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Analysis of larval antigens of *Cephalopina titillator* in the camel mucus for diagnosis of infestation

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Abstract: Serodiagnostic test for the diagnosis of infestation of camels by the camel nasal fly, *Cephalopina titillator* was examined. The enzyme-linked immunosorbent assay (ELISA) technique was used to analyze and compare the production of antibodies in the camel mucus against salivary gland contents (SGc), excretory-secretory products (ESP), digestive tract contents (DTc) antigens from 2nd and 3rd larval instars of *C. titillator* sampled in summer and winter. ELISA characterization of larval antigens showed that the presence of *C. titillator* larvae in camel, *Camelus dromedarius*, resulted in higher antibody titer in mucus samples of infested camels collected in winter than those collected in summer in both L2 and L3. In L2, the most antigenic secretion was SGc > ESP > DTc, but in L3 ESP > SGc > DTc. The antibody titers in camel mucus are affected by the intensity of infestation with *C. titillator.*

Key words: *Cephalopina titillator*; salivary gland contents; excretory secretory products; digestive tract contents; ELISA; camel mucus.

Introduction

The dromedary camel, *Camelus dromedarius* L., 1758 is an important species of livestock in arid and semi-arid environments (Shakerian et al. 2011). Nasopharyngeal myasis in camels is caused by the dipterous obligate parasitic species, *Cephalopina titillator* (Clark, 1816) (Family Oestridae) (Zumpt 1965; Hall & Wall 1995). The female fly darts towards the nostrils and deposits its larvae directly into the nasal cavity. The larvae crawl up to the nasopharynx and sometimes to the paranasal sinuses and molt twice while attached to the nasopharyngeal and paranasal mucus membrane of these organs for up to 11 months. During this period, they feed on mucosal debris and cause extensive irritation and tissue damage (Oryan et al. 2008; Shakerian et al. 2011).

Oestrids display complex host/parasite relationships. Their hosts also develop numerous but often ineffective strategies of expelling them by mucus hypersecretion and nasal discharge (Dorchies et al. 2006). The antigenic and some inflammatory products produced by larvae induce inflammatory and hypersensitive reactions including hypereosinophilia and mastocytosis (Angulo-Valadez et al. 2010).

Excretory secretory products (ESP) mainly from the salivary glands, and digestive tract contents (DTc) were found to be particularly interesting to study and investigate their diagnostic and protective value (Tabouret et al. 2001). The interaction between *C. titillator* developing larvae and host's immune system are still poorly investigated. Hence, a study concerning serological diagnosis of infestation in living animal mucus was carried out.

This study was aimed to (i) characterize different *C. titillator* larval antigens including digestive tract contents (DTc), excretory secretory products (ESP) and salivary glands contents (SGc) antigens of 2nd and 3rd instars, (ii) explore the usage of anti-*C. titillator* antibodies detection in camels mucus by ELISA in winter and summer in diagnosis of myiasis in further studies.

Material and methods

Collection of larvae

Cephalopina titillator larvae were recovered from naturally infested camel heads. The heads were collected from the local slaughterhouse of Elwaraq (Giza, Egypt) and split longitudinally. Second (L2) and 3rd (L3) instar larvae were washed several times in phosphate buffer saline (PBS).

Parasite proteins (antigens) preparation

Preparation of larval excretory secretory products (ESP)

Based on the method described by Tabouret et al. (2001b), a pool of 20 larvae of each L2 and L3 were washed with phosphate buffer saline (PBS), and incubated in sterile PBS (500 µl/larvae) for 24 h at room temperature in the dark. The solution collected was centrifuged at 10,000 rpm, and filtered through 0.8/0.2 µm sieves. Then, the ESP solution was lyophilized for more concentrated protein samples.

Preparation of digestive tract and salivary gland contents
Twenty larvae of each L2 and L3 were dissected in ice cold PBS (4–10 °C). The whole digestive tracts (DT) and the salivary glands (SG) were collected and centrifuged at 10,000 rpm at 4 °C to expel their contents (DTc and SGc). Then the supernatants were taken and stored at –70 °C until use.

Protein concentration measurement
Protein concentrations in ESP, SGc and DTc were determined spectrophotometrically according to the method described by Bradford (1976), using Bovine serum albumin as standard, and Coomasssie Brilliant blue (COBB) dye as a protein reagent. The optical density of the protein sample was measured at 595 nm.

