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Isolation and Characterization of gut bacteria from *Aspidomorpha milliaris*

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Abstract:

***Aspidomorpha milliaris* (tortoise beetles) are herbivores feeding on *Ipomoea carnea* (morning glory spp.), which is otherwise toxic to cattle and human beings due its poisonous nature as it contains toxic compounds such as calystegine and swainsonine. Hence, it is astonishing how these beetles feed and survive on this plant? Therefore, isolation and characterization of the gut bacteria of *Aspidomorpha milliaris* were studied to see its role in the digestion of plant extract.The bacteria were dissected out along with the insect gut in sterile condition and isolated by spread plate technique. After 24 hours of incubation, two types of bacterial colonies (white and yellow) were observed. To confirm whether these isolated bacteria were responsible for the beetle's survival on the toxic plant, the bacterial growth curve was studied in leaf extract by colorimetric assay. Absorbance was taken every 24 hrs for four consecutive days (96 hrs.). It was observed that growth of bacteria (absorbance) increased with increase in time (hrs) which indirectly proves that bacteria utilize or digest plant extract to grow. Bacteria were then characterized using various morphological, cultural and biochemical tests and finally classified as Klebsiella and Citrobacter species by identification key of unknown bacteria(Aneja, 2003). Therefore, the above species of bacteria might be helping in digestion of the toxic leaf by converting it into non toxic form before it is getting absorbed by the gut of the beetle; thus the tortoise beetle escapes the toxicity of the plant with the help of its gut flora.**

Keywords: *Aspidomorpha milliaris ,Ipomoea carnea,* Klebsiella species, Citrobacter species

1.0 Introduction:

Aspidomorpha milliaris, commonly known as tortoise beetles are from the Chrysomelidae family and are quite interesting to observe. The body of this tiny creature has an oval shape with flattened sides that give it the appearance of a miniature turtle, hence the name. Close observation of this small beetle will show that the brightly colored shell covering the wings is so all-inclusive that even the head is covered. In some species these beetles will even have spots or stripes on their outer shell, which is why some species are often mistaken for ladybugs. Tortoise beetles can alter color within a short time period, turning from brilliant gold to a dull, spotty reddish color. The gold color also fades when the insect dies. The gold color is caused by a thin layer of moisture between the cuticle and an inner layer of the elytra. Apparently the insect is able to "voluntarily" squeeze this layer, reducing its thickness and eliminating the gold color. This change also occurs involuntarily when the beetle is under moisture stress, and, of course, when it dies. As the life of this tiny creature fades, the bright color on the outer shell diminishes as well. Even so, enough of the shimmering iridescence is left behind that these tiny insects are often used to make jewellery. (Mason, 1929; Barrows E.M., 1979; Hatch and Melville H, 1971). Tortoise beetles are herbivores, feeding on and spending their entire lives on plants like sweet potato and closely related plants such as *Ipomoea spp.* especially *Ipomoea carnea*; and bindweed, *Convolvulus spp.* Only plants in the family Convolvulaceae are hosts. (Capinera J.L., 2001; Riley C.V., 1870; Riley E.G., 1986). Both larvae and adults feed on leaves causing them to be riddled with holes. This type of damage is most threatening to seedlings or newly set plant (Ghate, H.V et.al. , 2003).

Ipomoea carnea

Damaged leaves of *I.carnea*

One of the perennial morning glory species, it grows to a height of 5m. It grows in dense populations along riverbeds, riverbanks, canals and other waterlogged (wetland) areas. The leaves are light green, heart shaped or somewhat lanceolate and 10-25cm long. It acts as toxic to cattle. (Arnold T.H. and de Wet B.C., 1993; Austin D.F., 1977). It is reported to have stimulatory allelopathic effects. Roots are boiled to use as laxative and to provoke menstruation. Traditional healers for treatment of skin diseases have used it. The milky juice of plant has been used for the treatment of leucoderma and other related skin diseases. Only external applications have been recommended due to poisonous nature of the plant. It has depressant effect on central nervous system. Also shows muscle relaxant property (Adam *et al.,* 1973; Alroy *et al.,* 1985; Asano *et al., 1985;* Austin D.F. and Huaman Z, 1996). Experiments on *I. carnea* indicated that it contained a combined level of calystegines and swainsonine of 0.1%. It has been estimated that locoweeds containing at least 0.001% swainsonine are capable of producing neurological damage if consumed regularly over a sufficient period of time; the contentin *I. carnea* is therefore far in excess of the level necessary to induce poisoning.(Katalin *et al.,* 1999; Ikeda *et al.,* 2003; James L.F. *et al.,* 2004; Azzouz R *et al.,* 2008).

