Bacterial and Viral Pathogens Associated with The White Garden Snail *Theba Pisana* (Müller)

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ABSTRACT: The present study was performed during a periodical interval of nine months in (2014) at Alexandria Governorate, Egypt. Whereas, a laboratory trial was carried out to inspect the field collected dead adult land snails of*Thebapisana* (693 individuals). The inspection revealed that 19.0 % of these collected snails were infected with three pathogenic; bacteria; *Bacillus theuringiensis* (*B.t.*) (2.7%), *B. cereus* (0.1 %), and *Brevibacterium* sp. (10.1%) in addition to a polyhedrosis virus (2.7 %). Moreover, the made laboratory evaluation showed that the assessed daily cumulative mortality of snails as well as LC_{50} (Concentration of active ingredient responsible for killing 50% of the examined number animals) value of *B.t* post 13 days of snail treatment was ($7.3x10^8$ Visible spore (VS)/ml), while these determined values for *Brevibacteriumsp*. and polyhedrosis virus(P.V) were (7.6×10^8VSs/ml) and (3.3×10^6 Polyhedral Inclusion Bodies (P.I.Bs) /ml) after four and 16 days, respectively. The LT_{50} (Time in days, needed to kill 50% of the exposed pest) for *Brevibacterium* sp. ranged from 9.8 to 16.3 days for the different tested concentrations. The LT_{50} of lower concentration of polyhedrosis virus was much prolonged up to (10.6 days) compared to that of the highest concentration (1.5-day). Also, the LT_{50} values of *B.t* ranged from 10.2 to 17.5 day for the lowest and highest tested concentrations .The ease and simplicity of the followed bioassay procedures, and the reliability of the method described, as shown by the statistical evaluation of the obtained data, justify the use of the adopted method in the present study.

Keywords: land snails,*T.pisana*, *Bacillus thuringiensis*, *B.cereus, Brevibacteriumsp*., polyhedrosis virus, natural infection, bioassay, infectivity, microbial control.

INTRODUCTION

The land snails are considered as a serious agricultural animal- pests of worldwide (Speiser and Kistler; 2002).They attack plants causing serious damage (Godan, 1983). The economic damage caused by these mollusks is due to feeding and contamination with their faces and slime excretion leading to deterioration of the agricultural product quality (Iglesias *et al*., 2003). In Egypt, terrestrial mollusks infest crops, vegetables, ornamental and medicinal plants (Bishara*et al*.,1968, El- Okda 1980 and Eshra 2013). The land snail *T.pisana* was recorded in many Egyptian Governorates attacking various plantations (El-Deeb *et al*., 1996), Abu-bakr 1997, Eshra 1997,Eshra, 2004 and El-Shahaat *et al*., 2009). Osman and Mohamed (1991) reported on the molluscicidal activity of "*Thuricide*" insecticide (*Bacillus thuringiensis*) against some fresh water snails;*Bulinustruncatus* and *B. Alexandrian*, however *B.truncatus* was the most sensitive . The, increased exposure time resulted in increasing mortality. Low concentration of "*Thuricide*" caused a significant decrease in oviposition activity, size of egg-mass and the percentage of hatchability. Kienlen *et al*. (1996) studied the toxicity of some *B. thuringiensis* products and several unformulated strains on three species of slugs; *Derocerasreticulatum, Ariondistinotus* and *Limaxvalentianus* under laboratory conditions. Among the tested products, two contained beta-exotoxins; however, no

strain of *B. thuringiensis* was toxic to the slug species. Azzam and Belal (2002) studied the molluscidal activity of bacterial exotoxin (Victoback12 As) alone and in combination with *Rhabditi*s sp. El-Sabbagh *et al*. (2013) studied the biological control of species of land snails infesting citrus trees.

Therefore, the objectives of present study is devoted to identify the possibly existing or associating bacterial and / or viral pathogens in the collected dead adults of white garden snails; besides a performance of bioassay procedures to determine the toxic activities of some pathogens against the snail.