Mucus sampling
Mucus samples were collected using sterile cotton swabs from the nasal cavity of 36 and 53 slaughtered camels in winter and summer, respectively, at the abattoir of Elwaraq (Giza, Egypt). The collected samples were divided according to infestation intensity as follows: control (non-infested), low (1–10 larvae), medium (11–20 larvae) and heavy infested (more than 20 larvae). Each group contained 8–10 samples in winter and 10–15 in summer. All the swabs were then gently agitated in PBS for two hours at room temperature, then centrifuged at 3,000 rpm for 5 min, and stored frozen at –70 °C until used.

Enzyme-linked immunosorbent assay (ELISA)
An indirect ELISA was used as follows. Briefly, each well of a flat-bottom microtiter plate (Nunclon Distr. VWR International, Strasbourg, France) was coated with 100 µl of ESP, DTc or SGc at 20 µg ml^{-1} in sodium carbonate buffer (pH 9.6) for 2 h at 37 °C. The plates were washed three times in PBS, pH 7.2 containing 0.05% Tween 20 (PBS-T). To minimize non-specific binding of the antibody, the plates were incubated for 2 h, at room temperature with 200 µl of PBS-T with 2% bovine serum albumin powder (PBS-TBSA). Subsequently the plates were washed three times with PBS-T before adding 100 µl of mucus preparation (diluted 1 : 25 in PBS) to each well for 1 h at 37 °C (all mucus samples collected at necropsy date). After washing, 100 µl of protein A- peroxidase (HPR) from *Staphylococcus aureus* horse radish (1 : 80,000) in PBS-T, (Sigma Aldrish, Germany) was added in each wkell and incubated for 30 min at 37 °C. Then 100 µl of the HRP substrate (*O*-phenylene diamine 0.4 mg ml^{-1}) was added to each well, and incubated for 30 min at 37 °C. Plates were washed another three times in PBS-T, then 50 µl of stopping buffer was added into each well. Optical densities were recorded at 450 nm, using Dynatech microplate reader (Labsystem, Wynooski, Vermont, USA).

Statistical analysis
To evaluate the effect of intensity of infestation, antigenic tissue contents and season on antibody concentration in camel mucus samples the data were statistically analyzed using a three-way analysis of variance (ANOVA), and the Tukey's HSD test ($P \leq 0.05$). Statistical analysis was performed using SPSS (SPSS Statistics 2008). To evaluate the effect of season (winter and summer) (independent variable) on antibody titers in camel mucus samples, the student *t*-test ($P \leq 0.05$) (dependent variable) was used.

Results

ELISA analysis
The specific antibody titer in mucus of camels infested with *C. titillator* was determined using ELISA, coated with antigens from salivary gland contents (SGc), excretory-secretory products (ESP) or digestive tract contents (DTc) of the 2nd and 3rd larval instars (L2 and L3). Seasonal factor (winter and summer), and intensity of infestation were considered.

Effect of seasonal factor on antibody titer
Antibody titers in camel mucus against larval SGc
SGc of the 2nd instar larvae as a coating antigen (Fig. 1) showed a significant increase ($P \leq 0.05$) in antibody

