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Assessment of Antibacterial Activity of *Momordica charantia* Extracts and Antibiotics against Fecal Contaminated Water Associated *Enterococcus* spp.

Saiqa Andleeb[1]*, Tahseen Ghous[2], Nazia Riaz[1], Nosheen Shahzad[2], Summya Ghous[1] and Uzma Azeem Awan[1]

[1]Biotechnology laboratory, Department of Zoology, Azad Jammu and Kashmir University, Muzaffarabad 13100, Pakistan

[2]Biochemistry Laboratory, Department of Chemistry, Azad Jammu and Kashmir University, Muzaffarabad 13100, Pakistan

Abstract.- Antibacterial activity of extracts of *Momordica charantia* and several antibiotics were studied against *Enterococcus faecalis* and *Enterococcus faecium* isolated from water receiving fertilizers of animal origin by filter disc diffusion method. *E. faecalis* and *E. faecium* were found sensitive to gentamycin, ciprofloxacin and tetracycline, and resistant to penicillin G, trimethoprim and sulfomethoxyzol. *M. charantia* showed significant zone of inhibition in the case of n-hexane, ethanol and methanol extracts of green parts against A and C strains of *E. faecium*, while ethyl acetate and methanol extracts of seed parts of *M. charantia* indicated considerable inhibitory effect against both B and A strains of *E. faecium*. This study showed that fruit part of *M. charantia* could be a potential source of new antimicrobial agents.

Key words: Agar disc diffusion method, antibiotics, antibacterial activity, Enterococci, *Momordica charantia.*

Enterococci are considered part of the normal flora of food products, water, bowel, mammals, birds and urethra of humans (Reeves and Grant, 2004). Although, Enterococci are of relatively low virulence, opportunistic pathogens can cause serious humans diseases including diarrohoea, endocarditis, and bacterimia (Nannini *et al*., 2005). Enterococci are facultative anaerobic, Gram positive cocci that live as normal flora in the gastrointestinal tract of humans and animals (Kiem *et al*., 2003). *Enterococcus* species are indicators of animal and human fecal contamination in water and various food products (Moneoang and Bezuidenhout, 2009; Valenzuela *et al*., 2008).

More than twenty species of Enterococci have been classified. *Enterococcus faecium* and *Enterococcus faecalis* are the mostly indentified species in humans, animals, food products and water (Facklam, 2002). Fisher and Philips (2009) demonstrated that these pathogens would cause disease if the hosts immune system is suppressed. Hydrogen peroxide derived from *E. faecium* was shown to damage luminal cells in the colon of rats (Huycke *et al*., 2002). Infectious pathogens have been reduced using various medicinal plants such as *Momordica charantia* due to their potential antidiabetic, antihelmintic, antmicrobial, anti-cancerigenos and antioxidant activities (Costa *et al*., 2011). In the present study *Enterococcus* pathogens were isolated from animal fecal contaminated water, causing infections in human as well as in other mammals, to determine the pattern of antibacterial resistance. The aim of present work is to determine the antibacterial activity of *M. charantia* and to assess the efficacy of different antibiotics in use against *E. faecium* and *E. faecalis*.

Materials and methods

Sample collection

Water samples were collected from the different areas of contaminated river near Muzaffarabad city, Pakistan. A sample of 200 µl was spread on Slantez and Bartely medium (SBM) plates (Slanetz and Bartley, 1957), and incubated at 37°C for two days.

Isolation and identification of Enterococcus

Appearance of growth on SBM proved the presence of the members of genus *Enterococcus*. Two types of *Enterococcus viz., E. faecalis* and *E. faecium* were identified and confirmed by conventional microbiology and biochemical procedures performed in the Microbiology lab of Combined Military Hospital, Muzaffarabad, Pakistan. Screening of pathogens were carried out

* Corresponding author: drsaiqa@gmail.com
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by using various growths promoting bacterial media *i.e.*, MacConky agar, nutrient agar, nutrient broth, brain heart infusion agar, thiosulphate citrate-bile salts- sucrose agar, and eosin methylene blue agar purchased from Oxoid. The Gram staining was done to characterize the isolates. Various biochemical tests such as oxidase, catalase, coagulase, and API 10 were used for confirmation of pathogens (Clarke, 1953; Slanetz and Bartley, 1957).

Preparation of extracts of medicinal plants

M. charantia, purchased from the super market of Muzafarrabad, was air dried at room temperature for 10 days and then powdered with the help of a blender. The extracts of *M. charantia* (both seeds and green parts) were prepared consecutively with, n-hexane (N1, N6), chloroform (N2, N7), ethyl acetate (N3, N8), ethanol (N4, N9) and methanol (N5, N10) using a Soxhlet extractor for 48 h. All the extracts were concentrated using rotary flash evaporator and preserved at 4°C in airtight bottle until further use. All the extracts were tested for antibacterial activity assay. The extracts of seeds indicated as N1, N2, N3, N4, N5 whereas green parts of *M. charantia* as N6, N7, N8, N9, and N10, respectively.

Sensitivity test of standard antibiotics

Sensitivity of antibiotics against isolated pathogens was determined by filter paper disc method (Prescott *et al.*, 1990). Sensitivity was predictable with clear zone surrounding the disc. The potency of antibiotics per disc (5 mm in diameter) was as follows: amoxicillin (10 µg), streptomycin (10 µg), tobramycin (10 µg), gentamycin (10 µg), ciprofloxacin (5 µg), sulfomethoxyzol (25 µg), tetracycline (10 µg), penicillin G (10 µg), trimethoprim (5 µg), ampicillin (10 µg).

Filter paper disc diffusion method

Filter paper disc method was used for testing medicinal plant extracts against the selected pathogens (Alzoreky and Nakahara, 2003). The Nutrient agar was prepared, allowed to cool up to 40 °C, mixed with freshly prepared overnight culture, poured on autoclaved Petri plates and allowed to solidify under aseptic conditions. Whatman No. 1 filter paper disc (5 mm diameter) was impregnated with crude plant extracts and placed on nutrient agar which was previously swabbed with pathogens. Antibiotics were also used to check the susceptibility test. The methanol, ethanol, chloroform, ethyl acetate, and n-Hexane were used as blind controls. Finally the inoculated plates were incubated for 24 h at 37°C to allow the maximum growth of the microorganisms and the zone of inhibition was observed and measured in millimeters. Each assay in this experiment was repeated for three times. Growth inhibition (GI) was recorded as very high (++++), high (+++), medium (++) and low (+), which indicated zones of inhibition between 35-49, 21-34, 12-20, below 12 mm, and no activity indicated as zero mm. GI was measured as "zone of inhibition= inhibited area-disc size".

Results and discussion

Previous studies demonstrated that medicinal herbs are a new potential source of antibacterial agents even against some antibiotic-resistant strains (Kone *et al*., 2004). Results of this study confirmed the observation of earlier studies (Yuste and Fung, 2004).

Enterococcus is Gram positive bacterium, API 10, carbohydrate, and indole positive but negative for catalase test (Clarke, 1953; Slanetz and Bartley, 1957). Three different strains of *E. faecium* (A, B, C) and one strain of *E. faecalis* (D) were identified.

Table I shows the effect of various antibiotics on multiple drug resistant *Enterococcus* species (Courvalin, 2006). All the tested pathogens were resistant to pencilline G, trimethoprim and sulfomethoxyzol, and sensitive to tetracycline, ciprofloxacin, and gentamycin (Table I). It was observed that *E. faecalis* was sensitive to gentamycin, ciprofloxacin and tetracycline and resistant to remaining antibiotics (Table I). Our results are consistent with Arias *et al*. (2010) and Hooper and Wolfson (1991). Likewise, *E. faecium* (B) was sensitive to amoxylin, ampicillin, gentamycin, ciprofloxacin, and tetracycline, and resistant to the remaining antibiotics. Similarly, amoxylin, ampicillin, penicillin G, trimethoprim and sulfomethoxyzol inhibited the growth of *E. faecium*

(A and C) as indicated in (Table I). This finding is consistent with previous reports of non β-lactamase-producing penicillin-resistant enterococci (Acar and Buu-Hoi, 1988; Bush *et al*., 1989).

Table I.- Effect of antibiotics against three strains of *Enterococcus faecium* (A, B, C) and *Enterococcus faecalis* (D)

Antibiotics used against pathogens	*E. faecium*			*E. faecalis*
	A	B	C	D
Amoxylin (10 µg)	R	S	R	R
Ampicillin (10 µg)	R	S	R	R
Streptomycin(10 µg)	R	R	S	R
Tobramycin (10 µg)	R	R	S	R
Gentamycin (10 µg)	S	S	S	S
Ciprofloxacin (5 µg)	S	S	S	S
Pencillin G (10 µg)	R	R	R	R
Trimethoprim (5 µg)	R	R	R	R
Sulfomethoxyzol (25 µg)	R	R	R	R
Tetracycline (10 µg)	S	S	S	S

R, resistant; S, sensitive

Table II shows the effect of different organic solvent extracts of seeds and green parts of *M. charantia* on three strains of *E. feacium* and one strain of *E. faecalis*. *E. faecium* (C) was resistant to all extracts of seeds, whereas *E. faecium* (B) was resistant to all extracts of green parts of *M. charantia*. *E. faecium* (A) was highly sensitive (+++) to methanolic extract of seed, and methanolic and ethanolic extracts of green parts of fruit. *E faecium* (B) was highly sensitive (+++) to ethyl acetate extracts of seed, where *E. faecium* (C) is highly sensitive (+++) to n-hexane extract of green parts. *E. faecalis* (D) is however resistant to almost all the extracts of *M. charantia.*

The sensitivity of these pathogens may be due to the presence of phytochemical compounds of *M. charantia.* Our results are consistent with the previous studies that *M. charantia* have biological components included glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids that exhibit anti-bacterial activity (Costa *et al*., 2011; Abalaka *et al*., 2009; Budrat and Shotipruk, 2009). This study showed that fruit part of *M. charantia* could be a potential source of new antimicrobial agents.

