Global Veterinaria 10 (2): 158-164, 2013
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2013.10.2.71161

Immunological Evaluation of the Diagnostic Values of *Cephalopina titillator* Different Larval Antigens in Camels from Egypt

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Abstract: *Cephalopina titillator* larvae (Diptera: Oestridae) are considered an important nasal myiasis-producing agent of camels. Developing of an indirect ELISA as a monitoring assay for anti-*C. titillator* IgG detection in the living animals using different prepared *C. titillator* antigens (L3CE, L2CE, DTc, ESP and SGc) was the aim of the current study. Only 2nd and 3rd instars of *C. titillator* larvae were collected from nasopharyngeal cavities of slaughtered camels in Egypt. The apparent prevalence of infestation among camels using ELISA differed according to the used antigen. The higher reported values were 89.66% and 79.31% with L2CE and SGc antigens, respectively. However, low prevalence percentages; 53.45%, 46.55% and 13.22% were recorded with ESP, DTc and L3CE antigens, respectively. Results of L2CE and SGc antigens indicated that both induced the highest specificity and positive predictive value reported 100% for each. However, the recorded sensitivity and negative predictive value were (51.72% and 53.57%) and (6.66% and 13.33%), respectively. Moreover, DTc and ESP antigens showed 52.38%, 55.55%, 33.33% and 73.3% and 90.90%, 73.68%, 93.33% and 66.66% for specificity, sensitivity, positive predictive value and negative predictive value, respectively. On the other hand, L3CE antigen indicated the highest sensitivity and negative predictive value (100%) for each, while the lowest positive predictive value was 26.66% and specificity was 57.6%. In conclusion, the present study was considered a preliminary investigation of different ELISA measures; sensitivity, specificity, positive predictive value and negative predictive value of the different *C. titillator* prepared antigens used in diagnosis of *C. titillator* infestations among camels. The variations in seroprevalences demonstrated by different antigens may be attributed to differences in the immunogenic properties. The present study considered the salivary glands content antigen, the most immunogenic that had the capability to elicit IgG response against *C. titillator* larvae.

Key ward: Oestridae · Nasal Myiasis · *Cephalopina titillator* · ELISA · Camels · Egypt

INTRODUCTION

Cephalopina titillator (Diptera: Oestridae) is a common obligate parasitic-induced myiasis agent of domestic camel [1] causing economic losses in Egypt [2-5]. Larvae of this parasite infest the nasopharyngeal cavities of camels where they spend twelve months [4]. Through this time, the first larvae (L1), subsequently, molted to 2nd (L2) then 3rd (L3) larval instars. When L3 are fully developed they are expelled through the nasal openings to pupate on the ground [6]. The intensity of clinical signs depends on degrees of damage caused by migrating larvae. Unfortunately, interaction between *C. titillator* developing larvae and host's immune system are still poorly investigated, hence, little contributions were done concerning serological diagnosis of infestation in living animals [7]. Although, infestation seems to be less pathogenic and cause unspecific clinical signs to the

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host, nasal bot larvae induce significant morbidity, affect animal growth and greatly stimulate cellular and humeral immune responses because of the long period during which they interact with the host's immune system [7, 8]. The immune response to myiasis-causing larvae is related to animal health, larval biology, site of parasitism, nature of the antigen and specific immunological defense mechanisms triggered by designated larvae [9].

Traditional serological/immunological methods used for detection of immune response to many myiasis causing larvae have been developed as an alternative way to clinical parasitological examination in living animals [10-12, 21]. Crude 2nd larval instars of *C. titillator* were adopted as a reliable immunogenic antigen for diagnosis of such myiasis, obtained from heads of naturally infested camels [10-12]. Later on, the L1, L2, L3, haemolymph, tissues as fat body, mid gut, Malpighian tubules, salivary glands (SGc), cuticle, excretory secretory products (ESP) and larval secretory products (LSP) collected at 37°C for either 24hr or 48hr were used in diagnosis during anti-mortem stage [2, 13-20]. However, there are little available literatures on *C. titillator* serological diagnosis which indicated that the incidence of positive reactors was higher than that recorded by detection of larvae at post-mortem examination [3, 22]. Therefore, the present study attempted to evaluate the different *C. titillator* larval antigens including 3rd larval crude extract (L3CE), 2nd larval crude extract (L2CE), digestive tubule contents (DTc), excretory secretary products (ESP) and salivary glands contents (SGc) antigens, in anti-*C. titillator* antibodies detection in camels' sera by an indirect ELISA to determine the most informative antigen to be used in diagnosis of such myiasis.