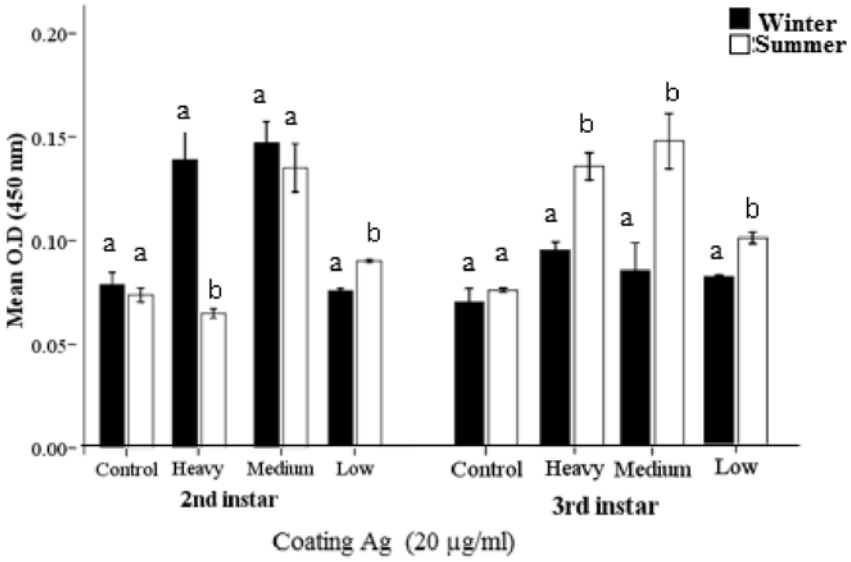

Fig. 1. Camel mucus antibodies in different seasons (winter and summer) using salivary glands content (SGc) of 2nd and 3rd instar *C. titillator* larvae as coating antigen. Different letters (a, b) within each concentration indicate significant change between different seasons (*t*-test, $P \leq 0.05$).

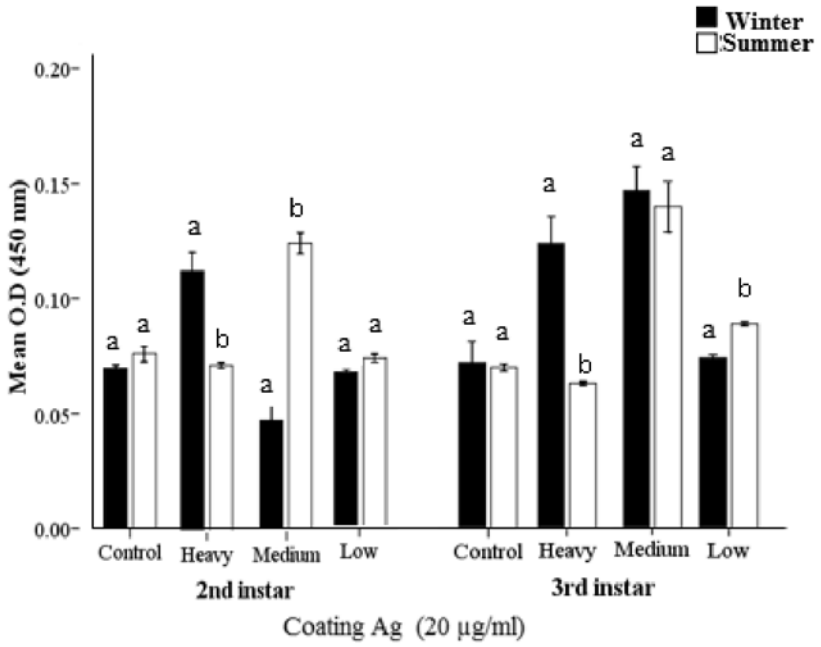

Fig. 2. Camel mucus antibodies in different seasons (winter and summer) using excretory secretory products (ESP) of 2nd and 3rd instar *C. titillator* larvae as coating antigen. Different letters (a, b) within each concentration indicate significant change between different seasons (*t*-test, $P \leq 0.05$).

