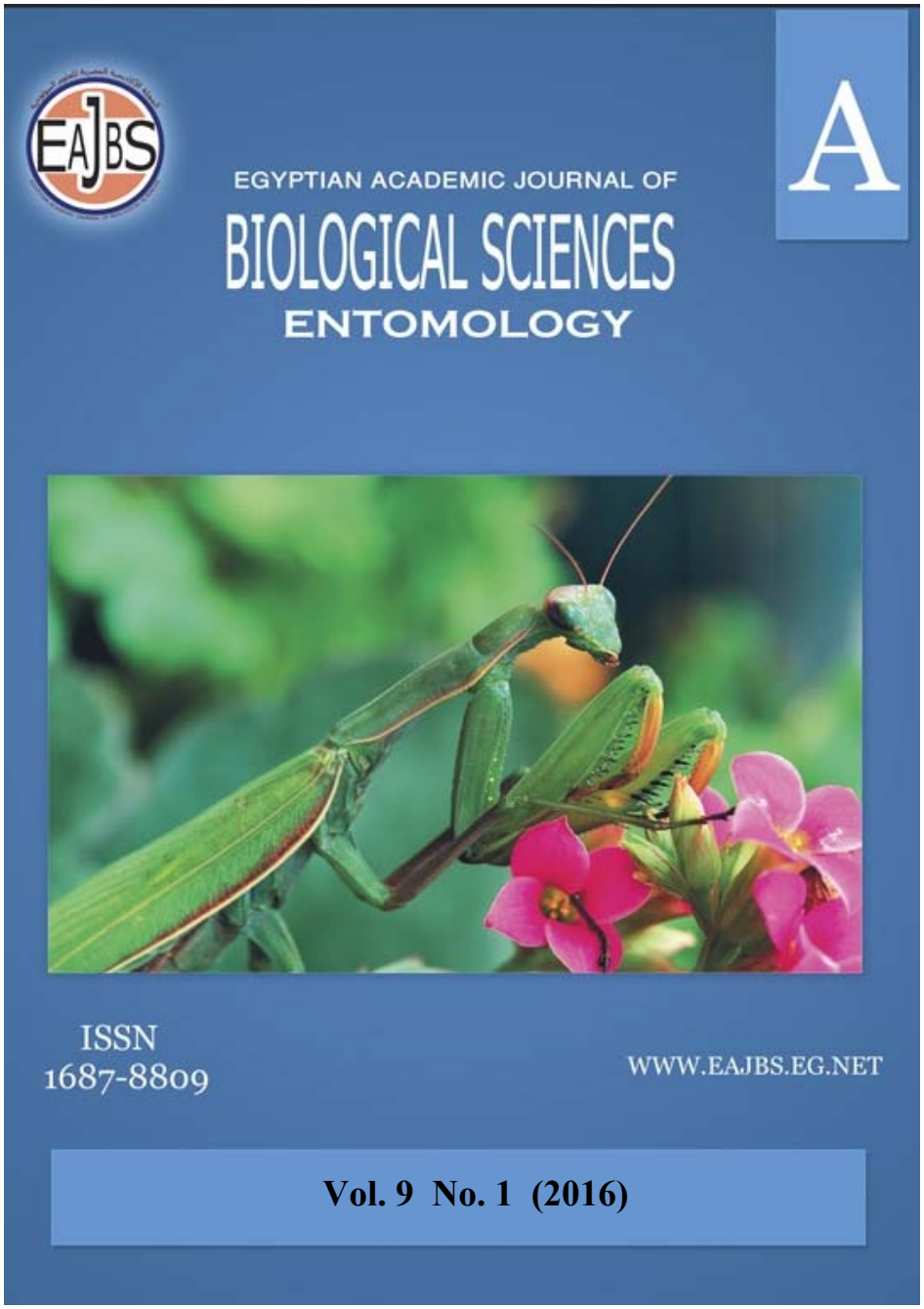

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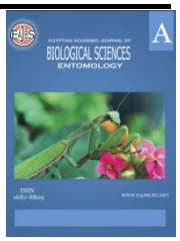

Virulence of Entomopathogenic Nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* Poinar *(*HP_{88} strain*)* Against the Black Cutworm, *Agrotis ipsilon*.

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ABSTRACT

Virulence of entomopathogenic nematodes, *Steinernema glaseri* and *Heterorhabditis bacteriophora* Poinar (HP88 strain) were studied against 3rd, 4th, 5th and 6th instar larvae of the black cutworm, *Agrotis ipsilon*. The observed mortality caused by the both tested nematodes at different time intervals was recorded. The establishment of nematodes in a host depends greatly on its ability to manage host defences so, the host haemocyte interaction with entomopathogenic nematodes were observed by encapsulation, nodule formation and phagocytosis processes. Phagocytosis is known to stimulate production of lysosomal enzymes. Acid phosphatase acts as a lysosome marker so; the activity and the pattern of acid phosphatase during the course of infection were recorded. Also, total protein and protein pattern were screened. Results indicated that, the two nematodes had variable significantly effects. *S. glaseri* recorded a highly significantly affect than *H. bacteriophora*. In addition, there are relationship between mortality rate and time exposure of *S. glaseri* and *H. bacteriophora* against 3rd, 4th, 5th and 6th larval instars of *A. ipsilon* i.e. the time exposure increased the susceptibility also increased. At the lower concentration, fourth and/or fifth instars were the most susceptible instars to *S. glaseri* ,while the 3rd and 4th instar larvae were most vulnerable instars to the infection with *H. bacteriophora*. Results was recorded that, six haemocyte types were identified in *A. ipsilon* 6th instar larvae: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, oenocytoids, and adipohaemocytes. *S. glaseri* in *A. ipsilon* escape encapsulation and overcome host immunity faster than *H. bacteriophora* which recognized by host haemocytes. Prohemocytes, plasmatocytes or granulocytes are phagocytic cells in *A. ipsilon* 6th instar larvae. Phagocytosis was expressed in *A. ipsilon* haemocytes infected with *H. bacteriophora* at a higher rate compared with *S. glaseri* infection. Data demonstrated both *S. glaseri* and *H. bacteriophora* induced a significant decrease in total protein contents after 24hrs from infection. While, there was significant increase in acid phosphatase activity after 6 hrs of infected groups of *S. glaseri* and *H. bacteriophora*. This increase may be due to phagocytosis processes which recorded in the present study at this time because phagocytosis is known to stimulate production of lysosomal enzymes. There is an acid phosphatise band detected only in *H. bacteriophora* and *S. glaseri* samples with Rf 0.86 after 6 and 12hrs from infection. This band may refer to nematodes exudates which contain high amount of this enzyme that the nematodes can easily penetrate and digest host tissues. There is a characterestic band of *S. glaseri* protein pattern with Mw 47KDa. It was identified as surface coat protein of *S. glaseri* which play important roles in defeating the host immune system by its antiphagocytic activity.