MATERIALS AND METHODS

1- Animal tested

The adult of *T.pisana* snails (Muller) (family: Hellicidae) were collected from pesticide-free garden at Elmontazah district, Alexandria Governorate, Egypt. Therefore, the snails were fully acclimatized for 15 days under the laboratory conditions prior to the conducted tests.

2- Isolation of pathogens associated with naturally dead *T.pisana* snails.

Under aseptic conditions, Koch's postulates were applied on the abnormal field-collected land snails shell. Snails were surface sterilized by alcoholic flaming, then passed through five separate washings of sterile distilled water (Campbell and Podgwaite, 1971;Hendi, 2003). The externally sterilized snails were transferred individually into sterile Petri dishes, each provided with a few drops of sterile distilled water and thoroughly smashed with the help of sterilized dissection scissors and flat-pointed needle. A loop full of each triturated snail suspension was spread over the surface of each of glycerol nutrient agar and potato dextrose agar in Petri dishes. Then, incubated at 30±2^{o}C for 1-7 days. Bacterial or fungal pure cultures were prepared and maintained on agar slants, then stored at 4^{o}C and periodically recultured to maintain the isolates for use in taxonomical studies and pathogenicity trials. On the other hand, a loop full of the rest of each triturated snail suspension was smeared on a clean microscopic slide and microscopically examined for evidences of viral, protozoan or other pathogens.

T. pisana snail was found to be naturally infected with each of *Bacillus thuringiensis, Brevibacterium* sp., and a polyhedrosis virus. The three pathogens were bio assayed against *T.pisana*, under laboratory conditions of 28.0± 0.3^{o}C, 81.0% RH, and a photo period of nearly (14hL.:10hD.) per day**.**

The stored pure cultures of the bacteria: *Brevibacterium* sp., and *B. thuringiensis* originally isolated from naturally dead snails of the *T.pisana* were used to culture 10-12 nutrient agar plates for each. After 24 hrs.incubation period at 30±2^{o}C, the bacterial growths on the surface of Petri plates of each test bacterium were aseptically scraped off by means of a sterile spatula and inoculated into 250-ml flask containing 100 ml sterile distilled water, then thoroughly mixed. The plate count technique was applied to determine the bacterial concentration in each flask. A suspension of polyhydrosis virus of *T.pisana* (*Tp*PV) was prepared by a thorough

remove of 43 naturally infected *T.pisana*. Manually hemogenied andput in 300 ml distilled water, then strained through a fine cotton cloth and filtered. The resulting suspension was quantified by making counts of the polyhedral inclusion bodies (P.I.Bs) with a haemocytometer. From these bacterial or viral stock suspensions, bioassay tests were carried out using a series of concentrations **(**Hendi, 2003**).**

3-Bioassay of *B.thuringiensis* isolates:

Four concentrations of each *B.t.* isolate were tested; 3.6, 6.0, 8.4, and 12.0 x 10^8 viable spores/ml of *TPB.t.* Snails were placed in plastic cups (12 cm diam.and 9 cm height) tightly covered with plastic sheet, then kept at laboratory conditions of 28.0±0.3^{o}C, 81.0±0.5 % RH, and a photoperiod of nearly 14hL. : 10hD. per day. Each concentration was replicated ten times. In check control treatments, the feeding-lettuce leaves was imbibed only distilled water .The tested of same size snails were randomly chosen of same sizes and starved for about 48 hours before assay. All cups were examined daily for mortality**.**

4- Bioassay of *Brevibacteriumsp*. isolate

In this trial, the same previous bioassay procedures and adopted conditions for *B.t*. were followed, but the tested concentrations were as follows: 2.6, 7.5, 13.0, 18.2, and 23.4 x 10^8 viable bacterial cells (VBCs)/ml.