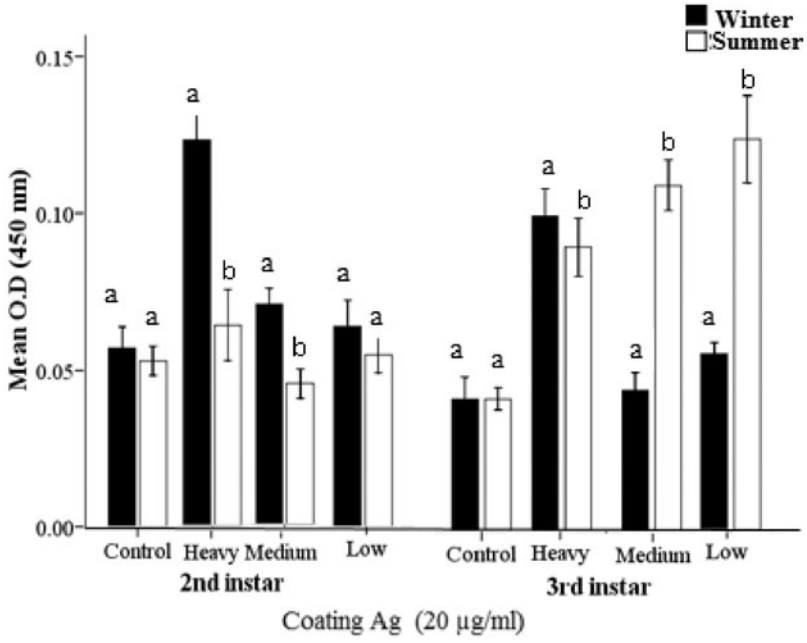

Fig. 3. Camel mucus antibodies in different seasons (winter and summer) using digestive tract content (DTc) of 2nd and 3rd instar *C. titillator* larvae as coating antigen. Different letters (a, b) within each concentration indicate significant change between different seasons (*t*-test, $P \leq 0.05$).

titer in the camel mucus at heavy and medium infestation in both winter and summer in comparison to control. The antibody titer at heavy infestation was higher in winter than in summer ($P \leq 0.05$). In SGc of 3rd instar larvae at different intensity of infestations, significantly higher antibody titers were measured in summer than in winter (Fig. 1).

Antibody titers in camel mucus against larval ESP

Significantly, higher antibody titers ($P \leq 0.05$) in the camel mucus in response to ESP of 2nd instar larval antigens at heavy infestation were revealed in winter than in summer (Fig. 2), while medium infestation resulted in a higher antibody titer in summer than in winter. In ESP of 3rd instar larvae (Fig. 2), at heavy

Table 1. ELISA antibody titers in camel mucus derived from animals with varying extent of infestation during two seasons and using three 2nd instar *C. titillator* larvae antigen sources.

Larval	Winter				Summer			
	Control	Heavy infestation	Medium infestation	Low infestation	Control	Heavy infestation	Medium infestation	Low infestation
SGc	0.079±0.005a A	0.139±0.015a B	0.147±0.009a B	0.075±0.001a A	0.074±0.003a AB	0.065±0.002a A	0.135±0.01a C	0.089±0.0007a B
ESP	0.069±0.002ab B	0.113±0.007a C	0.047±0.006b A	0.067±0.001a AB	0.076±0.003a A	0.072±0.001a A	0.124±0.004a B	0.073±0.0015b A
DTc	0.057±0.006b A	0.123±0.01a B	0.07±0.0045b A	0.064±0.007a A	0.053±0.004b AB	0.064±0.0097a B	0.045±0.004b AB	0.055±0.005c A

Explanations: Data are presented as mean ± SE. Means followed by different small letters within each column are significantly different (ANOVA, Tukey test, $P \leq 0.05$). Means followed by different capital letters within each raw in winter and within each raw in summer are significantly different (ANOVA, Tukey test, $P \leq 0.05$).

Table 2. ELISA antibody titers in camel mucus derived from animals with varying extent of infestation during two seasons and using three 3rd instar *C. titillator* larvae antigen sources.

Larval	Winter				Summer			
	Control	Heavy infestation	Medium infestation	Low infestation	Control	Heavy infestation	Medium infestation	Low infestation
SGc	0.06±0.005ab A	0.082±0.003a A	0.073±0.01a A	0.07±0.0005a A	0.065±0.0008a A	0.117±0.005a B	0.127±0.01a B	0.086±0.002a A
ESP	0.072±0.008a A	0.124±0.01b B	0.147±0.009b B	0.074±0.0012a A	0.07±0.0013a A	0.063±0.0007b A	0.14±0.0095a B	0.089±0.0007a C
DTc	0.042±0.006b A	0.1±0.0075ab B	0.045±0.005a A	0.056±0.003b A	0.042±0.003b A	0.09±0.008c B	0.11±0.007a BC	0.124±0.012b C

Explanations: Data are presented as mean ±SE. Means followed by different small letters within each column are significantly different (ANOVA, Tukey test, $P \leq 0.05$). Means followed by different capital letters within each raw in winter and within each raw in summer are significantly different (ANOVA, Tukey test, $P \leq 0.05$).

infestation, significantly higher antibody titer was measured in winter than in summer, but at medium infestation antibody titers were high and similar in both winter and summer.