Table II.- Zone of inhibition of extracts of seeds and green parts of *Mamordica charantia* against three strains of *Enterococcus faecium* and *Enterococcus faecalis.*

Extracts	*E. faecium*			*E. faecalis*
	A	B	C	D
Extracts of seeds				
N1 (n-Hexane)	0	0	0	0
N2 (Chloroform)	0	0	0	0
N3 (Ethyl acetate)	0	20(+++)	0	0
N4 (Ethanol)	0	5(+)	0	6(+)
N5 (Methanol)	20(+++)	0	0	0
Extracts of green parts				
N6 (n-Hexane)	0	0	22(+++)	0
N7 (Chloroform)	0	0	0	0
N8 (Ethyl acetate)	6(+)	0	0	0
N9 (Ethanol)	26(+++)	0	0	8(+)
N10 (Methanol)	26(+++)	0	0	8(+)

Growth inhibition was recorded: very high (++++) 35-62 mm; high (+++) 21-34 mm; medium (++) 12-20 mm; low (+) below 12 mm; no activity zero mm

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Record and Trophic Associations of Three Aphid Hyperparasitoids From Punjab Province of Pakistan

Imran Bodlah* and Muhammad Naeem
Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan

Abstract.- Three aphid hyperparasitoids, *Syrphophagus aphidovorus* (Mayr, 1876), *Pachyneuron aphidis* (Bouché, 1834) and *Asaphes suspensus* (Nees, 1834) are recorded for the first time from various districts of Punjab Province of Pakistan along with 14 new trophic associations and 31 new locality records.

Key words: Trophic associations, aphid hyperparasitoids.

* Corresponding author: imranbodlah@gmail.com

There are many controversial views about the role of hyperparasitoids. According to some workers they are considered as highly detrimental as they reduce the number of primary parasitoids (Hagen and Van den Bosch 1968, Carter *et al*., 1980), while Benett (1981) and Luck *et al*. (1981) rejected these views. It has been proven that under certain circumstances they may play a positive role in maintaining the balance between the primary parasitoids and their host species (Starý, 1970). The presence of hyperparasitoids may therefore enhance the stability of a given pest primary parasitoid system with the pest density constant in time (Pankanin-Franczyk, 1995).

Aphids being the hosts of hymenopterous primary parasitoids, which themselves are attacked by hymenopterous hyperparasitoids. Primary parasitoids of aphids fall in two groups of Hymenoptera: Braconidae (Aphidiinae; all genera of this subfamily) and Aphelinidae (*Aphelinus* and its related genera) (Sullivan and Völkl, 1999). There is solitary endoparasitoids of various groups of Aphididae. Aphid hyperparasitoids is a group of solitary endoparasitic or ectoparasitic parasitoids excepting a few gregarious species, belong to various families of Hymenoptera such as Figitidae, Encyrtidae, Pteromalidae and Eulophidae, Megaspilidae (Sullivan, 1988) and Tetrastichinae (Takada and Kamijo, 2012).

Lot of work has been done on aphid hyperparasitoid taxonomy, biology and their trophic associations in various parts of the world (Dessart, 1972, 1999; Evenhuis, 1972; Kamijo and Takada, 1973, Takada, 1973; Evenhuis and Barbotin, 1977; Starý, 1977; Andrews, 1978; González *et al*., 1979; Fergusson, 1980; Martines, 1985; Farooqi and Subba Rao, 1986; Carver, 1992; Gibson and Vikberg, 1998; Sureshan and Narendran, 2000; Kavallieratos *et al*., 2004). Most recent works on aphid hyperparasitoids include those of Krawczyk *et al*. (2009), Takada (2009), Westrum *et al*. (2010), Sæthre *et al*. (2011), Bouhachem (2011) and Takada and Kamijo (2011). In neighboring countries like India, Singh and Tripathi (1991) and Ahmad and Singh (1994), and in Iran Rakhshani *et al*. (2004), Talebi *et al*. (2009) and Mitroiu (2011) have done lot of work on aphid parasitoids. Due to lack of

basic research even on primary aphid parasitoids except that of Bodlah *et al*. (2011, 2012a,b,c) no extensive effort has been undertaken on hyperparasitoid taxonomy and their trophic associations in Pakistan. Keeping in view the paucity of information on parasitoids in Pakistan, a survey of various crops, ornamental plants and weeds in different districts of Punjab Province was undertaken.

Materials and methods

Samplings were carried out during 2005-2008 at different regions of Punjab, Pakistan including 22 localities from wheat, *Brassica*, wild spinach, pea, tobacco and *Hibiscus rosa- sinensis*. The parasitized aphids were collected together with plant materials and transferred to laboratory, maintained subsequently, until emergence of parasitoids and hyperparasites. Live aphids were preserved in 90% ethanol and 75% lactic acid in a ratio of 2:1 (Eastop and van Emden, 1972) for identification at a later date. Parasitoids and hyperparasitoids were identified and observed under Nikon™ microscope and coloured photographs were snaped. The emerged wasps were identified using the available keys and descriptions (Gibson and Vikberg, 1998; Trjapitzin, 2008). Aphids were identified using the keys of Blackman and Eastop (1984, 1994). Aphid parasitoids were identified using the keys of Starý and Schlinger (1967); Raychaudhuri (1990), and Starý and Ghosh (1983).

Syrphophagus aphidovorus (Mayr, 1876)

Aphidencyrtus aphidivorus Mayr, 1876
Microterys submetallicus Mercet, 1921

Material examined and trophic associations

Myzus persicae on *Brassica campestris* L. via *Diaeretiella rapae* , Rawalpindi, 28-iii-06, 25♀ and 12♂; Islamabad, 24-iii-06, 15♀ and 8♂; Attock, 28-iii-05, 15♀ and 12♂; Jhelum, 28-iii-06, 25♀ and 6♂; Gujrat, 26-iii-07, 4♀ and 3♂; Narowal, 22-iii-07, 5♀ and 3♂; Pakpatan, 28-iii-08, 15♀ and 6♂; D.G. Khan, 1-iv-08, 13♀ and 7♂; Lahore, 24-iii-05, 19♀ and 12♂; Faisalabad, 29-iii-06, 22♀ and 8♂; Bahawalpure, 24-iii-07, 15♀ and 8♂; Bahawalnager, 27-iii-07, 9♀ and 2♂; Gujranwala, 2-iv-08, 25♀ and 9♂; Layyah, 27-iii-07, 19♀ and 8♂; Bakher, 27-iii-07, 30♀ and 12♂; Mianwali, 1-iv-07, 29♀ and 12♂.

Myzus persicae on *Nicotiana tobacum* via *Diaeretiella rapae*, D.G. Khan, 29-iii-06, 35♀ and 18♂; Layyah, 27-iii-06, 35♀ and 8♂.

Brevicoryne brassicae on *Brassica campestris* L. via *Diaeretiella rapae*, Rawalpindi, 28-iii-08, 26♀ and 12♂; Islamabad, 28-iii-06, 35♀ and 4♂; Attock, 28-iii-05, 25♀ and 10♂; Jhelum, 28-iii-06, 35♀ and 18♂; Gujrat, 26-iii-07, 21♀ and 9♂; Narowal, 22-iii-07, 35♀ and 10♂; D.G. Khan, 1-iv-08, 18♀ and 10♂; Muzafar garh, 17-iii-07, 10♀ and 15♂; Lahore, 12-iv-05, 45♀ and 20♂; Faisalabad, 19-iii-06, 25♀ and 18♂; Khanewal, 24-iii-06, 35♀ and 24♂; Sahiwal, 18-iii-08, 22♀ and 8♂; Multan, 18-iii-06, 35♀ and 15♂; Bahawalpure, 21-iii-07, 45♀ and 18♂; Bahawalnager, 27-iii-07, 25♀ and 9♂; Gujranwala, 2-iv-08, 15♀ and 2♂; Layyah, 27-iii-07, 30♀ and 8♂; Bakher, 27-iii-07, 25♀ and 4♂; Mianwali, 1-iv-07, 39♀ and 20♂.

Brevicoryne brassicae on Brassica napus, via *Diaeretiella rapae*, Rawalpindi, 28-iii-08, 20♀ and 6♂; Islamabad, 23-iii-06, 14♀ and 12♂; Attock, 28-iii-06, 15♀ and 6♂; Jhelum, 28-iii-07, 15♀ and 4♂; Sahiwal, 18-iii-07, 18♀ and 2♂; Okara, 22-iii-08, 25♀ and 12♂; Multan, 28-iii-06, 35♀ and 19♂; Bahawalpure, 21-iii-07, 24♀ and 18♂; Bahawalnager, 27-iii-07, 39♀ and 16♂; Gujranwala, 7-iv-08, 35♀ and 18♂; Layyah, 27-iii-07, 30♀ and 16♂; Bakher, 27-iii-07, 20♀ and 15♂; Mianwali, 1-iv-07, 19♀ and 6♂.