1.1 Location:

This minute creature can be seen in a field that is lush with bindweed. They are present underneath leaves that appear to be chewed away due to numerous tiny holes and are no larger than 0.5 inch in diameter. Most tortoise beetles are wary of disturbances and will quickly fly away if disturbed.

1.2 Life Cycle and Description:

Very little biological information is available on this species, probably reflecting its slight economic importance. In the northern states there usually is only one generation annually. In New Jersey the beetles first appear in May or June, commence feeding on weeds, and deposit eggs soon thereafter. A new population of adults is evident in July (Koji Nakamura and Idrus Abbas,1987). The new adults feed briefly until the following spring. Development time from egg to adult requires about 40 days. The number of generations per year is unknown in Florida. The beetles vary slightly in color but invariably are orange colored, often golden metallic, and are sometimes called "gold bugs". (Barber H.S., 1960; Olmstead K.L. and Denno R.F., 1992, 1993).

1.3 Natural Enemies:

The wasp parasitoid *Tetrastichuscassidus* and the fly parasitoid *Eucelatoriopsis dimmocki* are known to attack golden tortoise beetle. Also known to cause

mortality, particularly among larvae, are such numerous ladybird beetle predators such as *Coccinella* spp. and *Coleomegilla* spp., but especially insects with piercing-sucking mouthparts such as damsel bugs, shield bugs, and assassin bugs (Baltazar E.P,1970). The anal fork carried by the larvae is somewhat effective against small predators, but large predators, especially those with long piercing-sucking mouthparts, are not deterred. (Barber H.S., 1960; Olmstead K.L. and Denno R.F., 1992,1993)

1.4 Management:

Larvae and adults are easily controlled by application of insecticides to the foliage, but this action is rarely warranted. (Bhuyan, et.al. 2008, Bhatia,S. et.al.,2007).

1.5 Gut Flora:

Gut bacteria of insects, millipedes and birds play an important role in pathogenesis of intestinal diseases since they are believed to protect against colonization of the intestine by pathogens and to stimulate their immune response (Visôtto L.E .et.al. 2009, Baluchamy, R and Periasamy, A. 2012 and Jangrang et.al. 2003). Isolation and characterization of bacteria from the gut of *Bombyx mori* (silk moth) , that degrade cellulose, xylan, pectin and starch and their impact on digestion were reported by Alwin, A et.al. in 2009. Insect associated bacterial diversity was also studied by Ryan Thomas Jones, Leticia Gonzales Sanchez and Noah Fiererin in 2013 . Isolation and characterization of gut bacteria from *Aspidomorpha milliaris* and its role in digestion of toxic plant (*Ipomoea carnea*) have been investigated in the present study to understand the survival of tortoise beetle on the above plant.

Tortoise beetle: Adult	Larvae	Pupa	Pupate

2.0 Materials and Methods:

2.1 Collection of Tortoise Beetles

Adult tortoise beetle were collected along with the leaves of morning glory from Fergusson College campus and allowed to feed on the same leaves by keeping the insects in a tray in the laboratory at room temperature.

2.2 Dissection of beetle

Under aseptic conditions in laminar flow, beetle was dissected with the help of fine scissor, Forceps and needle. The alimentary canal was isolated and placed in 0.9% saline in Petri plate.

2.3 Isolation of bacteria

The isolated alimentary canal was kept in 0.9% saline for few minutes and then centrifuged. Pellet was collected and re-suspended in 0.9% saline. Serial dilutions were made and suspensions were inoculated on nutrient agar plates by spread plate technique. These plates were incubated at 37 degrees for 24 hours.

2.4 Morphological, Cultural and Biochemical Assays of gut bacteria

To study the characteristics of the bacteria, morphological (shape, gram stain, spore stain,

motility) and cultural (color of colonies, growth, form, margin, elevation) tests were performed.
The isolated bacterial strains were identified using standard biochemical tests according to K.R Aneja's Experiments in Microbiology. The bacteria were identified through different biochemical tests such as carbohydrate fermentation tests, litmus milk reaction, starch, gelatine, lipid and casein hydrolysis, H_2s and indole production, methyl red, voges-proskauer, citrate utilization, nitrate reduction , urea hydrolysis and oxidation fermentation tests were performed. The results of the above assays were presented in Table 1 and Table 2.