MATERIALS AND METHODS

Animals: The present study was carried on two hundred fifty camels of both Egyptian and Sudanese breeds at El-Bassatin and El-Warrak abattoir, Egypt. Camels were in age range from 2.5 to7 years and samples were collected during different seasons of the years 2009-2010. Animals were examined to detect any clinical signs usually accompanying nasal bot myiasis-infestation and/or the expel of the larvae from nostrils.

***C. titillator* Larval Instars:** Different instars of *C. titillator* larvae were collected from nasopharyngeal cavities of camels after their being slaughtered [23]. The collected larvae were immediately washed several times with distilled water then 10mM PBS buffer (10mM di-sodium hydrogen phosphate, 137mM sodium chloride, 2mM potassium di-hydrogen phosphate and 2.7mM potassium chloride, pH 7.2, Sigma-Aldrich) containing antibiotics (penicillin 100IU/ml and streptomycin 100µg/ml, Invitrogen) and finally stored at -20°C till used.

Preparation of Blood Samples: Blood samples were collected from camels in clean tubes, centrifuged at 4000rpm for 15min (Shanghai Surgical Instruments, 80-2 Centrifuge) for serum separation. Serum samples were kept frozen at -20°C till used. Serum samples were identified as:

Parasitologically Infested Camels' Sera (PIS) with *C. titillator*: Fifteen blood samples were collected from camels where their nasopharyngeal cavities found infested with *C. titillator* at post-mortem inspection.

Parasitologically Non-Infested Camels' Sera (PNIS) with *C. titillator*: Fifteen blood samples were collected from camels where their nasopharyngeal cavities proved to be free from infestation with *C. titillator* larvae at post-mortem inspection.

Randomly Collected Camels' Sera: One hundred and forty-four blood samples were randomly collected at time of slaughtering of camels.

Control Negative Camels' Sera: Six blood samples were collected from young camels less than three months of age; proposed to be *C. titillator*-free, hence, were born in winter and before spring and/or summer seasons when the fly is detected.

Preparation of *C. titllator* Larval Antigens: Four different antigen preparations from *C. titillator* larvae were obtained according to procedures described by Tabouret *et al*. [16] with some modifications.

Excretory Secretory Products (ESP) Antigen: Larvae were incubated in PBS (1larvae/1ml, pH 7.2) containing antibiotics over night at 25°C in a dark bottle. The supernatant was clarified at 13000rpm for 20min. in cooling centrifuge (Labofuge- 400R, Heraeus, Germany) then collected, divided into aliquots and stored at -80°C till used.

Second and Third Larval Crude (L2CE and L3CE) Antigens: L2 and L3 were separately homogenized in PBS (0.25gm/1ml, pH 7.2). Each homogenate was centrifuged

at 13000rpm for 20min. in cooling centrifuge, then supernatants were collected, divided into aliquots and stored at -80°C until use.

Salivary Glands [SGc] and Digestive Tubules [DTc] (Organs Content) Antigens: Several larvae (L2 and L3) were dissected in cold PBS under a dissecting microscope where salivary glands (SG) as well as whole digestive tubules (DT) were collected separately then centrifuged at 13000rpm for 20min in cooling centrifuge, then supernatants were collected, divided into aliquots and stored at -80°C till used.

Preparation of Rabbit Hyperimmune Sera: Ten healthy white New Zealand males' rabbits weighed 1.5-2 kg each, were immunized (2 rabbits/antigen) with the different *C. titillator* antigens; L3CE, L2CE, DTc, ESP and SGc, in order to develop specific polyclonal hyperimmune serum against each of them according to procedures reported by Innocenti and coworkers [13]. Before the start, 3ml whole blood were collected from each rabbit to obtain the control negative serum for these injections (Neg-HIS) which was kept at -20°C till used. Later on at day 30, rabbit sera were tested by agar gel precipitation test (AGPT) to estimate the released immunoglobulins semi-quantitatively [24]. When the results were strongly positive, the rabbits were slaughtered and hyperimmune serum samples were individually collected, into aliquots and stored as rabbit-anti *C. titillator* L3CE (HIS-L3CE), rabbit-anti *C. titillator* L2CE (HIS-L2CE), rabbit-anti *C. titillator* DTc (HIS-DTc), rabbit-anti *C. titillator* ESP (HIS-ESP) and rabbit-anti *C. titillator* SGc (HIS-SGc) at -20°C until used.