Aphis fabae on wild spinach via *Aphidius colemani*, Rawalpindi, 23-iii-07, 34♀ and 16♂; Islamabad, 26-iii-05, 24♀ and 8♂; Attock, 29-iii-08, 35♀ and 16♂; Khushab, 27-iii-07, 35♀ and 14♂.

Rhopalosiphum padi on *Triticum aestivum* via *Aphidius colemani*, Rawalpindi, 29-iii-06, 35♀ and 16♂; Jhelum, 25-iii-05, 28♀ and 2♂; Muzafar garh, 24-iii-05, 10♀ and 3♂; Layyah, 9-iv-07, 42♀ and 23♂; D. G. Khan, 10-iv-06, 25♀ and 4♂; Attock, 22-iii-05, 22♀ and 13♂; Mianwali, 24-iii-07, 10♀ and 5♂; Lahore, 1-iv-07, 28♀ and 13♂; Khushab, 10-iv-08, 25♀ and 12♂.

Comments

This widely distributed Palaearctic species is

a secondary parasite of aphids (Hemiptera., Aphidoidea), common in India (Trjapitzin, 2008). Mahmood *et al*. (2002) reported this species from mummies of aphid, *Sipha maides* Passerine from Quetta. In present studies, it is reported for the first time from the Punjab Province. All new host plant and aphid, and primary parasite records have been recorded along with new locality records.

Asaphes suspensus (Nees, 1834)

Cyrysolampus suspensus Nees, 1834, Hym. Ichneum. Affin. Monog. 2: 127.
Asaphes suspensus Graham, 1969, Bull. Brit. Mus. (Nat. Hist.) Ent. Suppl. 16: 82.

Material examined and trophic associations

Aphis fabae on wild spinach via *Aphidius colemani,* Rawalpindi, 23-iii-07, 14♀ and 6♂; Islamabad, 26-iii-05, 15♀ and 4♂; Attock, 29-iii-08, 15♀ and 6♂; Khushab, 27-iii-07, 22♀ and 13♂.

Acyrthosiphum pisum on *Pisum sativum* via *Aphidius smithi,* Rawalpindi, 29-iii-07, 13♀ and 6♂; Islamabad, 25-iii-08, 6♀; Attock, 24-iii-06, 12♀ and 4♂.

Rhopalosiphum padi on *Triticum aestivum* via *Aphidius colemani*, Rawalpindi, 29-iii-06, 20♀and 6♂; Jhelum, 25-iii-05, 9♀ and 2♂; Muzafar garh, 24-iii-05, 8♀ and 3♂; Layyah, 9-iv-07, 12♀ and 9♂; D. G. Khan, 10-iv-06, 6♀ and 2♂; Attock, 22-iii-05, 12♀ and 3♂; Mianwali, 24-iii-07, 24♀ and 15♂; Lahore, 1-iv-07, 8♀and 3♂; Khushab, 10-iv-08, 5♀and 2♂.

Brevicoryne brassicae on *Brassica campestris* L. via *Diaeretiella rapae*, Rawalpindi, 28-iii-08, 15♀ and 6♂; Islamabad, 28-iii-06, 20♀ and 14♂; Attock, 28-iii-05, 25♀ and 4♂; Jhelum, 28-iii-06, 18♀ and 8♂; Gujrat, 26-iii-07, 10♀ and 9♂; Narowal, 22-iii-07, 5♀ and 1♂; D.G. Khan, 1-iv-08, 8♀ and 3♂; Muzafar garh, 17-iii-07, 10♀ and 5♂; Lahore, 12-iv-05, 15♀ and 2♂; Faisalabad, 19-iii-06, 25♀ and 18♂; Khanewal, 24-iii-06, 8♀ and 4♂; Sahiwal, 18-iii-08, 2♀ and 1♂; Multan, 18-iii-06, 16♀ and 5♂; Bahawalpure, 21-iii-07, 5♀ and 8♂; Bahawalnager, 27-iii-07, 5♀ and 9♂; Gujranwala, 2-iv-08, 12♀ and 5♂; Layyah, 27-iii-07, 3♀ and 1♂.

Aphis gossypii on *Hibiscus rosa- sinensis* via Genus *Binodoxys*, Rawalpindi, 29-iii-08, 9♀and 3♂; Islamabad, 24-iii-05, 13♀ and 7♂; Lahore, 27-iii-07, 10♀ and 3♂; Multan, 22-iii-06, 12♀ and 8♂; Attock, 28-iii-08, 9♀.

Comments

A. suspensus was first time recorded from India and Pakistan by Farooqi and Subba Rao (1986). Similarly (Gibson and Vikberg, 1998) also gave a comprehensive note on the same species from different bio-geographic regions of the world including Pakistan, which also confirms its distribution pattern under the present study. This species is recorded for the first time from various districts of Punjab. New host aphid and primary parasites records have been added. Specimens collected from Pakistan were compared with description given Gibson and Vikberg (1998) and found to be morphologically similar.

Pachyneuron aphidis (Bouché, 1834)

Diplolepis Aphidis Bouché,1834, Naturg. d. Insect. p.170.
Pachyneuron aphidis Reinhard,1859, Stettin. Ent. Ztg. 20: 195.
Pachyneuron aphidis: Graham,1969, Bul!.Brit. Mus. (Nat. Rist.) Ent. Supp!. 16: 842.
Pteromalus minutissimus Forster, 1841, Beitr. Monog. Pterom. 1: 28.
Pachyneuron minutissimum Delucchi, 1955, Z. Angew. Ent. 38: 137.
Pachyneuron gifuensis Ashmead, 1904, J. New York Ent. Soc. 12: 158. Syn. n.

Material examined

Aphis gossypii on *Hibiscus rosa- sinensis* via Genus *Binodoxys*, Rawalpindi, 29-iii-08, 14♀ and 8♂; Islamabad, 24-iii-05, 19♀ and 9♂; Lahore, 27-iii-07, 19♀ and 8♂; Multan, 22-iii-06, 22♀ and 14♂; Attock, 28-iii-08, 12♀ and 4♂. *Aphis fabae* on wild spinach via *Aphidius colemani,* Rawalpindi, 29-iii-08, 24♀ and 16♂; Islamabad, 29-iii-06, 12♀ and 4♂; Attock, 29-iii-08, 25♀ and 16♂; Khushab, 27-iii-07, 12♀ and 8♂. *Rhopalosiphum padi* on

Triticum aestivum via *Aphidius colemani*, Rawalpindi, 30-iii-06, 13♀ and 9♂; Jhelum, 25-iii-05, 19♀ and 12♂; Layyah, 9-iv-07, 12♀ and 9♂; Attock, 22-iii-05, 14♀ and 8♂; Lahore, 1-iv-07, 8♀ and 3♂; Khushab, 10-iv-08, 15♀ and 10♂.

Brevicoryne brassicae on *Brassica campestris* L. via *Diaeretiella rapae*, Rawalpindi, 29-iii 08, 8♀ and 2♂; Islamabad, 28-iii-06, 24♀ and 10♂; Attock, 28-iii-05, 5♀ and 4♂; Jhelum, 28-iii-06, 8♀ and 6♂; Gujrat, 26-iii-07, 11♀ and 6♂.

Comments

Sureshan and Narendran (2000) reported the distribution of *Pachyneuron aphidis* (Nees) from many countries including Pakistan. In Iran, it was reported as a hyperparasitoid of cereal aphid parasitoid (Rakhshani, 2005). *Pachyneuron aphidis* is a first record in Punjab as hyperparasitoid through *Binodoxys* and *Aphidius* genus. Mahmood *et al*. (2002) reported this species from mummies of aphid, *Sipha maides* Passerine from Quetta. In present studies, it is reported for the first time from the Punjab Province. All new host plant and aphid, and primary parasite records have been recorded along with new localities.

All three species can easily be differentiated from each other normally by their wing characters; Forewings with marginal vein distinctly longer than wide (*Syrphophagus aphidovorus*); fore wing with marginal vein not thickened (*Asaphes suspensus)*; marginal vein thickened throughout, short, at most one-third as long as submarginal vein (*Pachyneuron aphidis*) (Kamijo and Takada, 1973).

General discussion

Syrphophagus aphidovorus was mostly hyperparasitic via *Diaeretiella rapae* followed by *Aphidius colemani*. Parasitized aphids were *Myzus persicae, Brevicoryne brassicae, Aphis fabae* and *Rhopalosiphum padi* attacking on *Brassica* species, tobacco, wheat and wild spinach. Similarly *Asaphes suspensus* was hyperparasitic via *Aphidius colemani* followed by *Aphidius smithi*, *Diaeretiella rapae* and the genus *Binodoxys*. Aphids were *Aphis fabae, Acyrthosiphum pisum, Rhopalosiphum padi, Brevicoryne brassicae* and *Aphis gossypii*. These three species of hyperparasitoids appear to suppress the population of various parasitoids in the March and April months of the year in maximum numbers in various districts of Punjab as a complex. Kamijo and Takada (1973) reported aphid, parasitoid and host plant range for *Asaphes suspensus* and *Pachyneuron aphidis* from Japan as in our studies in Punjab. Talebi et al. (2009) also reported *Syrphophagus aphidivorus* (Mayr), *Pachyneuron aphidis* on medicinal plants in Iran as recorded in Punjab.

