml. Observed mortality from the combination of pathogens was consistent for that expected from additive effects. Forty-eight hours after treatment, there was no difference between the combined treatments and the *Lagenidium* control (Table 1), but a significant difference was found when the combined treatments that contained 5.7 and 11.4 ITU/100 ml were compared with their respective *B.t.i.* controls.

Dead mosquito larvae inspected for the presence of *Lagenidium* (infectivity rate) showed an inverse relationship with the dose of *B.t.i.* As the *B.t.i.* concentration increased, the number of dead larvae infected with *Lagenidium* decreased (Table 2). Significant differences in infectivity rate were found when the *B.t.i.* concentration was near or greater than the LC50 value of *B.t.i.* in the combined treatments. However, at the highest *B.t.i.* concentrations 34.2 and 45.6 ITU/100 ml, the mean percentages of body sectors invaded by the fungal mycelia (growth rate) were 40.8 and 33.4% respectively (Table 2). This particular inverse relationship was also significant at *B.t.i.* doses greater than the LC50 value. The observed infectivity rate in the combined treatment was less than the infectivity when the fungus was applied alone. Chi square test indicate that this antagonism was statistically significant.

Lagenidium applied as zoospores. Overall mortality 24 h after treatment was significantly higher in the combined treatments at all concentrations of *B.t.i.* applied than in the *Lagenidium* control (Table 3). When the combined treatments were compared with the *B.t.i.* controls, a significantly higher mortality was found in the combined treatments at *B.t.i.* doses of 11.4 and 22.8 ITU/100

TABLE 1
Percent mortality of *Culex quinquefasciatus* 3-day-old larvae after exposure to 20 mm^2 fungal mycelia of *Lagenidium giganteum* in combination with *B.t.i.*

B.t.i. (ITU/100 ml)	Mean percent mortality[a]	
	No fungus	Fungus present
	24 h postreatment[b]	
0.0	1.5 e	48.5 d
5.7	22.0 e	54.5 cd
11.4	59.5 c	71.0 bc
22.8	83.5 ab	88.5 ab
34.2	97.5 a	97.5 a
45.6	98.5 a	98.5 a
	48 h postreatment	
0.0	1.5 d	96.1 a
5.7	22.0 c	97.5 a
11.4	71.1 b	99.5 a
22.8	90.5 a	99.5 a
34.2	97.5 a	100.0 a
45.6	99.5 a	100.0 a

a. Means that are followed by the same letter within each time are not significantly different.
b. F = 53.15, df = 107; c. F = 169.37, df = 107; alpha = 0.05, Tukey's HSD.
Mean values are the result of 3 tests, each one had 3 cups/treatments and 20 larvae/cup.

TABLE 2
Percentage of 3-day-old *Culex quinquefasciatus* dead larvae infected with *Lagenidium giganteum* (infectivity rate) and relative ability of the fungus to develop in individual larvae (growth rate) after exposure to 20 mm^2 funal mycelia in combination with *B.t.i.*

B.t.i. dose (ITU/100 ml)	Mean infectivity rate (%) a,b	Mean growth rate (%) a,c
0.0	100.0 a	99.4 a
5.7	99.0 a	87.4 a
11.4	76.7 b	66.4 b
22.8	76.7 b	53.2 bc
34.2	55.6 c	40.8 cd
45.6	53.3 c	33.4 d

a. Mean within a column that are followed by the same letter are not significantly different. b. F = 21.08, df = 53; c. F = 34.55, df. = 53, alpha = 0.05, Tukey's HSD.
Mean values are the result of 3 tests, each one had 3 cups/treatment, and 20 larvae/cup.

