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Effect of *Bacillus thuringiensis* Var *Kurstaki* on the Ultra Structure of Mature *Fasciola gigantica* Flukes in Egypt

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Abstract: *Fasciola gigantica* adult flukes were treated *in vitro* with different concentrations (0.5, 1 and 2 mg) of *Bacillus thuringiensis* var *kurstaki*. The lethal concentration observed for the worms was 2 mg after 24 hours exposure, with LC_{50} value of 1.1 mg/ml. Scanning electron microscopy of adult worms treated with *B.t. kurstaki* showed upward shifting of the oral cone, with neck formation due to stretching of the worm tissue and herniation of the internal organs through oral suckers. The transverse folds of the rim of the oral suckers became lobulated, with loss of sensory papillae and gland pores. There were blebs of different sizes and erosions at the transverse folds of the oral suckers as well as in the mid-body regions. The blebs increased in size and burst, resulting in spine loss. Transmission electron microscopy showed different changes in the tegument, gastrodermis and vitelline cells of the treated *F.gigantica* adult worms. The apical plasma membrane of the tegument became infolded with irregular invaginations with marked reduction in the T2 secretory granules. The gastrodermis changes include detachment and disruption of cell-lamellae. The treated vitelline cells are vacuolated and the shell protein clusters are loosely packed with disruption some of them.

Key words: *Fasciola gigantica* · *Bacillus thuringiensis* · Biological control · Scanning and Transmission electron microscopy

INTRODUCTION

Tropical fasciolosis, which is caused by infection with *Fasciola gigantica,* is considered to be one of the most prevalent helminth infections among different animals, particularly ruminants, all over the world. In Egypt, the economic losses have reached 484.5 million Egyptian pounds per year according to the report of the Central Organization of Mobilization and Computation, Cairo (2000). The disease can also cross-infect humans, resulting in clinical and epidemiological health problems. Haseeb *et al.* [1] estimated that 830,000 individuals are currently suffering from fasciolosis in Egypt. Important strategies toward the control of fasciolosis have depended on chemical anthelmintics (fasciolicide). Triclabendazole (TCBZ) has been considered the drug of choice for treatment of fasciolosis [2]. Due to the world-wide use of anthelmintics, resistance has developed in some cases exhibits an undefined intensity [3]. An alternative effective treatment is required in order to avoid the drawbacks of anthelmintics on animal health, public health and the environment. Currently, biological control of fasciolosis represents a new trend in controlling parasitic infections in animals and humans. With regard to novel compounds for use as anthelmintics, there has been a growing interest in making use of natural plant products that have been used as traditional medicines in developing countries [4 - 10]. Herbal treatment for fasciolosis in animals is being considered both by researchers and pharmaceutical companies [11 - 15]. In addition, the non-pathogenic bacteria, *Bacillus thuringiensis,* has been widely used for the treatment of external and internal parasites [16 - 19]. The efficacy of the *Bacillus* strain is due to the production of two main types of bacterial toxins; delta toxins which are protein crystals and beta endotoxins which are adenine nucleotides with glucose [20, 21]. Hassanain *et al.* [18] found that *B. thuringiensis* var *kurstaki* was the most effective strain for treating sheep infected with *F. gigantica*. *In vitro* the light microscopy studies showed tegumental pathological

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changes with sloughing of spines and zenker necrosis in the muscle layer. The tegument is known to be a good model system for the estimation of drug effects due to its nutritive, immuno-protective and osmoregulatory functions [22 - 24]. The trematode tegument is a complicated syncytium comprising perinuclear and distal surface regions which are connected by cytoplasmic bridges. The tegumental cells are responsible for production of secretory granules To, T 1 & T 2 at different stages of worm development. Different vermicidal compounds were used against mature and immature *F. hepatica* (benzimidazole, triclabendazole, luxabendazole, nitroxynil and clorsulon). These compounds produced high efficacy against flukes, either *in vitro* or *in vivo* trials [25 - 28]. TEM pictures revealed disruption of microtubules and tegumental cells with more sever affection of gastro dermal cells.

The present research aimed to study the effects associated with *in vitro* treatment of adult *F. gigantica* worms with *B. thuringiensis* var *kurstaki* through scanning and transmission electron microscopy (SEM and TEM).

MATERIALS AND METHODS

Bacillus Thuringiensis Preparations: Dipel 2x (*B.t. Kurstaki*) Vectobac (*B.t. israelensis*) and HD703 (*B.t. thuringiensis)* were used in the present study and are products of Abott.

Worms Collection: *F. gigantica* adult worms were collected from condemned bovine livers obtained from the Cairo abattoir. Adult flukes were recovered from bile ducts and gall bladders under sterile conditions in a laminar flow cabinet, washed with sterile distilled water (30-32°C) and maintained in RPMI media supplemented with antibiotics (penicillin, 50 IU /ml; streptomycin, 50 ug /ml). The flukes were subsequently transferred to fresh culture medium containing 50% (v/v) heat denatured rabbit serum and 2% (v/v) rabbit red blood cells; as showed by Ibarra and Jenkins [29]. The collected worms were adjusted for the control and treated groups as described below.