In general the species (*Syrphophagus aphidovorus* and *Asaphes suspensus*) were hyperparasitic through *Aphidius colemani* and *Diaeretiella rapae* on *Rhopalosiphum padi* and *Brevicoryne brassicae* on wheat and *brassica* crop. Similarly *Pachyneuron aphidis* was hyperparasitic via *Aphidius colemani* followed by Genus *Binodoxys* and *Diaeretiella rapae*. Aphids those were parasitized; *Aphis fabae, Rhopalosiphum padi, Aphis gos*sypii and *Brevicoryne brassicae*. So these hyperparasitoids on one side regulates the population of aphids by reducing the effectiveness of primary parasitoids and on other side play a positive role in maintaining the balance of trophic association of host plant-aphid-primary parasitoid in wheat and brassica agro-ecosystems in Punjab. These agro-ecosystems of wheat and brassica are interacting with each other on the basis of trophic associations of these two hyperparasitoids (*Syrphophagus aphidovorus* and *Asaphes suspensus*). The knowledge trophic association is one of the main elements when developing integrated management strategies. Our results can become a basis for developing IPM strategies against mainly species of aphids on wheat crop and oilseed crops in Punjab.

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Appraisal of Lead and Cadmium Concentrations in Herbage and Milk of Ewes During Different Lactation Periods: A Case Study in Sargodha, Pakistan

Zafar Iqbal Khan[1*], Kafeel Ahmad[1], Muhammad Khalid Mukhtar[1], Abrar Hussain[4], Ameer Fawad Zahore[2], Anbreen Anjum[3], Muhammad Yousaf[2], Ghulam Hussain[5], Muneeba Shaheen[1] and Bushra Perveen[2]

[1]Department of Biological Sciences, University of Sargodha, Sargodha, Pakistan
[2] Department of Chemistry, Government College University, Faisalabad-38040, Pakistan.
[3] Department of Applied Chemistry, Government College University, Faisalabad
[5]Department of Chemistry, Government Islamia College, Chiniot, Pakistan
[4]Science and Technology Division, University of Education, Township Campus, Lahore, Pakistan

Abstract.- The present investigation was carried out to assess the concentration of cadmium (Cd) and lead (Pb) in 20 herbage and 80 ewe's milk samples collected on different intervals in Sargodha, Pakistan. The higher concentration of Cd in the herbage showed maximum concentration (0.063mg/kg) during September and lowest (0.031mg/kg) during October. The Pb was likewise highest (0.043 mg/kg) during September and lowest (0.028 mg/kg) during November These metal concentrations in the herbage were far lower than the toxic levels already established for livestock. In the milk samples the highest Cd concentration (0.253mg/L) was recorded in October and the lowest (0.193 mg /L) during December. The Pb concentration was highest (0.160 mg /L) during the month of November and the lowest (0.090 mg /L) during December. These results showed that metal concentration were lower than those recommended by the International Standards and Codex.

Keywords: Ewe milk, herbage, cadmium, lead.

* Corresponding author: zikhan11@gmail.com

Increase in industrial and agricultural processes have resulted in increased concentration of metals in the air, water and soil. These metals are taken in by plants and consequently accumulate in their tissues. Animals that graze on such contaminated plants and drink from polluted waters also accumulate such metals in their tissues and milk if lactating (Jelínek *et al*., 1993) and subsequently find their way into the food chain. This ever increasing pollution has given rise to concern on the intake of harmful metals in humans (Lopez *et al*., 2002). Metal levels in uncontaminated milk is generally low, but may accumulate along the food chain at different trophic levels (Gallo *et al*., 1996). The measurement of metal levels is helpful not only in ascertaining risk to livestock but, also to human health and environmental quality as well (Houpert *et al*., 1997). The levels of lead and cadmium in milk from animals grazing in pastures have been reported to exceed the permissible maximum limits (0.05 mg/kg body weight) recommended by Oskarsson *et al*. (1995) and may induce various complications in consumers and effects of that toxicosis are very pronounced in calves and lambs as well as in humans.

In Pakistan, at present, no data exists on heavy metal contamination of milk and dairy products from sheep. The aim of this work was to detect the concentrations of Pb and Cd metals in herbage in pasture and milk samples collected from ewes at a livestock farm at different intervals and to evaluate and anticipate any lethal effects on both public and animals health in this specific region.

Materials and methods

Twenty healthy, 5 years old ewes of Kajli breed, in their third lactation at Khizerabad sheep farm in Sargodha were used in this study for four months in 2010. In summer they were exposed to rotational pasture consisting of grasses and legumes. The ewes received salt lick and fresh water *ad libitum.*

Twenty herbage samples, mostly grasses and legume species in the main pastureland around the farm were obtained concurrently with 100 ml of milk samples for four months (September-December) during the cultivation season. The herbage samples were stored in polythene bags,

Table I.- Concentration of Cd and Pb in the herbage (mg/-kg) and milk (mg/L) samples during lactation period.

Metal	Herbage (mg/kg)				Milk (mg/L)			
	September (n=5)	October (n=5)	November (n=5)	December (n=5)	September (n=20)	October (n=20)	November (n=20)	December (n=20)
Cadmium	0.063±0.002	0.031±0.004	0.032±0.002	0.041±0.002	0.204^{c}±0.016	0.253^{a}±0.014	0.233^{b}±0.020	0.193^{d}±0.012
Lead	0.043^{a}±0.002	0.033^{c}±0.002	0.028^{d}±0.003	0.038^{b}±0.002	0.133^{b}±0.014	0.119^{c}±0.011	0.160^{a}±0.049	0.090^{d}±0.011

$^{a-d}$Means within row with different subscripts differ significantly (P< 0.05) from each other

which the milk samples were collected in small bottles. For lead and cadmium analyses, herbage samples were washed with distilled water, dried on a sheet of paper to eliminate excess moisture, weighed and oven-dried at 60ºC for 72 h. The dried sample were ground in a mortar, and sieved through 2mm mesh.

The pulverized (2.5 g) herbage samples were digested with 25 ml of concentrated HNO_3 for 32-45 min at 200-250^{o}C. After cooling the solution,10 ml of H_2SO_4 was added to the mixture and the digestion was continued till a colorless solution was obtained. Digested samples were filtered with 0.45 µm pore size cellulose nitrate membrane filter paper (Millipore) and the volume was increased to 50 ml with distilled water (Heckman, 1967). The milk samples were digested in a mixture of 1:1(nitric acid: perchloric acids) using a heating block (Swaileh *et al.* 2009). About one ml of digested milk sample was taken in a conical flask and heated on hot plate till a colorless solution was made. The digested samples were transferred into 50 ml flask, made up to the mark with distilled water and stored in 50 ml propylene bottles. Metal concentration in the digest was determined by Atomic Absorption Spectrophotometer using Shedmazu AA6800 apparatus with graphite furnace and background correction. Precision and accuracy of analysis was assured through repeated analysis of samples against National Institute of Standard and Technology, Standard Reference Material (SRM 1570) for both the heavy metals.

The data thus collected were subjected to statistical analysis following the procedure given by Steel and Torrie (1986) and significance between means were worked out at the level of 0.05, 0.01 and 0.001.

Results and discussion

Table I shows concentration of Cd and Pb in the herbage and milk samples during 4 months of lactation period. No significant (P >0.05) difference was observed in herbage Cd level, while Pb showed significant differences with respect to sampling periods (P<0.001). Cd ranged from 0.031 to 0.063 mg/kg and Pb 0.028 to 0.043 mg/kg across all the sampling periods. The highest herbage Cd was found during September and the lowest during October in this investigation, while herbage Pb was higher during the month of September and the lowest during the month of November (Table I).

The concentration of Cd and Pb in herbage reported in our study was ~~is~~ considerably higher than that reported previously by Ahmed *et al*. (2008) and Rodriguez *et al*. (1999) and it has reported that different plant parts contain variable amount of these elements (Mata *et al*., 1995). All forage samples in this study area had low level of Cd and Pb, which is not toxic for animals being reared at that specific farm. Presence of metal levels below the established maximum limits for livestock suggested that there is no toxicological risk for ewes grazing there in the pasture.

Analysis of variance of data for milk Cd and Pb showed significant effect (P<0.001) of sampling periods. The highest milk Cd was observed during October (0.253 mg/L) and the lowest (0.193 mg/L) during December, while milk Pb was the highest (0.160 mg/L) during November and the lowest (0.090 mg/L) during December (Table I).

A similar result of Cd concentration in milk above the permissible limit has also been reported by Ank *et al*. (1990). Kirova (1993) confirmed that ewe milk contain 1.5 times additional Cd compared to cow milk. Ewes extract a great deal of Pb through milk relative to cows (Mehennaoui *et al*., 1999).

Most other elements though toxic at high concentrations are actually required nutrient at lower levels. During second lactation period, the Cd and Pb levels were considerably ($P<0.01$). The relationships between metals in herbage and milk when worked out,a weak and negative relationships between Cd and Pb in herbage and milk with values of coefficients (-0.229) for Cd in herbage and milk (-0.260) was found during this investigation. To conclude, our results indicate that sheep milk and herbage for ewes and other animals from the specific livestock farm of Pakistan investigated in this study are safe for consumers.

