FEEDING PATTERN AND SPOROZOITE INFECTION IN ANOPHELINES AND BIOLOGICAL CONTROL OF MOSQUITOES

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ABBREVIATIONS

An.	Anopheles
BHC	Benzene hexachloride.
BTI	*Bacillus thuringiensis israelensis*
BS	*Bacillus sphaericus*
cc.	Cubic centimeter
Cont.	Control
CSP	Circumsporozoite protein
Cx.	Culex
DDT	Dichlorodiphenyltrichloroethane
dl.	Deciliter
fl.	Femtoliter
gm.	Gram
Hb.	Haemoglobin
Hrs.	Hours
MCH	Mean corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
mg.	Milligram
ml.	Milliliter
nm.	Nanometer
NP-40	Non-Idet P-40
P.	Plasmodium
PBS	Phosphate Buffer Saline
PCV	Packed Cell Volume
Pf capture	*Plasmodium falciparum* antibodies for coating the plate wells.

pH.	Hydrogen ion concentration
pg.	Picogram
Pos.	Positive
PR	Parasite rate
Pv capture	*Plasmodium vivax* antibodies for coating plate wells.
pvc plate	Polyvinylchloride plate
RI,RII,RIII	Replications 1,2,3.
Sq. m.	Square meter.
T20	Tween 20
TEC	Total erythrocyte count
TLC	Total leukocyte count
ul.	Microliter
UNICEF	United Nations Children's Fund
USAID	United States Agency for International Development
WHO	World Health Organization.

ABSTRACT

Anopheline surveys were conducted during June 1987 to May 1988 in Islamabad-Rawalpindi and its vicinities and fifteen different anopheline species were collected from the area under study. For bloodmeal identification enzyme-linked immunosorbent assay was used. 9.78 percent Anopheles culicifacies and 13.65 percent An. fluviatilis were found as positive to human blood. Sporozoite infections were detected in Anopheles fluviatilis that was earlier cosnidered as suspected malaria vector. Becillus thuringiensis H-14, Bacillus sphaericus 1593, Melia azadirachta L and Melia azedarach L. were tried against Pakistan Mosquito species. Bacillus thuringiensis H-14 was found as good for surface feeding anophelines and Bacillus sphaericus for bottom feeding culicines. Melia azadirachta L and Melia azedarach L extract in methanol was found as highly toxic to mosquito larvae. Considering the aquatic feeding habits domestic ducks and nine different fish were tried for mosquito control and it was observed that domestic ducks and chidu fish are suitable for mosquito control. Extracts from both, Neem and Bakain were also used in combination with Chidu fish and Bacillus thuringiensis H-14 and good larval control was achieved.

IMPORTANCE OF THE STUDY

One of the most vicious diseases of man is malaria. It has played a major role in shaping history, including the decline of civilization. Human malaria is known to have contributed to the fall of ancient Greek and Roman empires. Troops in both the civil wars of USA and the Spanish-American war were severely incapacitated by this disease. Human malaria is caused by the four species of malarial parasites, Plasmodium falciparum (Welch), Plasmodium vivax (Grassiand Feletti) Labbe, Plasmodium malariae (Laveran) Grassi and Feletti and Plasmodium ovale (Craig) Stephens . These malarial parasites are transmitted to humans by the bite of an infected anophelene mosquito. For the control of malaria, control of mosquito vector is very important.

An integrated malaria eradication programme was suggested to the World Health Organization in 1950 and this programme was accepted in 1955. By 1958 seventy six countries were planning, carrying out or had completed the eradication of malaria.

In Pakistan nationwide eradication programme was launched in 1961 under the auspices of World Health Organization and with the support of UNICEF and USAID. In 1960 slide positivity rate was 15.57%. In 1961 DDT was sprayed and due to reduction of vector species the

slide positivity rate went down to 3.64 percent. Due to malaria control operations during 1962 to 1970 the rate remained in the range of 0.91 to 2.8%. In 1971 incidence again reappeared and slide positivity rate was found as 5.36 in 1972 it was 14.5 and in 1973 as 14%. In 1972 BHC was also used alongwith DDT and thus in the coming years the spr again went down. In 1975 it was 7.43, in 1976 as 4.28%. The main insecticides used were DDT and BHC but again during 1973 and 1974 resistance against DDT and BHC was noticed in the two malaria vectors, Anopheles culicifacies and An. stephensi (WHO, 1976a). In 1976 Malathion and Sumithion were also used alongwith DDT and BHC. Sumithion was used from 1976 to 1978 but during 1978-80 it was not used. In 1978 spr reached down to 0.62%. In 1979 DDT was abandoned due to development of vector resistance and malathion and BHC were used during 1979-80. In 1981 spr again started increasing and reached to 1.26%. The spr during 1982 to 1989 was 1.71, 1.99, 2.27, 2.49 , 3.13, 2.16, 2.01 and 3.32% respectively (Table 1).

During 1980 sumithion and malathion were used but during 1981-82 only sumithion was used for vector control. In 1982 malathion was again included alongwith sumithion. In 1983 BHC was used alongwith malathion and sumithion but after 1984 only malathion is being used as residual insecticide for vector control in the country. This insecticide

has already produced resistance towards vector species (Rathore and Toqir, 1980; Rathore et al., 1985).

In foreign countries bulk of research has been done on malaria but very little attention has been paid to this effect in Pakistan. Faust and Russel (1957) have reported that at times of considerable importance in restricted areas every year one or more anopheline species enter into the category of malaria transmitters. Some of the anopheline species those are considered as suspected malaria vectors in our country i.e. Anopheles fluviatilis, An. annularis, An. maculatus and An. suspictus are the malaria vectors in neighbouring countries (Tempelis, 1975).

Some of these species have also been observed in the laboratory as having malarial parasite transmission capability (McCarthy and Clyde, 1968). It is also suspected that apart from the peak malaria transmission season of post monsoon rains, transmission also occurs during spring season, as has been observed in neighbouring India (Tempelis, 1975).

). This study has also not been yet confirmed in field conditions in Pakistan. Present study has got two parts. The overall objectives of the part one planned research was to measure the probability of human feeding and sporozoite infection to evaluate vector efficiency and incriminate secondary vestors. For this purpose a recently developed technique, the Enzyme-Linked Immunosorbent Assay was applied.

Monoclonal antibodies were used to detect the repititive epitopes for circumsporozoites of Plasmodium falciparum and Plasmodium vivax and to identify mosquito bloodmeal by the same technique for determining the host deviation in malaria vector species.

Aim of the part two research study was to reduce adult vector abundance by larval control below threshold level which interrupt and prevent the transmission of malaria and other vector borne diseases. This study was planned by considering the continuous and indiscriminate use of chemical pesticides and other related biohazards to the flora and fauna. Other problem with the chemicals is that improper training of spraymen and the residents where insecticide is being used resulted into some human poisoning. In 1976 alone, 2500 poisoning cases were reported from Pakistan with 5 fatalities (W.H.O., 1979). Therefore due to the increase in insecticide resistance and hazards to flora and fauna, the WHO expert committee on vector biology and control suggested that counter measures including the biological control should further be encouraged (W.H.O., 1980). Therefore two microbial pesticides, Bacillus thuringiensis serotype H-14 and Bacillus sphaericus, insecticidal extracts from two locally available trees, Neem, Melia azadirachta L. and Bakain, Melia azedarach L., the domestic ducks, Anas platyrhynchus L. and the larvivorous fish, Punctius ticto have been used against the larval stages of malaria vectors

and other mosquitoes. Bacillus thuringiensis H-14 has been used on trial basis against the Pakistan species of Anopheles culicifacies, An. stephensi and Culex tritaeniorhynchus (Rathore and Toqir, 1985) and Bacillus sphaericus has been used against Anopheles culicifacies and An. stephensi (Rathore and Toqir, 1987). These microbial agents have not been used at all against any other species of mosquitoes in Pakistan. Similarly, so far no work has been done on the effect of Leaf and fruit extract from both the trees of Melia azadirachta L. and Melia azedarach L. against mosquito species in Pakistan. The larvivorous fish and domestic ducks have close association with the breeding of mosquitoes and these have also not been tried in Pakistan for their role in reducing malaria vector and other mosquitoes. The approaches made during these studies are new to the science and the aim of these biological approaches was to see the possibility of shifting from the use of chemical pesticides towards biological and integrated mosquito control. Present studies are, therefore, anticipated to contribute it's share in malaria control programmes of the country.

STUDY AREAS

Keeping in view the availability of resources for conducting research studies, Islamabad-Rawalpindi and its vicinities were selected for anopheline surveys and other experiments. Area under present study has been described as hypoendemic for malaria

(Pervaiz and Shah,1988). Rawalpindi region is being selectively sprayed but in 1981 Islamabad was made a separate district and the then Rawalpindi Communicable Disease Control Department stopped their activities in this area. Since the creation of new district it has not been sprayed so far. Islamabad was established as federal capital city in the north east of Rawalpindi on February 24, 1960 and in 1981 it was made as a separate district. The study area forms the north eastern part of potwar plateau. Its north eastern side is bounded by Margalla hills. Soil is made of wind and water laid deposits and sedimentary rocks and is dark brown to yellowish brown fine textured, well-drained sandy loam topsoil to silt loam subsoil. The gullies are running in the area and the water from these gullies runs into popular soan river. There is a big lake known as Rawal Lake in the south-east of Islamabad city. The following five different places in Rawalpindi and Islamabad districts were selected for conducting the studies and these places are described here briefly.