***In vitro*- Treatment of Worms:** A total number of 45 *F.gigantica* mature flukes were used and replicates of 15 worms each were placed in petri dishes and exposed to three concentrations (0.5, 1 and 2 mg) of *B.t. Kurstaki*, *B.t. israelensis* and *B.t. thuringiensis,* with 3 replicates of each concentration. Adult worms were incubated with the bacterial toxin dissolved in tween-20 for 24 hours at 30-32°C and examined for the percentage of viability and mortality every 60 min with subsequent LC50 determination. The LC50 value was computed based on the mortality percentage recorded for toxin dilution through probity analysis within 95% confidence limits. The treated mature flukes were subjected to scanning and transmission electron microscopy.

Fresh Untreated Control Worms: Fifteen worms were used in the control group and were observed for 15 min in a separate Petri dish to ensure that worms were movable and active. Only one replicate was performed. The worms were then used directly for SEM.

Scanning Electron Microscopy (SEM): Both treated and untreated groups were washed with sterile distilled water and then fixed for 12 hours in a 3:1 mixture of 4% (w/v) glutaraldehyde 0.12M Millonig's buffer, pH 7.4 and 2% aqueous osmium tetroxide. The flukes were washed repeatedly in double–distilled water, dehydrated in acetone, critical point dried in carbon dioxide, fixed to alumonium stubs and coated with gold–Pallidium in a polaron E.5000 coating unit. The specimens were viewed with an ISI super Mini –SEM.

Transmission Electron Microscopy (TEM): Thin transverse sections were removed from the mid-body region of whole adult flukes. Tissue slices were processed intact. The material was fixed over night at 4°C in 4% (w/v) glutaraldehyde in 0.12 M Millonig's buffer, PH 7.4, containing 3% (w/v) sucrose and 0.5 mM calcuim chloride for 6 hours. This was followed by post-fixation for 1.5 hours in 1% osmium tetroxide in 0.12 M Millonig's buffer, PH 7.4 dehydration through alcohol and embedding in spurs low-viscosity resin (Spurs, 1969). Ultra thin sections were cut on an LKB ultra tom IV, mounted on uncoated copper grids and double-stained with alcoholic uranyl acetate, for 5 min and aqueous lead citrate for 8 min. Sections were viewed with a jeoll OO-Cx transmission electron microscope operated at 100 kv.

RESULTS

Effect of *Bacillus Thurengensis*, Different Strains on *Fasciola Gigantica* Mature Flukes *in Vitro*: *Fasciola* adult worms were treated *in vitro* with the three subspecies of *B. thuringiensis* and mortality percentages were recorded every 60 min. The viability of flukes did not change until 11 hr post treatment (Table 1).

Table 1: Mortality percentages of *F. gigantica* mature flukes treated with *B. thuringiensis* (in *vitro* studies)

	Mortality (%)								
	Dipel 2X			Vectobac			Hd 703		
Hours Post Treatment	0.5 mg	1 mg	2 mg	0.5 mg	1 mg	2 mg	0.5 mg	1 mg	2 mg
11	-	-	20	-	-	7	-	-	-
12	-	-	27	-	-	7	-	-	7
14	-	-	40	-	-	20	-	-	7
16	-	-	47	-	-	33	-	-	7
18	-	20	60	-	7	33	-	20	13
20	-	20	80	-	20	40	-	27	40
22	-	20	100	-	20	53	-	33	40
24	-	20	100	-	20	100	-	33	100

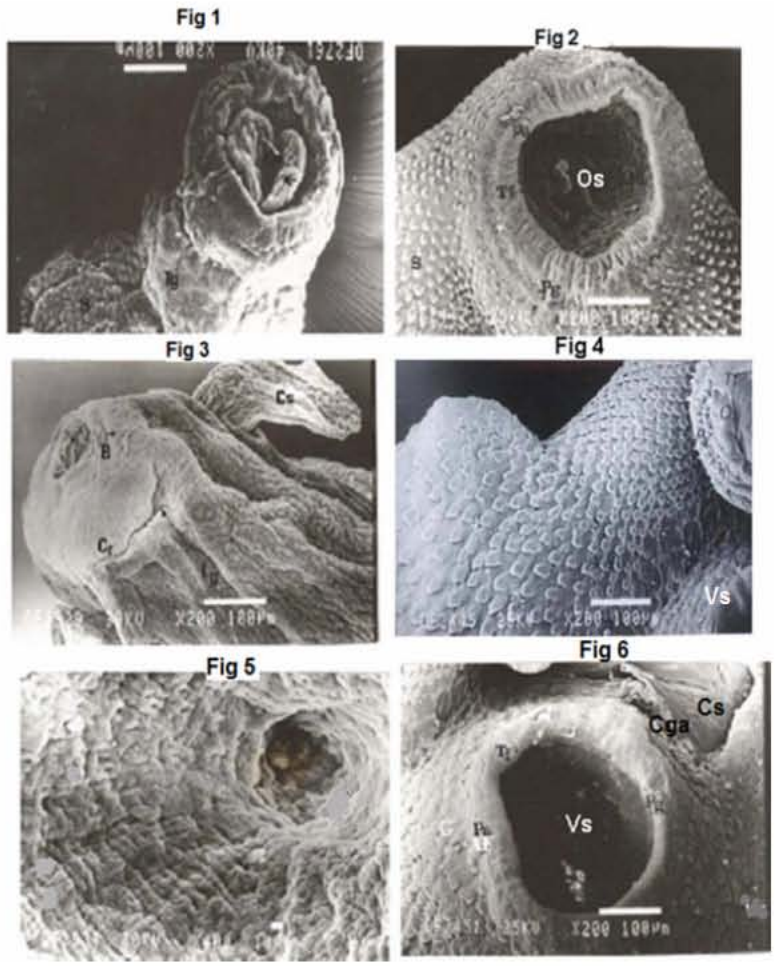
Based on the LC50 values demonstrating the potency of *B. t. kurstaki*, worms treated with this variety are subjected to investigation with scanning and transmission electron microscopy