INTRODUCTION

The black cutworm, *Agrotis ipsilon* (Hufnagel) are serious soil pests of many vegetable and field crops all over the world. Due to its soil-dwelling habits cutworm is difficult to control. It is often detected only when the plants are already severely damaged. In the past, several species of entomopathogenic bacteria, protozoa, fungi, and viruses were isolated and evaluated as possible biological control agents of cutworm pests (Ignoffo and Garcia, 1979). Entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* associated with their symbiotic bacteria *Xenorhabdus*, and *Photorhabdus*, respectively, are important biocontrol agents for soil dwelling insects (Kaya and Gaugler, 1993). They enter the haemocoel of the insect hosts via body openings and intersegmental membranes (Burnell and Stock, 2000) and then release their symbiotic bacteria (Dunphy and Webster, 1988). Symbiotic bacteria produce proteases and toxins that inhibit encapsulation, melanization and the production of antimicrobial peptides (Wang *et. al.,* 1995 and Gaugler *et al.,* 1997) *S. glaseri* releases its symbiotic bacteria, *Xenorhabdus poinarii*, 4–6h after its entry of the host haemocoel (Wang *et. al.,* 1995). *H. bacteriophora* symbiotic bacteria *Photorhabdus luminescens* can be detected as soon as 30 min after nematodes enter the haemocoel (Bowen *et. al.,*1998) Bacteria are known to overwhelm host defences 24–48hrs after nematode infection, but within the first few hours of penetrating a host, the nematodes themselves are thought to manipulate the host immune response (Brivio *et al.,* 2002). So, the establishment of a parasite in a host depends greatly on its ability to manage host defences (Else, 2005). Haemolymph of insects is a medium for several physiological processes like immune responses and intermediary metabolism. When an invader enters haemocoel of insects, haemocytes are engaged to remove non-self-target by phagocytosis, nodule formation, encapsulation, synthesis of antimicrobial peptides and reactive metabolites (Beckage, 2008). Encapsulation refers to haemocyte aggregation around larger pathogens like parasitoids and nematodes. The subsequent step of the process of encapsulation is the blackening of the capsule (melanisation) due to phenoloxidase activity. Finally, the invader is killed within the capsules (Lavine and strand, 2002). Phagocytosis is known to stimulate production of lysosomal enzymes. Acid phosphatase acts as a lysosome marker and free acid phosphatase activity have been determined histochemically in haemocytes and salivary glands (Armbruster *et al.,* 1986). In addition, free acid phosphatase is an indicator of cellular autolysis (Skelton and Bowen, 1987) and Cell death was shown associated with free acid phosphatase activity and the endonuclease released - histone group proteins (Gregorc and Bowen, 1997). The present work aims to evaluate the virulence of two different nematodes *S. glaseri* and *H. bacteriophora* Poinar (HP88 strain) and their role in control of the economically important insect pest *A. ipsilon* larvae and the interaction of the pathogenic nematodes with their symbiotic bacteria on the host haemocyte. In addition, the activity of acid phosphatase and protein pattern at different time intervals after infection to throw light on the possible enzymatic reactions of the black cutworm *A. ipsilon* in the present investigations.

MATERIALS AND METHODS

Experimental insects:

A laboratory colony of *A. ipsilon* was obtained from the Department of Cutworms, Plant Protection Research Institute, Agricultural Research Centre (ARC). The insect larvae were kept at 25 ºC, 60–70% relative humidity and 16:8 h day: night

photoperiod. Larvae were reared individually on castor leaves, *Ricinus communis*.

Nematodes:

Imported nematode species, *Steinernema glaseri* and *Heterorhabditis bacteriophora* Poinar (HP88 strain) were supplied by Dr. El-Sadawy, National Research Centre, Dokki, Giza, Egypt. For mass culturing of the used nematode isolates the last instar larvae of the greater wax moth, *Galleria mellonella* were used as hosts according to (Shamseldean *et al.,* 2009).

Bioassay test:

Experiments were carried out in plastic cups (4 X 5 cm) filled with 50 gm of sterilized sand and moistened with 20% water (v/w). Nematodes suspensions were prepared in serial concentrations of 12, 25, 50, 100, 200 and 400 infective juveniles (IJs)/ml/cup. Each of 3rd, 4th, 5th and 6th instar larvae of *A. ipsilon* placed in a separate plastic cup. Sixty cups, fifteen cups for each instar, of each concentration were conducted for each treatment. All cups were covered and kept at 25 ± 2 ^{o}C and checked after 24, 48, 72 and 96 hour to record mortality. Mortality percentages were corrected using Abbot´s formula (Abbott, 1927).

Host haemocyte interaction with entomopathogenic nematodes:

a- Haemocyte types: Drop of haemolymph from infected and / or healthy 6th instar larva was placed on microscopic slide, ten slides for each treatment, and allows drying for 10–20 min. then fixed with methanol for 5 min. and stained with 10 % Giemsa solution for 10 min and washed with distilled water (Gupta, 1972). Haemocyte populations were examined under light microscope. The time was chosen according to preliminary observations on penetration of both EPNs species.

b- Phagocytic activity is expressed as the ratio between the number of haemocytes with phagocytised particles and the number of all evaluated haemocytes according to (Berger and Jurčová, 2012).

Biochemical studies

Collection of haemolymph:

The haemolymph was obtained from 6th instar larvae, normal and infected with 50IJ/cup/ larva of *S. glaseri* and/or 400IJ/cup/ larva of *H. bacteriophora,* after 6, 12, 24, 48 hrs from infection. The haemolymph was obtained by amputation of one or two prothoracic legs of the larvae with fine needle.

Total protein was determined by the method of (Brad ford, 1976).

Acid phosphatase (AcP) was determined according to the method described by (Powell and Smith, 1954).

Separation of haemolymph Protein by Electrophoresis:

Proteins were separated by polyacrylamide gel electrophoresis (PAGE) according to the method of (Davis, 1964). After electrophoresis, the protein bands were visualized by staining with Coomassie Blue R250 stain then rinsed in distaining solution until the dark ground become colourless except blue protein bands.