Noorpur Shahan

This area is located on eastern side in the foot hills of Islamabad and is hypoendemic for P. vivax and P. falciparum. Lower grade workers and labourers live in the area and during day they go to Islamabad for work or business. A stream flows from almost the centre of the village. Stagnant or slow moving water on the sides of the stream and other ditches in and around the village serve as breeding places for mosquitoes. People sleep indoor

during winter and out side in the yards during summer. Domestic animals kept are cows, buffalloes, goats, dogs, cats and chickens and these animals are some times tide in the same rooms where people sleep during winter. Those who have cattle in their houses, during summer they sleep in the same yards where animals are kept. This brings a close contact between men, mosquitoes and animals. The area has not been sprayed with any insecticide since 1981.

Rawal Dam

It is situated in east south of Islamabad at a distance of about six kilometers from Noor Pur Shahan. Other features of the study area are almost similar to that of Noor Pur Shahan.

Tarnol, Rawat and Dhamial

Tarnol is situated about 15 kilometers north and Rawat as almost 20 kilometers towards south west of Rawalpindi. These places are comparatively warmer than Noor Pur Shahan and a pond is situated on west north corners of Tarnol & Rawat. Stagnant water due to rains and other sources at the peripheries of the village serve as breeding places. Dhamial is at almost six kilometers from Rawalpindi and all the features are similar to those of Rawat and Tarnol.

MAP OF ISLAMABAD-RAWALPINDI

ANOPHELINE SURVEY

INTRODUCTION

Out of the 25 anopheline species so far recorded from Pakistan (Aslam Khan, 1971; 72), An. culicifacies and An. stephensi are only incriminated vectors in Pakistan. An. fluviatilis, An. stephensi, An. annularis, An. subpictus, An. superpictus and An. pulcherrimus are suspected to be the vectors of malaria in different parts of the country. (Pal and Aziz (1985) surveyed this area during 1982 post monsoon season and collected seven different species of anophelines. They reported An. culicifacies, An. fluviatilis, An. stephensi, An. subpictus, An. pulcherrimus, An. annularis and An. nigerrimus. Complete information regarding the availability of different species in different months of the year is very important for entomologists and epidemiologists for forecasting malaria control strategies. These surveys were made during June 1987 to May 1988 for determining prevalent anopheline species, their density, bloodmeal identifications and sporozoite detection.

MATERIALS AND METHODS

Mosquitoes were collected with the help of mouth aspirator in flash-light during day from cattle sheds, houses, kitchens, stores etc. when they were resting during

day and by using pyrethrum in flit gun. Alive collections were transferred to paper cartons and brought into the laboratory, anaesthetized with ether and identified with the identification key prepared by Christophers (1933), Richard and David (1959) and personal communications with the malaria control department. Weather data was also recorded.

RESULTS AND DISCUSSION

The anopheline collections made round the year were identified and the following species were observed.

Anopheles culicifacies Giles, An. fluviatilis James, An. sergenti Theobald; An. Stephensi liston, An. subpictus Grassi, An. gigas Giles, An. superpictus Grassi, An. annularis Van der Wulp, An. nigerrimus Giles, An. pulcherrimus Theobald, An. maculatus Theobald, An. lindesayi Giles, An. splendidus Koidzumi, An. jamesi Theobald and An. barbirostris Van der Wulp.

Maxillary palpi of all the anopheline females are as long as proboscis In An. culicifacies costal wing margin has got four black spots, third longitudinal wing vein is

dark in colour and has got two white spots at the ends of veins

The third longitudinal wing vein in An. fluviatilis is white in colour and there are 5 white spots at the end of the veins. In An. culicifacies as well as An. fluviatilis the hind tarsi are black as well as with very narrow pale bands therefore there is a need to conduct chemotaxonomic study for determining genetic variability and genetic distance in these species. An. stephensi and An. subpictus has also got four black spots on anterior costal margin but the legs in An. stephensi are banded and spotted white while in An. subpictus banded legs are not spotted white

In An. annularis body and wings are very dark in colour and the hind tarsi are white in colour embracing atleast three and a half tarsal segments. An. pulcherrimus has got its tarsal segments as white embracing atleast four tarsal segments. It is a beautiful mosquito due to white specks on legs and body. In An. nigerrimus anterior margin of the wing has got less than four black spots and the base of front femur is enlarged. An. maculatus has got white tarsal segments embracing one and a half segment and having white specks on femur and tibia. An. splendidus is having three tarsal segments as white and the palpi and legs are speckled white. In An. gigas inner one quarter of costa is pale/white.

In An. sergenti third longitudinal wing vein is almost all dark. The hind femur in An. lindesayi has got a broad white band. In An. superpictus thorax has white scales on it. Female palpi is white pale at the tip. There are no white bands on front tarsi, the hind tarsi are unbanded and the tip of hind tarsi is dark in colour. An. jamesi thorax has white scales on it. Female palpi is white pale at the tip with two further narrow pale bands . There are no white bands on front tarsi, the hind tarsi are unbanded and the tip of hind tarsi is dark in colour. An. barbirostris has got dark tip in palpi without the pale bands and costal margin is all dark and thorax has got hairs or hair-like scale . An. fluviatilis remains as dominant species during February to July but from August onwards it declines in population. An. culicifacies takes this position till December. Man hour density was calculated with the formula:

$$\text{Man Hour Density} = \frac{n \times 60}{t \times p}$$

Where n = Total number of specimens collected
t = Time spent in minutes
p = Persons involved

Respective man hour density observed for Anopheles fluviatilis and Anopheles culicifacies (table 2) was 9 and 5 in February, 21.1 and 19.6 in March, 45.2 and 16 in April, 23.8 and 4.6 in May, 11.3 and 3 in June and 26 and 13 in July. From August onwards the population of An. culicifacies was found as higher than that of An. fluviatilis. The respective man hour density observed for An. fluviatilis

and An. culicifacies in August was 15.7 and 34.3, in September 19.8 and 54.6, in October 16.3 and 41.2, in November 4 and 19.5 and in December 3 and 8. January was very cold (table-3) and no anopheline were collected during collection efforts. The anopheline fauna of Kohat-Hangu valley (Naqvi and Qutbuddin, 1954; Qutbuddin, 1960) which is about 150 kilometers further north from the area under present study is almost similar to our findings because of similarity in climatic conditions. These authors reported An. culicifacies, An. stephensi, An. fluviatilis, An. maculatus, An. annularis, An. superpictus, An. barbirostris, An. splendidus, An. lindesayi, A. d'thali, An. pulcherrimus, An. subpictus and An. nigerrimus.

After anopheline identifications a simplified key to the species collected from the area under study was prepared which is presented here.

1. a- Palpi as long as proboscis---------Anophelines 2
   b- Palpi shorter than proboscis-------Culicines
2. a- Several hind tarsi all white---------- 3
   b- Hind tarsi not white, may be ringed with white---------- 4

3. a- Body densely coverd with white and grey scales, tip of hind tarsi white embracing 4 tarsal segemnts--------- An. pulcherrimus

b- Body and wings black and tip of the hind tarsi white embracing three and a half tarsal segments---------- An. annularis

c- Palpi and legs speckled white, three tarsal segments compl tely white---- An. splendidus

d- Palpi and legs speckled white, one and a half tarsal segment white--------- An. maculatus

e- Tip of the hind tarsi embracing 3 white segments, only one band in female palpi broad, other two bands narrow---------- An. jamesi.

4. a- Wings with 4 black spots, base of the femur not enlarged---------- 5

b. Wings with less than 4 black spots at anterior margin, base of the front femur enlarged, inner quarter of costa dark and palpi with distinct pale markings---------- An. nigerrimus.

5. a- Front tarsi ringed white---------- 7

b- Tarsal segments black without white rings or with very narrow pale bands---------- 6

