AIM OF THE WORK

The present work aims at:

1. Studying the impact of *B. alexandrina* snails' age on their compatibility patterns to *S. mansoni* infection using different parasitological parameters.
2. Detecting the effect of cytosolic superoxide dismutase enzyme (SOD_1) activity of young and adult *B. alexandrina* snails with different genetic origins on their compatibility patterns to *S. mansoni* infection.
3. SDS-PAGE electrophoretic analysis of young and adult *B. alexandrina* snails of different genetic backgrounds.

MATERIAL

I. *Schistosoma mansoni* Parasite:

Eggs:

Viable *S. mansoni* eggs were originally collected from stools of infected untreated children living in Abees village, Alexandria Governorate, Egypt.

Miracidia

S. mansoni miracidia were hatched from the parasite eggs to be used in infection of *B. alexandrina* snails. (34)

Cercariae

S. mansoni cercariae were shed from the infected *B. alexandrina* snails. They were used to infect the experimental animals in this study. (34) The parasite was then maintained in *B. alexandrina* snails and Swiss albino mice. This maintained cycle was the source of parasite eggs later on. (34)

II. Experimental animals:

Laboratory bred male Swiss albino mice four to six weeks old, each weighing 20-25 grams were obtained from the animal house, Medical Parasitology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt. Mice were kept under standard living conditions in the department and were maintained on a diet composed of wheat, bread and milk on alternate days.

III. Snails:

Laboratory bred *B. alexandrina* snails that were originally collected from a drainage ditch running along Abees village, Alexandria Governorate. These snails were kept under standard living conditions in the Medical Parasitology Department, Faculty of Medicine, Alexandria University.

The snails were grouped as follows:

a. Snails used for maintenance of *S. mansoni* life cycle:

Susceptible *B. alexandrina* snails were used for this purpose.

b. Experimental snails

These included; the infected and the non-infected snail groups:

1. **The infected group;**

These are susceptible and resistant groups, each included two subgroups; young and adult.

Group I: Susceptible snails:

Two hundred young susceptible **(subgroup Ia)** and two hundred adult susceptible **(subgroup Ib)** snails were used in this work. Young snails were infected on reaching the age of two months and the size of 3-4 mm in diameter (Before the start of egg laying). Adult snails were infected at the age of four months and the size of 8-10 mm in diameter (After the start of egg laying).

Group II: Resistant snails:

Two hundred young resistant **(subgroup II a)** and two hundred adult resistant **(subgroup II b)** snails were used in this work. Young snails were infected on reaching the age of two months and the size of 3-4 mm in diameter (Before the start of egg laying). Adult snails were infected at the age of four months and the size of 8-10 mm in diameter (After the start of egg laying).

2. **The non-infected group:**

One hundred non infected *B. alexandrina* snails served as controls for enzyme assay and electrophoretic studies. These included fifty young and fifty adult snails.

The experimental design of this study is shown in figures (6 &7).

IV. Reagents and equipment for:

a. Parasitological study

Snail rearing was done in suitable plastic containers, filled with de-chlorinated tap water (DTW) in a special well ventilated place, with good natural and artificial light sources. For maintenance of *S.mansoni* life cycle special palettes with separate wells were used for both snail infection and cercarial shedding. Additionally, dissecting microscope, sedimentation flasks, special sieves of mesh sizes 250 µm, 180 µm, 105 µm and 45 µm, centrifuge and centrifugation tubes, lugol's iodine for fixation of miracidia and cercariae, graduated pipettes and forceps for snail handling were used.

b. Enzyme assay

Super oxide dismutase enzyme assay kit, Biodiagnostic, Egypt was used. This kit contains 5 reagents (R);

R1: Phosphate Buffer pH 8.5 50 ml

R2: Nitroblue tetrazolium (NBT) dye 5 ml

R3: NADH 5 ml

R4: Phenazine methosulphate (PMS) 5 ml

R5: Extraction Reagent 25 ml

c. Electrophoretic study (SDS-PAGE) [179,181]

All chemicals and reagents used were purchased from Bio Rad, USA.

a) Equipment

The electrophoresis was performed using vertical slab gel apparatus. Additionally glass plates, spacers, combs and buffer reservoir of the gel apparatus were used.

b) Reagents

-Acrylamide

-Bisacrylamide

- *NNN'N'* tetra methylethylene-diamine (TEMED)

-Ammonium persulphate (Electrophoretic grade)

-SDS (Electrophoretic grade)

-β-mercaptoethanol

-Tris-base (Electrophoretic grade)

-Glycine (Electrophoretic grade)

-Coomassie blue R250

-Standard protein markers, Unstained, Wide molecular weight range 6.5-212 kDa.

Sample Preparation:

- **Buffers**

1- Phosphate buffer saline, (pH 7.2)

2- Sample buffer:

0.5 M Tris-HCl pH	6.8 1 ml
Glycerol 10%	1.6 ml
Bromophenol 1%	0.2 ml
Distilled water	3.2 ml

3- Running buffer

Tris base	15.1 gm
Glycine	72.0 gm
SDS	5.0 gm
Distilled, deionized water	1000 ml

The buffer is of 8.3 pH, was stored at 0-4°C when not used, SDS added only on use.

- **Gels**

1- **Separating gel, 10%:**

Acrylamide-bisacrylamide	3.3 ml
Distilled water	4.0 ml
Tris buffer	2.5 ml
Ammonium persulfate (APS)	10% 50 µl
TEMED	7.5 µl

2- Stacking gel, 2%:

Acrylamide-bisacrylamide mix	0.33 ml
Distilled water	1.4 ml
1.0 M TRIS (pH 6.8)	0.25 ml
APS 10%	0.02 ml
TEMED	0.002 ml
SDS 10%	0.02 ml

- **Stock solution**

1- Acrylamide-bisacrylamide solution.

Acrylamide	29 gm
Bisacrylamide	1 gm
Distilled water	100 ml

The solution was filtered and stored at 4°C in a dark bottle.

2- TEMED.

The solution was used as supplied, it is stable when stored at 4°C in a dark bottle.

3- Ammonium persulphate (APS) 10%.

APS	1 gm
Distilled water	10 ml

This solution is unstable and was freshly prepared before use.

4- Sodium- dodecyl sulphate (SDS) 10%

SDS	10 gm
Distilled water	100 ml

5- β-mercapto ethanol: used as supplied.

- **Protein staining**

1- Staining solution:

Coomassie brilliant blue R-250	0.05gm
Glacial acetic acid	10 ml
Methanol	50 ml
Distilled water	40 ml

2- De-staining solution:

Glacial acetic acid	7 ml
Methanol	5 ml
Distilled water	88 ml

- **Protein marker** [181]

Standard protein marker was obtained from Bio Basic Inc, Canada.

METHODS

Laboratory breeding of snails

Snails maintained in the laboratory were put in plastic aquaria that were supplied with well aerated aged DTW, at a density of 10-15 snails per liter. Water in these nursing aquaria was changed twice weekly with elimination of decayed food remains. They were maintained at temperature of 25°C. Snails were fed on fresh green lettuce leaves, supplemented with tetramine (fish food). Both of which were supplied with every water change. Pieces of chalk were placed in the water for calcium supplementation. Dead snails were regularly removed, except few shells that were left in the aquaria to provide calcium. Foam pieces were placed in the containers for egg deposition. Aquaria were covered with sieve-like nets to prevent escape of molluscs. Breeding of the snails was run under normal laboratory illumination. [(66,182,183)]

Selection of the susceptible and resistant snails

Selection of susceptible and resistant snails was achieved according to Zanotti-Magalhães, *et al.* [(184)] For selection of **susceptible group (group I)**, progeny of snails that yielded high infection frequencies, were isolated and reared singly for self-reproduction. For selection of resistant isolate, snails that remained uninfected after two parasitic exposures, each by (10 miracidia/snail) were isolated and reared singly for self-reproduction. Their progeny were selected to be the experimental **resistant group (group II)**. All snails were maintained in the above mentioned conditions. [(66,182,183)]

Collection of egg masses, inspection and rearing of the newly hatched snails

In order to obtain experimental snails used in this study, the newly deposited egg masses on the walls of the plastic aquaria, on the foam pieces, on lettuce leaves, and on other snail shells were carefully collected and were regularly removed gently using the blunt border of a scalpel. Then they were transferred to separate aquaria containing aged DTW. These egg masses were inspected daily till hatching.

After hatching, the baby snails laid by susceptible parents and resistant parents were reared for two months till being young, measuring 3-4 mm in diameter. The new snails belonging to each parental group were reared in separate containers. Two hundred snails from each group formed subgroups **Ia** (200 young susceptible snails) and **IIa** (200 young resistant snails) respectively. Other two hundred snails of those laid by susceptible parents and resistant parents were reared for four months till being adults, measuring 8-10 mm in diameter forming subgroups **Ib** (200 adult susceptible snails) and **IIb** (200 adult resistant snails) respectively.