5- Bioassay of the polyhedrosis virus isolate

The used *T.pisana* snails in this bioassay were starved for nearly 48 hours; then exposed singly to each test concentration as aforementioned in the *B.t.* trial. The bioassesed concentrations were: 1.7, 2.9, 4.0, and 5.8 x 10^6P.I.Bs/ml. Each concentration was replicated ten times. The untreated snails were fed on lettuce leaves treated only with distilled water. All the inspected snails in the conducted treatments and the check treatments had completely consumed the treated or/and untreated lettuce. The cups were kept under laboratory conditions of 28.0±0.3^{o}C and 81.0% RH, and covered with perforated plastic sheet with numerous holes was done for recording mortalities daily inspection of cups, for the treated and untreated snails. Tissue smears from all dead snails were microscopically examined for the presence of polyhedral, (Fig 2).

In each bioassay trial of the bacterial or viral pathogen (s). The % mortality was calculated according to following equation:

$$\% = \frac{\text{naturally dead snail number}}{\text{Total inspected no.}} \text{X}100$$

Also the results were statistically analyzed and interpreted using the probit analysis; statistical method of (Finney, 1952 and 1971).

RESULTS AND DISCUSSION

1- Pathogens associated with the abnormal naturally dead snail, *T. pisana*

Along a 9-month, extended from January 2014 till September 2014, to survey the natural microbial control agents of the white land snails *T. pisana*, in Alexandria; Egypt, it had been revealed that 19.0% of inspected 693 snails were found to be

naturally infected with the bacteria; Bacillus*thuringiensis* (6.1 %), *B.cereus* (0.1 %), and *Brevibacterium* sp. (10.1 %), and a polyhedrosis virus (2.7 %). The highest natural occurrence of the viral disease among the individuals of *T. pisana* snails was recorded in September (16.7%) and ranged; from 0.8 to 16.7 %; along the whole investigation period of 9-months, while the highest occurred rate of natural mortality % among the inspected snails due to bacterial infection by *B.t* .(25 %) and/or *Brevibacterium* sp.(26.2 %) was observed during May & August, respectively , and ranged, in respect, between 1 and 25 % and 1-26.2 % through the initiated experimental periods (Table 1).

Data in Table (1) also show that the pathogen*Brevibacteriumsp*. was the most frequently isolated pathogen (10.1 %) from naturally dead snails followed by *B.thuringiensis* (6.1 %), the polyhedrosis virus (2.7 %), and *B.cereus* (0.1 %) which recorded the lowest rate of occurrence. Herein, according the available literary information, the authors could state there are no previous reports on naturally occurring pathogen (s) in *T.pisana* snail; their findings give details on the firstly recorded natural bacterial pathogens in the abnormal & natural dead snails of *T. pisana*.

Table (1): The recorded rates of natural mortality of the white land snail *T. pisana*due to the detected associating pathogens during a period of 9- month extended from January 2014 till September 2014

Date of inspection	Natural mortality N.M. (%) / pathogen				
	B.thuringiensis	***B.cereus***	***Brevibacterium* sp.**	**Polyhedrosis virus**	**Mean (%) of recorded pathogens**
January	3.1	0.0	0.0	4.6	7.7
February	8.3	0.0	24.9	8.0	41.2
March	25.0	0.0	15.0	10.0	50.0
April	7.4	0.0	14.7	4.4	26.5
May	5.7	0.0	9.4	1.9	17.0
June	12.3	0.0	26.2	0.8	39.3
July	1.0	0.0	1.0	2.1	4.1
August	3.1	1.1	0.0	0.0	4.1
September	0.0	0.0	0.0	16.7	16.7
Average	6.1	0.1	10.1	2.7	19.0

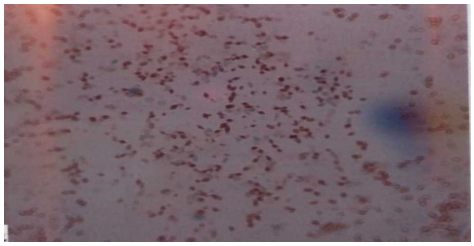
Total number of inspected snails = 693 snails