On that time, only 2 mg Dipel 2X (*B.t.kurstaki*) and 2 mg Vectobac (*B.t.israelensis*) resulted in 20% and 7% mortality, respectively. Then, the effect of different varieties began to increase with time. After 22hrs post treatment, all mature flukes treated with 2 mg Dipel 2X died, compared to only 53% and 40% mortality of worms treated with Vectobac and Hd 703 (*B. t. thuringiensis*), respectively. The Lc_{50} values of these varieties on mature flukes, at 22hrs post-treatment were 1.1 mg for Dipel 2X, 1.9 mg for Vectobac and 2.0 mg for Hd 703. After 24 hrs of exposure, all treated flukes with 2mg *Bacillus thuriengiensis* died with Lc_{50}1.1, 1.1 and 1.0 mg for Dipel 2X Vectobac and Hd 703, respectively. Worms treated with 0.5 mg of *B. thuringiensis* different strains, showed survival rates similar to the unexposed control ones.

SEM of Normal Fresh *F. Gigantica* Adult Flukes: Under scanning electron microscopy, the tegumental surface of the worm appeared rough due to the presence of numerous spins (S) and surface folding. The anterior and middle parts of the ventral surface showed the oral (OS) and ventral suckers (VS), with the common genital atrium (CGA) in between and also cirrus sac (Figs. 2, 4, 6). The oral cone comprises the smooth oral and ventral suckers, with thick rims covered with transverse folds (TF) and grooves in between. The transverse folds are not cover with spines and are arranged in two levels (doubling folds) surrounding the opening of the oral and ventral suckers. Both suckers are surrounded by rows of sensory papillae and in addition, there are pores of glands (pg) in between the transverse folds (Figs. 2,4). The spines were most numerous anteriorly and decreased in numbers posteriorly and toward the peripheral margins.

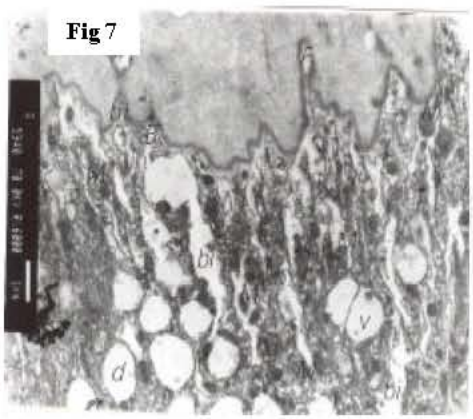
SEM of Adult *F. Gigantica* Treated with *B.t. Kurstiki in Vitro:* The bacterium, *B.t. Kurstaki,* produces inflammation with edematous swelling of the tegumental tissues of the worm. Consequently, the oral cone shifts upwards, leading to neck formation due to stretching of the tissues (Fig. 1). Additionally, separating tegumental folds were seen extending from the transverse folds of the oral sucker. The oral sucker became deformed, resulting in formation of transverse fissure or groove and swelling of cirrus sac (Fig. 3). Progressively, extensive damage of the tegumental fissure or crack (cr) separating the oral sucker from the oral cone was observed (Fig. 3). The oral suckers showed a protrusion or herniation of the internal contents, particularly the pharynx (Fig. 1). The transverse folds of the oral sucker rim were disturbed and became lobulated, with loss of sensory papillae and pores of glands (Fig. 1). The normal tegumental folds and grooves were lost, as well as the plateaus and valleys of the tegumental folds. Finally, the blebs in the mid body region increased in size and burst, causing lesions and loss of spines. Subsequently, the tegument peeled off, exposing the basal lamina beneath and producing a tegumental ulcer with progressive damage (Fig. 5).