Maintenance of *S. mansoni* life cycle

(A) Isolation of *S. mansoni* eggs from patients' stools:

Viable *S. mansoni* eggs were originally collected from stools of infected untreated children living in Abees village.The collected stools were dissolved in about ten times their volume of physiological saline, then the whole amount was shaken thoroughly in a stoppered flask. The suspension was strained through 2 layers of gauze and the filtrate was centrifuged at 500 rpm for 3 minutes. The supernatant was poured off and the sediment containing eggs of *S. mansoni* was used for infection of *B. alexandrina* snails. (185)

(B) Hatching of *S. mansoni* eggs:

The stool sediment containing eggs of *S. mansoni* was transferred to a flask containing one liter of warm (35°C) aged DTW. The flask was exposed to direct sunlight and was covered with aluminum foil leaving 3 cm of the flask neck uncovered and exposed to light for about 30 minutes to allow egg hatching. Depending on the fact that miracidia of schistosomes are phototropic, miracidia were seen after the allowed time with the naked eye as white specks in the uncovered area of the flask neck. With the aid of the dissecting microscope, eight to ten active vigorously swarming miracidia were aspirated using a fine capillary pipette, to be used for infection of individual snails used to maintain the cycle. (29,31,186)

(C) Exposure of the snails to *S. mansoni* miracidia:

Susceptible snails that were used for maintainance of *S. mansoni* life cycle, were exposed individually, to 8-10 vigorously swimming freshly hatched miracidia in palettes, each palette was with 6 wells, each well accommodated one snail with two ml of aged DTW. Palettes were directly exposed to sunlight for 3-4 hours, after being covered by glass covers to prevent water evaporation and also to prevent snails from crawling out of wells. (183) Four hours later, a drop from each well was aspirated and examined under a dissecting microscope to make sure that all miracidia in the well have penetrated the snail. Then the snails were returned to their original aquaria to be maintained in the previously mentioned conditions and they were kept in darkness for 4 weeks. (66,182,183)

(D) Cercarial shedding: (187, 188,189)

Four weeks post exposure, infected *B. alexendrina* snails were used to harvest cercariae. This was done by placing every ten snails in 200 ml beaker, containing 50 ml aged DTW, under direct sunlight for about two hours. Cercariae were seen by naked eye as white specks of about 500µm length swimming in the beakers.

After shedding, the snails were removed and the cercarial suspension was carefully shaken to obtain an even suspension for accurate count. Using a graduated pipette, 0.1 ml of this suspension was aspirated repeatedly, stained with lugol's iodine to kill cecariae to be easily counted, then cercariae were counted using a dissecting microscope. Total number of harvested cercariae was calculated, these were then used for animal infection.

(E) Animal infection: (90, 190-193)

Laboratory bred Swiss strain albino mice were infected using paddling technique. The mice were first placed in a large bowl containing water at room temperature adjusted to reach animal belly. This was done to stimulate mice to urinate and defecate to avoid urination and defecation during their immersion in the cercarial suspension that have a lethal effect on the cercariae.

After 30 minutes, mice were transferred to clean sedimentation flasks separetly. Five ml of aged DTW containing the cercarial suspension, which was obtained by pooling from infected snails were added to each flask. The infective dose of about 100 cercariae was used to infect each mouse. They were exposed to infection for about one hour at room temperature with animal tail immersed in the suspension. Then infected mice were returned to their dry cages, fed as usual and maintained for about seven to eight weeks. All experiments were done in compliance with the guide lines of national ethics committee for the use and care of Laboratory animals.

(F) Isolation of *S. mansoni* eggs from livers of the infected mice: (194,195)

These infected mice were the source of eggs, seven weeks after cercarial penetration. Mice were sacrificed by cervical dislocation seven to eight weeks post infection. This was done after the mice being anaesthetized for ethical issues. Livers were dissected, cut into small pieces, put in 100 ml normal saline and were homogenized using an electrical blender for two minutes. The homogenate was then strained by passage through a series of four sieves arranged in a descending order of mesh sizes 250 µm , 180 µm , 105 µm and 45 µm. Eggs retained in the last sieve were washed several times with saline, then the filtrate was centrifuged at 500 rpm, for 3 minutes. The supernatant was decanted and the sediment containing eggs was poured into one liter flask containing warm DTW at 35ºC.Then water was left in darkness for 30 minutes to ensure egg sedimentation. The eggs were then exposed to light to stimulate egg hatching, with subsequent miracidium release and collection. [186]

Infection of the experimental snails (66,182,183,186)

With the aid of the dissecting microscope and a fine capillary graduated pipette, 8-10 active vigorously swarming freshly hatched *S. mansoni* miracidia were used to infect each snail in the four experimental subgroups, this was done using palettes, each palette was with 6 wells, each well accommodated one snail with 2 ml of aged DTW, for 3-4 hours during which snails were directly exposed to sunlight. Two hundred young susceptible (**subgroup Ia)** and two hundred young resistant **(subgroup II a)** snails were infected on reaching the age of two months and the size of 3-4 mm in diameter (Before the start of egg laying). Two hundred adult susceptible **(subgroup I b)** and two hundred adult resistant **(subgroup II b)** snails were infected at the age of four months and the size of 8-10 mm in diameter (After the start of egg laying). After exposure of the eight hundred snails to miracidia, infected snails of each of the subgroups **Ia**, **Ib**, **IIa** and **IIb** were returned to separate plastic aquaria for each subgroup. Each aquarium contained about 3 liters of well aerated aged DTW in a density of about 15-20 snails per liter under the aforementioned conditions. So, snails of each experimental subgroup were accommodated in four plastic aquaria. Exposed snails of all subgroups were kept in darkness for about four weeks.

Later on, one hundred snails from each experimental subgroup were used for determination of parasitological parameters.The other one hundred snails were used for the enzyme assay and the electrophoretic studies.

Determination of susceptibility and resistance of the individual snails of the four subgroups (116,184,196-198)

Four weeks post exposure to infection, one hundred snails from each experimental subgroup were individually checked for cercarial shedding twice weekly, repeatedly for three weeks. To test shedding, snails were placed in palettes, each with 6 wells, each snail was placed in a well containing about two milliliters of aged DTW and exposed to direct sunlight for about two hours. Susceptible and resistant snails of each subgroup were separated and returned into different containers. Regarding resistant snails, they were returned together into a separate aquarium containing resistant individuals for each subgroup.

Susceptible individuals for each subgroup were isolated individually in separate small transparent incubating plastic containers. Each contained only one susceptible snail in 100 ml of aged DTW and was maintained under the same conditions described before and was kept in darkness, for maintenance throughout the shedding period. (66,182,183) Each container was labeled, the label showed the experimental subgroup, snail number, pre-patent period and number of shed cercariae every shedding time.

Non-shedding snails were returned to their aquaria, those were also tested twice weekly for cercarial shedding till 49 days post infection. Snails that died during the pre-patent period were squeezed between a slide and a coverslip and inspected under the microscope in search for sporocysts to verify its susceptibility status.(199,200)

1. **Parasitological study:**

The following parameters were determined for 100 snails in each subgroup:

A) Pre-patent period for each snail (PPP): (198)

It is defined as the period from the day of snail exposure to miracidia to the day immediately before its first shedding of cercariae. Starting from the 28th day post exposure to infection, all subgroups were examined twice weekly till the 49th day post infection, each snail was tested individually as mentioned above. (116,184,196-198)

B) Determination of the infection rate for each subgroup (IR): (166)

Percentage of susceptible and resistant snails in each subgroup was determined. All snails that died during the prepatent period were crushed between two slides and inspected under a microscope for immature parasite stages. (199) The snail's infection rate was calculated at the end of experiment by using the following equation:

$$\mathbf{IR} = \frac{\text{Number of shedding and positive crushed snails in each subgroup}}{\text{Number of exposed snails in each subgroup}} \times \mathbf{100}$$

C) Mean cercarial output per each susceptible snail (MCO): (119,200)

Number of cercariae that were shed from individual susceptible snails were counted twice weekly for three successive weeks. At the first day of detecting cercariae, positive snails were separated individually in plastic cups. The produced cercariae per snail were transferred to a small Petri dish by a Pasteur pipette, killed using lugol's iodine and counted under a stereomicroscope.

$$\mathbf{MCO} = \frac{\text{Sum of shed cercariae in all shedding times}}{\text{Number of shedding times}} \times \mathbf{100}$$

D) Mortality rate for each subgroup (MR): (198)

Number of dead snails for each subgroup was counted from the day of exposure of infection till the 49th day post infection. The number of dead snails in the breeding aquaria was twice weekly recorded. According to Yousif *et al.*, (198) the mortality rate was calculated using the following equation;

$$\mathbf{MR} = \frac{\text{the number of dead snails in each subgroup}}{\text{the total number of exposed snails in each subgroup}} \times \mathbf{100}$$