Antibody titers in camel mucus against 2nd larval DTc
By using DTc of 2nd instar larvae as a coating antigen (Fig. 3), a significant increase of camel mucus antibody titer was measured ($P \leq 0.05$) in winter in comparison to summer at heavy and medium infestation. In DTc of 3rd instar larvae, camel mucus antibody titer increased in response to DTc in winter and summer by the same range, but in cases of medium and low infestation, antibody titer was significantly higher in summer than in winter (Fig. 3).

Effect of antigenic tissue contents factor on antibody titer
The potential application of *C. titillator* larvae SGc, ESP and DTc as antigens in serological assays was analyzed by ELISA. By using tissue contents of 2nd instar larvae (Table 1) and of 3rd instar larvae (Table 2), different antibody titers were produced in camel mucus in winter and summer.

Antibody titer in camel mucus against 2nd instar larvae SGc, ESP and DTc in winter and summer
In winter, ELISA detection of camel mucus antibodies by using SGc, ESP and DTc of 2nd instar larvae as antigens (Table 1) revealed that the SGc had the highest antigenic effect ($P \leq 0.05$) at medium infestation. Camel mucus antibody titers against SGc, ESP and DTc were higher in case of heavy infestation. The antibody titers in camel mucus sampled in summer against SGc, ESP were not significantly different (Table 2). At medium and low infestation, SGc was the most antigenic tissue, followed by ESP and DTc.

Antibody titer in camel mucus against 3rd instar larvae SGc, ESP and DTc in winter and summer
Mucus antibodies using SGc, ESP and DTc of 3rd instar larvae as coating antigens (Table 2) revealed, in contrast to 2nd instar larvae, that ESP was the most antigenic secretion in all cases of infestation in winter. In summer, no significant changes were observed between different tissue products, except in case of heavy infestation, the camel mucus antibody titer against SGc was significantly higher than against ESP (Table 2).

Effect of infestation intensity by L2 and L3 larval stages on the antibody titer
Antibody titer in camel mucus against SGc of L2 and L3 in winter and summer
In winter, the antibody titers against 2nd instar larvae SGc antigen were significantly higher at heavy and medium infestation than in control and at low infes-

tation (Tables 2, 3). In summer, antibody titers were significantly higher at medium infestation than at the other intensities of infestation ($P \leq 0.05$).

Antibody titer in camel mucus against ESP of L2 and L3 in winter and summer

Mucus antibody titers against ESP of 2nd instar larvae at heavy infestation in winter and at medium infestation in summer were significantly higher than in the other cases of infestation (Table 2). Camel mucus antibody titer against ESP of 3rd instar larvae (Table 3) at medium infestation was the highest in both seasons ($P \leq 0.05$).

Antibody titer in camel mucus against DTc of L2 and L3 in winter and summer

Camel mucus antibody titers, using DTc of 2nd and 3rd instar larvae as coating antigens (Tables 2, 3), increased in winter non-significantly at different intensities of infestation. In summer, the antibody titers increased significantly at all intensities of infestation, even at low infestation in case of DTc of L3.

Discussion

ELISA technique was used to demonstrate the effects of different factors, such as season, antigenic tissue contents and the intensity of infestation of camels by L2 and L3 of *C. titillator,* on antibody production in camel mucus. Previously, ELISA technique was used to help in the diagnosis of camel infestation by *C. titillator* and *Rhinoestrus* spp. (Hendawy et al. 2013; Hassan 2014).

Previous studies on oestrosis or other myiasis demonstrated that contents of secretory organs of insects causing myiasis might provide target antigens useful for either diagnosis or protection of animals (Pruett et al. 1988; Innocenti et al. 1995, 1997; Frugere et al. 2000). Particularly, systemic IgG antibody production against *Oestrus ovis* (L., 1758) larval antigens has been used for disease diagnosis by ELISA (Suárez et al. 2005; Alcaide et al. 2005; Shakerian et al. 2011).

In winter, higher antibody titer was detected in camel mucus against ESP of 2nd instar larvae at high and medium infestation with *C. titillator*, while in summer, higher antibody titer was determined against the larvae SGc at high and medium infestation. Angulo-Valadez et al. (2008, 2010) found negative correlations among *O. ovis* larval establishment and/or larval development as well as intensity of local and systemic IgG responses in naturally infected ewes, depending on the season. The development of oestrids is closely related to season and the number of annual cycles depends on climatic conditions (Tabouret et al. 2001a; Angulo-Valadez et al. 2010). Angulo-Valadez et al. (2010) found that temperature above 12 °C induced activation of oestrid larvae and their feeding behaviour, which begins and reaches the optimum at 22–39 °C (L2 and L3 larvae are located under a relatively steady temperature and their development is continuous). For all species of oestrids, mating and seeking activities occur mainly during warm and sunny days and not windy days, at temperatures between 20 and 30 °C (Cepeda-Palacios et al. 1998).