Acid phosphatase pattern:

After electrophoresis, the gel was soaked in 100 ml of 50 mM Na-acetate buffer pH 5.0 containing 100 mg Fast blue BB salt, 100 mg α-naphthyl phosphate, 100 mg $MgCl_2$ and 100 mg $MnCl_2$ (Wendel and Weeden, 1989). 50 mM Na-acetate buffer pH 5.0 was prepared by adding 5.15 ml glacial acetic acid and 2.85 g sodium hydroxide to 500 ml distilled water).

Statistical analysis: Statistical analysis was carried out using Analysis of Variance (one wayANOVA) test through "SPSS-ComputerProgram". Means were compared using Duncan's Multiple Range test.

RESULTS AND DISCUSSION

The laboratory experiments conducted in the current investigation have screened nematodes *S. glaseri* and *H. bacteriophora* against 3rd, 4th, 5th and 6th instar larvae of the black cutworm, *Agrotis ipsilon* (Table 1).

Table 1: Bioassay of different instar larvae of *A. ipsilon* infected with different concentrations of Entomopathogenic nematodes, *S. glaseri* or *H. bacteriophora*.

Nematodes Conc.(IJs/ml) Larval instars			*S. glaseri*				*H. bacteriophora*				Control
			12	25	50	100	50	100	200	400	
3rd instar larvae	Corrected Mortality % after	24h.	0.0	0.0	6.67	86.67	13.33	13.33	33.33	33.33	0.0
		48h.	13.33	20	40	86.67	33.33	26.67	66.67	66	0.0
		72h.	26.67	40	66	100	40	46.67	80	80	0.0
		96h.	26.67	53.33	66.67	100	53.33	73.33	86.67	93.33	0.0
	Pupation%		20±0.0	6.67±3.1	0.0	0.0	0.0	0.0	0.0	0.0	93.3±3.8
	Adult Emergences%		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	86.6±3.8
4th instar larvae	Corrected Mortality % after	24h.	20	26.67	0.0	80	0.0	0.0	0.0	0.0	0.0
		48h.	35.71	21.43	71.42	100	0.0	21.43	35.71	49.99	0.0
		72h.	64.28	63.57	78.57	100	14.28	35.71	63.57	71.42	0.0
		96h.	64.28	64.28	78.57	100	35.71	42.86	64.28	78.57	0.0
	Pupation%		13.3±3.8	20±6.67	0.0	0.0	0.0	0.0	0.0	0.0	93.3±3.8
	Adult Emergences%		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	93.3±3.8
5th instar larvae	Corrected Mortality % after	24h.	0.0	0.0	0.0	86.67	0.0	0.0	0.0	0.0	0.0
		48h.	46.67	66	53.33	100	0.0	6.67	26.67	46.67	0.0
		72h.	73.33	86.67	80	100	13.33	13.33	46.67	66	0.0
		96h.	73.33	93.33	93.33	100	13.33	26.67	46.67	66	0.0
	Pupation%		13.3±3.8	0.0	0.0	0.0	40±6.67	40±11.55	33.3±3.8	20±0.0	100±0.0
	Adult Emergences%		0.0	0.0	0.0	0.0	20±6.67	13.33±3.82	20±0.0	6.67±3.14	93.3±3.85
6th instar larvae	Corrected Mortality % after	24h.	0.0	0.0	0.0	26.67	0.0	0.0	0.0	0.0	0.0
		48h.	13.33	40	33.33	86.67	0.0	6.67	13.33	33.33	0.0
		72h.	40	53.33	80	93.33	6.67	6.67	40	46.67	0.0
		96h.	40	66	86.67	100	6.67	20	40	53.33	0.0
	Pupation%		20±6.67	13.3±3.8	6.67±3.1	0.0	60±6.67	40±0.0	33.3±3.8	13.3±3.8	100±0.0
	Adult Emergences%		0.0	0.0	0.0	0.0	40±0.0	20±6.67	13.3±3.8	6.67±3.1	100±0.0

The nematode, *S. glaseri* recorded a highly affect than the other one nematode, *H. bacteriophora*. At the lowest concentration 12IJs/ml of *S. glaseri*, the fourth and/or fifth instars were the most vulnerable instars i.e. the mortality reach to 64 and 73 % after 96 hours from infection, respectively. While at the highest concentration 100 IJs/ml causes 100% mortality for all instars. The 6th instar larvae were the most tolerant instar to the infection with *H. bacteriophora*. Our results confirm these of (Mogahed and Abbas, 1998) who found out that 4th instar larvae were more susceptible to *S. Carpocapsae* and *H. bacteriophora* than 6th instar larvae of *A. ipsilon*. In lepidopteran insects, older larval instars and pupae are less susceptible to entomopathogenic nematodes (Glazer & Navon, 1990; Shannag *et al.,* 1994 and Baur *et al.,* 1998). On the other hand, (Power *et al.,* 2009) state that older scarab larval instars are less susceptible, possibly owing to stronger evasive and aggressive behavior of the older than the early instars. In *A. ipsilon* larvae, aggressive behavior of 6th instar larvae may be responsible for the lower susceptibility of this instar to both tested nematodes. Also, as shown in Table (1) there are a relationship between the mortality rate and time exposure of *S. glaseri* and *H. bacteriophora* against 3rd, 4th, 5th and 6th larval instars of *A. ipsilon* i.e. time exposure increased the susceptibility also increased. These results confirm these of (Ben-Yakir *et al.*, 1998)

who reported that, *Ostrinia nubilalis* (Hūbner), larval death rate gradually increased and was significantly higher after 9hrs of exposure than after 3 or 6hrs to *S. carpocapsae* and *H. bacteriophora*, at an infestation rate of 500 IJs per Petri dish. Data also recorded a reduction in both of percentage of pupation and percentage of adult emergence.

Identification of haemocytes

Six haemocyte types were identified in *A. ipsilon* 6th instar larvae by morphology: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, oenocytoids, and adipohaemocytes as shown in Fig. (1).

Prohemocytes are small rounded cell with variable sizes. Plasma membrane was generally smooth, and nucleus was large, centrally located, almost filling up the whole cell. They have deeply stained cytoplasm that is rich in ribonucleic acid Fig. (1-A).

Plasmatocytes are oval or spindle in shape and variable in size. The majority of the plasmatocytes of the 6th larval instar are round dense cells having pale nuclei that elongate or lobate nucleus exhibit variable sizes and is centrally localized, showing scattered chromatin masses. Dense round plasmatocytes are found singly, in pairs, and occasionally in small clusters Fig. (1-B).