2-Pathogenicity of the bacteria and virus isolated from the land snail, *T.pisana* to its natural host

T.pisana snails were found to be infected by *TpB.t, Brevibacterium* sp., and the polyhedrosis virus when they had been fed upon lettuce contaminated with different concentrations of each tested pathogen, under laboratory conditions (28.0± 0.3^{o}C, 81.0±-0.5 %RH). All of the dead snails in the *B.t*., and/or in the viral treatments contained masses of typical *B.t*. rods, crystals, spores or sporangia, (Fig.1), and / or the viral polyhedral, (Fig.2).

A-*B.t.* bioassays

Data in Table (2) indicated that the LC_{50} value of *B. thuringiensis* var. *kurstaki*, originally isolated from naturally dead *T. pisana* snail, at 13 days post-treatment (7.3 x 10^8 VSs/ml) was insignificantly lower, than the corresponding detected higher value at 12 days post-treatment (8.8 x 10^8 VSs/ml), as it was indicated by the increasing slope value (from 3 to 3.6; (Table 2). Dulmage (1981) mentioned that the different isolates of *B.thuringiensis* of the same variety can produce different endotoxins, to which the pathogenicity to a susceptible host is attributed, and the extent of the potency of the different varieties of *B.t*. depends upon the isolate itself rather than the variety or serotype. Additionally, Table (3) reveals that the time to obtain 50 % mortality of the treated *T. pisana* snail was influenced by the source of the *B. thuringiensis*var. *Kurstaki*where; the LT_{50} values ranged from 10.2 to 17.5 days for the different concentrations of the *TpB.t.k* isolate. Also, the data prove that the increase in *B.t*. concentration significantly shortened the survival time of *T. pisana* lethally *B.t*.-infected snail by 10.2, instead of 17.5, days when the subject snail fed on lettuce treated with tested concentrations ranging from 12.0 to 3.6 x 10^8 VSs/ml in the case of the *TpB.t* isolate. On the other hand, daily cumulative mortality values for the *TpB.t* showed that the highest test concentration of 12 x 10^8 VSs/ml showed that 14 days were needed to produce 100 % mortality; while for the lowest concentration of 3.6 x 10^8 VSs/ml, 20 days were required to achieve the same mortality level.



Figure(1): Naturally-occurring bacterial pathogen of the land snail *T. pisana* (*B. thuringiensis* var. *kurstaki*; *Brevibacterium* sp.).

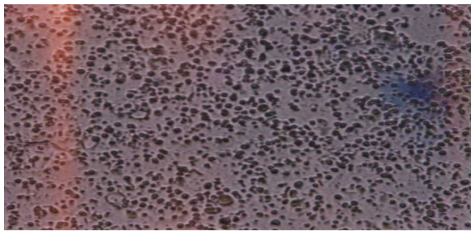


Figure (2): Naturally-occurring viral pathogen of the land snail *T. pisana* (polyhedrosis virus).

B- *Brevibacterium* bioassay

The LC_{50} for the *Brevibacterium* sp. at 16.0 days post-treatment was $7.6x10^8$ viable bacterial cells/ml, (Table 2) with concentrations ranging from 2.6 to $23.4x10^8$ VBCs/ml (Table 3) while the LT_{50s} ranged from 9.8 to 16.3 days for the different tested concentrations (Table3). At concentration below 13 x 10^8VBC$_s$/ml, the LT_{50} value had been shortened by 12.8 days only. On the other hand, daily cumulative mortality data for the highest bacterium concentration (23.4 x 10^8 VBCs/ml) indicated that a 100 % mortality of *T. pisana*tested snail was achieved after a period lasted about 16 days post-infection, and with the lowest test concentration ($2.6x10^8$VBC$_s$/ml), 22 days were required to attain the same mortality level of the subject snail.