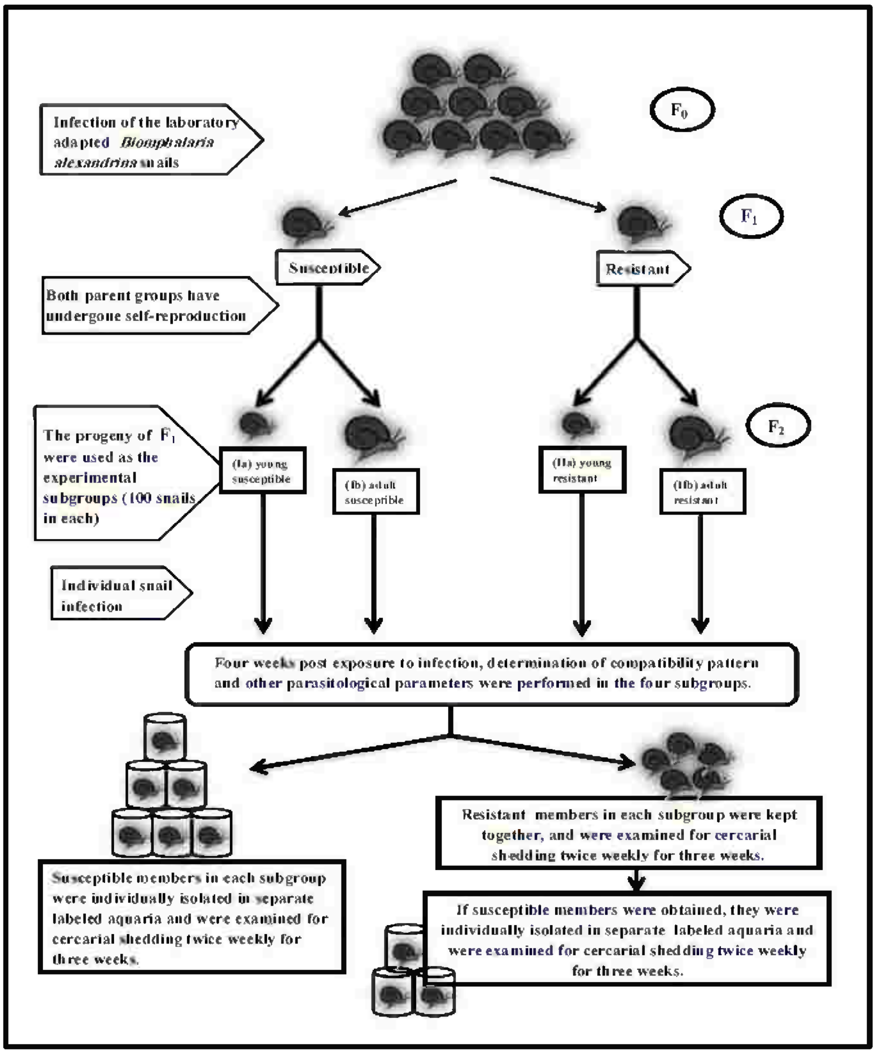

Figure 6: Experimental design of the parasitological study.

2. Biochemical study:

For biochemical studies (enzyme assay and SDS-PAGE), 8 susceptible and 8 resistant snails from the one hundred snails dedicated for biochemical studies in each experimental subgroup were selected. As for susceptible snails, they were obtained after the first shedding of the snail at the 28th day post exposure to infection. For non shedding snails at the 28th day post exposure to infection, they were cleaned, gently crushed between two clean glass slides to be examined under the microscope to make sure whether they contain any sporocysts, [200] those that did not show any sporocysts under the microscope were considered resistant. Moreover, 8 uninfected young, together with 8 uninfected adult snails were added in this analysis as 2 additional control groups.

Whole tissue sample preparation (Homogenization step)

Snails were cleaned well by gentle scrubbing using a soft tooth brush and distilled water. They were dried well using tissue paper, then each snail was crushed between two clean glass slides which were changed for each snail, bony particles were removed using a fine forceps. Soft tissue of the crushed snail was perfused with 0.9% Nacl, blotted on a tissue paper, then was weighed thereafter, then it was homogenized with 1 ml of phosphate-buffer saline (PBS, pH 7.2) by sonic circulation for 2 minutes in a glass test tube embedded in a beaker containing ice cubes to prevent temperature elevation in the sonicated tissue aiming at protection of snail tissue enzymes from deactivation by heat. The homogenized tissue was centrifuged at 500 rpm for 4 minutes. The supernatant containing the proteins was stored in labeled aliquots at - 20°C until used. This was repeated for each snail separately.

These collected supernatants were used later for SOD enzyme assay and Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by thawing in the room temperature, then immediate examination.

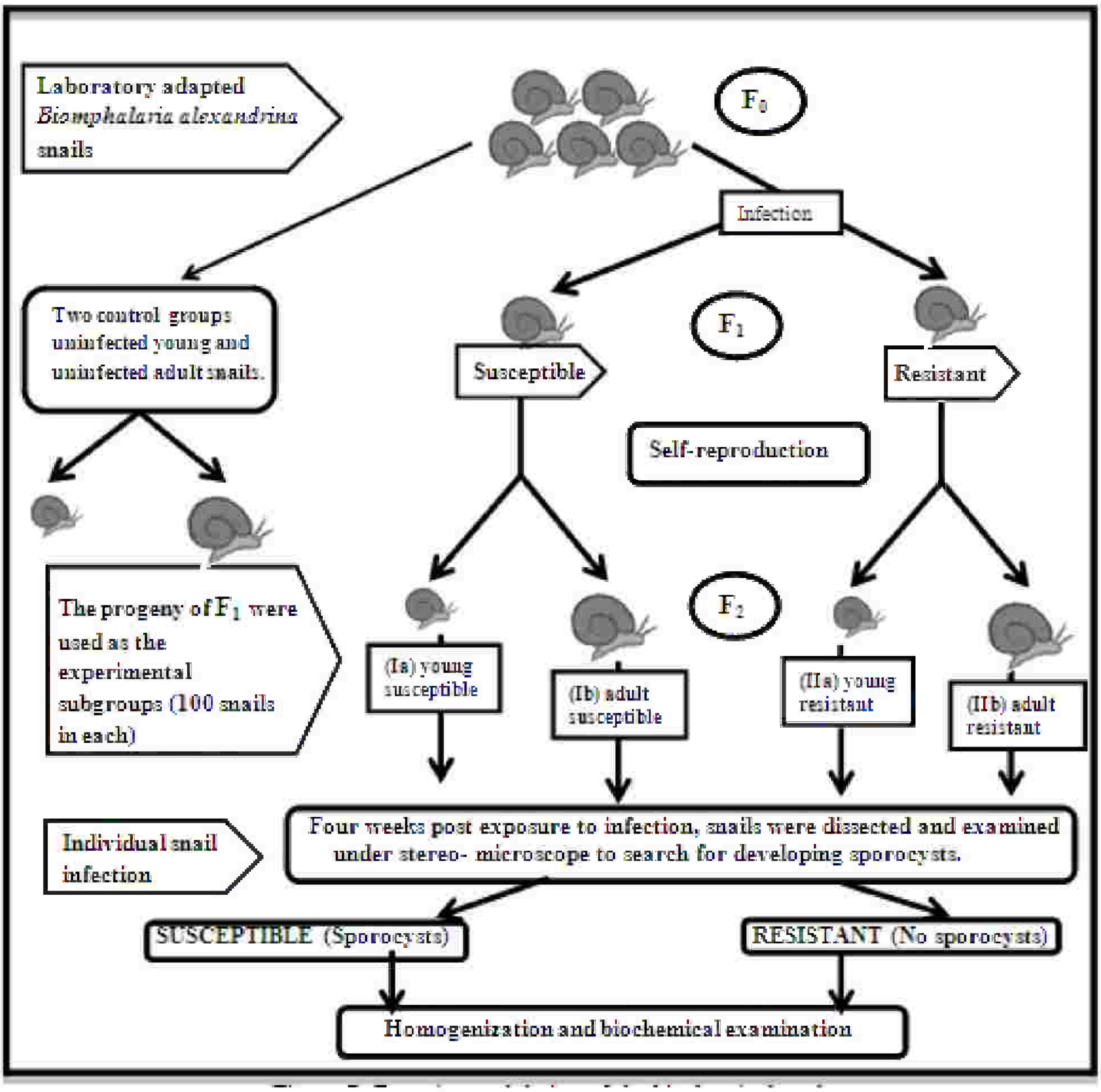

Figure 7: Experimental design of the biochemical study.

A) Enzyme assay:

Superoxide dismutase activity was determined by adding the substrate of the enzyme to the snail tissue homogenate containing the SOD. The increase in absorbance with time during the reaction was read using spectrophotometer.(107) Homogenate supernatants targeted for enzyme assay first have undergone determination of protein concentration for each sample using Lowry's method. (201)

This assay relies on the ability of SOD enzyme to inhibit the Phenazine methosulphate-mediated reduction of Nitroblue tetrazolium dye.

Preparation of solution:

R1, Ready for use.

R2, reconstitute in 5 ml distilled water.

R3, reconstitute in 5 ml distilled water.

R4, reconstitute in 5 ml distilled water, dilute 1000 times immediately before use (10 µl +10ml distilled water), discard after use.