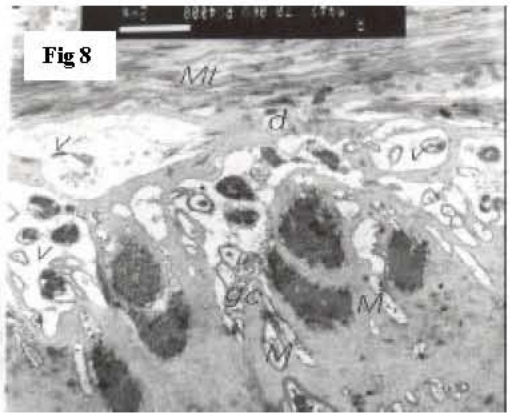
TEM of Normal Untreated *F. Gignatica* Adult Flukes: The tegument is a nucleate cytoplasmic syncytuim, with basement membrane below. The apical tegumental plasma membrane (apm) is somewhat straight rather than folded. It is also provided with small number of surface pits (Sp) and invaginations. The apical zone of the cytoplasm was more electron dense granules and is free of mitochondria expect at the lower part of the syncytium are stacked horizontally. Whereas, numerous mitochondria (M) are



Figs. 1-6: Comparative SEM of *in vitro* treated and fresh normal *F. gigantica* flukes: (1) Scanning electron micrograph of treated worm showing herniation of pharynx (arrow), swelling tegument (Tg), oral sucker (Os) and disruption of spines (S). Bar = 100 µm. (2) Scanning electron micrograph of normal *F. gigantica* oral cone showing oral sucker (Os), pharynx, transverse fold and grooves (Tf), sensory papillae (Pa), pores of glands (Pg) and spines (S). Bar = 100 µm. (3) Scanning electron micrograph of treated worm showing blebs (B, arrow) at rim folds of oral sucker, destructed cirrus (Cs), cracks (Cr) and longitudinal grooves (Lg) in the tegument. Bar = 100 µm. (4) Scanning electron micrograph of normal *F. gigantica* oral sucker (Os), ventral sucker (Vs) and shoulder region with characteristic pattern of spines (S) distribution, transverse folds and grooves (Tf), sensory papillae (Pa). Bar = 100 µm. (5) Scanning electron micrograph of treated worm showing tegumental ulcer (large arrow)) with complete destruction of spines. Bar = 10 µm. (6) Scanning electron micrograph of normal *F. gigantica* ventral sucker (Vs) showing transverse folds and grooves (Tf), sensory papillae (Pa), pores of glands (Pg), common genital atrium (Cga) and cirrus (Cs). Bar= 100 µm. m.

found near or adjacent to the basement membrane with underlying muscle layer (Ml) (Fig. 9). The tegumental granules of *F. gigantica* adult flukes are of two types; T1, T2 (Fig. 10). There are few of the T1-type tegumental secretory granules or vesicles within the tegument. The T 2-type is more predominant and dense. T 1 and T2 granules express their contents into the tegumental surface as contribution to glycol calyx. The fine structure of the gastrodermis in the present study is that of cell-type lining the main caeca of *F. gigantica* (Fig. 12). The cells have small pyramid-shaped extensions of apical cytoplasm and are provided with micro villi (gm), cross sectioned lamellae (csl) and pseudopodia like bodies (ps) distributed in the parenchyma. Concerning vitelline cells, the immature and mature types of cells in their corresponding follicles were present (Fig. 15).





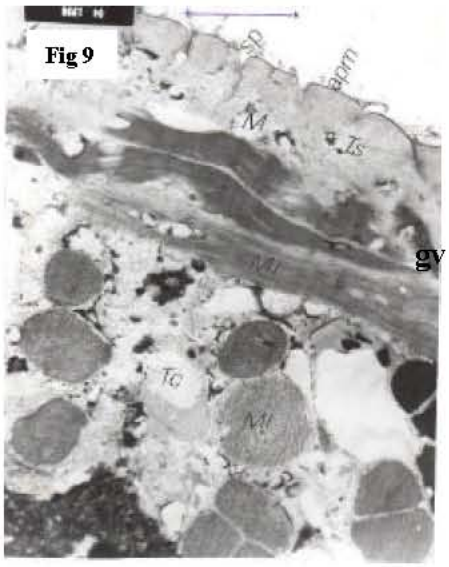
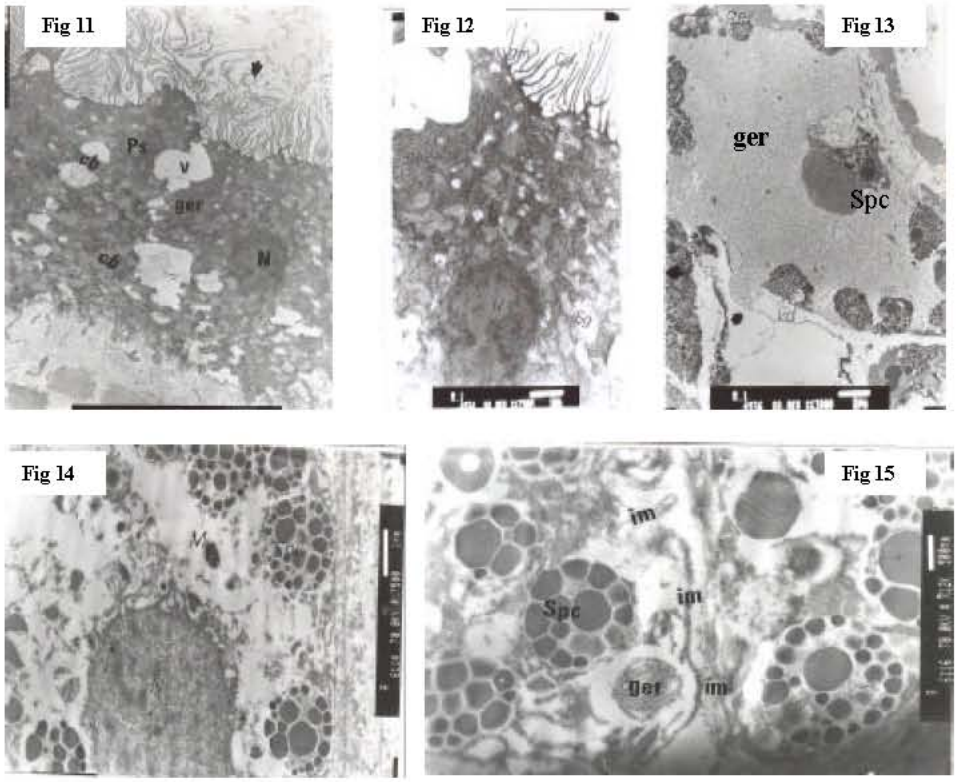