Positive diagnosis of *Brevibacterium* sp. was confirmed through Koch's postulates, for all dead *T.pisana*snail in the treated samples (Campbell and Podgwaite, 1971). The present results provide evidence, about lethality of the *Brevibacterium*sp. to the land snails *T.pisana*. The first evidence that the genus *Brevibacterium*is lethal to insects when ingested as reported by Bucher (1963); also, Alfazairy (1983) recorded that the natural mortalities ranged from 24.7 to 47.8% among the individuals of stored product insect pests, *Sitophilus granaries* (L.), *S.oryzae*(L.), *Rhizoperthadominica*(F.), *Oryzaephilussurinamensis*L., and*Laemophloeusferrugineus* (Steph.). The LC_{50} values of *Brevibacterium* sp. for these insect pests were extremely high and had ranged from 3.8 x 10^9 to $1.9x10^{10}$ VBCs/5 g food, estimated at 4 - 16 days post-treatment. *Brevibacterium* –infected snail gradually turned into soft red-colored cadavers with some deformities.

C-Polyhedrosis virus bioassay

Daily cumulative mortality data for each tested virus concentration showed that death of the virally infected *T.pisana* snail usually occurres during 2 - 23 days after infection. Light microscopic examination of all *T.pisana* virally-infected cadavers confirmed the presence of large numbers of polyhedral (Fig.2). Infected snails become flaccid and gradually turn into brown-or black –colored cadavers. The results of performed bioassay established the pathogenicity of the *T.pisana*polyhedrosis virus to its natural host ;(Table 2). The relatively low LC_{50} value at 4 days post-infection indicated a high degree of infectivity, as it was also confirmed by the too much low LT_{50} value (1.5 - day) for the highest concentration of 5.8 x 10^6P.I.Bs/ml (Table 3).

Table (2): Probit analysis data of mortality of *T. pisana* snail separately infected with the pathogens, *Bacillus thuringiensis, Brevibacterium* sp., and polyhedrosis virus

Pathogen	Days post-treatment	Regression equation	LC_{50} (VSs/ml) (VBCs/ml) (PV/ml)	95 % confidence limits	Slope	Fit of the drawn Lc-p lines		
						Chi^2	P	Degrees of freedom
B.thuringiensis	12	Y = - 27.2795 + 3.0495 x	8.8×10^8	7.9×10^8 to 9.8×10^8	3.0	0.815	0.665	2
	13	Y = - 31.9373 + 3.6036 x	7.3×10^8	6.7×10^8 to 7.9×10^8	3.6	2.634	0.268	2
Brevibacterium sp.	16	Y = -21.2680 + 2.3942 x	7.6×10^8	6.7×10^8 to 8.7×10^8	2.4	27.524	0.186	5
Polyhedrosisvirus	4	Y = - 20.6592 + 3.1702 x	3.3×10^6	3.0×10^6 to 3.6×10^6	3.2	0.814	0.666	2

Table (3): LT_{50} values; their 95 % confidence limits for *T.pisana* infected separately with different concentrations of the pathogens, *B.thuringiensis, Brevibacterium* sp., and a polyhedrosis virus

Concentration (V.Ss/ml) (V.B.Cs/ml) (PV/ml)	LT_{50}(days)	95 % confidence limits	Slope
B.thuringiensis			
3.6×10^8	17.5	17.5 to 17.6	0.12
6.0×10^8	13.9	13.8 to 13.9	0.11
8.4×10^8	12.7	12.6 to 12.7	0.10
12.0×10^8	10.2	10.1 to 10.2	0.10
***Brevibacterium* sp.**			
2.6×10^8	16.3	16.1 to 16.4	0.04
7.5×10^8	13.5	13.4 to 13.6	0.04
13.0×10^8	12.8	12.7 to 12.9	0.05
18.2×10^8	10.7	10.6 to 10.8	0.04
23.4×10^8	9.8	9.2 to 10.3	0.04
Polyhedrosis virus			
1.7×10^6	10.6	10.6 to 10.7	0.03
2.9×10^6	8.0	7.8 to 8.1	0.04
4.0×10^6	6.4	5.9 to 6.9	0.04
5.8×10^6	1.5	1.4 to 2.8	0.03

The viral disease mortality data recorded in this laboratory bioassay trial may reflect a promise able efficient pathogenicity of such polyhedrosis virus towards same snail species of its natural host (*T. pisana*), where these tested land snail were found to be considerably susceptible to their naturally occurring virus.