Evaluation of Immunological Activities of *C. titillator* Antigens by Indirect Enzyme Linked Immunosorbant Assay (ELISA): Procedures were done according to the modified technique of standard indirect solid phase ELISA that was reported by Allam [25]. Where carbonate-bicarbonate pH 9.6 buffer (Sigma-Aldrich) was used as coating buffer, 10% non-fat dry milk (NFDM) in PBS pH 7.4 (Sigma-Aldrich) was used as blocking buffer, 0.5% NFDM in PBS pH 7.4 was used as dilution buffer and 5% sodium dodecyl sulphate (Sigma-Aldrich) was used as stopping buffer. Optimum antigen concentration, serum and conjugate dilutions were determined by checker board titration [25]. Antigens coating was by 100ml/well at dilutions 1:25, 1:50 and 1:100 for L2CE, ESP and L3CE, DTc and SGc antigens, respectively. While randomly collected PIS and PNIS sera were diluted 1:50, 1:25, 1:400 and 1:100 specifically for each of the following antigens L3CE, L2CE, DTc and ESP and SGc, respectively in dilution buffer and applied as 100ml/well. Peroxidase conjugated protein-A (Sigma-Aldrich) at dilution 1:1000 was used as anti-species and tablets of substrate solution (one tablet containing 20mg/ml O-phenylenediamine, Fluka, Biochemika) was dissolved in 50ml substrate buffer (0.1M citric acid and 0.2M di-sodium hydrogen phosphate, pH 5 and 25µl 30% H_2O_2, Sigma Aldrich). Finally, the optical densities (OD) were read at 450nm (ELISA reader; BIO-TEK, INC. ELx, 800UV). The cut-off value based up on 20% of the positive control serum was selected to discriminate between positive and negative tested samples [26].

The percentage of antibodies was calculated for each serum samples by comparison with positive and negative control sera. Additionally, the sensitivity, specificity, positive predictive value, negative predictive value and apparent prevalence were calculated for each antigen as mentioned elsewhere [16, 26].

$$\text{Antibodies}(\%) = \frac{\text{OD}(\text{serum sample}) - \text{OD}(\text{negative control})}{\text{OD}(\text{positive control}) - \text{OD}(\text{negative control})} \times 100$$

$$\text{Apparent prevalence}(\%) = \frac{\text{Total of positive}}{\text{Total number of animals}} \times 100$$

$$\text{Sensitive}(\%) = \frac{\text{True positive}}{\text{Ture positive + False negative}} \times 100$$

$$\text{Specificity}(\%) = \frac{\text{True negative}}{\text{Ture negative + False positive}} \times 100$$

$$\text{Positive predictive value}(\%) = \frac{\text{True positive}}{\text{True positive + False positive}} \times 100$$

$$\text{Negative predictive value}(\%) = \frac{\text{True negative}}{\text{True negative + False negative}} \times 100$$

RESULTS

Clinical Examination of Sampled Camels: During Anti-mortem examination no clinical signs that usually accompanying infestation with nasal bot myiasis could be noticed on any of the slaughtered camels nor the existence of expelled larvae on boundaries of nasal openings. Moreover, only the 2nd and 3rd instars of *C. titillator* larvae were collected from nasopharyngeal cavities of camels during post-mortem inspection.

Table 1: Immunological and diagnostic values of *C. titillator* larval antigens in epidemiological analysis of infestation with regards to ELISA parameters.

	Types of Antigen									
	L3CE		L2CE		Dtc		ESP		Sgc	
Item	+ve	- ve	+ ve	- ve	+ve	- ve	+ ve	- ve	+ ve	- ve
PIS sera	4/15	11/15	15/15	0/15	5/15	10/15	14/15	1/15	15/15	0/15
PNIS Sera	0/15	15/15	14/15	1/15	4/15	11/15	5/15	10/15	13/15	2/15
Random Sera	19/144	125/144	127/144	17/144	72/144	72/144	74/144	70/144	110/144	34/144
Total Animals Number	23/174	151/174	156/174	18/174	81/174	93/174	93/174	81/174	138/174	36/174
Apparent prevalence %	13.22		89.66		46.55		53.45		79.31	
Sensitivity %	100		51.72		55.55		73.68		53.57	
Specificity %	57.6		100		52.38		90.90		100	
Positive predictive value %	26.66		100		33.33		93.33		100	
Negative predictive value%	100		6.66		73.3		66.66		13.33	
% of antibodies	0.00 : 84.8		8.43 : 179.5		0.18 : 99.8		0.57 : 128.1		0.50 : 287.9	
Positive Controls OD Values										
A- Rabbits (10)	0.4 - 0.8		0.29 - 0.36		1.09 - 2.48		0.24 - 1.09		0.16 - 0.7	
B- Camels (5)	0.195		0.4		2.4		1.00		0.79	
Negative Controls OD Values										
A- Rabbits (10)	0.06 - 0.08		0.12 - 0.14		0.2 - 0.3		0.06 - 0.07		0.05 - 0.06	
B- Camels (6)	0.06 - 0.07		0.1 - 0.2		0.2 - 0.3		0.08 - 0.12		0.06 - 0.08	