Fig. 7-10: Comparative TEM of *in vitro* treated and fresh normal *F. gigantica* flukes: (7) Transmission electron micrograph of treated *F. gigantica* tegumental syncytium. The apical plasma membrane becomes infolded in some sites and protrudes upward (F), swelling of basal infoldes (bi) and shifting upward, blebs (B), mitochondria (M), vacuoles(V) and lipid (d) Bar = 1 µm. (8) Transmission electron micrograph of treated *F. gigantica* tegumental cells. The cells are vaculated (v) and cytoplasm is filled with swollen mitochondria (m), lipid droplets (d), the golgi complex (gc) and muscle layer (MI) Bar = 2 µm. (9) Transmission electron micrograph of normal *F. gigantica* golgi vesicles (gv). Mitochondria (M) are stacked horizontally at the lower part of syncytium, muscle layer (Ml), tegumental cells (tc) and tegumental syncytiun (Ts). Bar = 5 µm. (10) Transmission electron micrograph of normal *F. gigantica* syncytium at higher magnification. Secretory granules (T1 &T2) and apical plasma membrane (amp). Bar = 1 µm.

Free ribosomes (r) distribute through the cytoplasm and some granular endoplasmic reticulum (ger) is situated at the distal parts of the cytoplasm. In mature vitelline cells, the granular endoplasmic reticulum becomes limited to the cell periphery, besides more lipids (d), ribosomes (r) and glycogen. In addition, there are shell protein globules (Spc) accumulated in spherical clusters with micro vesicles of glycogen and yolk globules.

TEM of Adult *F. Gigantica* Treated with *B. T. Kurstaki in Vitro:* The apical tegumental plasma membrane (apm) becomes infolded with irregular invaginations and in some sites protrudes (Fig. 7). The produced invaginations contain blebs (B) just below the tegumental surface and extend over the tegumental surface. The cytoplasm is extensively vacuolated, disrupted and many myelin whorls with lipid droplet (d) are present.



Figs. 11-15: Comparative TEM of *in vitro* treated and fresh normal *F. gigantica* flukes: (11) Transmission electron micrograph of treated *F. gigantica* gastrodermis. Detachment and disruption of cell lamellae with increased secretory granules (arrow), nucleus (N), granular endoplasmic reticulum (ger), vacuole (v), pseudopodium-like bodies (Ps) and cytoplasmic bodies (cb) Bar = 2 µm(12) Transmission electron micrograph of normal *F. gigantica* gastro dermis. Cross-sectioned lamellae (csl), gastro dermis microvilli (gm), pseudopodium - like bodies (ps), golgi complex (gc), granular endoplasmic reticulum (ger), nucleus (N), secretory granules(sg) and vacuoles (v). Bar = 1 µm. (13) Transmission electron micrograph of treated *F. gigantica* mature vitelline cell with higher magnification. shell-protein globule clusters (Spc) and nucleus(N) Bar = 0.5 µm. (14) Transmission electron micrograph of treated *F. gigantica* mature vitelline cell with higher magnification. Shell-protein globule clusters (Spc), cistern of granular endoplasmic reticulum (ger) and interstitial material (im) Bar = 0.5 µm. (15) Transmission electron micrograph of normal *F. gigantica* vitelline cell development. parenchymal cell (P), vitelline duct(vd), nurse cell cytoplasmic extension (ne), granular endoplasmic reticulum (ger), shell-protein globule clusters (Spc) and ribosomes (r) Bar = 2 µm.