6. a- Third longitudinal wing vein black, wing margin with 2 fringe white spots---- An. culicifacies

b- Third longitudinal wing vein white, wing margin with 5 white spots------ An. fluviatilis

c- Third longitudinal wing vein all dark---------- An. sergenti

d- Inner quarter of costa pale or white---------- An. gigas

7. a- Femur, tibia and portions of palpi with white specks---------- An. stephensi

b- Femur, tibia and palpi without white specks---------- An. subpictus

8. Palpi with dark tip, tarsi in front legs unbanded and thorax with hairs or hair-like scale----------An. barbirostris

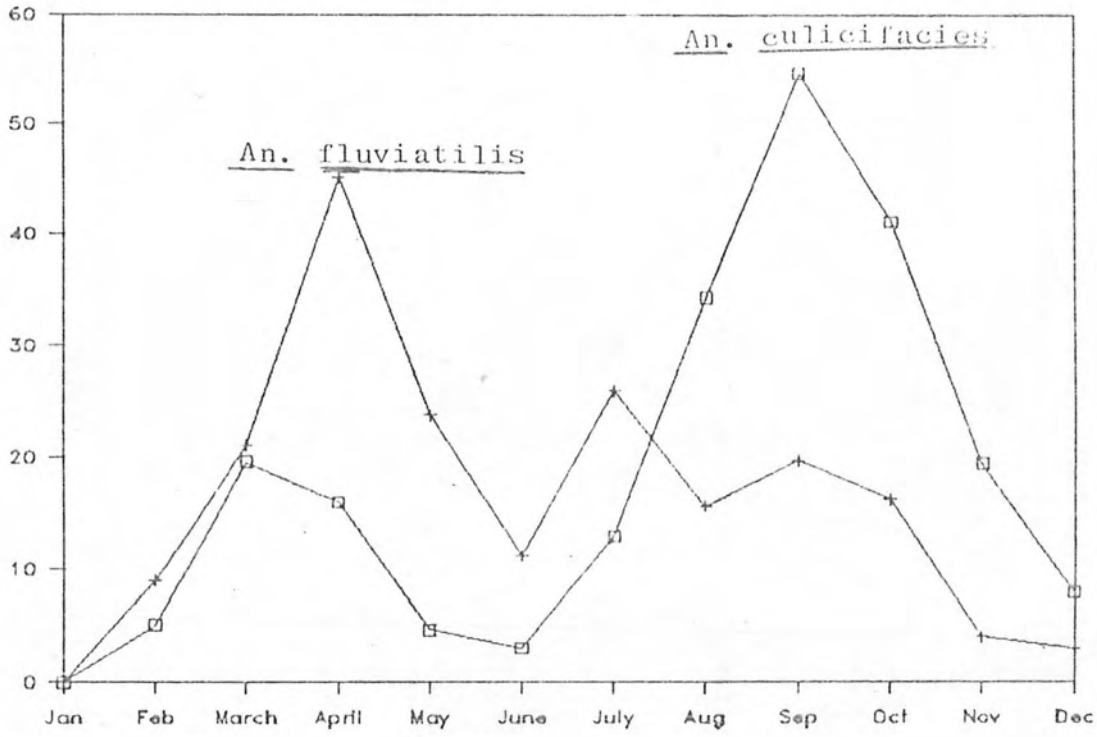
9. Tip in female palpi white, thorax with white scales and no white bands in front tarsi----------An. superpictus

10. Hind femur has got a broad white band----------An. lindesayi

From the area under study fifteen different species have been recorded during June 1987 to May 1988. During post monsoon season of 1982 seven anopheline species were recorded from the same area by the same investigator. From the present studies it has been observed that Anopheles fluviatilis remains dominant species during March to July and An. culicifacies from August to December (Fig-1) therefore major emphasis was paid to An. fluviatilis on its potential as malaria vector in the area. All other species were found as low indensity. The parasite reservoir is present in the area and all the socio-economic factors and climatic conditions favour malaria transmission.

FIG- 1

MAN HOUR DENSITY OF ANOPHELES FLUVIATILIS AND AN. CULICIFACIES.



BLOODMEAL IDENTIFICATION

INTRODUCTION

Bloodmeal identification is an important tool in applied field research, epidemiological surveillance and malaria control programmes of a country. In our local conditions houses and cattle sheds are located very closely and in many cases both the houses and cattle sheds are even shared together by domestic animals and humans. Under these situations mosquitoes may feed on cattle and then rest in human dwellings or vice versa. Since cattle are the prefered hosts of mosquitoes (Kuntz et al., 1982, Snow, 1983) it is therefore, important to determine the source of bloodmeal mosquito has fed upon. In the past several serological techniques have been practiced (Weitz, 1956; Tempelis, 1975; Boreham, 1975; Reisen and Boreham, 1976; Chow et al., 1980; Reisen and Boreham, 1982; Nasci, 1982; Collins et al., 1986). Todate immunofluorescence and passive agglutination have been widely used for this purpose (WHO, 1976). Van Weemen and Schuurs (1971) introduced the idea of using enzymes conjugated with antibodies or antigen for the detection of antigens or antibodies respectively. Engvall et al. (1971) devised enzyme-linked immunosorbent assay (WHO, 1976) ELISA is being widely used in foreign countries (Voller et al., 1974; 1975).

ELISA has been compared with IFA and found as specific (Edrissian et al., 1979; Quakyi, 1980; Washino and Tempelis, 1983; Kumar et al., 1987). Mixed blood feedings by malaria vectors have been identified (Burkot et al., 1988) and the effectiveness of zooprophylaxis in mosquito control has earlier been studied (Sota and Mogi, 1989; Motala et al., 1989).

Modern research based on immunology and the development of good quality reagents has improved the specificity and economy in bloodmeal identifications and has resulted into simple ways in getting appropriate informations regarding mosquito hosts by using ELISA (Burkot et al., 1981; Edrissian and Hafizi, 1982; Washino and Tempelis, 1983). New ELISA methods have been developed for the identification of host blood meals in arthropod vectors. The test has advantages over the other tests because large number of specimens can be processed at a time, sporozoites can be identified upto species level, both fresh and dried or frozen specimens can be used and the sensitivity of the assay is greater even for light infections. In the present study indirect sandwitch elisa and dipstick elisa were used for bloodmeal identifications. The specific objectives of the study were

1. To establish the elisa techniques for bloodmeal identification and sporozoite detection for use by the national malaria control programme.

2. To determine the feeding pattern of anophelines and its effect on vectorial capacity and the applications of these studies in forecasting malaria transmissions.

MATERIALS AND METHODS

Reagents and solutions were prepared as below:-

Phosphate buffer saline

One liter of PBS was made by weighing 0.7 gm of potassium dihydrogen phosphate and 1 gm of disodium hydrogen phosphate and adding into 1000 ml of distilled water. This was then dissolved uniformly by stirring with the help of magnetic stirrer for about 15 minutes. pH of the saline was fixed at 7.4

To avoid contaminations, all the times only 1 liter of PBS was made. 500 ml of this saline was used for reagent mixing etc. and into the remaining 500 ml, 0.1 ml tween-20 was added and was then used for washing the plates during assays. This amount of PBS was enough for running three plates.

Plate washing and abdomen grinding solution.

0.1% Tween-20 was added in PBS for this purpose. Tween-20 was dissolved by using magnetic stirrer for almost 30 minutes.

Coating Solution

Antibodies were coated in Phosphate Buffer Saline having pH-7.4.

Antigen/Conjugate diluent.

1.5% fish protien (Fisher Scientific Company) was dissolved in PBS (pH-7.4) for coating the microtitration polyvinyl chloride plates and antigen/conjugate dilutions. These were then throughly mixed by stirring for about 30 minutes each (Bovine Serum Albumin gave cross reactions with bovine bloodmeal assays).

Abdomen grinding suspension.

Mosquito abdomen was ground in 50 ul of PBS T20 (0.1%) and then 250 ul of the same solution was added into 1.5 ml specimen vials to make the total volume of 300 ul.

Storage of Reagents.

All the lyophilized reagents were stored at -70°C. After reconstitution all 1 ml of reagents were transfered

as 200 ul each into small vials. One vial of each reconstituted reagents was stored at 4 degree centigrade while the remaining all vials were kept at -70°c for later use.

Principle of ELISA.

As illustrated in the figure 2 antibodies are coated on polyvinyl chloride plates. The antigen is incubated in the same wells, then peroxidase labelled antibodies are added and incubated. The antibodies are labelled in such a way that the immunological and enzymatic activities of both the antibodies and enzyme are maintained. The enzyme acts on the substrate and produces green colour which can be seen visually (Fig-3) and was later on measured with elisa reader.

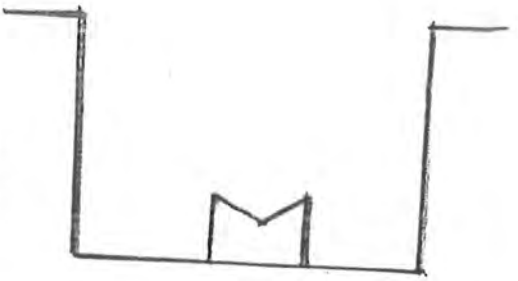
Procedure for bloodmeal ELISA.

After identification mosquitoes were cut from the middle of thorax and abdomen. After salivary gland dissections thorax plus head were preserved separately in small 1.5 ml plastic vials for sporozoite detection and abdomen was used for bloodmeal identification.

Reagents used were obtained from, Kirkgaard and Perry laboratories, 2-Cessna Court, Gaithersburg, Maryland, U.S.A.