2.5 Growth Curve of bacteria

The growth of the bacteria in leaf extract was also studied by colorimetric assay. Absorbance of leaf extract containing bacterial suspension incubated at 37^0C for 24 hours was measured at 620 nm using the leaf extract as the blank. After every 24 hours absorbance was measured and plotted graphically for four consecutive days (96 hrs). (Graph 1)

3.0 Result and Discussion:

3.1 Isolation of bacteria: After incubation, plates were observed for growth of bacteria. Two types of colonies- yellow and white were observed, indicating presence of two types of bacteria. (Fig.1 &2)

3.2 Growth curve of bacteria: Colorimetric assay revealed that absorbance increases with increasing growth of bacteria in the leaf extract. This observation clearly indicates that the bacteria utilize the leaf extract to grow and hence, help the beetle to digest the toxic extract of plant. The growth of bacteria was plotted using a graph from 0 hours to 96 hours (four consecutive days) against the absorbance at 620nm (Graph 1). The growth of bacteria was directly proportional to the absorbance at 620nm. It revealed that the bacteria helped in digestion of the leaf extracts and thus the beetle escapes the toxic action of the leaf.

3.3 Morphological and cultural characteristics of bacteria: The morphological characteristics were studied by performing gram staining and motility test. Both the bacteria were rod shaped. Also both the microorganisms were gram negative in nature, even non-spore former. But bacterium 1 which was yellow in color was non-motile whereas the white colonies proved to be motile in nature.(Table1) The cultural characteristics of bacterial colonies were studied by observing the color, growth, form, margin and elevation. The bacterium 1 was yellow in color whereas the bacterium 2 was white in color (Table1, Fig.1). The growth of both the colonies was moderate in agar media while form and margin were circular and entire respectively. But elevation of the colonies indicated that bacterium 1 was raised in nature while bacterium 2 was flat. (Table 1)

3.4 Biochemical Characteristics of bacteria: Further characterization of the isolated bacteria from the gut of the beetle was done by performing various biochemical assays. (Table 2). Carbohydrate (Glucose, Sucrose, Lactose and Mannitol) fermentation tests showed that both the bacteria were positive except bacterium 1 was negative to sucrose fermentation test. Litmus milk reaction proved to be positive for both. The results of starch, Gelatin, Lipid and Casein hydrolysis tests indicated that bacterium 1 and 2, both were positive for starch and lipid hydrolysis but both were negative for gelatin and casein hydrolysis. Urea hydrolysis assay revealed that bacterium 1 (yellow colony) was positive while bacterium 2 (white colony) was negative in nature. H_2S production and Indole production tests were positive for both the bacterium. (Table 2). The results of methyl red test, citrate utilization test and oxidation fermentation test indicated that both the bacteria were positive, whereas the results of voges-proskauer and nitrate reduction tests proved to be negative in nature. (Table 2).

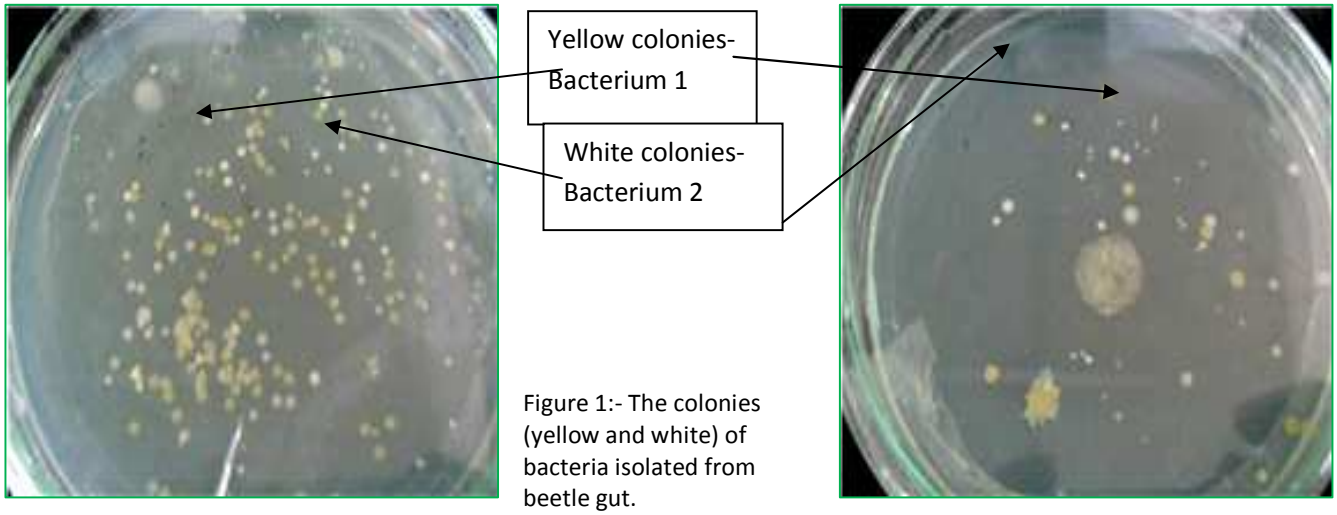


Figure 1:- The colonies (yellow and white) of bacteria isolated from beetle gut.