There is a marked reduction in the T 2 secretory bodies through the tegumental sanctum. The basal lamina membrane is disrupted with sever swelling of the basal in folds which is shifting upwards almost to the apex of the tegument (Fig.7). There are aggregations of mitochondria (m) all over the tegumental syncytium which do not restricted to the basal region as in the normal tegument (Fig. 7). The TEM shows swollen, diffuse, electron lucent appearance of the sub tegumental musculature (swollen, diffuse and lucent blocks of circular and longitudinal muscles (M I), (Fig. 8). The tegumental cells are extensively broken down and show spaces between individual cells and the surrounding parenchyma. The cytoplasm is filled with swollen mitochondria, lipid droplets, vacuoles and the Golgi bodies are more swollen and irregular than normal with decrease in numbers of secretory bodies (Fig. 8). Concerning the changes in the gastrodermis due to the effect of *B. T. Kurstaki,* the obtained results revealed the detachment and disruption of cell lamellae with increased secretory granules. No changes in the granular endoplasmic reticulum with heavy increased of cytoplasmic bodies (cb) and vacuoles (v) with different sizes at different levels of the cytoplasm (Fig. 11). The

treated vitelline cells are vacuolated and the nucleus of the cell is swollen and condensation of its chromatin occurred (Fig. 13). The shell protein globule clusters (Spc) within the mature vitelline cells are loosely packed. The yolk globules and glycogen deposits become fewer than normal with disruption to some shell globules (Fig. 14). The granular endoplasmic reticulum and Golgi cistern are swollen and disrupted. Also there is a marked decrease in the ribosomal covering.

DISCUSSION

The present study revealed promising results for the successful use of the non-pathogenic bacteria, *B. T. var kurstaki,* as a vermicidal product against *F. gigantica* during *in vitro* trials. The Lc_{50} value of the bacterial compound was 1.1mg/ml. The mechanism of action of the biological material is due to bacterial toxins [16, 17, 20 and 21], as proven by the scanning electron microscopy of the *in vitro* treated adult flukes. The mentioned damages in the present research would undoubtedly disrupt the physiological processes associated with the tegument; osmoregulation, nutrient uptake and immunoprotection [2]. Some of these changes, particularly the surface blebbing and the microvillus are common features of the anthelimintic-treated parasitic *F. hepatica* [15, 30 - 35]. Concerning the transmission ultra structure in the present investigation, the lethal effect of the bacterium on the tegument structures, gastrodermis and vitelline glands was shown. Somewhat similar results were shown by Rew *et al.* [36], Fairweather *et al.* [37] and Anderson and Fairweather [38] who studied the effect of diamphenethide free amine (DPT-FA) and deacylated (amine) metabolite of diamphenethide (DAMD) on *F. hepatica*. They revealed an accumulation of T 2 secretory bodies at the apical surface of the tegumental syncytium, together with blebbing of the surface membrane and marked swelling of the basal infolds and edema. These authors explained the action of diamphenethide as an inhibitor of protein synthesis, may help to explain the drug higher efficacy against juvenile than adult *F. hepatica* flukes and the disruption of its osmo-regulatory system. Also in agreement, Skuce and Fairweather [23], Ledger and Tanzer [29] and Dunphy and Rothman [40] stated that monensin; sodium ionophore produced osmotic swelling of the Golgi and an inhibition of the intercellular transport of secretory products with swelling of subtegumental musculature.

In the tegumental cells, there were distinct accumulations of T1 secretory bodies around the Golgi complexes. Tubulozole-treated flukes showed more sever effects with initial accumulations of secretory bodies, both at the tegumental apex and base [41 - 43].

The *in vitro* effect of *B. t. Kurstaki* against *F. hepatica* tegument was the same did with flukicide; clorsulon [27]. The drug produced sever swelling of the basal infolds in the tegumental syncytium and large cytophagic vacuoles. Also swollen mitochondria with fewer Golgi complexes are found. The changes in the gastrodermal cells were evident; swelling of the cisternae of the endoplasmic reticulum, an increase in the number of autophagic vacuoles, a reduction in the number of secretory bodies and disruption of the lamellae projecting from the surface of the cells. The changes in the gastrodermis and vitelline glands of *F. gigantica* adult flukes in the present study were similar to those obtained by Stitt and Fairweather [30, 42].

The present study revealed that, bacterium *B. t. Kurstaki* produces sever changes in the tegument, gastrodermis together with immature and mature vitelline cells. So, it could be concluded that this non pathogenic *Bacillus* has biological vermicidal effect on *F. gigantica* flukes.