Stability of the solution

R1 is stored, stable at 4ºC (refrigerator).

R2, R3 and R4 are stored at -20 ºC or below (deep freezer).

R3, after reconstitution is stable for 24 hours in a closed container at -20 ºC or below.

Procedure

- Samples were diluted to give an inhibition percent between 30 and 60.
- R4 was diluted 1000 times immediately before use (10 µl +10ml distilled water), was discarded after use.
- Working reagent was prepared by mixing R1+ R2+R3 mixed in a ratio of 10+1+1, immediately before use.

	Control	Sample
Working reagent	1 ml	1 ml
Sample	-	0.1 ml
Distilled water	0.1 ml	-
	Both were mixed well. Then the reaction was initiated by the addition of:	
R4	0.1 ml	0.1 ml

- The increase in absorbance at 560 nm for 5 minutes for control ($\Delta A_{control}$) and for sample (ΔA_{sample}) was measured at 25 ºC.

Calculation:

Percent inhibition= $\frac{\Delta A\ control - \Delta A\ sample}{\Delta A\ control} \times 100$, where;

$\Delta A_{control}$ = The change in absorbance at 560 nm over 5 minutes following the addition of R4 to the reaction mixture in the absence of the sample.

ΔA_{sample} = The change in absorbance at 560 nm over 5 minutes following the addition of R4 to the reaction mixture in the presence of the sample.

Purified SOD was shown to inhibit the initial rate of photo activated Phenazine methosulphate mediated reduction of O_2^- to O_2 which then reduced Nitroblue tetrazolium 1.5 Units/ assay of the purified enzyme produced 80% inhibition.

SOD_1 activity:

Units/ gram tissue used = % inhibition × 3.75 × $\frac{1}{\text{gram tissue used}}$

B) Elecrophoretic study: [56,202,203]

This was performed for differentiation of total protein composition in different subgroups. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. Protein extracts from snail tissue homogenate were applied to SDS-PAGE to identify the electrophoretic patterns of protein and to estimate their molecular weights (MW) using standard wide range protein markers.

Procedure:

- The glass plates, spacers, combs, and buffer reservoir of the gel apparatus were cleaned with detergent and rinsed in tap water.
- The casting apparatus was assembled and the height to which the resolving gel was to be poured was determined by inserting a well-forming comb between the glass plates making the outer plate 1-2 cm below the teeth of the comb.
- For electrophoresis, the monomer solution for the appropriate resolving gel was prepared by combining all of its reagents. Then it was poured between the gel plates up to the mark delimiting the resolving gel. The solution was then immediately overlaid with water to exclude air, which might inhibit polymerization.
- The gel was allowed to polymerize for 45 minutes to one hour. Polymerization was evidenced by the appearance of a sharp interface beneath the overlay.
- The top of resolving gel was rinsed with water and dried with filter paper.
- Stacking gel monomer (2%) solution was prepared. The stacking gel solution was poured on top of the resolving gel, the comb was aligned in its proper position, polymerization of the stacking gel occurred in about ten minutes.
- The electrophoresis cell was assembled. The upper and lower reservoirs were filled with running buffer and the comb was removed from the stacking gel.
- The prepared samples were loaded into the wells in the stacking gel using a micropipette.
- Leads were attached to the unit and connected to the power supply.
- The electrophoresis was performed using mini-PROTEAN II cell (Bio-Rad) at 125V through stacking gel for about two hours till the end of electrophoresis.
- After electrophoresis, the glass plates were separated and the gel was removed.
- The stacking gel was cut off.
- The separating gel was stained with Coomassie blue R-250 (0.1%) for two hours. Then it was properly de-stained.
- Protein MW determination was done by using standard protein markers. [181]

Protein staining: [180]

It was done using Coomassie Brilliant blue R-250.

- The staining solution was prepared by mixing its ingredients.
- Then it was filtered after the dye had dissolved. It was stored at room temperature.

- The gel was placed in an excess of staining solution for 30 minutes.
- Then it was placed in de-staining solution, which was changed several times until the background was almost removed.

Statistical analysis of the data: (204, 205)

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum) mean, standard deviation and median.

Comparison between the different studied subgroups was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Monte Carlo correction. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test. Moreover Histogram and QQ plot were used for vision test. When it revealed normal data distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used.

For normally distributed data, comparison between different studied subgroups were analyzed using F-test (ANOVA) and Post Hoc test (Scheffe) for pair wise comparison. Significance test results were quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

The following tests of significance were used;

a- **Mean value** $(\bar{X}) = \frac{X}{n}$, where;

X = the sum of all observations, n = the number of observations.

b- **Standard deviation (SD)** = $\sqrt{\frac{\sum(X - \bar{X})^2}{n-1}}$

c- **(t) test,** was used for comparison between means of different subgroups.

d- **Chi-square test, was** used to detect difference between two or more proportions.

e- **ANOVA test (Analysis Of Variance) (F-test),** was used to compare more than two arithmetic means. The level of significance chosen for this study was P ≤ **0.05**.

f- **Correlation coefficient** (linear relationship between two quantitative variables).

RESULTS

In the present study, the following results were obtained:

1. Parasitological study:

A) Pre-patent period (PPP):

Snails of each subgroup were examined for cercarial shedding, individually, every four days, starting from the 28 th day after infection till the 49 th day. Results for PPP were summarized and statistically analyzed in table (1) and figure (8).

In Subgroup Ia (Young susceptible) members, the majority of the shedding snails were recorded by the 28th day being 37 snails. 27 shed cercariae for the first time at the 32nd day, 16 shed at the 36th day. Only 5 shed at the 40th day, no more snails showed cercariae at the 44th day nor at the 49th day. Regarding subgroup II a (Young resistant), 2 snails shed cercariae for the first time at the 28th day, 3 shed at the 32 nd day, 5 shed at the 36th day, 7 shed at the 40th day, 9 shed at the 44th day and 11 snails shed at the 49th day. Subgroup I b (Adult susceptible) individuals showed the following results; 11 snails shed cercariae for the first time at the 28th day, 14 shed at the 32 nd day,16 shed at the 36th day, 17 shed at the 40th day, 9 shed at the 44th day and 7 snails shed at the 49th day. Completely resistant snails were obtained from subgroup II b (Adult resistant), so, no PPP was recorded.

Statistical analysis of PPP results revealed significant differences between subgroup Ia members, subgroup IIa members at all except the 40th day. Moreover, subgroup Ia members showed significant differences with those of Ib members at all days except the 36th day. Moreover, subgroup Ia members showed significant differences with those of IIb members at the 28th, the 32 nd and the 36th days of shedding. Significant differences were also noted between subgroup IIa and subgroup Ib members at days 28th, 32 nd, 36th and 40th. Additionally, significant differences were recorded between subgroup Ib and subgroup IIb members at all days of shedding. Subgroups Ib and IIb showed differences at the 40th, the 44th day and the 49th day.

Table 1: Number of shedding snails in the examined pre-patent period days:

PPP (n=100)	28th day	32nd day	36th day	40th day	44th day	49th day
Ia	37	27	16	5	0	0
IIa	2 [a]	3 [a]	5 [a]	7	9 [a]	11 [a]
Ib	11 [ab]	14 [ab]	16 [b]	17 [ab]	9 [a]	7 [a]
IIb	0 [ac]	0 [ac]	0 [ac]	0 [bc]	0 [bc]	0 [bc]
χ^2	79.451*	45.965*	23.200*	22.716*	18.848*	20.710*
p	<0.001*	<0.001*	<0.001*	<0.001*	^{MC}p<0.001*	^{MC}p<0.001*

PPP: pre-patent period; n: number of members in each subgroup; Ia: young susceptible subgroup; IIa: young resistant subgroup; Ib: adult susceptible subgroup; IIb: adult resistant subgroup.
a: Significance with subgroup Ia; b: Significance with subgroup IIa; c: Significance with subgroup Ib.
χ^2: value for Chi square; MC: Monte Carlo test. *: Statistically significant at $p \leq 0.05$

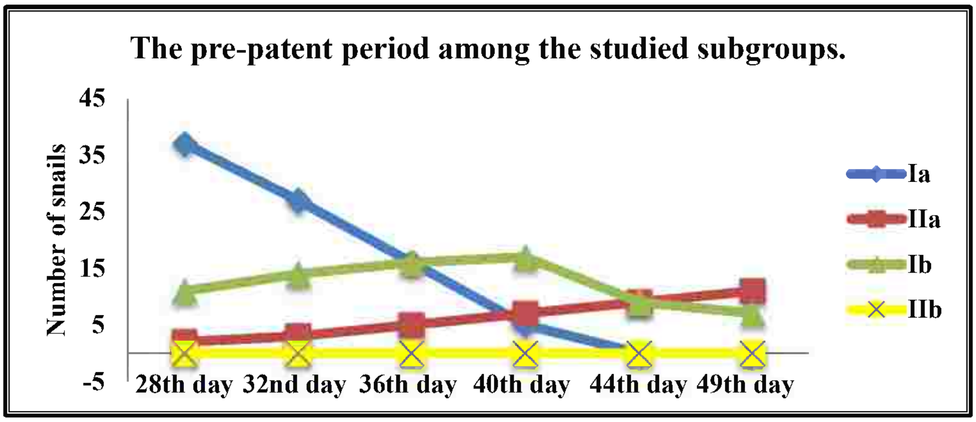

Figure 8: The pre- patent period among the studied subgroups.