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Distribution and Roosting Habitats of Some Microchiropteran Bats in Rawalpindi District, Pakistan

Muhammad Sajid Nadeem[1*], Sara Zafar[1], Amjad Rashid Kayani[1], Muhammad Mushtaq[1], Mirza Azhar Beg[1], and Muhammad Farooq Nasir[2]

[1]Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Murree Road, Rawalpindi

[2] Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University, Murree Road, Rawalpindi

Abstract.- This study was aimed at knowing the microchiropteran fauna of urban, suburban and rural human settlements of Rawalpindi district. A total of 50 specimens of bats belonging to five species were collected with hand nets; *Scotophilus heathii* (52%), *Pipistrellus pipistrellus* (30%), *Pipistrellus javanicus* (10%), *Pipistrellus tenuis* (6%) and *Rhinolophus lepidus* (2%). The majority of the bat specimens were captured from urban localities (58%), followed by sub-urban areas (28%) and rural areas (14%); the capture success being 0.935, 0.667 and 0.368 per hour, respectively. These specimens were taken from recesses present in bridge structures (10%), crevices in buildings (70%), tree cavities (18%) and fissures in rocks (2%).

Key words: Human habitation, microchiroptera, microhabitat, *Pipistrellus*, *Scotophilus*, roosts.

Bats are the second largest order of mammals worldwide and constitute a quarter of known mammal species of Pakistan (Roberts, 1997). They may be found in a variety of habitats, including forests, urban, sub-urban and agricultural areas. Anthropogenic disturbances reduce bat populations by changing or destroying roosting and foraging habitats (Racey and Entwistle, 2003; Maron and Fitzsimons, 2007).

Bats of subtropical and temperate zones spend greater part of the day in roost during the

* Corresponding author: sajidnm@uaar.edu.pk

summer months. Roosts are one of the most essential features of a bat's environment. They offer protection against both harsh weather and from predators and thus they have a major impact on their survival and fitness (Vaughan, 1987; Vonhof and Barclay, 1996). The particular type of roost site preferred by different bat species may be determined by the morphology of the bat, the temperature or humidity within the roost, and the proximity of the roost to appropriate foraging and drinking sites (Entwistle *et al*., 1997).

Relatively little attention has been given to the urban ecology of bats in spite of Taylor's (1926) observation that some species are apparently flourishing in disturbed areas. Although previous studies have indicated that urbanization had a negative effect on the activity and abundance of bats (Gehrt and Chelsvig, 2003), in recent times built structures, especially dwellings, have been widely used by bats; some species like *Pipistrellus pipistrellus* roost almost exclusively in these sites (Thompson, 1992).

This study presents information on bats body size, roost sites and distribution of five species of microbats among three types of human habitations found in Rawalpindi district.

Study area

The samples were collected from Rawalpindi city (33.59° N and 73.04° E), its suburbs and some widely scattered villages. Some of these villages were served by metalled roads and some by dirt roads. The climate of Rawalpindi district is subtropical type in its southern part and warm temperate type in its northern part. Scattered through the region are patches of mostly rain fed agricultural areas surrounded by scrub vegetation and remnants of tropical thorn forest characterized by *Acacia modesta* and *Olea cuspidata*. North to the Rawalpindi district lies Margalla hills, the foothills of the Himalayas. Rawalpindi district is the north eastern district of the Pothwar plateau which is characterized by flat areas frequently dotted with eroded sedimentary hills, gullies, ravines and nullahs (Anonymous, 2004).

Materials and methods

From March to November 2010 some urban, suburban and rural areas of Rawalpindi district were sampled for bats. Hand nets and gloves were used for capturing and handling the bats. The date, time and sampling site of each specimen were recorded along with the hours spent on active search for the bats in urban, suburban and rural habitations; the search periods being 31, 21 and 19 hours long, respectively. Body length and weight of the specimens were also recorded. All potential roosting sites namely, tree hollows and cavities, under roof recesses of houses, fissures, cracks and crevices in built structures including bridges were checked for the presence of bats. The four types of roosts used by the bats were defined as follows: Long recess= Long narrow hollow spaces left between the concrete pillars supporting the overhead bridges for the passage of vehicles. Crevices= Slits between walls and girder supporting the ceilings of verandas and rooms of houses and other buildings. Tree cavities= Deep cavities or long hollows in the trunks or limbs of trees. Fissures= Long deep cracks in rocks.

Results

Five species of microbats were recorded from three types of human habitations (*viz.*, urban, suburban and rural) in Rawalpindi district. A total of 50 individuals of microbats were captured; 26 of these bats belonged to *Scotophilus heathii*, 15 to *Pipistrellus pipistrellus*, five to *Pipistrellus javanicus*, three to *Pipistrellus tenuis* and just one specimen to *Rhinolophus lepidus*. With the exception of *S. heathii*, all these bats were relatively of small size. The average head and body length of *S. heathii*, was 7.6 cm and average body weight was 33.9 g, while these measurements for the other species were in the range of 3.3- 4.0 cm and 2.4-3.4 g, respectively.

Capture success

Scotophilus heathii were caught most frequently than all other species with a capture success of 0.366 h^{-1} of the active search. *P. pipistrellus* had a capture success of 0.211 h^{-1}, while the other three species namely, *P. javanicus*, *P. tenuis* and *R. lepidus* had much lower capture success *viz.* 0.072, 0.042 and 0.014, respectively. The combined capture success for all the five

species was 0.704 h^{-1} (Table I). In the urban sub-habitat, capture success was 0.935 h^{-1} as compared to 0.667h^{-1} and 0.368 h^{-1} of the sub-urban and rural sub-habitats (Table I). Thus the urban area was relatively more densely populated while the rural sub-habitat was thinly populated with the bats. The capture success for the three sub-habitats averaged to 0.704h^{-1}.

Utilization of roost types

All the fifty specimens of bats were captured while they were resting in their diurnal roosts. The majority of the specimens (70%) were taken from crevices found in human dwellings and other buildings from all the five species of bats. As such, the sample of bats obtained from the crevices evidenced greatest diversity both in terms of species and abundance. The only specimen of *R. lepidus* was also taken from a crevice. Tree cavities were apparently the next best roost type (18%, Table II). From the recesses found in the bridges, four species were recorded, while from rock fissures only one specimen of *S. heathii* could be recorded. More than 50% of the specimens of bats caught from the various types of roosts belonged to *S. heathii*, while 30% belonged to *P. pipistrellus*, 10% to *P. javanicus*, 6% to *P. tenuis* and just 2% to *R. lepidus* (Table II).

Discussion

The three essentials for the life of the microchiropteran bats are suitable roosts for protection from harsh weather and from predators, suitable foraging grounds, and water (Vaughan, 1987; Russ and Montgomery, 2002). The human influences the lives and habits of these volant mammals mainly through his activities related to the development of built areas and expansion of agriculture. These activities generally result in reduction of bat populations by destroying or degrading their foraging and roosting habitats (Racey and Entwistle, 2003; Maron and Fitzsimons, 2007).

In Pakistan, the country side is being lost fast to built areas and sprawls that are influencing the life and habits of the bats. But, many species of bats have managed to live in or near human dwellings. These anthrophillic species have adopted to take up abode in anthropogenic shelters (Prater, 1971). These shelters have facilitated the establishment and spread of the bats (Payne and Francis, 1998).

Bats communities in urban areas are often dominated by a few common species that have adapted well to urban environments (Ulrey *et al*., 2005). In our study area, *S. heathii*, *P. pipistrellus* and *P. javanicus* were common in urban localities, while *P. tenius* was relatively less in the urban as compared to suburban environment of Rawalpindi; while *R. lepidus* was recorded just once from the rural habitat roosting in a wall crack. *S. heathii* seemed to have relatively successful in exploiting the resources and roosting niches as it inhabited all the three sub-habitats of our study area and used all the four roost types, though not with equal frequency. According to our data this bat has numerical superiority over other species in all the three anthropogenic sub-habitats. It is known to use a variety of habitat types both in urban and non-urban areas (Bates *et. al*. 2008). It can also roost among crowns of palms, in hollows of trees and among leaves of banana (Bates and Harrison 1997). Its wide adaptability is thought to have enhanced its distribution (Payne and Francis, 1998).

P. pipistrellus was present in all the three sub-habitats types of our study area and was recorded from all the four roost types except for the cracks in rocks. It seemed to be less partial to urban sub-habitat than *S. heathii*. Lawlor (1979) reported that this bat was very common in both towns and cities and that it could also be found in parks and forests. During the summer months, it could be found in cracks on the outside of buildings, behind shutters, and in unfinished houses (Schober and Grimmberger, 1997). It had been discovered in winter roosting in churches, limestone mines, in crevices and cracks in walls, cliffs, cellars and caves and possibly holes in trees might also be used (Vaughn *et al*., 2000; Hutterer *et al*., 2005).

In our sample, *P. tenuis* was represented by only three specimens from crevices and a tree hole. But it has been reported by Roberts (1997) abundant in older towns of Punjab, where it roosts in cracks between bricks, spaces behind the pipes, under roof tiles, and narrow tree holes. Heaney *et al*. (1998) considered it to be a largely forest species. *P. tenuis* is adapted to highly disturbed habitats, gardens, and

Table I.- Abundance/Capture success h^{-1} of the microchiropteran bats in three sub-habitats in Rawalpindi district.