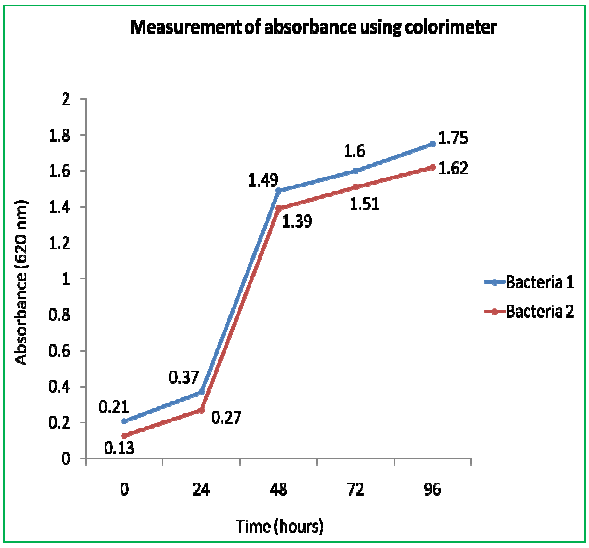
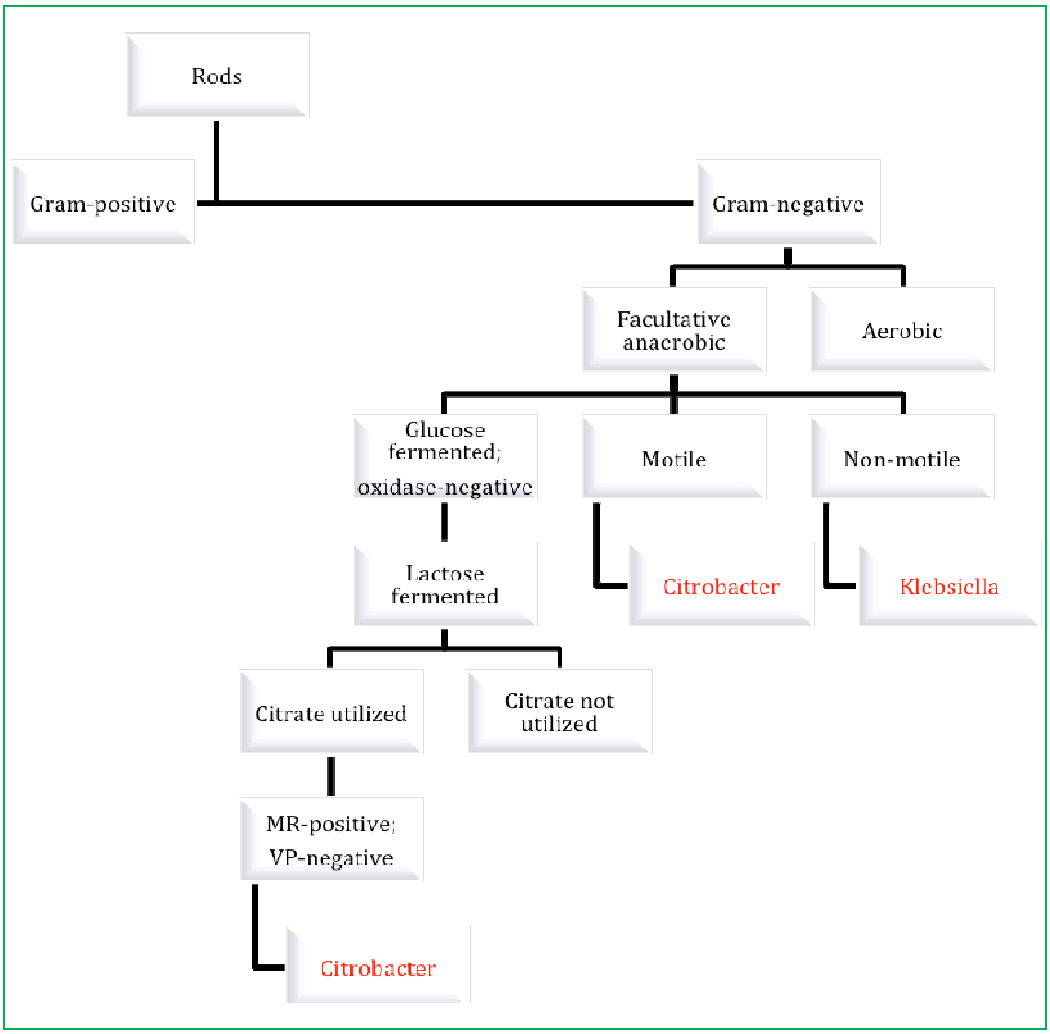