TABLE 3
Percent mortality of *Culex quinquefasciatus* 3-day-old larvae when exposed to 1200 zoospores/ml of *Lagenidium giganteum* for 1 h in combination with *B.t.i.* for 1 h

B.t.i. (ITU/100 ml)	Mean Percent Mortality[a]	
	No fungus	Fungus present
	24 h postreatment c	
0.0	0.0 g	17.5 g
11.4	5.0 g	28.5 ef
22.8	8.5 g	32.5 ef
45.6	42.5 cde	37.5 de
68.4	56.0 bc	48.7 cd
91.2	81.0 a	66.2 ab
	48 h postreatment c	
0.0	0.0 d	75.0 b
11.4	7.5 d	82.5 ab
22.8	11.0 d	83.5 ab
45.6	58.5 c	82.5 ab
68.4	58.5 c	86.0 ab
91.2	85.0 ab	93.5 a

a. Means followed by the same letter within each time are not significantly different. b. F = 54.39, df = 43; c. F = 109.32, df = 43; alpha = 0.05, Tukey's HSD.
Means are based on 1 test that had 4 cups/treatment and 20 larvae/cup.

ml/1 h, but this difference was not found at higher levels of *B.t.i.* At the concentrations mentioned, 5% and 17.5% mortality was caused by *B.t.i.* and *Lagenidium* respectively; a combination of the two caused 28.5% mortality. Observed mortality from the combination of pathogens did not exceed those expected as additive effect. Forty-eight hours after exposure, the combined treatments with the highest *B.t.i.* dose and its *B.t.i.* control did not show significant difference in overall mortality, although the mortality observed was significantly higher than in the *Lagenidium* control. None of the other combined treatments showed differences with the *Lagenidium* control. The combined treatments that contained *B.t.i.* doses ranging between 11.4 and 68.4 ITU/100 ml did show significant higher mortality than their respective *B.t.i.* controls.

The activity of high dosages of *B.t.i.* is shown in the infectivity and growth rates that were found in the same experiment. These rates decreased as the *B.t.i.* concentration increased (Table 4). The observed infectivity rate did not exceed the expected values for additive interaction; however, an antagonistic effect was found for the variable growth rate.

Infection of 1-day-old larvae in the water samples taken 24 h after treatment was significantly higher in the combined treatments at all *B.t.i.* doses than in the *Lagenidium* control; however, the larvae exposed to water samples taken 48 h after treatment only showed a significant difference from the control at the *B.t.i.* dose of 91.2 ITU/100 ml (Table 5). At this level of *B.t.i.*, infection was reduced in comparison to the *Lagenidium* control and was also lower than in all other combined treatments of the pathogens, as it was seen previously (Table 4).

Effect of *B.t.i.* on *Lagenidium* persistence. Tukey's test was used to compare treatment means at each time after reflooding buckets. *B.t.i.* applied at a rate of 15.4 ITU/100 ml in two application schedules (single and daily) with *Lagenidium* did not show an adverse effect on the ability of the

TABLE 4
Percentage of *Culex quinquefasciatus* dead larvae infected with *Lagenidium giganteum* (infectivity rate) and relative ability of the fungus to develop in the larval body (growth rate) when exposed to 1200 zoospores/ml during 1 h in combination with *B.t.i.* for 1 h

B.t.i. dose (ITU/100 ml)	Mean infectivity rate (%) a,b	Mean growth rate (%) a,c
0.0	100.0 a	98.6 a
11.4	93.3 a	78.0 ab
22.8	100.0 a	91.4 a
45.6	100.0 a	90.0 a
68.4	73.3 b	63.4 b
91.2	56.7 c	38.6 c

a. Means within a column that are followed by the same letter are not significantly different. b. F = 29.77, df = 17; c. F = 21.36, df = 17; alpha = 0.05, Tukey's HSD. Mean values are the result of 1 test that had 4 cups/treatment and 20 larvae/cup.

TABLE 5
Percentage of *Culex quinquefasciatus* 1-day-old larvae infected with zoospores present in water samples from cups containing larvae treated with 1200 zoospores/ml of *Lagenidium giganteum* in combination with *B.t.i.*

	Mean % infected larvae at given times[a]	
B.t.i. (ITU/100/ml/1 h)	24 h post-treatment[b]	48 h post-treatment[b]
0.0	0.0 d	100.0 a
11.4	10.4 bcd	100.0 a
22.8	8.3 cd	100.0 a
45.6	22.9 b	100.0 a
68.4	14.6 bc	100.0 a
91.2	37.5 a	33.3 b

a. Means within a column that are followed by the same letter are not significantly different. b. F = 19.22, df 23; c F = 262.80, df 23; alpha = 0.05, Tukey's HSD. Mean values are the result of 1 test that had 48 wells/treatment and 1 larvae/well.

fungal pathogen to produce and maintain epizootic events during the time after reflooding that the experimental buckets were monitored (Table 6). However, there were significant differences between the combined treatments and the *B.t.i.* controls. No evidence was found for recycling of *B.t.i.* in any of the treatments.