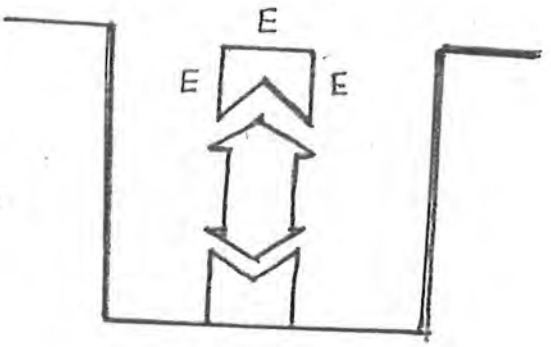
BLOODMEAL IDENTIFICATION BY ELISA

A. Anti-human monoclonal antibody absorbed to pvc plate.



B. Mosquito abdomen triturate added to the wells.

C. Peroxidase labelled anti-human monoclonal antibody added.



D. Peroxidase substrate added.

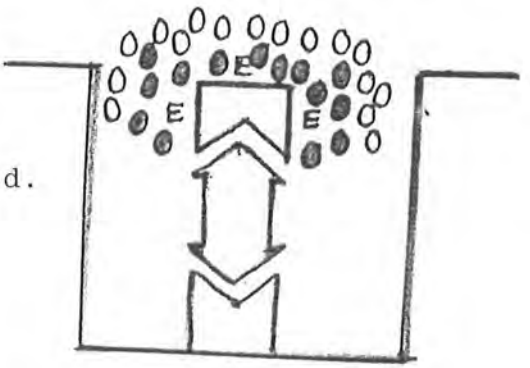


Fig - 2

1. 15 ml disposable plastic vials were labelled with a marker for adding human and bovine immunoglobulins. 10 ul (1:1000) of affinity purified monoclonal antibodies to both human (Lot No. HG02-1) and bovine (Lot.No.HJ14-1) immunoglobulins were added separately into 10 mililiter of PBS and were mixed by shaking the tubes. For both the human and bovine tests pH of coating solution (PBS) was 7.4. This suspension was then added into a sterilized petri dish and with the help of multichannel pipette 100 ul of this suspension was added into each well of the microtitration polyvinyl chloride plates and the plates were incubated overnight at room temperature.

2. Next morning the contents were thrown out carefully, plates were dried with the help of kimwipes disposable wipers and all the wells were completely filled with blocking buffer (1.5 % Gelatin in PBS pH. 7.4) to block the remaining sides of plate wells. The plates were incubated for 30 minutes at room temperature, emptied and dried.

3. 100 ul of diluent (1.5% Gelatin in PBS pH. 7.4) was added into each well. 10 ul Mosquito abdomen suspension already ground in PBS T20 was added in each well of pvc plates for bovine and human respectively, except blank, negative and positive controls. In positive control human and bovine serum was used and nothing was added in the negative controls

and blank. Well A1 was kept as blank, B1 to D1 wells as negative controls and wells E1-F1 & G1-H1 as human & bovine positive controls. Then the plates were incubated for two hours.

4. After two hours plates was washed thrice with PBS T20 at pH 6.0. Washings were done with the help of multi-channel pipette. Wells were filled with PBS T20, left for 2 to 3 minutes, emptied, then again filled and emptied. After 3 washings the plates were dried by using kimwipes disposable wipers.

5. 15 ul (1:666) of peroxidase labelled affinity purified monoclonal antibody both to human (GH19-1) and bovine (HM24-1) immunoglobulins was mixed separately in 10 ml diluent and 100 ul of each were added in each well in respective plates and incubations were done for one hour. All the above incubations were done at room temperature.

6. After one hour the plates were washed thrice with PBS T20 at pH 6.0 and then dried.

7. The substrate solutions, 2.2-azino-di-(3-ethyl-benzthiazoline sulfonate (Lot. No.HJ22) and hydrogen peroxide (Lot. No.JC07) were mixed in 1:1 ratio and 100 ul was added in each well.

8. Results were observed visually and with the help of Dynatech Microelisa Reader at 405 nm after 60-90 minutes.

The above quantity of reagents required for better results was determined by serial dilutions and by trial and error methods. Human antibodies were tested with bovine blood and vice versa for their specificities. These were also tested against horse, goat, sheep, mice and swine blood for determining their purification.

There were 3 negative & 2 each of human & bovine positive comparison controls in each plate. All the data was fed to the computer and analysed statistically.

Considering the reagents used up in titer determinations and those available the specimens were tested only once. However the specimens used in first two tests were retested by dipstick elisa for the confirmation of the results and for evaluation of dipstick elisa technique.

Dipstick ELISA.

176 samples already tested in plate no.1 and 2 were retested with dipstick elisa for the confirmation of

above results and for evaluation of this method for field application. Since the reagents available were in small quantities therefore the specimens tested with the method were less. Assay was run as follows.

i. 1 ul of affinity purified antibodies to both the human and bovine immunoglobulins were added separately on cellulose acetate strips and kept for 5 minutes to dry.

ii. 1 ul of mosquito abdomen suspension ground in PBS T20 was added on the strip at the same place where antibodies were added.

iii. The strips were kept for 30 minutes at room temperature for incubation.

iv. 1 ul of both the human and bovine conjugates were added on the strips and left for 10 minutes.

v. Substrate solutions 2.2-azino-di-(3-ethyl-benzthiazoline sulfonate and hydrogen peroxide was mixed in 1:1 and 1 ul of this suspension was added on these strips.

vi. Results were read visually.

RESULTS

Cut-off value was calculated for separating the positive results from negative ones and for interpretation of results. For this purpose arithmatic mean and standard deviation of 3 negative controls was calculated and the mean was then multiplied with 3 standard deviations (Beier et al.,1987). This was kept as cut-off value and any value above this but approximately the optical density of plus 0.1 was considered as suspected positive and below this as negative for the test. Any value above mean plus three standard deviations plus + 0.1 was thus considered as clear positives.

When monoclonal antibodies to human were tested against bovine, goat, sheep, horse, mice and swine blood no reactions were observed. Similarly bovine antibodies also did not react with other animals blood which means that these were highly specific. 2538 anophelines of different species were tested for their bloodmeal source. 1370 specimens were Anopheles culicifacies. 134 Anopheles culicifacies gave elisa positive results for human, 687 were bovine positive and 556 specimens were found as non human non-bovine (Fig- 4.) (table 4 ,5 , 6). When the data was checked in percentages, 9.78 percent were human, 50.14% bovine and the remaining 40.58 percent An. culicifacies were neither human nor bovine