In the present work, the analysis of antibody titers in camel mucus samples against different tissue antigens (SGc, ESP and DTc) of 2nd instar larvae of *C. titillator* showed that SGc were the most antigenic secretory products, followed by ESP and DTc. These results were relatively similar to those observed by Hendawy et al. (2013) for *C. titillator,* and for *O. ovis* (Innocenti et al. 1995; Tabouret et al. 2001b). The variations in seroprevalences demonstrated by different larval antigens may be attributed to the differences in their immunogenic properties (Hendawy et al. 2013), e.g., the mucus and serum immunoglobulins reacted weakly with *O. ovis* gut soluble proteins (Tabouret et al. 2001b, 2003). Tabouret et al. (2001a) reported that in case of *O. ovis* infections, local IgG and IgA recognize mainly salivary gland antigens whose functions are not known.

Millillo et al. (2010) and Hassan (2014) stated that the salivary glands of *Rhinoestrus* spp. larval stages induced intense antibody response compared to that stimulated against whole larval extracts and that they contain the major immunogens in horse nasal bot.

Abd-El-Meguid et al. (2013) investigated the ultrastructure of salivary glands of 2nd larval stage of *C. titillator* and found that the lumen of the cells were fully packed with secretory vacuoles extruded from the epithelial cells. ELISA showed that SGc, ESP and DT of *C. titillator* larvae could be considered as immunogenic antigens for investigation of myiasis. Nassar & Youssef (1991) found that crude extracts of 2nd instar larvae of *C. titillator* obtained from the head of camels were adopted as a reliable immunogenic antigen for diagnosis of myiasis in naturally infested camels. Also hemolymph, fat body, midgut, Malpighian tubules, salivary glands (SGc), cuticle, excretory secretory products (ESP) and larval secretory products (LSP) of L1, L2, L3 collected from *O. ovis*, *C. titillator* and *Rhinoestrus* spp. at 37 °C for either 24 h or 48 h, were used as antigens in diagnosis of myiasis during anti-mortem stage (Innocenti et al. 1995, 1997; Tabouret et al. 2001, 2003; Suárez et al. 2005; Milillo et al. 2010; Hendawy et al. 2013).

The present work revealed that the stimulation of the immune response in camels was related to the intensity of infestation and the severities of lesions were mainly related to the presence of early L2 and early L3 larval stadia of *C. titillator*. According to Tabouret et al. (2001a), ELISA data correlated with L2 and L3 capacity to produce higher quantities of antigenic proteins related to the increased size of larval secretory organs. In addition, it was found that pathological damages in the infected animals were more severe in sinus cavities and were associated with the presence of L2 and L3 larvae (Tabouret et al. 2003).

The camel mucus antibody titer against ESP of L3 of *C. titillator* at medium infestation level was higher than at heavy infestation in both seasons ($P \leq 0.05$). This may be due to decrease of feeding activity and

development of L3 due to crowding in case of heavy infestation, but in case of medium infestation L3 increase nutrient intake and feeding activity, resulting in increase of both production of ESP and antibody response.

According to Innocenti et al. (1997) proteins derived from the larval cuticle are capable to stimulate the host immune system, therefore shedding of old cuticle and the new cuticle should be considered in the pathological effect that may be associated with larval molting. This process may lead to the strongest immune stimulation provoked during or immediately after larval molt.

Conclusion

The results of this study showed that by using ELISA technique antibody titers against both L2 and L3 antigens of *C. titillator* measured in camel mucus were higher in winter than in summer. L2, the most antigenic tissue was SGc > ESP > DTc, while in L3 it was ESP > SGc > DTc. Antibody titers in mucus increased as L3 numbers increased. Further studies will be carried out to produce a suitable technique that will help to diagnose the infestation of camels with *C. titillator*.

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