TABLE 6
Mean number of *Culex quinquefasciatus* pupae produced during 15 days after drying and reflooding buckets of treatments containing various combinations of 20 mm^2/100 ml mycelia discs of *Lagenidium giganteum* and 15.4 ITU/100 ml of *B.t.i.*

Treatments		Mean number of pupae produced per bucket[a]				
		Days after reflooding buckets				
B.t.i. *applic*	Lagenidium applic	7 b	9 c	11 d	13 e	15 f
daily	once g	9.3 a	27.7 a	10.7 a	2.7 a	0.3 c
once	once	11.3 a	15.7 a	9.3 a	0.3 b	0.0 c
daily	–	12.7 a	23.3 a	19.7 a	11.0 a	19.0 a
once	–	10.7 a	21.3 a	14.0 a	8.3 ab	19.3 ab
–	once	2.7 a	8.3 a	4.0 a	0.0 b	0.0 c
–	–	3.7 a	22.3 a	15.7 a	11.7 a	22.7 a

a. Means within a column that are followed by the same letter are not significantly different.
b. $F = 2.10$, $df = 17$; c. $F = 1.97$, $df = 17$; d. $F = 1.86$, $df = 17$; e. $F = 6.83$, $df = 17$; f. $F = 223.81$, $df = 17$, alpha $= 0.05$, Tukey's HSD.
Mean values are the result of 1 test that had 3 buckets/treatment.

DISCUSSION

Lagenidium applied as mycelia. Charles and de Barjac (1981) and Lahkim-Tsror et al. (1983) demonstrated that mosquito larvae will die shortly after ingestion of a large dose of the *B.t.i.* delta-endotoxin; however, amounts as small as the lowest used in this experiment produced delay in the onset of death (Lacey and Lacey, 1981). Mortality measurements were increased 48 h after exposure to the pathogens when *Lagenidium* was applied with low dosages of *B.t.i.* Normally, this pathogenic fungus requires 24–48 h to fill the head, thorax, and anterior abdominal segments with mycelia and to kill mosquito larvae (Guzman and Axtell, pers. comm.). At high dosages of *B.t.i.* delta-endotoxin, fewer larvae were infected by the fungal zoospores and/or showed restricted mycelial growth within the cadavers. Recently, it has been found that *B.t.i.* endospores can germinate and produce vegetative growth in the cadavers of the mosquito larvae (Aly, 1985). Thus, mycelial growth could be diminished by a depletion of the nutrients; also, opportunistic microorganism infections could occur, consuming the tissues in the moribund larvae (Aly et al., 1985).

It has been demonstrated that the main targets of the *B.t.i.* delta endotoxin are the gastric coeca and the midgut (Charles and de Barjac), 1981; Lahkim-Tsror et al., 1983); and under normal conditions, the main portal of entry of *Lagenidium* zoospores to the mosquito larvae is the peribuccal area (Domnas, 1981; Koethe, 1982). However, in the experiments in which SFE-agar discs containing *Lagenidium* mycelia or zoospores were tested against several concentrations of *B.t.i.*, a clear relationship regarding the mode of action of these pathogens within a particular *B.t.i.* concentration was not apparent. It is possible that with the *Lagenidium* doses used, encystment of the zoospores occurred at the same level in each *B.t.i.* concentration applied. However, differences were observed in relation to the *B.t.i.* concentration gradient as discussed before.