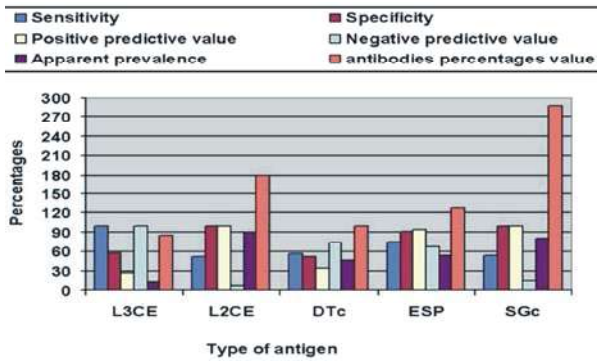

Chart 1: Reactivity of different C. titillator larval antigens during indirect ELISA

Reactivity of Different *C. titillator* Larval Antigens during Indirect Enzyme Linked Immunosorbant Assay (ELISA): One hundred and seventy-four camel's sera were tested by ELISA assay to detect *C. titillator* antibodies levels in the living animals as well as to evaluate and compare sensitivity, specificity, positive predictive value, negative predictive value and apparent prevalence percentages to the different *C. titillator* larvae antigens prepared during the present study.

ELISA results indicated that percentages of *C. titillator* antibodies were highly varied among different serum samples in their reactivity with different *C. titillator* larval antigens. The highest percentage of anti-*C. titillator* antibodies was 287.9% estimated against SGc antigen, however the lowest percentage was 84.8% when L3CE antigen was used (Table 1 and Chart 1). Moreover, apparent prevalence of *C. titillator* infestation among camels differed according to the used antigen. The highest values were 89.66% and 79.31% with L2CE and SGc antigens, respectively (Table 1 and Chart 1). Nevertheless, apparent prevalence of 53.45%, 46.55% and 13.22% were recorded when ESP, DTc and L3CE antigens were used, respectively (Table 1 and Chart 1).

The obtained results of the highest specificity and positive predictive values were reported with L2CE and SGc antigens recording 100% for each (Table 1 and Chart 1). On the other hand, DTc antigen gained the lowest specificity and positive predictive values, 52.38% and 33.33%, respectively (Table 1 and Chart 1). While, L3CE antigen showed lower specificity and positive predictive value than ESP antigen, 57.6%, 26.66% and 90.90%, 93.33%, respectively (Table 1 and Chart 1).

Concerning the sensitivity of ELISA and negative predictive value related to different prepared *C. titillator* larval antigens; the highest percentage were 100% when the test was performed using L3CE antigen (Table 1 and Chart 1). While sensitivity percentages were 73.68%, 55.55%, 53.57% and 51.72% with ESP, DTc, SGc and L2CE antigens, respectively (Table 1 and Chart 1). Moreover, negative predictive values were 73.3%, 66.66%, 13.33% and 6.66% with DTc, ESP, SGc and L2CE antigens, respectively (Table 1 and Chart 1).

DISCUSSION

Recently, ELISA has been developed as large scale quantitative and qualitative monitoring assay for immunoglobulin as well as designated antigens in the living animals, respectively [9]. Therefore, allows easy and cost effective diagnosis of many diseases including myiasis-causing infestation; even when larvae are still migrating or undetectable in the animal body [9, 20]. However, the previous studies didn't prepare corresponding antigens, only crude preparations from different larval instars to be used for serodiagnosis of *C. titillator* infestations [3, 10-12, 21, 22].