B) Infection Rate (IR):

Percentage of susceptible and resistant snails in each subgroup was determined. Results of infection rate in different subgroups are listed and analyzed in table (2) and figure (9). Results showed that 92 snails of subgroup Ia, 37 snails of subgroup IIa and 74 in subgroup Ib were susceptible, whereas subgroup IIb contained only resistant members. Statistical analysis of the results of different subgroups showed significant difference between subgroups Ia – IIa, Ia – Ib, Ia–IIb , Ib- IIa, Ib – IIb and IIa – II b.

Table 2: Infection rate among the studied subgroups:

	Ia	IIa	Ib	IIb	χ^2	p
IR%	92.0	37.0	74.0	0.0	200.315*	<0.001*
Sig	Ia – IIa*, Ia – Ib*, Ia – IIb*, Ib- IIa*, Ib – IIb*, IIa – II b*					

IR%: infection rate; Ia: young susceptible subgroup; IIa: young resistant subgroup; Ib: adult susceptible subgroup; IIb: adult resistant subgroup. *: Statistically significant at $p \leq 0.05$ Sig: Significance between subgroups using chi square test; χ^2: Chi square test.

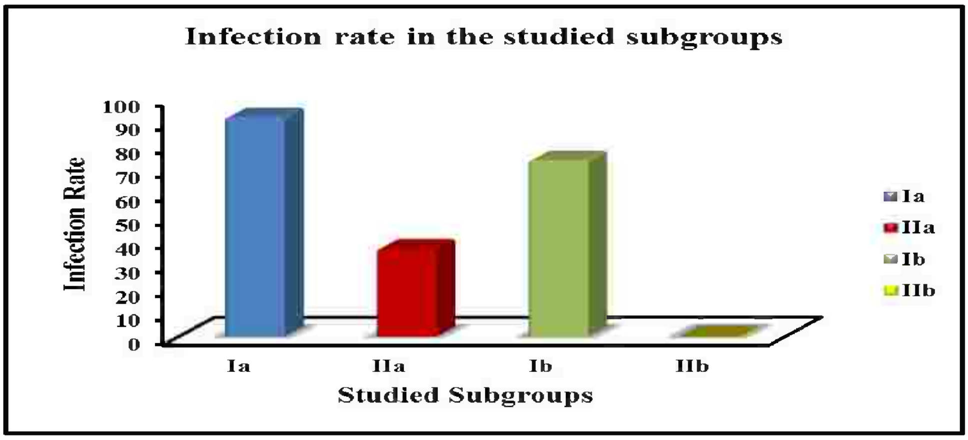

Figure 9: Infection rate among the different studied subgroups.

C) Total cercarial production (TCP) and mean cercarial output (MCO) per susceptible snail in each subgroup:

Total number of cercariae produced by the susceptible snails in each subgroup was counted over a period of three weeks, every four days. Mean number of cercariae shed by each susceptible snail in the different studied subgroups were calculated. Results are shown and analyzed in tables (3), (4) and figure (10).

The total number of cercariae produced by all snails over the three weeks tested for shedding (TCP) was 151002 from subgroup I a , and 9877 from subgroup IIa. On the other hand, TCP values of subgroup Ib was 41732, while subgroup IIb showed no cercarial production at all.

The highest mean cercarial production per snail per shedding time was recorded in subgroup Ia, followed by subgroups Ib, and IIa, being 298.1 ± 132.13, 94.13 ± 44.17, and 44.49 ± 32.35 respectively. Minimum, maximum, median, mean and standard deviation of the number of cercariae shed by all subgroups were calculated. Significant differences were noted between all subgroups.

Table 3: Cercarial production in the studied subgroups:

Subgroups	Number of shedding snails	TCP	MCO
Ia	85	151002	298.1 ± 132.13
IIa	37	9877	44.49 ± 32.35
Ib	74	41732	94.13 ± 44.17
IIb	0	0	0.0 ± 0.0

TCP: Total number of cercariae/ subgroup; MCO: Mean cercarial output (Mean number of cercariae/ snail/ shedding time); Ia: young susceptible subgroup; IIa: young resistant subgroup; Ib: adult susceptible subgroup; IIb: adult resistant subgroup.
Normally distributed data were expressed in mean ± SD (standard deviation).

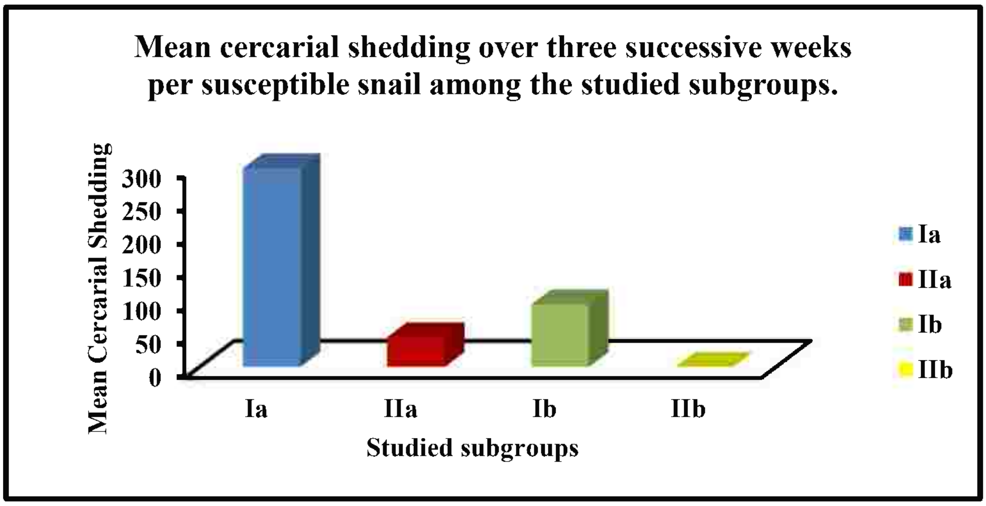

Figure 10: Mean cercarial shedding over three successive weeks per susceptible snail among the different studied subgroups.

Table 4: Mean cercarial shedding over three successive weeks per susceptible snail among the studied subgroups:

	Ia	IIa	Ib	IIb
Min. –Max.	**97 – 594**	**18 – 173**	**20 – 214**	**0 – 0**
Mean ± SD.	**298.1 ±132.1**	**44.5 ± 32.35**	**94.13 ± 44.2**	**0.0 ±0.0**
Median	**271.0**	**33.50**	**90.50**	**0.0**
F	**234.333***			
p	**<0.001***			
Sig	**Ia–IIa***, Ia – Ib***, Ia –IIb ***, IIa – Ib*, Ib- IIb***, IIa – IIb***			

Ia: young susceptible subgroup;
IIa: young resistant subgroup;
Ib: adult susceptible subgroup;
IIb: adult resistant subgroup.
Normally distributed data were expressed in mean ± SD (standard deviation) and was compared using F test (ANOVA).
Sig: Significant difference between subgroups using Post Hoc Test (Scheffe).
*: Statistically significant at $p \le 0.05$
**: Statistically significant at $p \le 0.01$
***: Statistically significant at $p \le 0.001$

D) Mortality Rate (MR):

Number of dead snails for each subgroup was counted from the day of exposure to infection till the 49th day post infection. Mortality rate for each subgroup was 47%, 27%, 35%, 14% for subgroups Ia, IIa, Ib and IIb respectively. Results are listed in table (5) and figures (11,12).

All the 47snails that died in subgroup Ia were susceptible, 7 of them died before the 28th day, 2 more snails died before 36th day, 5 before the 40th day, 14 before the 44thday, and 19 before the 49th day.

The susceptibility pattern of the 27dead snails in subgroup IIa were as follows; the 2 snails that died before the 28th day, and the 3 snails that died before the 32nd day were resistant, one of the 3 snails that died before the 36th day was resistant while 2 were susceptible. Moreover, two snails out of the 5 snails that died before the 40th day were resistant while 3 were susceptible. 6 snails died before the 44thday, 2 of them were resistant and the remaining 4 were susceptible. Furthermore, 8 snails died before the 49th , they were equally divided into 4 resistant and 4 susceptible.

In subgroup Ib the 35 snails that died were as follows; snails that died before the 32nd day, snails that died before the 36th day and those before 40th day were all susceptible. Furthermore, the 6 snails that died before the 40th day were also susceptible. Only one out of the 11 snails that died before the 44th day was resistant, while two out of the 15 snails that died before the 49th day were resistant.

Regarding subgroup IIb, all the dead snails were resistant; 1 died before the 28th day, another 1 died before the 32nd day, 2 snails died before the 36th, 3 snails died before the 40th day, 3 died before the 44th, and 4 snails died before the 49th day. No mortality was recorded in the uninfected control snails during the experiment time.