When the *Cx. quinquefasciatus* 3-day-old larvae were exposed to *B.t.i.* after previous exposure to *Lagenidium* zoospores, the overall mortality pattern was not different from a previous experiment in which exposure to the infective stage of the pathogenic fungus was achieved 4–8 h after the *B.t.i.* delta endotoxin was applied to the bioassay cups. Although higher infectivity rates were obtained, the shortest exposure times to the pathogens produced lower overall mortality. However, the reduced infectivity and extension rates found in the treatments in which *B.t.i.* was applied at rates 68.4 and

91.2 ITU/100 ml resulted in a faster zoosporogenesis (24 h after exposure), but the amount of zoospores produced was lower as indicated by the lower transmission of the mycosis. This information is not surprising since Guzman and Axtell (pers. comm.) found the zoospore production (time and number of zoospores produced) from larval cadavers is proportional to the size of the larvae. In the present study, variation in fungal infectivity and extention rates in the larval carcasses due to the action of the *B.t.i.* delta-endotoxin could be equivalent to the variation due to larval size.

Mortality was higher when mosquito larvae were exposed to a combination of *Lagenidium* mycelia or zoospores and *B.t.i.* that when exposed to either pathogen alone. The increase in mortality observed when the pathogens were applied simultaneously was similar to the change observed when the pathogens were applied sequentially. This increased mortality appeared to be an additive effect of the two pathogens; similar results were observed when Richter and Fuza (1984) studied the combined effects of *Anticarsia gemnatalis* nuclear polyhedrosis virus and *B.t.* in *A. gemnatalis* larvae.

The method of application of the pathogens affected the nature of the interaction when infectivity rate was measured. The interaction *B.t.i.-Lagenidium* was antagonistic when the fungus was applied as mycelia, but additive when applied as zoospores. This change in response might be due to the timing of zoosporogenesis when the fungus was applied as mycelia.

When zoospores were formed 4–8 h after larvae were exposed to the *B.t.i.*, some larvae had already been killed by the bacterial toxin, therefore prevented the zoospores from infecting the host. However, when larvae were first exposed to the fungal zoospores and then to *B.t.i.*, there was no evidence for antagonism in the infectivity rate, and results were consistent with an additive interaction between pathogens. For growth rate the interaction was antagonistic for both methods of application of the pathogens. Although *Lagenidium* could infect mosquito larvae, the extent of this infection was probably reduced by the rapid death of the larvae. It is also possible that germination of *B.t.i.* spores and subsequent vegetative growth and/or presence of secondary invaders could consume and deplete nutrients in the moribund larvae (Aly, 1985; Aly, et al., 1985).

Production of epizootic waves by *Lagenidium* was not inhibited after short term simulated drought and when *B.t.i.* delta-endotoxin was used in two different application schedules. However, total control of pupal production was achieved two days earlier in the treatments that contained a single application of *B.t.i.* and in the *Lagenidium* control than in the treatments that were simulating a slow release application of *B.t.i.* The simulated sustained-release of *B.t.i.* delta-endotoxin in the aquatic environment could permit active competition for the host and reduce the mycelial growth within larval bodies, as was seen earlier. Sustained released of the toxin could also inhibit partially or delay zoosporogenesis as it was demonstrated by Lord and Roberts (1985).

Aly (1985) observed germination of the *B.t.i.* endospore and vegetative growth in the gut of *Ae. aegypti* and *Ae. vexans* larvae. Aly et al. (1985) found that *B.t.i.* can grow, sporulate, and produce toxin in cadavers of mosquito larvae. However, the *B.t.i.* control treatments did not reveal *B.t.i.* delta-endotoxin activity during the days that the experiment was monitored. Perhaps in the conditions used in this experiment toxin production was not sufficiently high and/or the presence of scavenger organism or secondary invaders destroyed the cadavers before the toxin was formed (Aly et al., 1985). Also this study failed to detect residual activity of *B.t.i.*

CONCLUSION

In the present study it was found that under the conditions tested, *B.t.i.* does not affect in a detrimental fashion the ability of the mosquito fungal pathogen *Lagenidium giganteum* to produce and maintain epizootics. Furthermore, it was found that in certain cases, the co-invasion of the mosquito larval body by *B.t.i.* and *Lagenidium* triggers a faster zoosporogenesis; however, the fungal infectivity obtained from treatments containing high dosages of *B.t.i.* was lower.

Due to the fast mode of action of *B.t.i.* delta-endotoxin, the number of dead larvae infected with

the fungus and the *Lagenidium* mycelial growth within the larval body showed an inverse relationship with the *B.t.i.* concentration.

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