REFERENCES

1. Haseeb, A.N., A.M. El-shazly, M.A. Arafa and A.T. Morsy, 2002. A review on fascioliasis in Egypt. J. the Egyptian Society of Parasitol., 32: 337-354.
2. Fairweather, I. and J.C. Boray, 1999. Fasciolicides: efficacy, actions, resistance and its management. Veterinary J., 158: 81-122.
3. Overend, D.J. and F.L. Bowen, 1995. Resistance of *Fasciola hepatica* to triclabendazole. Australian Veterinary J., 72: 275-276.
4. Buenz, E.J., D.J. Schnepple, B.A. Bau-er, P.L. Elkin, J.M. Riddle and T.J. Motley, 2004. Techniques: Bio prospecting historical herbal texts by hunting for new leads in old tomes. Trends Pharmacology Sci., 25: 494-498.
5. Buenz, E.J., B.A. Bauer, H.E. Johnson, G. Tavana, E.M. Beekman, K.L. Frank and C.L. Howe, 2006. Searching historical texts for potential new drugs. British Medical J., 333: 23-30.
6. Crump, A., 2006. New medicines from nature's armamentarium. Trends Parasitol., 22: 51-54.
7. Newman, D.J. and G.M. Cragg, 2007. Natural products as sources of new drugs over the last 25 years. J. Natural Products, 70: 461-477.

8. Toner, E., G.P. Brennan, K. Wells, J.G. McGeown and I. Fairweather, 2008. Physiological and morphological effects of genistein against the liver fluke, *Fasciola hepatica*. Parasitol., 135: 1189-1203.
9. Haçariz, O., G. Sayers, M. Mc Cullough, M. Garrett, J. O'Donovan and G. Mulcahy, 2009. The effect of Quil A adjuvant on the course of experimental *Fasciola hepatica* infection in sheep. Vaccine, 27: 45-50.
10. Shehab, N.G., A.R. Abdel Monem, S.E. Hassan and N.I. Toaleb, 2009. Botanical study of *Meryta denhamii seem* and its anthelmintic activity against *Fasciola gigantic*. J. the Egyptian Society of Parasitol., 39: 269-288.
11. Muddathir, A.K., G. Balansard, D.P. Timon, A. Babadjamian, A.K. Yag-oub and M.J. Julien, 1987. Anthelmintic properties of polygonum galbrum. J. Pharmacia and Pharmacol., 39: 296-300.
12. Shiramizu, K. and M. Abu, 1991. The preventive investigation of bovine fascioliasis. The effectiveness of a fern as anthelmintics in crude drug recipe. Yamaguchi J. Veterinary Medicine, 18: 39-45.
13. Shiramizu, K., T. Tuchida and M. Abu, 1993. Anthelmintic effect of a crude preparation containing the root of Matteuccia orientalis on bovine fascioliasis. J. Japanese Veterinary Medical Association, 46: 561-564.
14. Abdel-Rahman, E.H., O.M. Kandil and K.N. Abdel-Megeed, 1998. Comperative studies of lethal effect of *Bacillus thuringiensis*, *Allium sativum* and Nerium olunder on Tricostrogylidae parasites.Egyptian J. Zool., 30: 65-70.
15. Hegazi, A.G., F.K. Abdel-Hady and H.A. Shalaby, 2007. An *in vitro* effect of propolis on adult worms of *Fasciola gigantica*. Veterinary Parasitol., 144: 279-286.
16. Bottjer, K.P., L.W. Bone and S.S. Gill, 1985. Nematode: Susceptibility of the egg to *Bacillus thuringiensis* toxin. Experimental Parasitol., 60: 234-241.
17. Hassanain, M.A., M.F. Garhy, F.A. Abdel-Ghaffar, A. El-sharaby and K.M. Abdel-Megeed, 1997. Biological control studies of soft and hard ticks in Egypt. 1-The effect of *Bacillus thuringiensis* varities on soft and hard ticks (ixodidae). Parasitol. Res., 83: 209-213.
18. Hassanain, M.A., E.H. Abdel- Rahman and K.N. Abdel-Megeed, 1998. Vermicidal effect of *Bacillus thuringiensis* on *Fasciola gigantica*. J. Egyptian Veterinary Medical Associatio, 58: 251-267.
19. Hassanain, M.A., M.M. Abdel-Aziz and N.T. Abu- Eiezz, 1999. Nematocidal effect of *Bacillus thuringiensis* on *Trichostrongylus colubriformis* (Nematode). Egyptian J. Veterinary Sci., 33: 19-27.
20. Carberg, G. and R. Lindstrom, 1987. Testing fly resistance to thuringiensis produced by *B. thuringiensis* serotype H.1. J. In veterinary Pathol., 49: 194-197.
21. Jespersen, J.B. and J. Keiding, 1990. Bio control of arthropods affecting life stock and poultry (ed). Donald A. Rutz and Richard S. Patterson West view Press Boulder, San Francisco Oxford.
22. Hanna, R.E.B., 1980. *Fasciola hepatica*: an immuno-fluorscent study of antigenic changes in the tegument during development in the rat and sheep. Experimental Parasitol., 50: 155-170.
23. Skuce, P.J. and I. Fairweather, 1989. *Fasciola hepatica*: the effect of the sodium ionophore monensin on the adult tegument. Parasitol. Res., 75: 223-232.
24. Stitt, A.W. and I. Fairweather, 1991. *Fasciola hepatica*: the effect of the microfilament inhibitor cytochalasn B on the ultrastracture of the adult fluke. Parasitol. Res., 77: 675-685.
25. Stoitsova, S. and L. Gorchilova, 1994. Effect of Luxabendazole on the tegument of *Fasciola hepatica*. J. Helminthol., 68: 73-80.
26. Stoitsova, S. and L. Gorchilova, 1997. Effects of colchicine on the number of Tz vesicles in the tegument of *Fasciola hepatica*. Folia Parasitol., 44: 283-288.
27. Meaney, M., S. Haughey, G.P. Brennan and I. Fairweather, 2005. Ultra structural observations on oral ingestion and trans tegumental uptake of clorsulon by the liver fluke, *Fasciola hepatica*. Parasitol. Res., 95: 201-212.
28. Mckinstry, B., G.P. Brennan, L. Halferty, A.B. Forbes and I. Fairweather, 2007. Ultrastructural changes induced in the tegument and gut of *Fasciola hepatica* following *in vivo* and *in vitro* drug treatment with nitro xynil (Trodax). Parasitol. Res., 101: 929-941.
29. Ibarra, O.F. and D.C. Jenkins, 1984. An *in vitro* screen for new fasciolicidal agents. Zeitschrift fiir Parasitenkunde, 70: 655-661.
30. Stitt, A.W. and I. Fairweather, 1993a. *Fasciola hepatica*: The effect of the microtubule inhibitors colchicine and tubulozole-c on the ultra structure of the adult fluke. Parasitol., 07: 297-309.