Granulocytes were the most frequently observed cell type in larvae and were spherical cells. The nucleus was round, centrally located, with scattered chromatin masses and nucleolus the cytoplasm is basophilic and contains large number of acidophilic granules that vary in size within the same cell. The nuclei vary in shape from spherical to ovoid and may be centric or eccentric Fig. (1-C).

Spherulocytes were characterized by their inclusions and membrane-bound spherules took up almost all the cytoplasm. The cellular surface was homogenous but exhibit cytoplasmic protrusion corresponding to the spherules. The nucleus was small, eccentric, mostly deformed by the spherules Fig. (1-D)

Oenocytoids are very large cells with an eccentric small spherical nucleus. They are rounded or oval shaped cells, darkly stained nucleus and clear, uniform weakly basophilic cytoplasm when stained with Geimsa stain. The cytoplasm has spherical inclusions called granular cells Fig.(1-E)

Adipohaemocytes are spherule cells characterized by refringent fat droplets and other inclusions Fig. (1-F).

Host haemocyte interaction with entomopathogenic nematodes:

Encapsulation:

Encapsulation is the response of haemocytes to large targets such as parasites, protozoa, and nematodes. Haemocytes bind to the target in multiple cell layers until they form a capsule around the invader. The capsule is normally melanized at the end (Ling *et al.*, 2006). Finally, the invader is killed within the capsules (Lavine and Strand, 2002).

In the present study insect haemocytes with nematodes were observed every 3h. Granulocytes and plasmatocytes play the major role in encapsulation reactions and haemocytic aggregation. *S. glaseri* was not recognized by host haemocytes (Figs. 2-C, D & E); however, *H. bacteriophora* was recognized and haemocytes were attached to the whole body of the nematodes Figs. (2-A & B). Most *H. bacteriophora* were recognized, but few members of this species were free-moving. Our current data agree with a study by (Wang *et al*., 1995) who showed that *S. glaseri* in *P. japonica* escape encapsulation and overcome host immunity. Results declared that *H. bacteriophora* shed the IJ-cuticle Fig. (2-F) it could be considered a strategic mechanism to escape from host immunology. Our results confirm these of (Peters *et*

al., 1997) who stated that, the loss of the external layer of the body surface of *H. bacteriophora* could be considered a strategic mechanism to escape from host immunological surveillance.

Phagocytosis

Phagocytosis is probably the oldest defence mechanism against microorganisms. During phagocytosis the target particle is first recognized by phagocytic receptors that activate various signalling pathways in the cell interior (Jones *et al.*, 1999). These signals lead to dramatic changes in the dynamics of the plasma membrane and the cytoskeleton. The membrane extends pseudopods around the particle, forming a cup that moves into the cell. Within few minutes the membrane closes at the distal end, leaving a new plasma membrane-derived phagosome (Yeung *et al.*, 2006). In insects, phagocytosis is performed by a subset of haemocytes in the haemolymph (Strand, 2008). In the present study the phagocytic activity of haemocytes was evaluated by ingestion of bacteria associated with tested nematodes (Table 2). Bacteria were ingested by prohaemocytes Fig. (3-A), plasmatocytes Figs.(3-B, C and D) and granulocytes Figs.(3-E,F,G and H). Prohaemocytes had a phagocytic activity lower than plasmatocytes and granulocytes in both tested nematodes. No phagocytosis was observed in the spherulocytes, oenocytoids and adipohaemocytes. In *A. ipsilon* 6th instar larvae, plasmatocytes and granulocytes consider the main phagocytic cells. Phagocytosis was expressed in *A. ipsilon* haemocytes infected with *H. bacteriophora* at a higher rate 18.58±0.62 % compared with *S. glaseri* infection 10.52±0.48% as shown in Table (2).

Table 2: Phagocytic activity % of *A. ipsilon* haemocytes against *S. glaseri* and *H. bacteriophora* infection

Nematodes / Haemocytes	Phagocytic activity %	
	S. glaseri	*H. bacteriophora*
Prohaemocytes	4.21±0.77	9.21±1.16
Plasmatocytes	12.79±0.15	21.46±0.26
Granulocytes	14.57±0.51	25.07±0.45
Spherulocytes	0.0±0.0	0.0±0.0
Oenocytoids	0.0±0.0	0.0±0.0
Adipohaemocytes	0.0±0.0	0.0±0.0
Mean of phagocytic cells	10.52±0.48	18.58±0.62

The data presented here on *A. ipsilon*, are close to similar data on other insect species, phagocytes in Diptera and Lepidoptera have also been described as plasmatocytes and granular haemocytes, respectively (Lavine and Strand 2002). In agreement with this, plasmatocytes or granulocytes are the main phagocytic cells in most insects (Meister, 2004; Castillo *et al.*, 2006; Lamprou *et al.*, 2007; Lemaitre *et al.*, 2007 and Garcia-Garcia *et al.*, 2009).

Nodulation

Nodulation is a predominant quantitative cellular defence mechanism against bacterial, infection (Miller *et al,* 1994; Stanley *et al*., 1998). It consists of formation of multicellular haemocyte aggregates that entrap large numbers of bacteria (Figs. 4-A and B) on the other hand, the toxin produced by these bacteria attack *A. ipsilon* haemocytes (Figs. 4- C, D, E, and F).

Biochemical studies

Changes in *A. ipsilon* haemolymph protein after infected with entomopathogenic nematodes.

Data presented in the Table (3) demonstrated both *S. glaseri* and *H. bacteriophora* induced a significant decrease in total protein contents after 24hs from infection.

Table 3: Changes in total protein of *Agrotis ipsilon* 6th larval instar haemolymph after infected with entomopathogenic nematodes at different time intervals.

Treatment		Total protein mg protein /ml			
		Hours post infection			
		6 hrs	12 hrs	24 hrs	48 hrs
S. glaseri	Mean±SE	9.04±0.34	8.94±0.1	7.33±0.1	5.04±0.09
	%Change	0.0	-1.08	-20.59	-49.51
	p	a	a	c	b
H. bacteriophora	Mean±SE	9.42±0.33	9.04±0.62	8.19±0.1	5.80±0.67
	%Change	4.15	0.0	-11,27	-41.28
	p	a	a	b	b
Control	Mean±SE	9.04±	9.04±	9.23±	9.61±0.34
	p	a	a	a	a

P = Probability value
Mean with the same letter within the same coelom is not significantly different (P≤0.05).