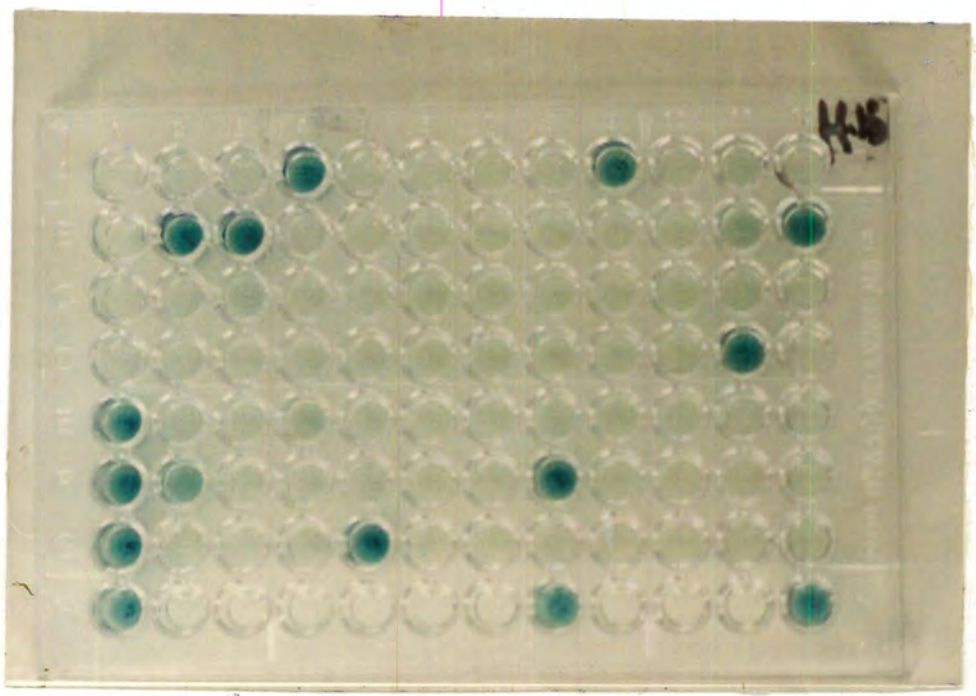


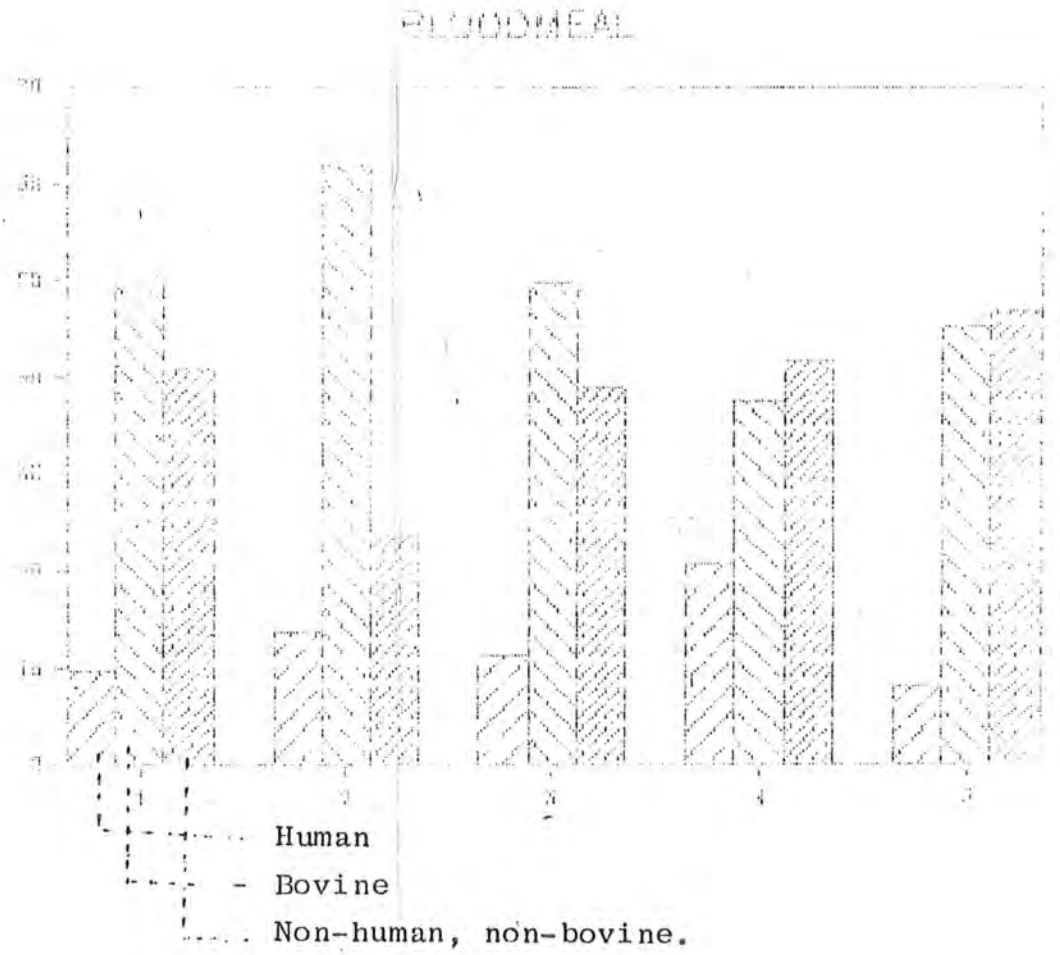
Fig. 3 : Bloodmeal test plate. Well A1 is Blank.
Wells B1 to D1 are Negative Controls. Wells E1, F1 are human positive controls, wells G1, H1 are Bovine positive controls. Specimens in green wells are positives.

which means that these 40.58% feedings were on other animals like cat, dog, goat, sheep, chicken, rat etc. present in the vicinity. Most of the bovine positive An. culicifacies were also good positives and their optical densities were observed as above 1.00.

Total numberof Anopheles fluviatilis tested were 834. 114 were positive for human immunoglobulin, 516 to bovine and the remaining 197 were found as negative to both human and bovine groups. This means that 13.66 percent were positive to human source, 61.87 percent to bovine and the remaining 23.62 percent were non-human non-bovine.

Depending upon the collections available 175 An. stephensi were tested for both human and bovine blood source and 20 out of these specimens were found as positive to human host and 87 for bovine source. This means that 11.43 percent An. stephensi had human bloodmeal, 49.71 percent that of cattle and the remaining 38.85 percent had neither human nor cattle blood. There was significant difference in the feedings of An. culicifacies and An. fluviatilis ($X^2=7.8$, df=1, $P < 0.05$), but between An.culicifacies and An. stephensi ($X^2=0.469$, df=1, $P < 0.05$) and the An.fluviatilis and An. stephensi ($X=0.630$, df=1, $p < 0.05$) there was non-significant difference in human feedings. As regards the bovine feedings there was significant difference in the

FIG- 4



1. Anopheles culicifacies
2. Anopheles fluviatilis
3. Anophelese stephensi
4. Anopheles subpictus
5. Anopheles splendidus

feeding of An. culicifacies and An. fluviatilis (X^2=283.3, df=1, $p < 0.05$) and between An. fluviatilis and An. stephensi (X^2=8.8, df=1, $p < 0.05$) on human host but there was significant difference between the feeding of An. culicifacies and An. stephensi (X^2=0.011, df=1, p=0.05).

24 Anopheles subpictus were tested, 5 were found as positive to human blood source, 9 to bovine and 10 were neither human nor bovine or otherwise 20.83 percent of the tested specimens of An. subpictus were positive for human blood, 37.5 percent for bovine and the remaining 41.66 percent were neither human nor bovine.

Five An. nigerrimus were tested and two specimens, one each for human and bovine were found as positive.

Twelve An. superpictus were tested, two were positive to human blood, five to cattle and five were neither human nor bovine. When compared in percentages 16.7 percent were human positive, 41.67 to bovine and 41.66 neither to human blood nor to bovine. Out of 23 An. maculatus, one was positive for human blood, 13 for bovine blood and 9 were neither human nor bovine, i.e. 4.35 percent positive to human, 56.52 percent for bovine and 39.13 percent were neither human nor bovine.

19 An. annularis were tested, 2 were positive to human IgG, 13 to bovine and 4 were neither human nor bovine, i.e. 10.53 percent to human blood, 68.42 percent to bovine and 21.05 percent to animals other than man and cattle.

14 specimens of An. sergenti were tested, one was positive to human blood, 5 to bovine and 8 neither to human nor to bovine. When checked in percentage 7.14 percent were humans, 35.71 percent bovine and 57.14 percent as none of these two hosts. An splendidus tested were 62. Five were positive to human blood, 28 to bovine and 29 negative to both the human and bovine blood giving a percentage of 8.06 percent human, 45.16 percent to bovine and the remaining 46.77 percent negative both to human and bovine blood.

All samples with elisa values 0.4 were retested to separate suspected positives from clear negatives. Bovine results are presented on plates B31 and B32 and human in H31 and H32. Out of 112 bovine samples only eight were found as slightly positive for bovine blood showing absorbance value from 0.165 to 0.322. These samples were also positive for human blood. 162 females were suspected from human tests and therefore these were assayed again. Their results showed 15 females as slightly positive for human blood. Seven of them were positive for bovine as well which means that these mosquitoes had fed both on human and animals.

The results obtained with dipstick elisa were similar with those of multiwell plate method (table 7,8), 21 An. culicifacies 101 An. fluviatilis, 29 An. stephensi, 4 An. subpictus, 3 An. superpictus and 18 An. splendidus were tested during these assays. 3 An. culicifacies, 16 An. fluviatilis, 4 An. stephensi and 1 An. splendidas which were detected positive for human blood source with plate method were also found as positive with dipstick elisa. Same specimens were then again tested separately for bovine bloodmeal by dipstick method and like that of pvc plate method 10 An. culicifacies, 58 An. fluviatilis, 15 An. stephensi, 2 An. subpictus, 2 An. superpictus and 8 An. splendidas were again detected as bovine positive.

It was observed that within 40 to 50 minutes it was possible to identify the source of mosquito bloodmeal by dipstick method but the quantity of reagents utilized by these assays was comparatively large.

DISCUSSION

Anopheles culicifacies, Anopheles stephensi and Anopheles fluviatilis are the proven malaria vectors in our country. It is, therefore, concluded that if the infections are present and the temperature and humidity also favours, there will be the possibility of malaria outbreaks in this area

due to 9.78, 11.43 and 13.66 percent human feedings by the vector species An. culicifacies, An. fluviatilis and An. stephensi. This high percentage of human feedings could be due to very close living habits of the people alongwith animals. During summers they sleep very close with the animals in a small yard and whenever there is need to sleep inside due to rains, hot sun etc. they sleep in the same room where cattle are tied. This gives an equal chance to the mosquitoes to feed on animals as well as on man. For estimating the malaria transmission host diversion is of utmost importance.

An. fluviatilis is the second dominating species in Rawalpindi-Islamabad area and this species has also been observed as infected with the sporozoites during the present ELISA studies. An. fluviatilis density remains highest from March onwards till July. From July onwards its density comes down as next to An. culicifacies. Density of An. stephensi in the area under study was found as very low. According to Bellanca (1986) longer collection periods are required for the collection of Anopheline species because the number present may be low and that the index for this purpose should be bites per man per night and that the index for good malaria vectors may be less than one bite per man per night. According to him this could still produce a significant malaria rate under favourable conditions even if the percent of

infective malaria vectors were only one in 100. Present studies regarding the anthropophilic and zoophilic habit of feedings are very helpful in forecasting malaria transmissions. 50.14 percent bovine feedings by An. culicifacies, 61.87 percent by An. fluviatilis and 49.71 percent feedings by Anopheles stephensi on cattle show that these malaria vectors are highly zoolphilic in their feeding habits. These studies can thus be applied in malaria control programs in deviating the route of malaria vectors towards cattle, provided these cattle are kept at a distance of about 150 tO 200 yards away from human dwellings at the peripheries of the villages and cities. This will help for less feeding on humans and thus less malaria transmission and other vector borne diseases.

It has also appeared from the present studies that the elisa techniques are very helpful in saving time by processing large number of mosquitoes at a time. The dipstick elisa took 40 to 45 minutes for getting the results. It has appeared that the technique is very accurate and simple for fieldwork applications. The reagents required for dipstick method are considerably more in quantity, however if these reagents could be prepared locally, the technique will serve the purpose for field work programmes and further research studies.

SPOROZOITE DETECTION

INTRODUCTION

In Pakistan principal malaria vector is Anopheles culicifacies and secondary malaria vector is Anopheles stephensi(Rehman and Mutlib,1967; Reisen and Boreham,1982). Some of the anophelines those are considered as suspected malaria vectors in our country are the vectors of malaria in neighbouring countries (Tempelis 1975).