Table 5: Mortality rate among the studied subgroups at the different studied pre-patent periods with compatibility status of the dead snails:

MR %	Before 28th day	Before 32nd day	Before 36th day	Before 40th day	Before 44th day	Before 49th day
Ia (47%)	7 **(7S)**	0	2 **(2S)**	5 **(5S)**	14 **(14S)**	19 **(19S)**
IIa (27%)	2 **(2R)**	3 **(3R)**	3 **(1R, 2S)**	5 **(2R, 3S)**	6 **(2R,4S)**	8 [a] **(4R,4S)**
Ib (35%)	0 [a]	0	3 **(3S)**	6 **(6S)**	11 **(10S, 1R)**	15 **(13S, 2R)**
IIb (14%)	1 **(1R)**	1 **(1R)**	2 **(2R)**	3 **(3R)**	3 [ac] **(3R)**	4 [ac] **(4R)**
χ^2	27.085*	11.897*	0.410	1.05	9.368*	13.461*
P	<0.001*	$^{MC}p = 0.015^*$	$^{MC}p = 1.000$	$^{MC}p = 0.874$	0.025*	0.004*

MR %: mortality rate;
Ia: young susceptible subgroup;
IIa: young resistant subgroup;
Ib: adult susceptible subgroup;
IIb: adult resistant subgroup;
S: susceptible; R: resistant.
a: Significance with subgroup Ia;
b: Significance with subgroup IIa;
c: Significance with subgroup Ib.
χ^2: value for Chi square; MC: Monte Carlo test. *: Statistically significant at $p \leq 0.05$

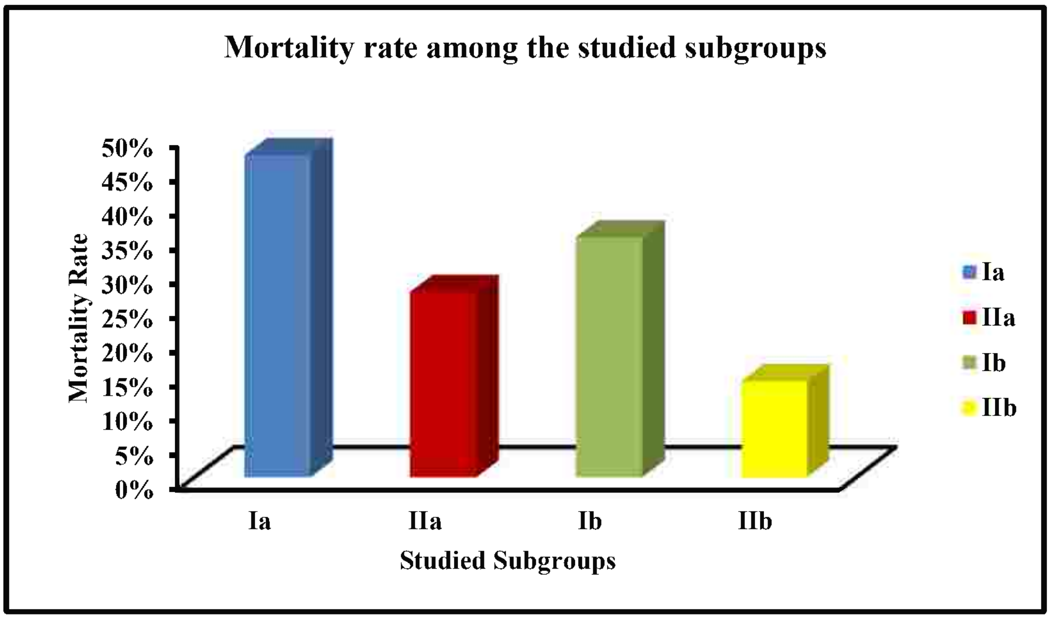

Figure 11: Mortality rate among the studied subgroups.

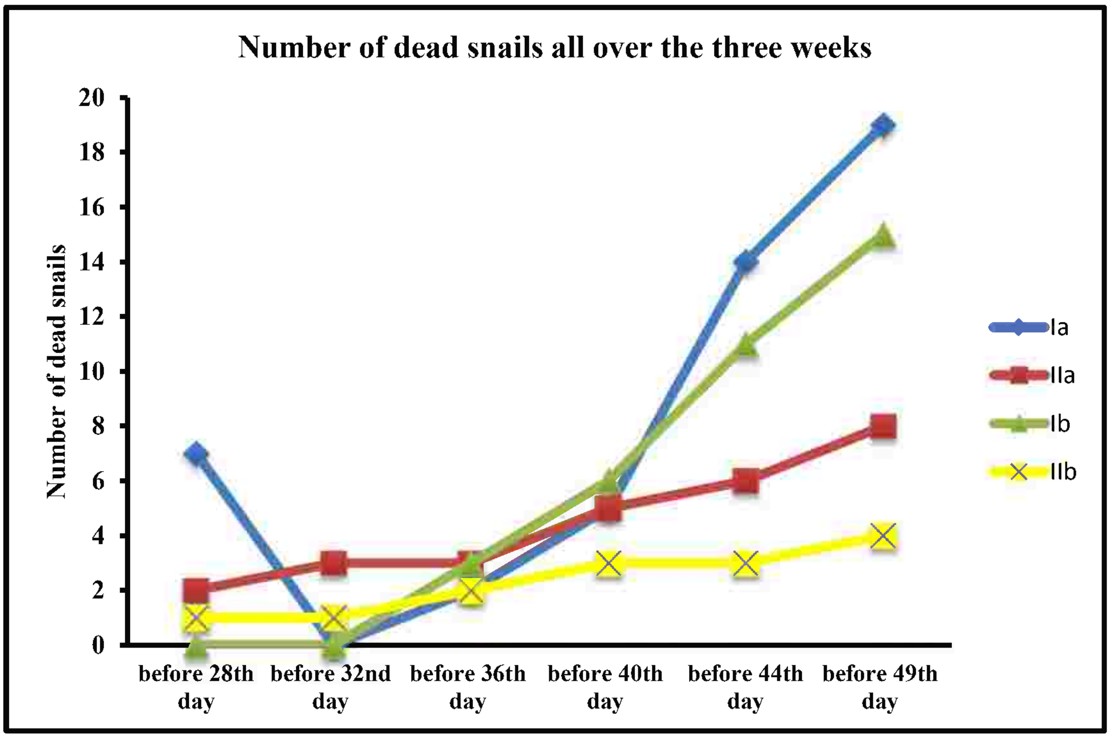

Figure 12: Number of dead snails all over the three weeks of shedding.

Correlation between both infection rate and mortality rate of each subgroup was done and represented in table (6) and figure (13).

Table 6: Correlation between infection and mortality rates in the studied subgroups:

Correlation	IR %	MR%	χ^2	p
Ia	92%	47%	47.765*	<0.001*
IIa	37%	27%	2.298	0.130
Ib	74%	35%	30.668*	<0.001*
IIb	0%	14%	15.054*	<0.001*
r (p)	0.983 (0.017*)			

MR %: mortality rate; IR %: infection rate;
Ia: young susceptible subgroup;
IIa: young resistant subgroup;
Ib: adult susceptible subgroup;
IIb: adult resistant subgroup.
χ^2:Chi square test p: p value for Chi square test.
r: Pearson coefficient.
*: Statistically significant at $p \leq 0.05$

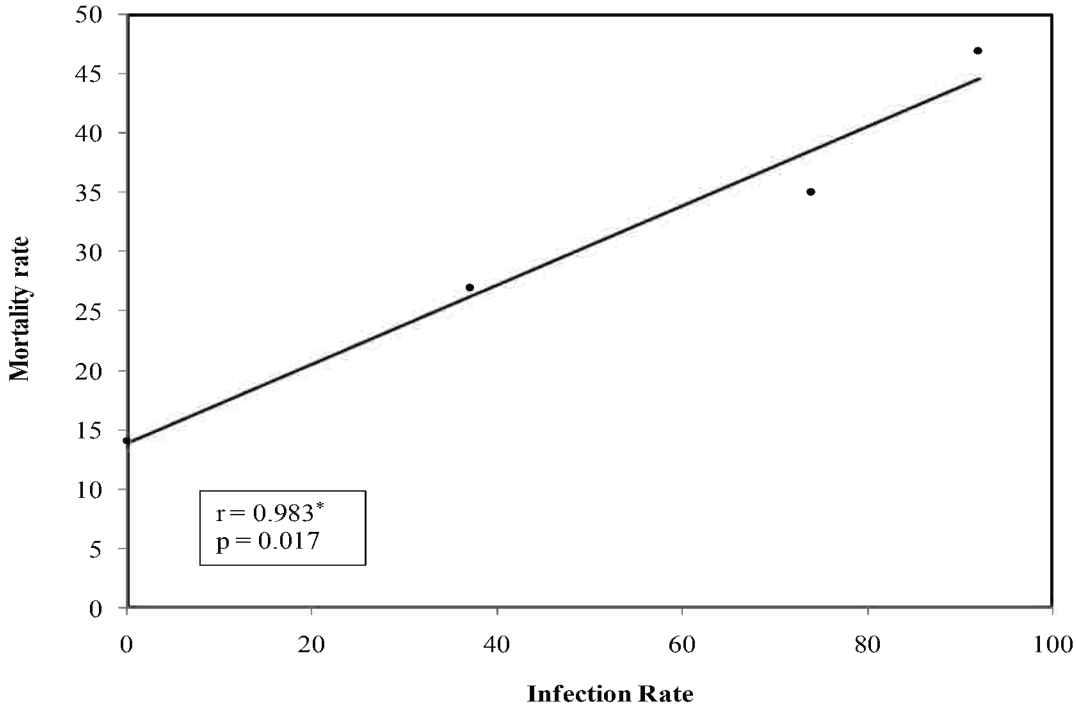

Figure 13: Correlation between infection rate and mortality rate among the studied subgroups.