It was also found that, the highest decline was recorded at 48 hrs post infection with *S. glaseri* and *H. bacteriophora* 5.04±0.09 and 5.80±0.67 with percentage changes -49.51 and -41.28 respectively. The data also declared that, the infection with *S. glaseri* induce more decrease in total protein content than *H. bacteriophora.* Data from the current study is consistent with that obtained by (El-Sadawy *et al.,* 2009) in *Parasarcophaga. aegyptiaca* and *Argas (persicargas) persicus* when infected with *H. bacteriophora* and *S. riobrave*. Also, (El-Bishry, 1989) reported that haemolymph protein of *S. littoralis* was markedly reduced 30 hrs post-nematode infection. Author attributed this reduction to the proteolytic activity detected in the haemolymph of infected larvae, this activity was believed to be the main cause of the host quick death.

Changes in acid phosphatase activity oh *A. ipsilon* haemolymph after infected with entomopathogenic nematodes.

The results revealed that, there was significant increase in acid phosphatase activity after 6 hrs of infected groups of *S. glaseri* and *H. bacteriophora* 7.20±0.23 with % change 41.03 and 5.86±0.13 with % change 15.38 respectively, compared with control groups 5.07±0.13. This increase still until 12hrs from infection and then decrease to reach no significant differences were observed between different treatment groups and control group at 24hrs (Table 4).

Table 4: Changes in Acid Phosphatase activity of *A. ipsilon* haemolymph after infected the 6th larval instar with entomopathogenic nematodes at different time intervals.

Treatment		Acid Phosphatase µg Phenol released/ min / ml			
		Hours post infection			
		6 hrs	12 hrs	24 hrs	48 hrs
S. glaseri	Mean±SE	7.20±0.23	7.33±0.27	4.20±0.42	4.53±0.13
	%Change	41.03	43.59	-21.79	-17.95
	p	a	a	a	b
H. bacteriophora	Mean±SE	5.86±0.13	6.13±0.13	4.33±0.18	4.93±0.13
	%Change	15.38	20.51	-15.38	-10.26
	p	b	b	a	b
Control	Mean±SE	5.07±0.13	5.07±0.35	5.33±0.35	5.46±0.13
	p	c	c	a	a

P = Probability value
Mean with the same letter is not significantly different (P≤0.05).

This increase may be due to phagocytosis processes which recorded in the present study at this time because phagocytosis is known to stimulate production of lysosomal enzymes of which acid phosphatase is a key component. Also, (Walter,

2008) stated that the activities of both acid and alkaline phosphatase were very high in *S. carpocapsae* exudates. The high amount of these enzymes is an indication that the nematodes can easily penetrate and digest host tissues. In addition, *S. carpocapsae* exudate they maybe active on phospholipids their targets being cell membranes which would thus limit haemocyte adhesion. Lysosomal acid phosphatase plays an important role in regulating metabolic processes by indirectly providing information concerning the intra-cellular digestion processes (Arme, 1966). On the other hand the decrease level of acid phosphatase after 48hrs from infection may be according to decrease the rate of metabolism. (Senthil Nathan *et al.,* 2005) suggest that, the decreased levels of acid phosphatase activity reduced phosphorous liberation for energy metabolism, decreased rate of metabolism as well as decreased rate of transport of enzyme regulation.

Separation of protein bands by electrophoresis.

Protein electrophoretic pattern of the samples is given in Table (5) and Fig. (5). A maximum number of 12 bands, which were not necessarily present in all of the studied samples , were detected at approximately Rf ranging between 0.08 with Mw 126.7 KDa and 0.99 with Mw 13 KDa . The maximum number of bands was nine and observed in control, 6hrs of *H. bacteriophora* (*H.b*) and 6hrs & 12hrs of *S. glaseri* (*S. g*) whereas the minimum number was two and recorded in 24hrs and 48hrs of *H. bacteriophora* and *S. glaseri*. The resulted profile comprises three unique bands and scored as follows: one in control samples (C.) at Rf value of 0.93& Mw 36.13 KDa; one in samples of *S. glaseri* at Rf value of 0.65& Mw 47KDa and one in samples of *H. bacteriophora* at Rf values of 0.57 & Mw 46KDa.

Table 5: Relative fragmentation (Rf) and Molecular weight (Mw) of haemolymph protein pattern for both *H. bacteriophora* and *S. glaseri* infected and healthy samples of 6th larval instar of *A. ipsilon*.

Parameters			6 hours			12 hours			24 hours			48 hours		
Band No.	Rf	Mw	Cont.	*H.b*	*S.g*	Cont.	*H.b*	*S.g*	Cont.	*H.b*	*S.g*	Cont.	*H.b*	*S.g*
1	0.08	126.7	+	+	+	+	+	+	+	+	+	+	+	+
2	0.14	97.07	+	+	+	+	+	+	+	+	+	+	+	+
3	0.25	78.12	+	+	+	+	+	+	+	-	+	+	-	+
4	0.32	72.96	+	+	+	+	+	+	+	-	-	+	-	-
5	0.42	65.28	+	+	+	+	+	+	+	+	+	+	+	+
6	0.51	52.50	+	+	-	+	-	-	+	-	-	+	-	-
7*	0.56	47.00	-	-	+	-	-	+	-	-	-	-	-	-
8*	0.57	46.10	-	-	-	-	-	-	-	-	-	-	+	-
9	0.63	42.22	+	+	+	+	+	+	+	+	+	+	+	+
10	0.93	36.13	+	-	-	+	-	-	+	-	-	+	-	-
11	0.94	30.96	-	+	+	-	+	+	-	+	+	-	+	+
12	0.99	13.00	+	+	+	+	+	+	+	+	+	+	+	+
Total			9	9	9	9	8	9	9	6	7	9	6	7

* Asterisks indicator to characteristic band
(+) = Present band
(-) = Absent band

The characteristic band of *S. glaseri* with Mw 47KDa was identified by (Liu *et al.,* 2012) as surface coat protein of *S. glaseri* which play important roles in defeating the host immune system by its antiphagocytic activity. 47 kDa protein with high sequence identity to enolases from different species of nematodes. *S.g*-ENOL was

detected in the host haemolymph after infection of *G. mellonella* with *S. glaseri*, indicating that *S.g*-ENOL was secreted into the insect haemocoel and was involved in infection. This is the first report of the characterization of a surface coat protein in an entomopathogenic nematode.

Acid phosphatase pattern

Electrophoretic pattern of acid phosphatase was obvious through three bands, with Rf ranged from 0.056 to 0.86 as shown in Table (6) and Fig. (6). Acid phosphatase was recorded in the haemolymph as one or two subunit of enzyme at control and infected samples. One common band appeared in different samples. This band was number one with Rf value 0.56. On the other hand, band number two detected only in *S. glaseri* (*S. g*) samples at 24hrs with Rf 0.76. The band number three detected only in *H. bacteriophora* (*H. b*) and *S. glaseri* (*S. g*) samples with Rf 0.86 after 6 and 12hrs from infection. This band may refer to nematodes exudates which contain high amount of this enzyme that the nematodes can easily penetrate and digest host tissues. This result agrees with (Walter, 2008).