Anopheles fluviatilis has been observed in the laboratory as showing the transmission capability (McCarthy and Clyde 1968). So far, dissection is being done for sporozoite detection in our country and no immunological approach has been made for the identification of sporozoites. In foreign countries sporozoites detection has been done by indirect flourescent antibody (Gupta et al.,1981; Knobloch et al.,1982; McBride et al.,1982; Boyle et al.,1983; Ramsey et al.,1983; Warren et al.,1984; Cochrane et al.,1984; Campbell et al.,1984; 1984; Mendis et al.,1984; David et al., 1984), by indirect haemagglutinization test (Gupta et al.,1981, Mathews and Dondero,1982a; 1982b) by Immunoelectron microscopy (David et al.,1984), by micro semiautomative assay (Cowen et al., 1983), by Immunoradiometric assays (Avraham et al.,1982; Zavala et al.,1982; Boyle et al.,1983; Jeje et al.,1983), by two dimensional electrophoretic analysis and western blot analysis (Kemp et al.,1983; Cochrane et al.,1984) and by SDS-Polyacrylamide gel electrophoresis (Deans et al.,1983).

Enzyme-Linked Immunosorbent Assay for the detection of circumsporozoite protein is a variation of immunoradiometric assay that was developed by Zavala et al.(1982; 1983). Elisa technique for the detection of sporozoites was then devised by Burkot et al. (1984). ELISA has advantages over other methods for using in field conditions, the technique is very sensitive, the reagents have long shelf life, are easy to transport and large number of fresh, frozen or even dried specimens can be processed at a time with this method (Perrin et al.,1980, Mahajan et al., 1982, Ramsey et al.,1983; Stanley et al., 1984; Collins et al., 1984). Elisa is being applied for identifying sporozoites from infected mosquitoes world over (Voller et al., 1974; Voller 1975; Pollack et al., 1985; Kumar et al., 1986). Similarly lot of work has been done on the production and characterization of malarial parasite antibody and enzyme immunoassays (Hollingdale and Leland, 1982; Cochrane et al., 1982; Denforth et al., 1982; Lundgren et al., 1983; Kemp et al., 1983; Monjour et al., 1983; Gabrielsen et al., 1983; Anders et al., 1984; Campball et al., 1984; Adreshir et al., 1985; Pirson and Perkins, 1985). Work has been done on the detection of malaria in man and for blocking the action of malarial parasites in man (Clyde et al.,1973; Carter and Chen, 1976; Nardin et al.,1979; 1982; Mackey et al., 1982; Orjih et al., 1982; Avraham et al., 1982; Collins et al., 1984; Nussenzweig and Nussenzweig, 1984; Harti et al., 1985; Zavala et al., 1982; 1985; Pollack et al., 1985; Rosenberg, 1985; Lim, 1988 ; Cattani, 1989; Chulay, 1989; Schofield,

1989; Hollingdale and Rosario, 1989).
In the recent past work has been done on the sporozoite detection by a number of research workers (Campbell et al., 1987a; 1987b; Giudice et al., 1987; Saleha et al., 1987; Romerio et al., 1987; Burkot et al., 1988; Webster et al., 1988; Lee et al., 1988; Nussenzweig and Nussenzweig, 1989; Lowel et al., 1989; Vaughan et al., 1990). The effect of antisporozoite antibodies on sporogony has been studied by Elisa (Vaughan et al., 1989). Recently new immunological methods for the quantitation of antisporozoite immunoglobulins in the hemolymph of Anopheles stephensi after blood feeding were used (Habluetzel et al., 1989; Vaughan et al., 1990). Antibody response with sporozoite antigen has also been studied by using synthetic peptides (Meuwissen, 1989; Pessi et al., 1990; Wijesundera et al., 1990).

Inspite of lot of studies on different immunological aspects of malaria in foreign countries, no similar study has been made in Pakistan so far. Present studies were therefore planned to detect the circumsporozoite protein by using elisa technique. The specific objectives of this study were:

1. To confirm the status of incriminated malaria vectors.
2. To incriminate the suspected malaria vectors.
3. To observe malaria transmission during spring season.
4. To extend malaria control activities to the target

anopheline vector species.

MATERIALS AND METHODS

Salivary gland dissections.

All anophelines collected during post monsoon malaria transmission season i.e. in the months of August to November were dissected for detecting the presence of sporozoites in salivary glands.

For dissections the wings and legs of the females were separated and these specimens were placed on a clean slide under a dissecting microscope with its head pointing towards right. A drop of 0.85% normal saline was added gently on the slide near mosquito. Thorax was pressed slightly and head pulled with the help of right hand needle to pull out the glands. The glands were then separated from other body tissues and were transfered to clean side of the same slide, covered with a coverglass and were pressed gently. The body of the mosquito was removed from the slide, abdomen was transfered to specimen vials and frozen for bloodmeal identifications and the salivary glands were observed at 100 X to see the presence of sporozoites.

After observing under microscope these salivery glands alongwith head and thorax were transfered very

carefully to specimen vials for elisa studies. These vials were stored at -20°C.

Production of sporozoites for checking the quality of reagents and confirmation of results

Sporozoites of Plasmodium falciparum and Plasmodium vivax for this purpose were produced as follows:

1. Anopheles culicifacies females were kept in paper cages and starved for 2-4 days.
2. These starved females were then transfered to two small size paper cartons having 100 each for P. falciparum and P. vivax. A water soaked cotton pad was placed over the mesh cloth for providing moisture and for avoiding death due to high temperature and the females were then carefully taken to the field.
3. Plasmodium falciparum and P. vivax cases were searched by making thick and thin smears, staining with 10% Giemsa stain for 10 minutes and examination of the slides in the field.
4. Positive cases of both P. falciparum and P. vivax revealing heavy infection with gametocytes were selected.
5. The females were allowed to feed on the arm by exposing them through the mesh cloth untill

females were fully engorged with blood. The patients was requested to exercise patience during feeding.

6. The females were then carefully brought into the laboratory by placing soaked cotton over the mesh cloth and were carefully transfered to the small size insect cages.
7. These females were kept at 25-28 degree centigrade and 70-80 percent relative humidity for completion of sporogony.
8. A cotton pad soaked in 10 percent sugar solution was placed daily over the mesh cloth from two days of feeding the females on patients onwards.
9. Salivary gland dissections of few females were started after 12 days in case of P. falciparum and after 7 days in case of P. vivax. The sporozoites had developed after 14 days in Plasmodium falciparum and 9-10 days in Plasmodium vivax.
10. Upon detection of sporozoites in the salivary glands, these salivary glands were carefully washed with the help of micropipette into the wells of the 96 well plate and marked.
11. These specimens were kept at -20 degree centigrade. Elisa assays were then run for confirmation of the quality of the reagents and the results with field collected specimens.

Preparation of reagents and solutions

Phosphate Buffer Saline

One liter of PBS was made by weighing 1.42 gm of sodium phosphate dibasic, 8.76 gm of sodium chloride and adding into 1000 ml of distilled water. This was then dissolved uniformly by stirring with the help of magnetic stirrer for about 15 minutes. pH of the saline was fixed at 7.4 by using 1M sodium phosphate monobasic. To avoid contaminations, always only 1 liter of PBS was made. 500 ml of this saline was used for reagent mixing etc. and into the remaining 500 ml tween-20 was added and was then used for washing the plates during assays. This amount of PBS was enough for running three plates.

Coating Buffer

1 percent bovine serum albumin was dissolved in PBS (pH-7.4) for coating the microtitration polyvinyl chloride plates. These were then thoroughly mixed by stirring for about 30 minutes each.

Blocking Buffer/Conjugate Diluent

10 gm of bovine serum albumin, 0.1 gm thimerosal and 0.01 gm phenol red was added into 1,000 ml of phosphate buffer

saline pH-7.4.

Mosquito Grinding Solution

500 ml of the BB was taken and 2.5 ml NP-40 was added into it.

Plate Washing Solution

0.05% tween 20 was added in PBS for this purpose. Tween 20 was dissolved by using magnetic stirrer for almost 30 minutes.

The elisa assays were run on the year round collection as well as on dissected specimens.

Sporozoite elisa procedure

1. Head plus thorax preserved in the refrigerator were used for sporozoite detection. For this purpose individual mosquitoes were ground in 50 ul of the grinding solution in 1.5 ml capped polypropylene vials with the help of a small size pestle and after grinding head plus thorax 250 ul of the grinding solution was added into this 50 ul to make the total quantity of the antigen as 300 ul.

2. 15 ml disposable plastic vials were separately labelled with a marker for adding capture Plasmodium vivax

and Plasmodium falciparum antibody. 2.5 ul/5 ml (1:2000) of Pv capture(NSVS lot no. HD14) and Pf capture(2A10 lot no. HD13) were added separately into the vials and mixed with the help of magnetic stirrer. Considering the small quantity of the reagents available, 50 ul was used for coating and was added into each well of 96 well plates and incubated overnight at room temperature.

3. Next morning the contents were thrown out carefully, plates were dried with the help of kimwipes disposable wipers and all the wells were completely filled with blocking buffer. The plates were incubated for 30 minutes at room temperature, emptied and dried.

4. 50 ul of antigen diluent was added into each well. 15 ul Mosquito suspension already ground in PBS T20 was added in each well of pvc plates coated for P. falciparum and P. vivax respectively except blank, negative and positive controls. In positive controls synthetic P. falciparum and P. vivax supplied in the kit were used and nothing was added in the negative controls and blank. Well A1 was kept as blank, B1 to D1 wells as negative controls and wells E1 to H1 were kept as positive controls. for P. vivax and P. falciparum.The plates were incubated for two hours.