Sub-habitat	No. searched hours	Capture success h^{-1} (No. of bats)					
		Scotophilus heathii	*Pipistrellus pipistrellus*	*Pipistrellus javanicus*	*Pipistrellus tenuis*	*Rhinolophus lepidus*	Total
Urban	31	.548(17)	.258(8)	.096(3)	.032(1)	-	.935(29)
Sub-urban	21	.286(6)	.190(4)	.095(2)	.095(2)	-	.667(14)
Rural dwelling	19	.158(3)	.158(3)	-	-	.053(1)	.368(7)
Combined	71	.366(26)	.211(15)	.071(5)	.042(3)	.014(1)	.704(50)

Table II.- The types of diurnal roosts used by different species of microbats in Rawalpindi District.

Roost type	No. of bat specimens					
	Scotophilus heathii	*Pipistrellus pipistrellus*	*Pipistrellus javanicus*	*Pipistrellus tenuis*	*Rhinolophus lepidus*	Total (%)
Long recesses in bridge structures	01	04	-	-	-	05 (10)
Crevices in building	20	07	05	02	01	35 (70)
Tree cavities	04	04	-	01	-	09 (18)
Fissures in rock	01	-	-	-	-	01 (2)
Total specimens	26	15	05	03	01	50 (100)

mangrove forests (Bates *et al*., 2005). In South Asia it is common in woodlands and urban and rural landscapes both in arid and humid areas.

At least four of the five species of bats of our study area seem to have adapted to live as commensal of man. They utilize the under roof niches, wall cracks and crevices in human dwellings for diurnal roosting. Outside the human dwellings, they roost in tree hollows and cavities. Naturally, all these roosting niches are available in human settlements, built structures and in nearby hollow trees, where some sources of water are also present making the human habitations of Rawalpindi suitable for the bats roosting and foraging.

In this study, a single specimen of *R. lepidus* was recorded from the rural sub-habitat only. This could be attributed to its preference for the undisturbed areas (Struebig *et al*., 2011). This might be limiting its distribution in anthropogenic habitats. However, this highlights the need to investigate the occurrence and distribution of microchiropteran fauna associated with human habitations more intensively.

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Targeting of GH Gene at the Proximal End for Identification of Markers for Breast Cancer Among Pakistani Women

Adnan Farooq and Amtul Jamil Sami*
Institute of Biochemistry and Biotechnology, University of the Punjab Lahore 54590 Pakistan

Abstract. - Chromosome 17 is one of the 23 pairs of chromosomes in humans. Spans more than 81 million base pairs) and represents between 2.5 and 3 % of the total DNA in cells. Chromosome 17 likely contains between 1,200 and 1,500 genes. It also contains GH1 gene which was targeted for mutations related to breast cancer at the promoter region in the proximal end of the gene. DNA was isolated from blood of normal female subjects and proximal end of GH1 gene was amplified by PCR, using a set of primers. The amplified DNA was sequenced and sequences of the amplified products showed variations in the 5' flanking region of GH-N (accession no. M13438.1). In the promoter region two changes were noticed one was change of A to G at position -6 and the second was change of A to T at position -1. One variation in signal peptide where G replaces A was also found in all four samples but this is a silent mutation as it has no effect on amino acid sequence. It was noticed that there were 6 mutations found in local population related to increase or decrease risk of breast cancer. This is the first study aimed at the identification of genetic markers for link to breast carcinoma risk in Pakistani woman.

Key words: GH1 gene, breast cancer marker, chromosome 17, Promoter region.

Corresponding author: amtuljamilsami@yahoo.com

GH gene is located on chromosome 17 which has been studied for molecular genetic markers and their impact in breast cancer, as reviewed by Zhang and Yu (2011). Gene marker identified in the link included epidermal growth factor, gene for DNA topoisomerase, RDMI (involved in DNA double strand break and repair and recombination), P53 (respond to diverse cellular stress), BRCA 1 (role in maintaining genome stability), HIC-I (Hyper methylation in cancer I), the TAU gene for microtubule associated protein (function to keep the cell in shape). Among the others GH1 gene also had been associated with breast cancer (Wagner *et al.* 2005; Canzian, 2005; Le Marchand, 2002; Ren, 2004). Horan *et al.* (2003) studied 16 SNPs in the human growth hormone proximal promoter region. Estaban *et al.* (2007) establish a map of 25 SNPs as present in over 1% of individuals, whereas 29 other sequence changes (single or multiple nucleotides) are present in less than 1% of subjects. Giordano (2006) reported eight SNPs in the promoter and 5′UTR regions, Wagner *et al.* (2005) identified 16 SNPs in the promoter to intron 1. Polymorphism and mutations are reported to be related to breast cancer. There are at least 6 mutations in the proximal end of GH1 gene which are identified in relation to breast cancer (Wagner *et al.* 2005), Here we report a study to identify the mutations on the proximal end of GH gene related to breast carcinoma in Pakistan. Chromosome 17 has been identified for the location of a number of genetic markers related to cancer as reviewed by (Zhang

and Yu, 2011). There are reports on the role of mutations in GH gene that are related to breast carcinoma (Wagner *et al.* 2005; Canzian, 2005; Le Marchand, 2002; Ren, 2004). Furthermore, Le Marchand in 2002 reported a positive association between the P-1 polymorphism and colorectal neoplasia. Estaban *et al.* (2007) investigated the relation of this polymorphism, along with four common SNPs in the proximal promoter region of the *GH1* gene, to breast carcinoma risk. The promoter and the proximal region are highly polymorphic, with 9 single-nucleotide polymorphisms (SNPs) reported within a 310–base pair (bp) stretch (Chen *et al.*, 1989). Studies on the growth hormone gene with reference to cancer could be helpful in curing cancer at its early stages. Horan *et al.* (2003) located 36 haplotypes in control subjects of the British population, which would result from the combination of 15 of the previously reported SNPs. Cherbonnier *et al.* (2002) reported Human growth hormone gene transfer into tumor cells may improve cancer chemotherapy. As breast cancer is one of the major killer diseases in Pakistan due to its diagnosis at later stages in Pakistan a study at the genetic level of the proximal end of growth hormone gene to locate the mutations related to breast cancer, could be helpful in the early diagnosis and cure of disease that could save a number of lives. In the region there is no such study has been launched, so far. The presented work reports on a preliminary study on the promoter region of GH gene to identify the already reported markers for breast cancer in Pakistani woman.

Materials and methods

Blood samples (1 ml) were obtained from healthy female subjects and DNA was isolated using FavorPrep™ Blood Genomic DNA Extraction Kit (Favorgen biotech crop.), according to the manufacturer's instructions. To check the purity of genomic DNA, it was run on 1% agarose electrophoresis gel. For PCR amplification after two primers were used to align at positions -173 to -154 for forward primers (5'-AGCACAAGCCCGTCAGTGGC -3') and +40 to +60 for the reverse (5'-GGACGCTGCCTCTCCCCTCA -3') sequence of growth hormone 1 gene. Primers were checked for specificity with NCBI primer blast tool and found specific for the selected DNA sequence (Fig. 1). The region between the two primers about 240 bases was amplified as follows, using a Promega™ master mix. 25µl of the 2x master mix was added in a PCR tube, 10µl of DNA and then 4µl of each forward and reverse primer and finally 7µl of distilled water was added. Then the conditions were set on the

Fig. 1. Graphical view of primer blast results from NCBI Primer blast tool. Primer positions and length of product is shown in blue line. (www.ncbi.nil.nih.gov/tools/primer-blast).

thermocycler as denaturation at 94°C for 30sec, annealing at 58°C for 30sec, extension at 72°C for 30 sec and then final extension at 72°C for 5 minutes. The number of cycles was set 30. After PCR, the products were run on 1.5% agarose gel to visualize the amplified DNA. Sequence of the amplified DNA was determined and studied for mutations linked to breast cancer.

Results and discussion

DNA isolated from blood of a normal female subject was amplified for proximal end of the GH gene region between the -173 to +67 and PCR products of 240 base pairs were obtained, as shown in Figure lanes 2-5. Results are summarized in Table I. The amplified DNA was sequenced and a number of variations were recorded (Figs. 3-4). In the promoter region two changes were noticed one was change of A to G in position -6 and the second was change of A to T at position -1 (Fig. 3). Earlier

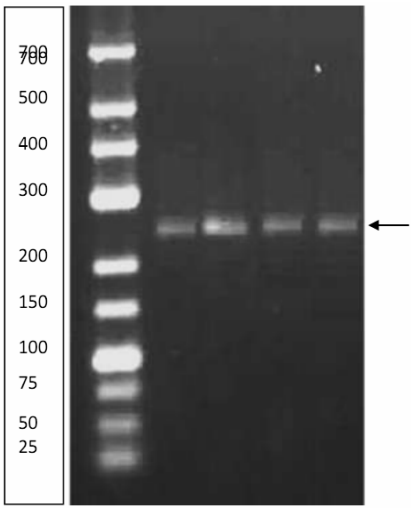

Fig. 2. Lane 1 showing Marker; Lanes 2-5 showing PCR product of 240 bp with human DNA, using a set of primers binding at the proximal end of GH1 gene for the amplification of DNA.

Table I.- Mutations in nucleotide sequences of GHEXSEQ1 to 4 (from total 12 samples), as compared to human GH-N (accession no. M13438.1). Mutations related to increasing or decrease risk for breast cancer were mentioned with reference to Wagner *et al.* (2005).