In the present study, the apparent prevalence of *C. titillator* infestations among camels varied according to the used antigen which roughly indicated variation in their capability to trigger the humeral cascade of immune system [5]. The higher values were recorded 89.66% and 79.31% with L2CE and SGc antigens, respectively, (Table 1 & Chart 1). These results were relatively similar to the previous records (85%) reported by Nassar and Youssef [11] when used L2CE antigen of *C. titillator* in diagnosis. However, higher apparent prevalence percentages of infestation among camels were reported; 93.5%, 97.9% and 85.6% using IHAT as previously published, respectively [10, 12, 22]. In correlation to *O. ovis;* the closest *Oestrinae* nasal bot larvae, the high noted prevalence results; 91.43%, 80.95%, 83% and 83.2%, were in agreement with those reported previously by Alcaide *et al.* [27], Mohran [22], Tabouret *et al.* [16] and Suárez *et al.* [19], respectively, in goats and sheep using crude and ESP antigens of 2nd instar larvae. Moreover, analogous apparent prevalence for *O. ovis* larvae using L2CE, L2SGc and L3SGc was recorded 78.4%, 90.6% and 79.6% in sheep by ELISA, respectively [28]. In contrary, the present study demonstrated low apparent prevalence, 53.45%, 46.55% and 13.22%, when ESP, DTc and L3CE antigens were used, respectively. These results were similar to previous studies reported 50.3% and 57% prevalence of *O. ovis* infestation in sheep during summer season when L2CE was used [16, 26]. Moreover, low seroprevalences were recorded, 60% and 42%, during summer and winter seasons when 28 kDa Sgc antigen was applied for detection of anti-*O. ovis* antibodies in sheep [16].

The present study proved that SGc antigen is the most immunogenic followed by L2CE antigen since both achieved similar specificity and positive predictive values recorded 100% while percentage of antibodies were 287.9% and 179.5%, respectively, (Table 1 & Chart 1). On the other hand, both types of antigens recorded the lowest sensitivity and negative predictive values (51.72% and 53.57%) and (6.66% and 13.33%), respectively (Table 1 & Chart 1). ESP antigen is also considered with a significant antigenicity and immunogenicity, hence, high specificity, positive predictive value and percentage of antibodies 90.90%, 93.33% and 128% were recorded, respectively, (Table 1 & Chart 1). DTc and ESP antigens, however, showed 52.38% and 90.90% for specificity, 33.33% and 93.33% positive predictive value, 55.55% and 73.68% sensitivity and 73.3% and 66.66% negative predictive value, respectively (Table 1 & Chart 1). Comparable previous results of closely related nasal bot larvae (*O. ovis*) were in agreement with present obtained results which demonstrated that SGc antigen provokes a strong humeral antibody response in experimentally infected sheep [17, 29]. On the other hand, disagreement results were reported by Frugére *et al*. [15] who showed a higher antigenicity for L3ESP compared to L2CE when applied in ELISA.

On the other hand, the odd contradictory results of L3CE antigen which achieved highest sensitivity and negative predictive value (100%) while lowest positive predictive value and specificity (26.66% and 57.6%) (Table 1 & Chart 1), have decreased the immunogenicity and usefulness of this antigen for serodiagnosis, despite that it was thought-out as the most immunogenic modulator for *C. titillator* during the early stage of the experimental design of this study. Hence, Sabah [3] declared that the 3rd larval antigen is the best to be used in diagnosis which could detect the highest titer of immunoglobulin when ELISA was used for characterization and rough quantitative estimation of antibodies in experimentally inoculated rabbits with the three larval instars antigens of *C. titillator*. However, El-khateeb [21] had proved that 2nd larval instar of *C. titillator* antigen is the only immunogenic when utilized the three larval instars of *C. titillator* by agar gel diffusion test. These results of high specificity and high positive predictive value of L2CE, ESP and SGc may be explained by long developing time displayed by 2nd larval instar [16, 30], excretory secretory products of enzymes used in preparation of larval nutrient [16, 19], as well as the salivary glands content secreted onto nasal mucosa [16, 28, 29], respectively, thus enhancing the host's immune response to stimulate IgG production. On the other hand, the highest sensitivity and negative predictive value of L3CE antigen may be attributed to suggestion of non specific reaction, hence, 3rd larval instars directed their metabolic

activity for completion of its full maturity and expulsion outside the body to complete their life cycles and/or the sampled individual of the examined camels may be actually infested by the smallest 1st larval instar which are difficulty detected at necropsy [12]. Moreover, the lowest specificity showed by DTc antigen may be returned to that digestive tubules are not in contact with the host immune system during natural infestation and its secretions in minute amount haven't got enough immunogenicity nor antigenicity to stimulate the host' humeral immune response for IgG production [9,16, 31]. In conclusion, the present study introduces a preliminary investigation of different *C. titillator* prepared antigens used in diagnosis of infestations among camels. Despite ELISA is considered a valid tool for diagnosis of such myiasis, the variations in seroprevalences demonstrated by different prepared antigens may be attributed to differences in host-larval interaction that mostly depend on immunogenic properties of the used antigens which in turn trigger camels' immune response during larval developmental stages. The present preliminary assessment considered the salivary glands content antigen, the most immunogenic one that had the capability to elicit the best humeral immune response against *C. titillator* larvae and represented the basic step towards further studies particularly IgM and local secretory IgA release. Finally, further characterization of different proteins of each antigen is required.

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