2. Enzyme Assay study:

For the enzyme assay study, 8 susceptible and 8 resistant snails from the one hundred snails dedicated for biochemical studies in each experimental subgroup were selected. Moreover, 8 non-infected young, together with 8 non-infected adult snails were added in this analysis as 2 additional control groups.

Each snail was crushed, and bony particles were removed. Weights of young snails ranged from 0.159 to 0.192 grams, while that of adult snails ranged from 0.267 to 0.290 grams.

The highest level of SOD_1 enzyme was obtained in subgroup IIb (adult resistant) giving mean of 0.90 ± 0.07 U/gram tissue. This was followed by subgroup Ib (resistant members), subgroup Ib (resistant members), subgroup Ia (resistant members), subgroup IIa (susceptible members), subgroup Ib (susceptible members), subgroup Ia (susceptible members), and non-infected adult being; 0.76 ± 0.05, 0.68 ± 0.04, 0.55 ± 0.07, 0.40 –0.03, 0.34 ± 0.07, 0.32 ± 0.03 and 0.30 ± 0.03 Units/gram tissue respectively. The least enzyme level was given by non-infected young group being 0.26 ± 0.04 Units/gram tissue. Results were summarized and analyzed in table (7) and figure (14).

Table 7: The level of SOD_1 enzyme per snail's gram tissue in Units/gram tissues among the studied subgroups:

SOD_1 Units/gm	Ca	Cb	Ia s	Ia r	IIa s	IIa r	Ib s	Ib r	IIb
Min -Max	0.20 – 0.31	0.25 – 0.35	0.25 – 0.35	0.43 – 0.63	0.34 – 0.45	0.66 – 0.83	0.21 – 0.44	0.61 – 0.73	0.79 – 0.99
Mean ± SD.	0.26 ± 0.04	0.30 ± 0.03	0.32 ± 0.03	0.55 ± 0.07	0.40 ± 0.03	0.76 ± 0.05	0.34 ± 0.07	0.68 ± 0.04	0.90 ± 0.07
Median	0.26	0.30	0.32	0.58	0.40	0.77	0.36	0.67	0.91
F	**209.018***								
p	**<0.001***								
Sig				a- c.	a, b, d.	a-e.	d, f.	a-e, g.	a- h.

SOD_1: Cytosolic superoxide dismutase enzyme.
Ca: young non-infected; Cb: adult non-infected;
Ia s: young susceptible subgroup (susceptible members);
Ia r: young susceptible subgroup (resistant members);
IIa s: young resistant subgroup (susceptible members);
IIa r: young resistant subgroup (resistant members);
Ib s: adult susceptible subgroup (susceptible members);
Ib r: adult susceptible subgroup (resistant members);
II b:adult resistant subgroup.
a: significance with group uninfected young;
b: significance with group uninfected adult;
c: significance with subgroup I a (susceptible members);
d: significance with subgroup I a (resistant members);
e: significance with subgroup II a (susceptible members);
f: significance with subgroup II a (resistant members);
g: significance with subgroup I b (susceptible members);
h: significance with subgroup I b (resistant members);
i: significance with subgroup II b.
Normally distributed data were expressed in mean ± SD and was compared using F test (ANOVA). Sig: Significance between subgroups was done using Post Hoc Test (Scheffe).
*: Statistically significant at $p \leq 0.05$

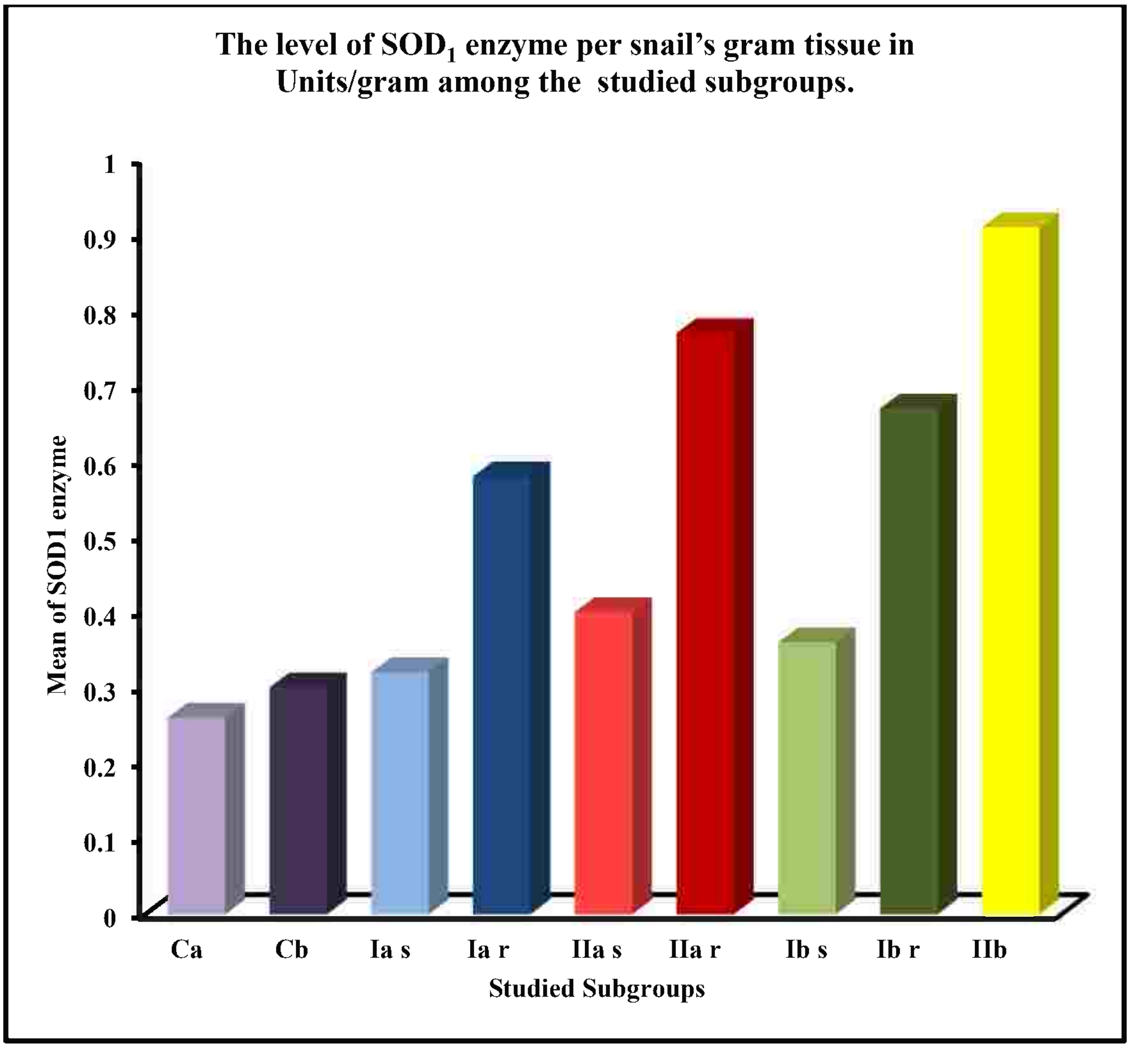

Figure 14: The level of SOD_1 enzyme per snail's gram tissue in Units/gram among the studied subgroups.

Ca: young non-infected; Cb: adult non-infected;
Ia s: young susceptible subgroup (susceptible members);
Ia r: young susceptible subgroup (resistant members);
IIa s: young resistant subgroup (susceptible members);
IIa r: young resistant subgroup (resistant members);
Ib s: adult susceptible subgroup (susceptible members);
Ib r: adult susceptible subgroup (resistant members);
II b:adult resistant subgroup.

3. Electrophoretic study:

The results of the SDS-PAGE electrophoresis were summarized in tables (8, 9) and figures (15, 16).