5. After two hours plates were washed thrice with PBS T20 at pH 6.0-6.4. After 3 washings the plates were dried

by using kimwipes disposable wipers.

6. 7.5 ul (1:666) of peroxidase labelled affinity purified monoclonal antibody both to P. vivax and P. falciparum circumsporozoite protein was mixed separately in 5 ml conjugate diluent and 50 ul of each were added in each well in respective plates and the plates incubated for one hour.

7. After one hour the plates were washed thrice with PBS T20 at pH 6.0 -6.4 and then dried.

8. The substrate solutions, 2.2-azino-di-(3-ethyl-benzthiazoline sulfonate and hydrogen peroxide were mixed in 1:1 ratio and 100 ul was added in each well.

9. Results were observed visually and with the help of dynatech elisa reader at 405 nm after 30-45 minutes.

The reagents and positive controls were checked and compared by using laboratory produced sporozoites in only 20 wells of the 96 well plate because of small quanity of the reagents. All samples showing elisa positives for circumsporozoite protein were reassayed.

RESULTS

Cut-off value to differentiate between elisa positives and negatives was calculated similarly as for bloodmeal assays. From the experience of present elisa assay studies the best cut-off value was calculated as one quarter of positive controls.

None of the anopheline dissections were found as sporozoite positive. The dissections were made only during postmonsoon malaria transmission months. The number of dissections made for An. stephensi were less because of low density of this species in the area under study.

Sporozoite elisa studies gave positive results only with An. culicifacies and An. fluviatilis. Results are depicted in table no. 9, 10 and figure 5-6.

In August 1987, 2 An. culicifacies were detected with Plasmodium vivax and one An. culicifacies was detected P. falciparum sporozoite positive. One An. fluviatilis revealed sporozoite positive results for P. vivax. None of the An. fluviatilis were detected positive for Plasmodium falciparum during the month of August.

In September 3 An. culicifacies revealed P. vivax positives and two for P. falciparum. An. fluviatilis revealed

two positives for P. vivax. An fluviatilis did not reveal any positives for P. falciparum from the collections of September.

In October two An. culicifacies were found as positive for P. vivax and two for P. falciparum. An. fluviatilis revealed one positive for P. vivax and one for P. falciparum.

In March 1988 one each of An. culicifacies and An. fluviatilis were found as positive for P. vivax and in May one Anophenels fluviatilis was detected as elisa positive for P. vivax.

In August 1987 two An. culicifacies positive for P. vivax were collected from Rawaldam cattle sheds. One An. culicifacies positive for P. falciparum was also collected from cattle shed from Noor Pur Shahan. One An. fluviatilis positive for P. vivax was collected as house resting from Noor Pur Shahan.

During September 1987 three An. culicifacies positive for P. vivax were collected from a cattle shed from Rawaldam. Two An. culicifacies positive for P. falciparum were also from cattle sheds from Noor Pur Shahan. Two An. fluviatilis positive for P. vivax were collected from cattle shed from Rawaldam.

FIG- 5

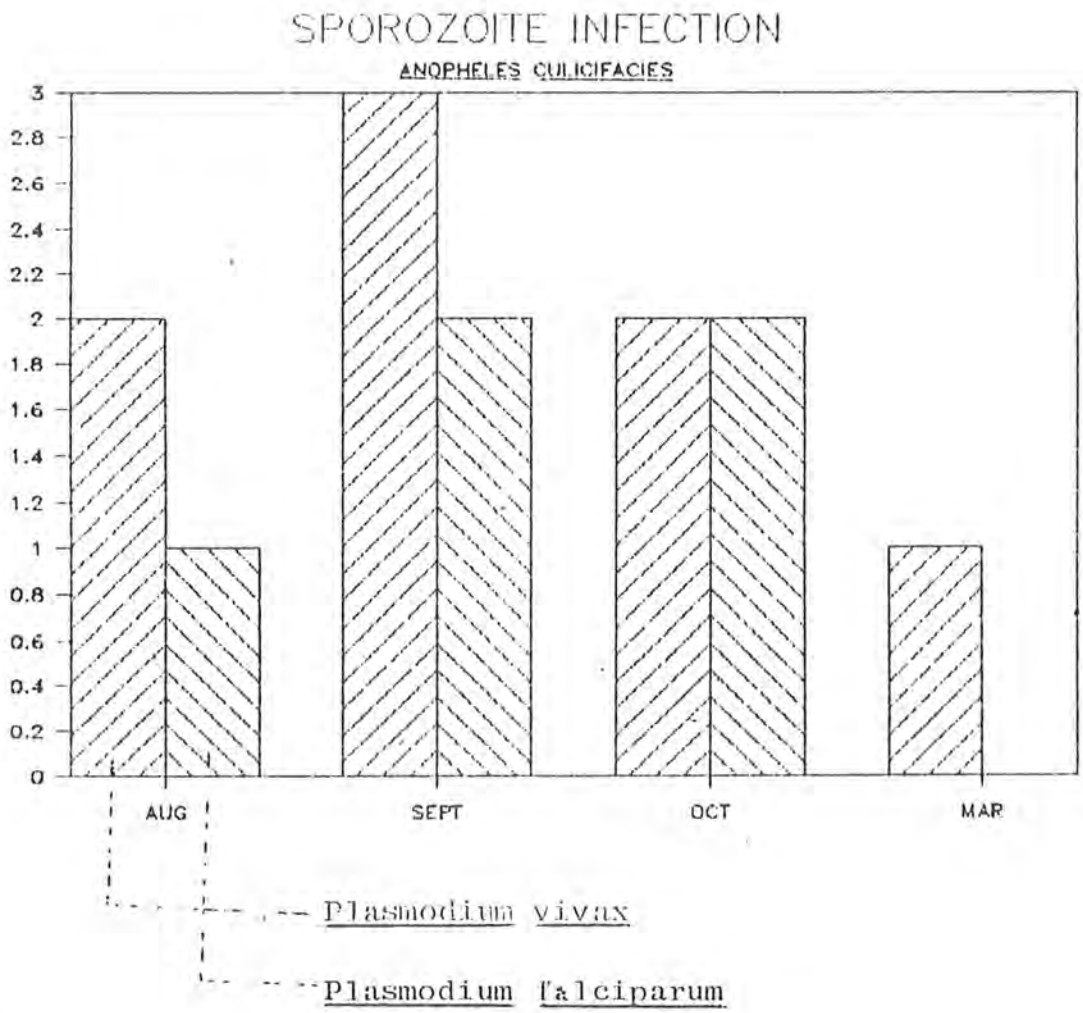
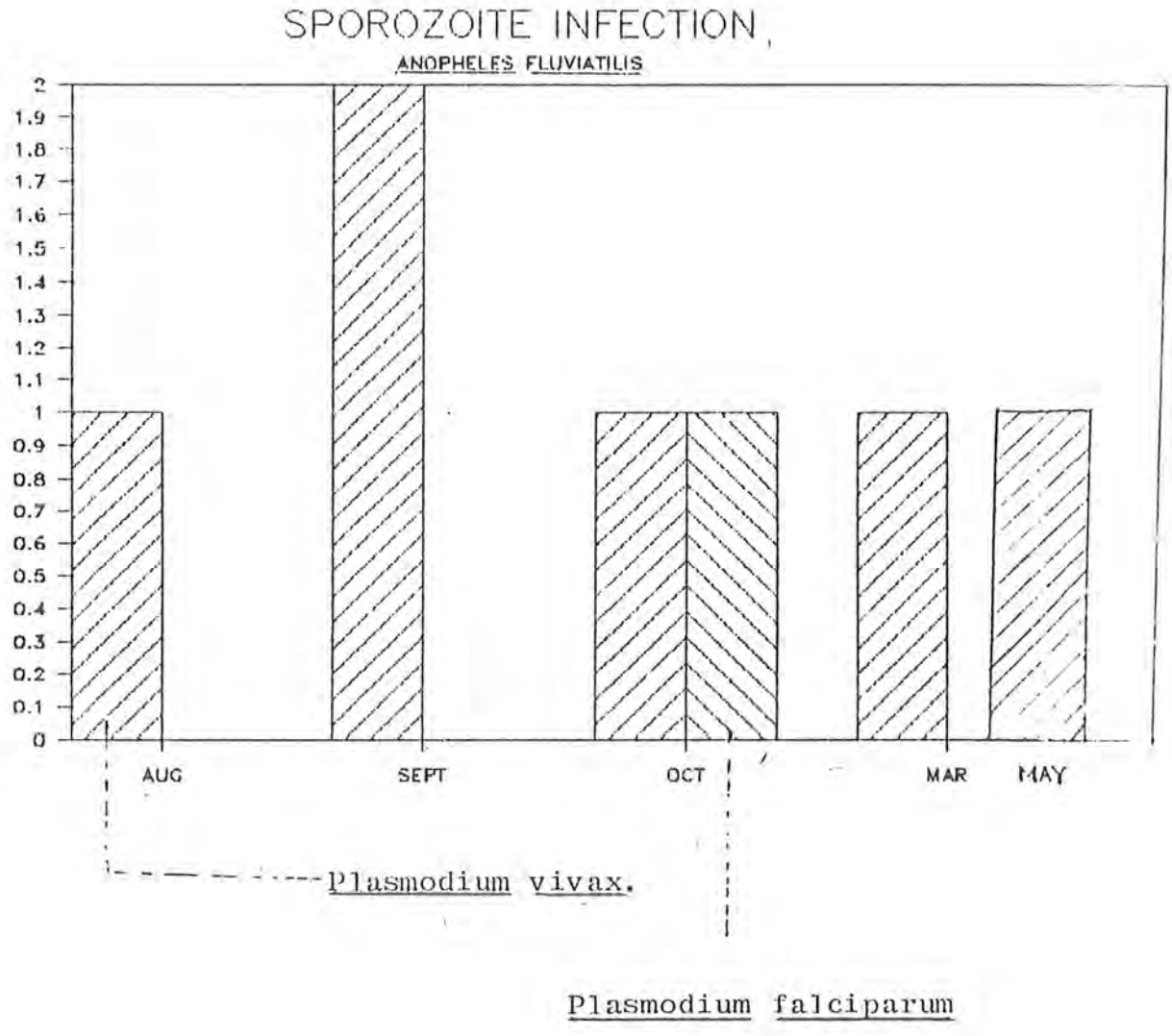