Mutation	Position	Region	Probability	References	Risk
A→G	+69	Signal peptide	33%	Adkins, 2005; Esteban *et al*., 2007	Decrease
T→G	+59	5' UTR	33%	Wagner *et al.*, 2005; Giordano, 2006, Horan *et al*., 2003; Adkins, 2005; Esteban *et al*., 2007	Decrease
C→T	+58	5' UTR	33%	---------	
A→T	-1	Promoter	25%	Wagner *et al*., 2005; Giordano, 2006; Horan *et al*.,	Increase
A→C			8.0%	2003; Adkins, 2005; Esteban *et al.*, 2007	
A→G	-6	Promoter	8.0 %	Wagner *et al*., 2005; Giordano, 2006; Horan *et al*., 2003; Adkins, 2005; Esteban *et al.*, 2007	Increase
G→A	-48	Promoter (VDRE)	33%	Esteban *et al*,.2007	
T→G	-50	Promoter (VDRE)	33%	---------	
A→G	-54	Promoter (VDRE)	16%	---------	
C→G	-56	Promoter (VDRE)	8.0%	---------	
T→G	-57	Promoter (VDRE)	33%	Wagner *et al*., 2005; Giordano, 2006; Horan *et al*., 2003; Adkins, 2005; Esteban *et al.*, 2007	Decrease
T→G	-62	Promoter	33%		
C→A	-72	Promoter	8.0%		
G→C	-75	Promoter (Pit-1)	25%	Wagner *et al*., 2005; Giordano, 2006; Horan *et al*., 2003; Adkins, 2005; Esteban *et al.*, 2007	Decreased
DEL A	-76	Promoter (Pit-1)	33%	---------	
T→A	-77	Promoter (Pit-1)	8.0%	---------	
T→A	-83	Promoter (Pit-1)	16%	---------	
C→A	-85	Promoter (Pit-1)	8.0%	---------	
T→G	-87	Promoter (Pit-1)	33%	---------	
G→C	-93	Promoter (Pit-1)	16%	---------	
T→G	-95	Promoter (Pit-1)	33%	---------	
T→G	-99	Promoter	8.0%	---------	
C→G	-101	Promoter	8.0%	---------	
C→A	-107	Promoter	8.0%	---------	
A→C	-108	Promoter	25%	---------	

Fig. 3. Alignment of 5' promoter region of our four nucleotide sequences (GHEXSEQ1 to 4) of hGH with human GH-N Accession no. (M13438.1). Variations in sequences were highlighted in turquoise color, start codon (ATG) and part of signal peptide was highlighted in gray color, TATA Box was highlighted in yellow color, the consensus binding site for Pit-1 (proximal) and VDRE were shown in boxes. SNPs also reported by other workers are marked with arrows and labeled.

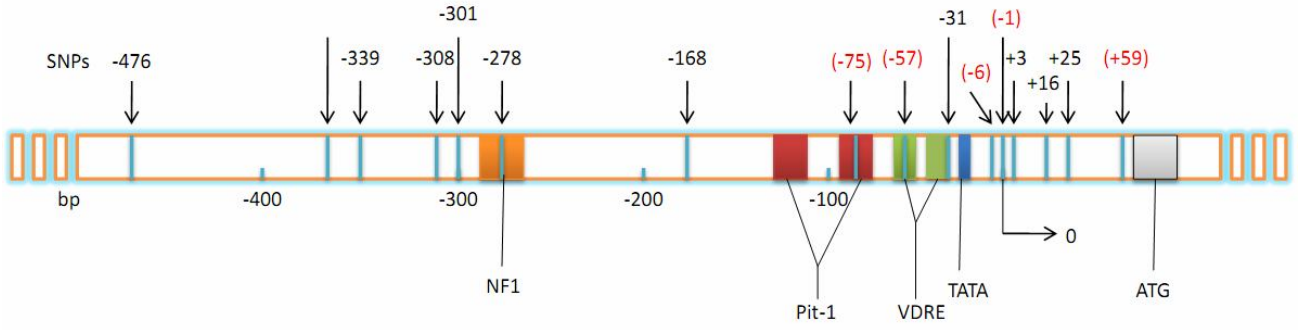

Fig. 4. Positions of 16 SNPs were shown (-476 to +59) in the GH1 promoter region relative to transcriptional start site. SNPs found in GHEXSEQ1-4 are shown in red color in brackets. Gray box represents exon 1,colored boxes shows the positions of binding sites for transcription factors, nuclear factor 1 (NF)1 in orange color, Pit1 in brown color , vitamin D receptor (VDRE) in green color and TATA box in blue color. Translational initiation codon ATG is also shown (Numbering based on *Horan et al*., 2003).

both the changes had been reported by Wagner *et al.* (2005) and are related to an increase the risk of breast cancer (Table I). The proximal end of GH gene had been prone to mutations not only in human (Wagner *et al.* 2005; Canzian, 2005; Le Marchand, 2002; Ren, 2004; Esteban *et al.*, 2007; Giordano, 2006; Horan *et al.* 2003; Adkins, 2005) but also in other mammals (Ferraz *et al.*, 2006; Sami *et al.*, 2011), possible due to the location of a number of important sites for expression and binding. Estaban *et al.* (2007) established a map of 25 SNPs as present in over 1% of individuals, whereas 29 other sequence changes (single or multiple nucleotides) are present in less than 1% of subjects. Giordano (2006) reported eight SNPs in the promoter and 5′UTR regions, Wagner *et al.* (2005) identified 16 SNPs in the promoter to intron 1, while Horan *et al.* (2003) located 36 haplotypes in control subjects of the British population, which would result from the combination of 15 of the previously reported SNPs. There's no such study for Pakistani population. This is the first study on the identification of a genetic marker for breast cancer in local population in Pakistan. Variation in this gene region has been reported previously for a number of populations, and associated with various diseases, including breast cancer, has been suggested. An equivalent study in Pakistan is potentially of interest.

The work presented here identified genetic markers related to breast cancer in local population, there could be a survey for the identifications of markers at the genetic level in the local population. The presented work provides a foundation for such study in Pakistan.

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Patterns of Insecticides used on Cotton Before Introduction of Genetically Modified Cotton in Southern Punjab, Pakistan

Muhammad Razaq[1], Anjum Suhail[2], Muhammad Aslam[3], Muhammad Jalal Arif[2], Mushtaq A. Saleem[4] and Hammad Ahmad Khan[5]

[1]Department of Entomology, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan
[2]Department of Agri. Entomology, University of Agriculture, Faisalabad, Pakistan
[3]COMSATS Institute of Information Technology, Mailsi Road, off Multan Road, Vehari, Pakistan
[4]Institute of Molecular Biology and Biotechnology, The University of Lahore, 1-Km Defense Road (off Raiwind Road) Lahore, Pakistan
[5]Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan

Abstract.- A complex of sucking and bollworm pests attack cotton crop in Pakistan. Cotton jassid, *Amrasca devastans* (Dist.), cotton whitefly, *Bemisia tabaci* (Genn.), and thrips, *Thrips tabaci* (Lind.) are regular sucking insect pests, whereas spotted bollworms, *Earias spp.*, Pink bollworm, *Pectinophora gossypiella* (Saund.) and American bollworm, *Helicoverpa armigera* (Hub.) are regular bollworm insect pests. Insecticides were the only option to manage these insect pests before introduction of *Cry* I Ac toxic gene harboring cotton. After the introduction of *Bt* cotton the insecticides are mainly applied for sucking insect pests. We report patterns of insecticides used before introduction of *Bt* cotton from 2001 to 2003 cropping years of Multan, Dera Ghazi Khan and Bahawalpur districts of Punjab. Average numbers of sprays were 4.64, 6.65 and 8.90 per unit area during crop years 2001, 2002 and 2003, respectively. Insect pests received diversity of insecticides. New insecticides were applied in the highest proportion and reached 56% during 2003. Imidacloprid, acetamiprid and diafenthiuron were the dominant insecticides to manage sucking insect pests and ranged 26.07 to 33.44% among all the insecticides used during three years.

Key words: Cotton, sucking insect pests, *Bt* cotton

* Corresponding author: mrazaq_2000@yahoo.com

Cotton has been known worldwide having notorious insect pests and pest management problems. About 1326 species of insects and mites attack cotton crop around the world, of which 145 species are in Pakistan (Huque, 1994). Cotton jassid, *Amrasca devastans* (Dist.), cotton whitefly, *Bemisia tabaci* (Genn.), and thrips, *Thrips tabaci* (Lind.) are regular sucking insect pests, whereas spotted bollworm, *Earias* spp., pink bollworm, *Pectinophora gossypiella* (Saund.) and American bollworm, *Helicoverpa armigera* (Hub.) are regular bollworm insect pests.

Cotton accounted for 22.5% of all crop insecticide sales worldwide in 1994, which has entailed development of resistance in the insect pests (Castle *et al.*, 1999). *H. armigera* was first time reported resistant to endosulfan, chlorpyrifos, thiodicarb and cypermethrin during 1991 to 1993 in Pakistan (Ahmad *et al.*, 1995, 1998). Changes in susceptibility were also reported for fipronil, chlorfenapyr, indoxacarb, spinosad, abamectin and emamectin benzoate. *A. devastans*, *Aphis gossypii* and *B. tabaci* have also been reported to develop resistance against pyrethroids, *B. tabaci* against methamedophos (Razaq, 2006), *Spodoptera litura* against conventional and new insecticides (Ahmad *et al.*, 2008; Saleem *et al.*, 2008; Shad *et al.*, 2010).