Table 8: SDS-PAGE analysis, showing number of peptide bands and molecular weights of the studied uninfected young group and infected young subgroups:

Fraction	Molecular Weight (KDa)					
	Protein marker	(Non-infected young) Lane 1	subgroup Ia (susceptible members) Lane 2	subgroup Ia (resistant members) Lane 9	subgroup IIa (susceptible members) Lane 5	subgroup IIa (resistant members) Lane 4
1	212.000					
2				196.739		
3					174.426	174.426
4					124.635	124.635
5	120.000					
6		115.819	115.819	115.819		
7	97.400					
8		93.600	93.600	93.600	93.600	93.600
9		67.596			69.002	
10	66.200					63.452
11			59.448	59.448		
12					44.009	44.009
13						34.680
14	31.000	30.349				
15			26.100	27.014		
16	20.000	20.758	20.380	20.380	20.124	20.124
17			18.655			
18						
19		17.471	16.873	16.873	16.547	16.547
20	15.000	15.099			15.062	15.161
21	14.400					
22	6.500	7.297	7.297	6.999	6.999	6.999
23	2.972	2.930	3.158	3.158	3.128	3.128
Number of Bands		9	9	9	10	11

Table 9: SDS-PAGE analysis, showing number of peptide bands and molecular weights of the studied non-infected adult group and infected adult subgroups:

Fraction	Molecular Weight (KD)				
	Protein marker	(Non-infected adult) Lane 7	subgroup Ib (susceptible members) Lane 3	subgroup Ib (resistant members) Lane 6	subgroup IIb (adult resistant) Lane 8
1	212.000				
2		204.353		196.739	196.739
3			140.989		
4					129.700
5				124.635	
6	120.000	120.000			
7			103.290		
8	97.400				94.111
9		93.600	93.600	93.600	93.600
10					
11				82.839	82.839
12		74.663			
13	66.200	64.818	64.818		
15		55.597	55.597	55.597	55.597
16		45.073	44.009		
17					39.962
18	31.000				
19				28.565	28.565
20		24.189	24.189		
21	20.000	19.523	20.380	20.380	
22				18.554	18.861
23		17. 391	17.391		
24			16.738	16.738	16.738
25	15.000		15.119		
26				14.962	14.951
27			14.846	14. 846	14. 846
28	14.400		14.652		
29				14.169	14. 169
30			13.920		
32				12.991	12.991
33	6.500	6. 424	6.799	6.424	6. 424
34		4.145		4.373	4.373
35					3.006
36	2.972	2.930	2.930	2.930	
Number of Bands		13	16	16	17

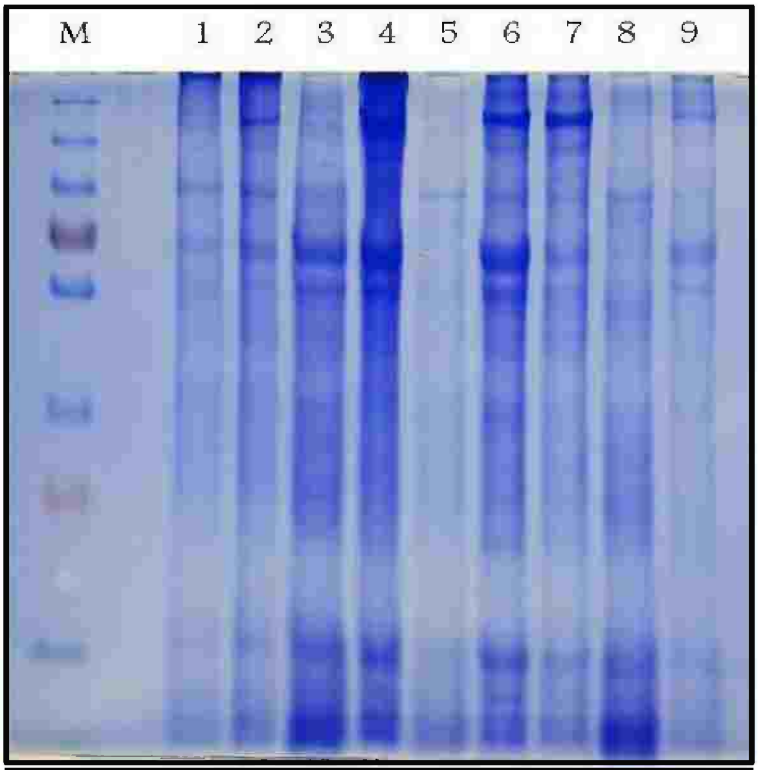

Figure 15: Peptide mapping of snails' tissue of the studied subgroups using SDS-PAGE.

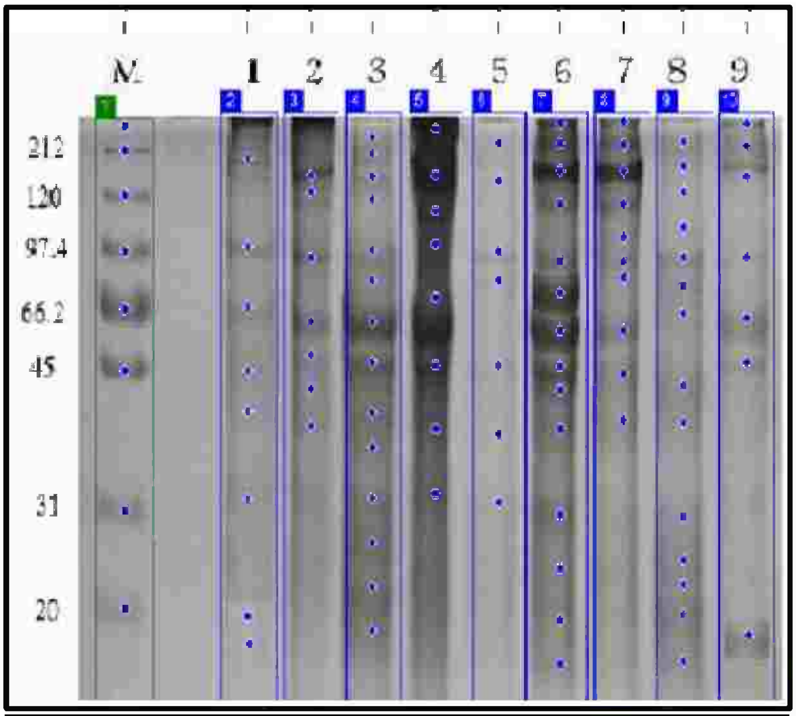

Figure 16: Total lab analysis for SDS-PAGE of the studied subgroups.

Using wide range molecular weight protein marker (6.5- 212) kilo daltons (KDa), SDS-PAGE revealed the protein pattern of the nine studied subgroups. Then gel was photographed. Enlarged photographs of gels were examined, principal bands were scored. Molecular weights of these bands were analyzed using Total Lab Analysis software program. Then to calculate percentage band sharing, bands in a given lane were compared with those in other lanes. A similarity index was constructed on the basis of the presence/absence of bands. Band sharing between all possible pairs was constructed using Dice similarity coefficient, [206] with the following formula:

S= 2a / 2a+ b+c Where;

S; is the degree of identity between the 2 compared samples.
a; is the number of common shared bands in the 2 compared samples.
b; is the number of excess bands present in sample 1, but not in sample 2.
c; is the number of excess bands present in sample 2, but not in sample 1.
S value of 1.0 denotes complete identity in the electrophoretic profile of both subgroups, while value of <1.0 indicates a variation in the polypeptide profile between the 2 compared samples.

The highest similarity coefficient was found between subgroup IIb adult resistant & subgroup Ib resistant members, being 0.89. Moreover, coefficients of 0.85 and 0.84, were calculated between (subgroup Ia resistant and susceptible members) and (subgroup IIa resistant and susceptible members) respectively. On the other hand, the least similarity index was observed between the most resistant subgroup in our experiment (IIb adult resistant) and the most susceptible subgroup (Ia susceptible members) being 0.4. Similarity indices between different subgroups were calculated. These are shown in table (10).

Table 10: Calculation of Dice similarity coefficient between all studied subgroups:

	non-infected young	subgroup Ia susceptible members	subgroup Ia resistant members	subgroup IIa susceptible members	subgroup IIa resistant members	non-infected adult	subgroup Ib susceptible members	subgroup Ib resistant members	subgroup IIb adult resistant
non-infected young	1	0.63	0.67	0.7	0.74	0.75	0.67	0.6	0.52
subgroup Ia susceptible members	0.63	1	0.84	0.6	0.56	0.57	0.6	0.48	0.4
subgroup Ia resistant members	0.67	0.84	1	0.63	0.56	0.52	0.48	0.64	0.45
subgroup IIa susceptible members	0.7	0.6	0.63	1	0.85	0.64	0.69	0.64	0.52
subgroup IIa resistant members	0.74	0.56	0.56	0.85	1	0.6	0.58	0.6	0.62
non-infected adult	0.75	0.57	0.52	0.64	0.6	1	0.7	0.6	0.6
subgroup Ib susceptible members	0.67	0.6	0.48	0.69	0.58	0.7	1	0.75	0.6
subgroup Ib resistant members	0.6	0.48	0.64	0.64	0.6	0.6	0.75	1	0.89
subgroup IIb adult resistant	0.52	0.4	0.45	0.52	0.62	0.6	0.6	0.89	1