31. Robinson, M.W., A. Trudgett, E.M. Hoey and I. Fairweather, 2002. Tri-clabendazole-resistant *Fasciola hepatica*: β-tubulin and response to *in vitro* treatment with ticlabendazole. Parasitol., 124: 325-338.
32. Meaney, M., I. Fairweather, G.P. Bernnan, P. Ramasamy and P.B. Subrama-nian, 2002. *Fasciola gigantica*:tegumental surface alteration following treatment *in vitro* with the sulphoxide metabolite of triclalbendazole. Parasitol. Res., 88: 315-325.
33. Meaney, M., I. Fairweather, G.P. Bernnan, L.S.L. McDowell and A.B. For-bes, 2003. *Fasciola hepatica*: effects of the fasciolicide clorsulon *in vitro* end *in vivo* on the tegumental surface and a comparison of the effects on young and old-mature flukes. Parasitol. Res., 91: 238-250.
34. Buchanan, J.F., I. Fairweather, G.P. Bernnan, A. Trugett and E.M. Hoey, 2003. *Fasciola hepatica*: surface and internal tegumental changes induced by treatment *in vitro* with the sulphoxide metabolite of albendazole(valbazen), Parasitol., 126: 141-153.
35. McMinstry, B., I. Fairweather, G.P. Brennan and A.B. Forbes, 2003. *Fasciola hepatica*: tegumental surface alteration following treatment *in vivo* and *in vitro* with nitroxynil (Trodax). Parasitol. Res., 91: 251-263.
36. Rew, R.S., R.H. Fetterer and T.C. Martin, 1983. Fasciola hepatica: Effects of diamfenetide tree amine on *in vitro* physiology, biochemistry and morphology. Experimental Parasitol., 55: 159-167.
37. Fairweather, I., H.R. Anderson and L.E. Threadgold, 1986. *Fasciola hepatica*: Tegumental changes in the adult fluke induced by the deacetylated (amine) metabolite of diamphenethide *in vitro*.Experimental Parasitol., 62: 336-348.
38. Anderson, H.R and I. Fairweather, 1995. *Fasciola hepatica*: ultra structural changes to the tegument of juvenile flukes following incubation *in vitro* with the deacetylated (amine) metabolite of diamphenethide. International J. Parasitol., 25: 319-333.
39. Ledger, P.W. and M.L. Tanzer, 1984. Monensin-a perturb ant of cellular physiology. TIBS, 9: 313-314.
40. Dunphy, W.C. and J.E. Rothman, 1985. Compartmental Organization of the Golgi Stack. Cell, 42: 13-21.
41. Stitt, A.W. and I. Fairweather, 1994. The effect of the sulphoxide metabolite of Tric1abendazole (Fsinex) on the tegument of mature and immature stages of the liver fluke (*Fasciola hepatica)*. Parasitol., 108: 555-567.
42. Stitt, A.W. and I. Fairweather, 1996. *Fasciola hepatica*; Disruption of the vitelline cells *in vitro* by the sulphoxide metabolite of Tric1abendazole. Parasitol. Res., 82: 333-339.
43. Stitt, A.W. and I. Fairweather, 1993b. *Fasciola hepatica*: tegumental surface changes in adult and juvenile flukes following treatment *in vitro* with the sulphoxide metabolite of ticlabendazole (Fasinex). Parasitol. Res., 79: 529-536.