Table 6: Relative fragmentation Rf of haemolymph Acid phosphatase pattern of *A. ipsilon* after infected the 6th instar larvae with entomopathogenic nematodes.

Parameters		**6 hours**			**12 hours**			**24 hours**			**48 hours**		
Band No.	**Rf**	**Cont.**	***H.b***	***S.g***	**Cont.**	***H.b***	***S.g***	**Cont.**	***H.b***	***S.g***	**Cont.**	***H.b***	***S.g***
1*	**0.56**	+	+	+	+	+	+	+	+	+	+	+	+
2	**0.76**	-	-	-	-	-	-	-	-	+	-	-	-
3	**0.86**	-	+	+	-	+	+	-	-	-	-	-	-

*** Asterisks indicator to common band**
(+) = Present band
(–) = Absent band

In conclusion, results revealed that both nematodes can act as bio-control agent for *A. ipsilon* larvae but *S. glaseri* are more virulent than *H. bacteriophora*. *S. glaseri* escape from encapsulation and overcome host immunity faster than *H. bacteriophora* which recognized by host haemocytes. Phagocytosis was expressed in *A. ipsilon* haemocytes infected with *H. bacteriophora* at a higher rate compared with *S. glaseri* infection because *S. glaseri* secrete enolase to host haemocoel during infection. Enolase locates on both nematode cuticle and surface coat of *Steinernema glaseri*. Enolase identifies as anti-immune surface coat proteins of *Steinernema glaseri* infective juveniles**.** So, we suggest inserting *S. glaseri* anti-immune, surface-coat protein gene into immune-susceptible *H. bacteriophora,* thereby increasing it's pathogencity to *A. ipsilon.*

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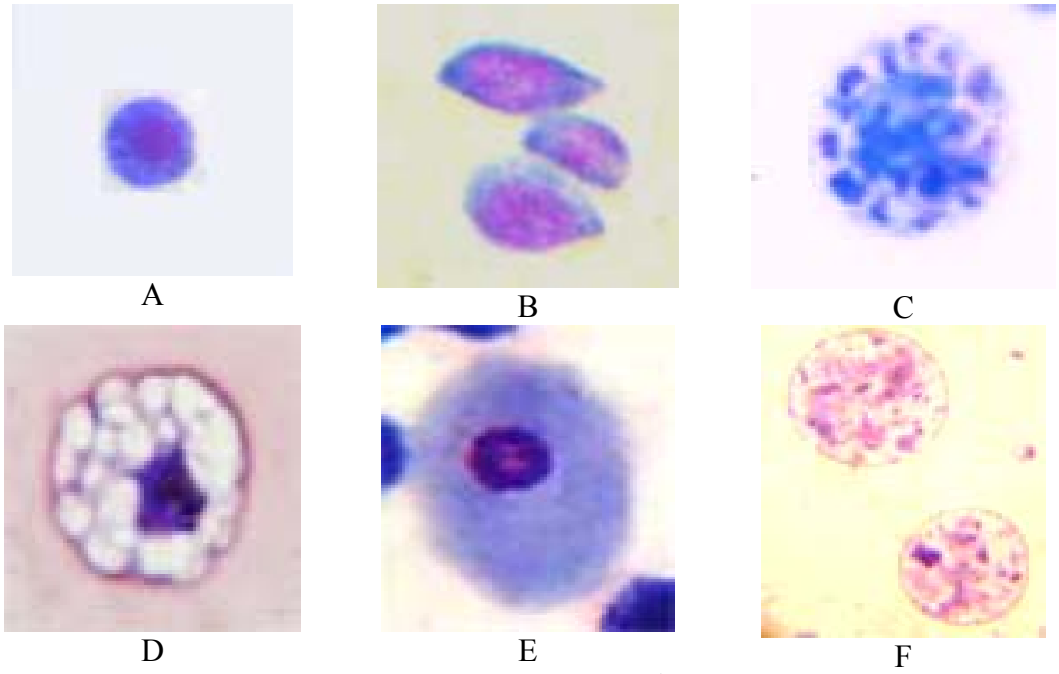

Fig. 1: Photomicrographs of normal haemocytes of *A. ipsilon* 6th *instar* larvae.