The obtained results of conducted bioassay tests also show that the described method for assaying test pathogens give reproducible results and can easily be adopted for routine bioassay work with such land snail *T. pisana*, which is known, to a large extent. The ease and simplicity of the procedure, and the reliability of the method, as emphasized by the statistical analysis of the obtained data, justify the use of the method adopted here. Unfortunately, no previous literary

reports were available on *T. pisana* bioassay method to discuss the present results; which could be considered as firstly recorded bioassay trial.

In the light of all data recorded in these bioassay tests, it seems also that the newly isolated bacterial and viral pathogens can provide much promise able microbial control agents for this land snails *T.pisana*. Therefore, this study suggests that the direct spray of any of the pathogens listed above onto the host trees might have some value in *T.pisana* control procedure, curatively or preventively. Confirmation or rejection of the latter suggestion certainly needs further investigation. Also, Shairra and Nabil (2012) mentioned that the immature stages of white garden snails were greatly sensitive than adult stages for nematodes infection.

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الملخص العربى

الممرضات البكتيرية والفيروسية المرتبطة بقوقع الحدائق الأبيض

رضا عبد السميع هندى، السيد حسن عشره و سعاد شعيرة

معهد بحوث وقاية النباتات – مركز البحوث الزراعية– مصر

تم إجراء الدراسة الحالية خلال فترة بحثية (٩ اشهر) فى موسم ٢٠١٤ فى محافظة الإسكندرية حيث أجريت تجربة معملية على أفراد من قوقع الحدائق الأبيض ميته طبيعيا أو ذات الشكل الغير طبيعى***Thebapisana*** تم جمعها من الحقل (٦٩٣ فرد). حيث أوضحت النتائج أنه تم عزل ثلاث مُمرضات بكتيريه (*B. thuringiensis*، *Brevibacteriumsp., B. cereus)* وأخر فيروسىPolyhedrosis virus. وقد سجل المُمرض البكتيرى*Brevibacterium* sp. أعلى نسبة موت طبيعية (١٠,١ %) بين القواقع التى تم فحصها يليها فى ذلك المُمرضالبكتيرى*B.thuringiensis* (٦,١%)ثم الممرض الفيروسى Polyhedrosis virus (٢,٧ %) وأخيرا المُمرض البكتيرى*B.cereus* (٠,١ %). وقد كشفت النتائج المعملية للتقييم الحيوىbioassay لكل من المُمرض البكتيرى*B.thuringiensis*،*Brevibacteriumsp.* والمُمرض الفيروسى متعدد الأوجه Polyhedrosis virus على أساس نسب الموت اليومية بين القواقع المُعامله وكذلك قيم الـ LC_{50} (التركيز اللازم لقتل ٥٠% من اليرقات المعامله) والـ LT_{50} (الوقت اللازم لقتل ٥٠% من الأفراد المُعامله) أن هذه الممرضات – المعزوله أصلا من قوقع ***T. pisana***– أظهرت قدرة ممرضة تراوحتمابين المنخفضة والعالية ضد عائلها الأصلى***T.pisana***. ومن ناحية أخرى أوضحت نتائج التقييم الحيوى للمُمرضات المُختبره بعد إختبارها إحصائيا – مدى نجاح ومصداقية وسهولة وبساطة الإجراء العملى المُستخدم فى تجارب التقييم الحيوى التىأُجريت فى هذا البحث والذى يمكن إستخدامها فى تجارب التقييم الحيوى لقوقع ***T. pisana***