The development of resistance and selection for different resistance mechanisms depend upon the selection pressure exerted by application of insecticides. Resistance can be lowered by reducing the selection pressure by judicious application of insecticides. Resistance mechanisms conferring high resistance factors like nerve insensitivity can also be reverted to easily manageable mechanisms like metabolic resistance. For this purpose the evaluation of pattern of use of insecticides becomes necessary. The most effective strategy of managing resistance to insecticides remains strict control of their use (McCaffery, 1998; Forrester *et al*., 1993).

Genetically modified cotton has been introduced since the last several years and is wide spread in Punjab and Sindh provinces of the

Pakistan. The population of lepidopterous pests like bollworms has reduced, ultimately leading to reduced applications of insecticides (Ali *et al*., 2010). Farmers have observed a high incidence of armyworm (*S. litura* and *S. exigua*) and sucking pests in *Bt* cotton (Arshad *et al*., 2009). Herein we report pattern of use of insecticides before introduction of *Bt* cotton and discuss risks involved for development of insecticide resistance in sucking insect pests. No coordinated study has ever been conducted on this aspect of cotton in Punjab, so far.

Materials and methods

Farmers from the intensive cotton growing areas *viz.*, Multan, Dera Ghazi Khan and Bahawalpur were interviewed for the pattern of insecticides used from 2001-2003. Extension workers were first approached to contact farmers of the area. Farmers having nearly same farm area were included in the survey to avoid biased figures towards small holdings. Each farmer was asked to document total area of cotton sown and insecticides sprayed from beginning to the end of crop season. Data for generic insecticides and branded insecticides was pooled. Total numbers of farmers interviewed were 250, 240 and 250, in years 2001, 2002 and 2003, respectively. There were minor differences only in insecticide mixtures, so data on insecticide use from three districts was also pooled. To determine average number of insecticides applied, total numbers of insecticides applied by all farmers were divided by total numbers of farmers interviewed.

Results and discussion

Period of time for application of insecticides

Non *Bt* cotton is a summer crop and is planted in May/June in the surveyed areas of Punjab. The incidence of the insect pests followed the same pattern. *A. devastans* and *T. tabaci* received the first application of insecticides. Farmers started application of insecticides in the first or second week of July for *A. devatans*, *T. tabaci* and for *B. tabaci* in the mid of August. Sprays for bollworms were also started in the mid of August during surveyed years. In the month of September insecticide applications continued for *B. tabaci* and bollworms. There were negligible applications of insecticides after mid October. Except for spotted bollworm, *Earias* spp. (as it also attacks terminal shoots of cotton plants) all cotton bollworms appeared at flowering stage.

Total insecticides used

Average numbers of sprays were 4.64, 6.65 and 8.90 per unit area during crop years 2001, 2002 and 2003, respectively. During crop year 2001 and 2003, 12.58% and 12.85% mixtures were applied as compared to 20.05% during 2002 out of total insecticides. The mixtures of pyrethroids and organophosphates (OPs) were dominant. During 2001 and 2003 mixture of new chemical insecticides were in high proportion that is 11.24% out of 12.85%. Mixtures of new chemical insecticides occupied highest proportion (12.23% out of 20.05%) during 2002.

Chemical insecticides used

During cotton season 2001, new insecticides accounted for 31.38% followed by OPs 28.8% and pyrethroids 14.99%. Acetamiprid use was maximum (15.95%) followed by imidacloprid (13.1%) and diafenthiuron (2.33%) during 2001 (Table I). Including mixtures of insecticides, use of imidacloprid was the maximum (18.27%) followed by cypermethrin (16.98%) (Table I).

The use of pyrethorid insecticides was maximum (27.89 %) which was almost equal to new insecticides (27.32%) followed by OPs (22.49%) during 2002. Immidacloprid use was maximum (15.04%) followed by diafenthiuron (6.27%) and acetamiprid (4.76%). Among the pyrethroids use of cypermethrin was maximum in alone (18.30%) as well as in mixtures (11.37%) followed by bifenthrin (8.4%) and deltamethrin (1.19%) during the year 2002. While considering over all use of insecticides based upon chemical groups, use of cypermethrin (pyrethroids) was maximum (29.67%) followed by imidacloprid (new insecticides) (27.26%) and chlorpyrifos (15.60%) (Table I).

The use of new chemical insecticides was about two folds during crop year 2003. The use of Acetamiprid (11.24%) and diafenthiuron (11.1%), which are recommended to control sucking insect pests especially whiteflies was equal. These insecticides effectively control whiteflies as the pest

Table I.- Percent use of insecticides and their mixtures during 2001-03 by the farmers in cotton growing areas of Punjab, Pakistan (data pooled for generic and branded insecticides and also for Multan, Bahawalpur and Dera Ghazi Khan Districts).

Group	Insecticides	Years		
		2001	2002	2003
Organo chlorines	Endosulfan	11.64	0.00	0.00
Organophosphate	Quinalphos	0.60	0.00	0.09
	Chlorpyrifos	5.60	9.46	12.67
	Profenofos	13.80	8.27	2.56
	Methamedophos	1.9	0.00	0.00
	Triazophos	6.9	4.76	0.45
Carbamates	Furathiocarb	0.43	0.44	0.00
	Methomyl	0.00	0.31	0.18
Pyrethroids	Cypermethrin	5.17	18.30	5.26
	Beta cyfluthrin	0.00	0.00	2.47
	Cyhalothrin	0.00	0.00	1.08
	Deltamethrin	1.72	1.19	3.69
	Fenvalrate	0.34	0.00	0.00
	Bifenthrin	7.76	8.40	0.00
New Chemicals	Abamectin	0.00	0.00	5.71
	Emamectin	0.00	0.00	1.21
	Acetamiprid	15.95	4.76	11.10
	Imidacloprid	13.1	15.04	11.24
	Diafenthiuron	2.33	6.27	11.10
	Spinosad	0.00	0.00	6.52
	Indoxacarb	0.00	1.25	9.12
	Buprofezin	0.17	0.00	0.00
IGRs	Decorafluron	0.00	1.50	2.70
Pyrethroids + New Chemistry	Cyper + Imida	5.17	4.95	0.00
	Delta + Imida	0.00	7.02	0.00
	Bifen + imida	0.00	0.25	0.00
OP+NC	Chlor + Aceta	0.00	1.00	0.90
P+OP	Cyper + Chlor	0.43	6.14	0.22
	Cyper + Profeno	2.33	0.00	0.00
	Cyper + Aceta	0.00	0.19	0.00
	Cyper + Metha	3.88	0.19	0.00
	Cyhalo + profe	0.00	0.31	0.27
	Bifen + Metha	0.17	0.00	0.00
	Delta + Metha	0.60	0.00	0.00
N+N	Spino + Imida	0.00	0.00	11.24
P+P	Fenpro + Cyper	0.00	0.00	0.22

developed resistance to most of conventional insecticides. (Razaq, 2006). Use of carbamate and IGRs insecticides on cotton during 3 years of study was quite low compared to other insecticides. Imidacloprid use was maximum (11.24% alone and 11.24% in mixtures) like previous years during 2003 (Table I).

From the results it is clear that insect pests of cotton received complex of insecticides with respect to their chemical nature. Applications of new chemical insecticides increased followed by pyrethroids and OPs. Use of imidacloprid followed by acetamiprid and diafenthiuron (alone and in mixtures) was dominant during all the three years of study before introduction of *Bt* cotton. All the insect pests are exposed to diversity of insecticides. Ultimately insect pests will be selected by diverse mechanisms. C*horistoneura rosaceana* has been found cross-resistant to spinosad and conventional insecticides before introduction of this insecticide in USA (Ahmad *et al.*, 2002).

Australian IRM for pyrethroids and endosulfan strategy proved effective for twelve years by strict insecticide use based upon the rotational strategy *i.e.* exposing one generation of the pest with single mode of action of insecticides. Control failures were observed in *H. armigera* due to pyrethroids in late 1990s (McCaffery, 1998). But strategy extended the useful life for about 12 years.

More than 50% of the global cotton area is now under genetically modified cotton. In India, area under *Bt* cotton has increased to 8.4 million hectares in 2009 exceeding that of China's 3.4 million hectares (Ali *et al.*, 2010). Survey regarding *Bt* cotton in the Punjab proved that farmers were aware of the major insect pests of cotton and reported a low incidence of cotton bollworms and a high incidence of armyworm (*S. litura and S. exigua*) and sucking pests in *Bt* cotton (Arshad *et al.*, 2009). However, in India two years of research proved that densities of sucking insects, of the foliage feeder *Myllocerus undecimpustulatus* were similar on *Bt* and conventional cultivars (Mann *et al.*, 2010).

One of the major concerns for genetically modified cotton is the sucking insect pest. This cotton is planted very earlier *i.e.*, February/March than conventional cotton, thus providing more time

and ultimately continuous food to sucking insect pests. Cotton which was normally available to insects in the mid of July is now available in March.

Caution is needed in application of insecticides for managing sucking insect pests to avoid development of resistance. Moreover, continuous monitoring of insecticide resistance and insecticides use patterns is needed. In the recent laboratory study after selection of *B. tabaci* for eight generations with acetamiprid, resistance to acetamiprid increased to 118-fold compared with susceptible population. Selection also increased resistance to imidacloprid, thiamethoxam, thiacloprid, nitenpyram, endosulfan and bifenthrin (Basit *et al*., 2011). Attempt therefore, should be made not to select sucking insect pests repeatedly with new chemical insecticides to avoid development of resistance.

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