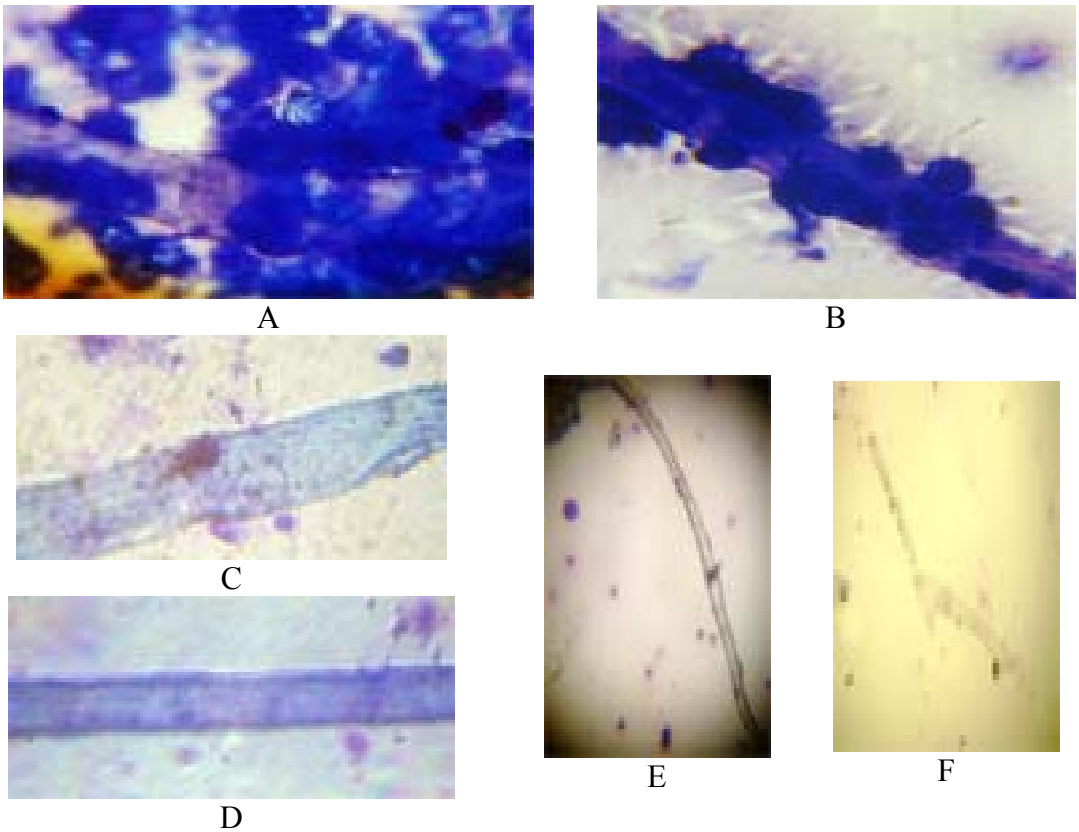

Fig. 2: Photomicrographs of host haemocyte interaction with entomopathogenic nematodes by encapsulation.

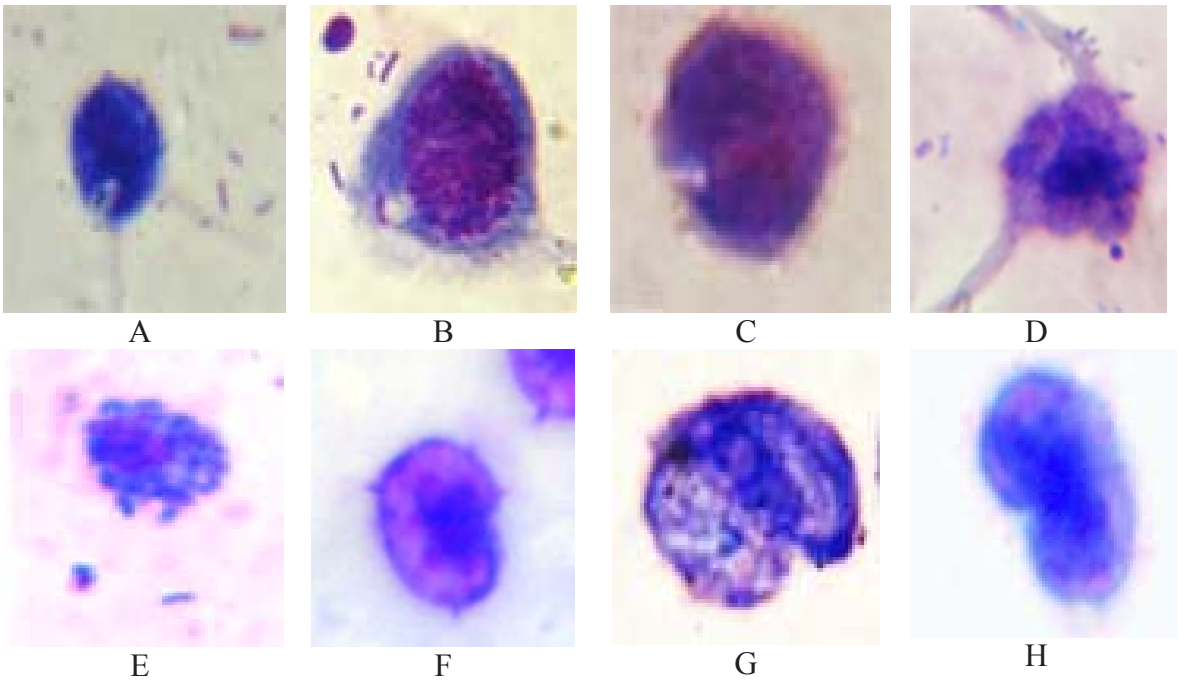

Fig. 3: Photomicrographs of host haemocyte interaction with entomopathogenic nematodes by phagocytosis.

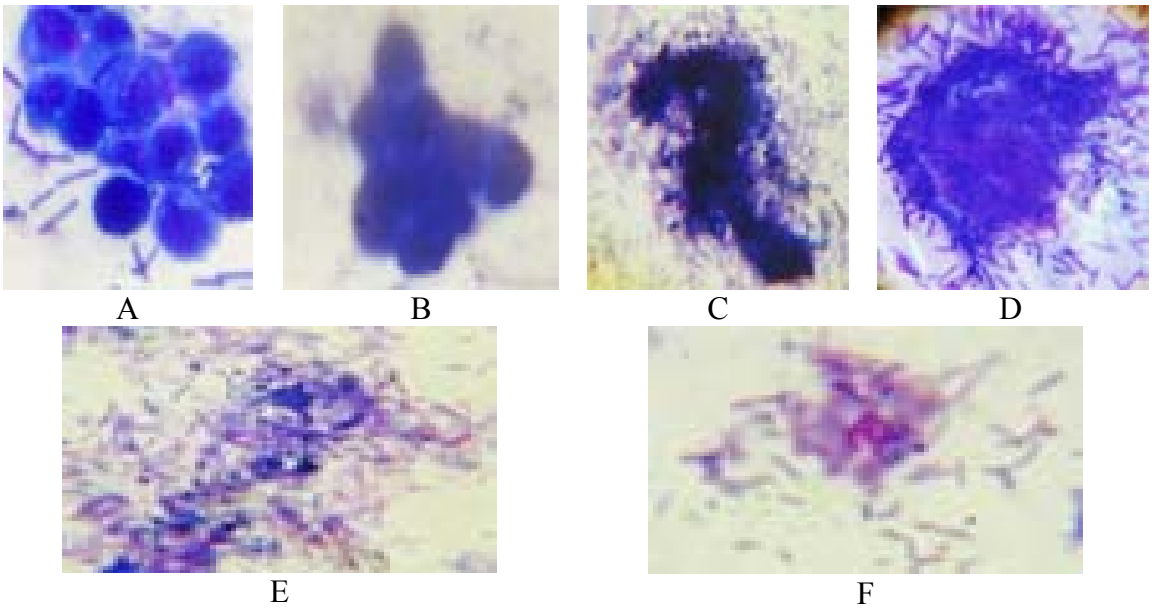

Fig. 4: Photomicrographs of host haemocyte interaction with entomopathogenic nematodes by nodulation.