Fig. 1:- Growth curve of bacteria

Table 1:- Morphological and cultural characteristics of bacteria.

Characteristics	***Bacterium 1***	***Bacterium 2***
Morphological		
Shape:	Rods	Rods
Gram stain:	Negative	Negative
Spore stain:	Non-spore former	Non-spore former
Motility:	Non-motile	Motile
Cultural		
Colonies:	Yellow	White
Growth (agar media):	Moderate	Moderate
Form:	Circular	Circular
Margin:	Entire	Entire
Elevation:	Raised	Flat

Table 3:- Identification Key of Unknown Bacteria (K.R. Aneja, 2003).



Based on this hierarchy and the results obtained from the various tests done on the bacteria, the tentative identification of the bacteria was concluded to be: **Bacterium 1: Klebsiella species and Bacterium 2: Citrobacter species**

The colonies of bacteria were identified on the basis of traditional tests which determine phenotypic characteristics and metabolic characteristics of the bacteria by performing series of tests according to K. R. Aneja's standard bacterial taxonomy. Preliminary microscopic examination revealed that both the bacteria 1 and 2 are gram negative rod type. Relationship of bacteria to oxygen proved both the bacteria as facultative anaerobic, which provides useful clues to the identification of the microorganisms. Further characterization revealed that bacterium 1 is non-motile whereas bacterium 2 is motile and both come under facultative anaerobic according to identification key of K.R. Aneja, 2003 (Table 3). Moreover, the above isolated gram negative rod shaped bacteria were glucose fermented, lactose fermented, citrate utilized, MR+ve and VP-ve type, therefore it could be Citrobacter species (Aneja, 2003) (Table 3). Motile nature of bacterium 2 under gram negative rod and facultative anaerobic group further classify it as Citrobacter species (Aneja 2003). While non-motile bacterium 1 under facultative anaerobic and gram negative rod could be classified as Klebsiella species (Table 3). The bacterial growth curve which increased gradually with time (0hrs – 96 hrs) indirectly indicated that the bacteria utilized the leaf extract during its growth period.

Table 2:- Biochemical Characteristics of bacteria.

Biochemical tests	***Bacterium 1***	***Bacterium 2***
Carbohydrate fermentation test: Glucose Sucrose Lactose Mannitol	 + - + +	 + + + +
Litmus milk reaction	+	+
Starch hydrolysis	+	+
Gelatin hydrolysis	-	-
Lipid hydrolysis	+	+
Casein hydrolysis	-	-
H_2S production	+	+
Indole production	+	+
Methyl red test	+	+
Voges-Proskauer test	-	-
Citrate utilization test	+	+
Urea hydrolysis	+	-
Nitrate reduction test	-	-
Oxidation fermentation test	+	+

Thus the bacteria inhibiting in the gut of the tortoise beetle might be helping in digestion of the toxic leaf extract and converting it into the non toxic form which is finally absorbed by the tortoise beetle; thus the beetle escapes the toxicity of the plant with the help of its gut flora. Characterization of microbial gut flora of insects and its role in digestion of plant extract were studied by Willaims, L and Roane in 2006, Alwin Prem Anand et.al. and Visôtto L.E. et.al. in 2009. Recently, insect associated bacterial diversity was also studied by Ryan Thomas Jones et.al. in 2013. .

4.0 Conclusion:

- Bacterial colonies inhibiting the gut of tortoise beetle can be classified as Klebsiella species (Bacterium 1) and Citrobacter species (Bacterium 2) according to the identification key of K. R. Aneja (2003) on the basis of various morphological, cultural and biochemical assays.
- The above bacteria plays a significant role in the survival of *Aspidomorpha milliaris* on the toxic leaf of *Ipomoea carnea* by digesting the plant extract and probably by converting the toxic compounds of plant extract into non - toxic form before it is getting absorbed by the gut of the beetle.

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