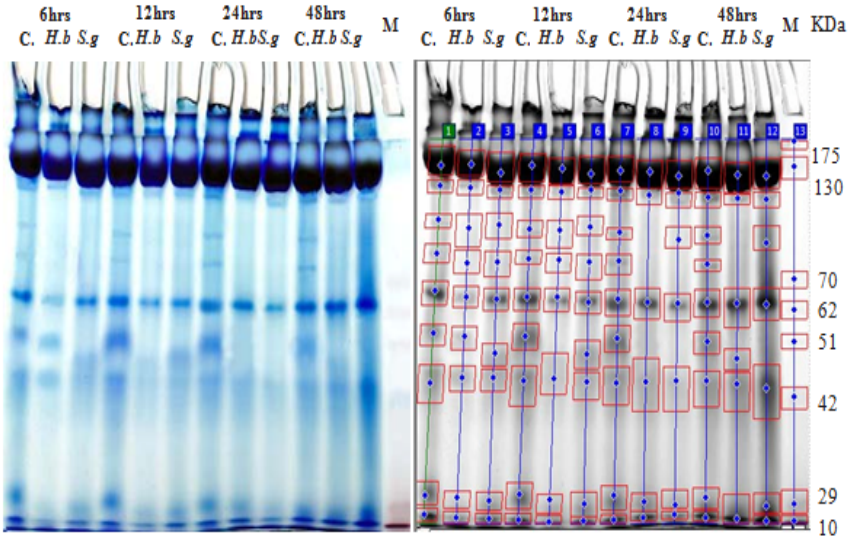

Fig. 5: Electrophoretic protein pattern of *A. ipsilon* haemolymph after infected the 6th instar larvae with entomopathogenic nematodes.

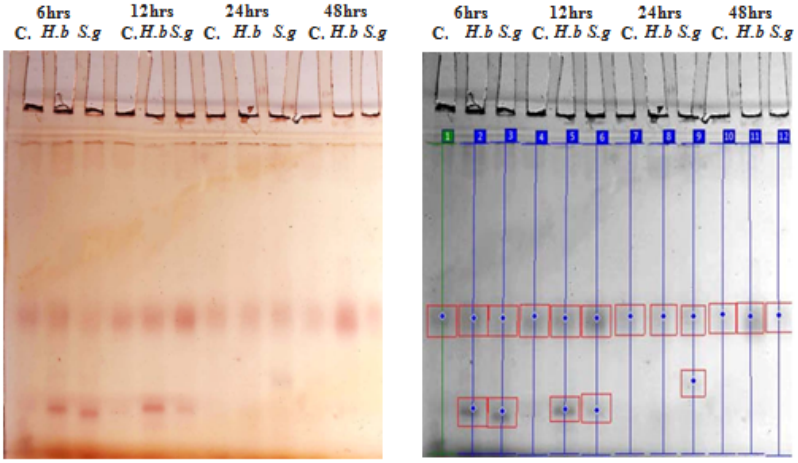

Fig. 6: Electrophoretic acid phosphatase pattern of *A. ipsilon* haemolymph after infected the 6th instar larvae with entomopathogenic nematodes.

ARABIC SUMMERY

القدره المرضية للنيماتودا الممرضه للحشرات *Steinernema glaseri* و *Heterorhabditis bacteriophora Poinar (HP$_{88}$ strain)* ضد الدودة القارضة *Agrotis ipsilon*

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تمت دراسة القدره المرضية للنيماتودا الممرضه للحشرات *Steinernema glaseri* و *Heterorhabditis bacteriophora Poinar (HP$_{88}$ strain)* ضد العمر اليرقى الثالث والرابع والخامس والسادس للدودة القارضة *Agrotis ipsilon* و ذلك *من* خلال تسجيل نسبة الموت للاعمار التى تم اصابتها بالنيماتودا المختبرة على فترات زمنية مختلفة . وحيث ان قدرة النيماتودا تعتمد الى حد كبير على قدرتها على التغلب على الجهاز المناعى للعائل لذلك فقد تم متابعة التغييرات المتبادلة بين كل من الجهاز المناعى (خلايا الدم) للحشرة والنيماتودا المختبرة وذلك من خلال تسجيل عملية الكبسلة والابتلاع وتشكيل العقيدات. وبما ان عملية الابتلاع تعمل على تحفيز انزيمات الليزوزومات والتى منها انزيم الفوسفاتيز الحمضى لذلك فقد تم تسجيل التغييرات الحادثة فى كل من نشاط انزيم الفوسفاتيز الحمضى الكمى والكيفى وكذلك التغييرات فى المحتوى الكلى والنمطى للبروتين. وقد اشارت النتائج الى ان كل من *S.glaseri* و *H. bacteriophora* لهما تأثير معنوى واضح على الحشرة المختبرة وان كانت *S.glaseri* لها التأثير الاكبر. كما اوضحت النتائج ان هناك علاقة بين نسبة الموت والفترة الزمنية التى تتعرض لها الحشرة للنيماتودا المختبرة فكلما زادت الفترة الزمنية كلما زادت نسبة الموت. وقد كان العمر اليرقى الرابع والخامس اكثر حساسية تجاه *S. glaseri* عند التركيزات المنخفضة بينما التركيزات المرتفعة سجلت اعلى نسبة موت 100% لجميع الاعمار بعد فترة تعرض 96 ساعه بينما كان العمر اليرقى الثالث والرابع اكثر حساسية تجاه *H. bacteriophora*. وقد تم التعرف على ستة انواع من خلايا الدم فى دم العمر اليرقى السادس للدودة القارضة وهم الخلايا الاولية و البلازمية و المحببة والمستديرة و النبيذية والخلايا غير المتحركة. وقد كان معدل العملية الابتلاعية فى خلايا دم الدودة القارضة المصابة ب *H. bacteriophora* اعلى من المصابة ب *S. glaseri* كما سجلت النتائج نقص معنوى فى المحتوى الكلى للبروتين فى كل من المعاملتين بعد 24 ساعة من الاصابة بينما كانت هناك زيادة معنوية فى نشاط انزيم الفوسفاتيز الحمضى بعد 6 ساعات من الاصابة بالنيماتودا المختبرة . وقد فسرت هذه الزيادة نتيجة للعملية الابتلاعية التى تم تسجيلها فى هذه الدراسة حيث ان العملية الابتلاعية تكون مصحوبة بزيادة فى انزيمات التحلل. وقد لوحظ ظهور حزمة انزيمية مميزة للعينات المصابة بالنيماتودا وقد ترجع هذه الحزمة الى الافرازات الانزيمية التى تفرزها النيماتودا لكى تستطيع اختراق وهضم الخلايا. كما سجلت حزمة بروتينية وزنها الجزيئى 47 كيلو دالتون متخصصة ل*S. glaseri* وقد تلعب هذه الحزمة دور هام فى تثبيط الجهاز المناعى للحشرة.