SPOROZOITE INFECTION
ANOPHELES CULICIFACIES
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AUG
SEPT
OCT
MAR
Plasmodium vivax
Plasmodium falciparum

FIG- 6


SPOROZOITE INFECTION
ANOPHELES FLUVIATILIS
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
AUG
SEPT
OCT
MAR
MAY
Plasmodium vivax.
Plasmodium falciparum

During October 1987 two An. culicifacies positive for P. vivax were collected from a house in Noorpur Shahan and out of the two positives for P. falciparum from Rawaldam were collected one each from a cattle shed and a house respectively. One An. fluviatilis detected positive for Plasmodium vivax was collected from a cattle shed from Noorpur Shahan and one for Plasmodium falciparum were collected from cattle shed from Rawaldam. In November no positives could be detected.

In March 1988 one each of An. culicifacies and An. fluviatilis positive for P. vivax were collected from Rawaldam. During May 1988 one An. fluviatilis positive for P. vivax was collected from house.

The density of An. stephensi in the study area was found as very low and therefore none of them were found as positive for sporozoite elisa. All other species were similarly found as in small numbers and none of them were also found as positive for sporozoite assays. Only An. culicifacies and An. fluviatilis were found as positive for P. falciparum and P. vivax circumsporozoite protein. The mosquito infections were detected in postmonsoon months i.e. August, September, October and November and in spring season i.e. March and May 1988. In case of patient fed specimens the females with comparatively large number of sporozoites showed higher optical density.

DISCUSSIONS

From the present studies it has appeared that not only An. culicifacies and An. stephensi (Rehman and Mutalib, 1967) but An. fluviatilis which was earlier considered as suspected malaria vector has also been observed as carrying sporozoites. Earlier laboratory studies have shown An. fluviatilis as capable of developing and transmitting parasites (McCarthy and Clyde, 1968). The dissections of field collected specimens did not reveal sporozoite but the elisa studies has revealed the presence of sporozoites in both An. culicifacies and An. fluviatilis. The elisa positives were dectected from the collections of August, September and October during post-monsoon rains as well as during March and May in spring season. In case of patient fed specimens the females with comparatively large number of sporozoites showed higher optical density when compared with small number of sporozoites. It is thus concluded that the optical density observed in sporozoite elisa positive specimens increased with higher number of sporozoites and was less with less number of sporozoites.

The number of mosquitoes assayed with Anopheles stephensi which is also a malaria vector in Pakistan and An. maculatus and An. pulcherrimus that are considered as suspected vectors was low because of their low density. Con-

sidering the recommendations of Bellanca (1986) collection efforts were increased. According to him as mentioned in his booklet, "Mosquito Surveillance Guide" that for the collection of Anopheline species longer collection periods are required because the number present may be low and that the index for this purpose should be bites per man per night and that the index for good malaria vectors may be less than one bite per man per night. According to him this could still produce a significant malaria rate even if the percent of infective malaria vectors were only one in 100. But inspite of increasing our collection efforts it was observed that the density of An. stephensi, An. maculatus and An. pulcherrimus was found to be very low in the area under study.

The number of sporozoite elisa positive specimens has appeared as low. It is very important to consider here that only one macrogametocyte is required for developing an oocyst and thus one or few oocysts go away without detection under the microscope. Faust and Russel (1953) has reported that at times of considerable importance in restricted areas every year one or more anopheline species enter into the category of malaria transmitters. According to Shute and Maryon (1957) mosquitoes can be infected with P. vivax even if the gametocytes are not seen in thick films but under such circumstances small number of mosquitoes with very small number of oocysts may be infected. It is concluded that collection efforts should comparatively be more in the

houses than in the cattle sheds while making collections for sporozoite detection. This technique is very useful in detecting even small number of sporozoites present in the salivery glands. Macdonald (1957) reports that experimentally mosquitoes having more than 50 oocysts develope infections in half the persons they bite and if the oocysts are less than 50, then comparatively the mosquitoes will infect more less.

The dissections can only be done when the specimens are fresh whereas elisa tests can be performed even if the specimens are frozen, dried or fresh. The dissections required are always too many and if one or two mosquitoes are found as positive the sporozoite rate appears as very low, usually less than even 1% (Warren, 1975) and thus usually the technicians feel dissection procedure as laborius and the sporozoite positive specimens may get missed unless dedicated technicians are available. With elisa large number of specimens can be tested at any time after the field surveys. The whole body of a mosquito should never be tested with this method. The tests should always be conducted on head plus thorax and not on the abdomen. The presence of oocysts may reveal positive elisa results but the sporozoites may not reach salivary glands and the mosquitoes may not transmit infections to humans.

Since entomological innoculation rate which is the product of sporozoite rate plus human biting rate is important for estimating malaria transmission in an area and predicting malaria epidemics (Onori and Grab, 1980) thus the human feedings by 9.78 percent An. culicifacies, 13.66 percent An. fluviatilis and 11.43 percent. An. stephensi can be applied for determining the above rate. The animals kept in the vicinity were also observed as low at the peripheries of the federal capital. Animal keeping is not allowed in these areas. However inspite of all these restrictions people at the peripheries of these cities do keep the animals for milk and small farmings. The feedings on man seem comparatively high. This is because of very close association and sleeping habits of men in the animal yards and sheds. For estimating the malaria transmissions this host diversion is very important.

During postmonsoon rain season the number of infections detected were comparatively more than those during March. In March one An. culicifacies and one An. fluviatilis and in May one An. fluviatilis were detected and these positives were those of P. vivax. No P. falciparum infected mosquito

was observed during spring season. Considering these findings it has been concluded that malaria vector control efforts may also be directed towards Anopheles fluviatilis as well because this species dominates during February to July in northern areas and plays its role in malaria transmission. It has also appeared that though the major malaria transmission season is the postmonsoon season but the transmission is also there during spring season as well though it is less when compared with the post monsoon season.

Only An. culicifacies and An. fluviatilis revealed sporozoite positive results during these assays. These positive mosquitoes were collected from Noor Pur Shahan and Nawaldam. Both of these villages are located outside Islamabad City where enough chances are available for anopheline breeding, the houses are made of stone plus earth or bricks with and without cement. The two villages are right at the periphery of the city. Cows, buffaloes, goats, sheep and chicken etc. are kept by the villagers and the people are low income dwellers either working in these respective villages or they go to the city for low paid jobs and labour. No mosquito control measures are being implemented in these areas. The rains in this area are usually during the cold months of December-January and heavy rains are during July-August which supply

scattered breeding places for anopheles. The breeding also occurs in slow moving shallow water streams going through the villages. Since the present studies has revealed sporozoite positive An. fulviatilis during these studies and this species prevail in most of the northern areas of the country, it is therefore suggested that in malaria vector control strategy, this species may also be considered as a target species alongwith An. culicifacies and An. stephensi. Secondly at this stage only one round of 2 gm per sq.m. malathion is being sprayed as indoor residual house sprays. The present studies has also revealed malaria transmission in spring season which was not confirmed earlier and it is therefore also suggested that vector control activities may also be done for cutting the spring transmission as well. Our findings regarding spring malaria transmission season has confirmed the suggestions of Zulueta et al., (1980). The writers earlier discussed the past data of malaria control programme and concluded the possibility of malaria transmission in spring season alongwith the peak transmission of pastmonsoon rains.

MALARIAL PARASITE SURVEY

Islamabad is located in hypoendemic zone and high endemicity has been recorded from time to time. (Pervaiz and Shah, 1988). Considering the detection of circumsporozoite protein during spring season as well as during postmonsoon season parasite survey was made during September-October 1988 and March-April 1989 to determine the parasite situation in the area under study.

MATERIALS AND METHODS

Slide Preparation:

Both thick and thin blood smears were made from different age group persons from the villages Noor Pur Shahan and Rawaldam. The slides were dried, numbered, wraped in paper and brought into the laboratory for further processing. Thick smear was dehaemoglobinized for 5 minutes in distilled water and thin smear was fixed by immersing in methyl alcohol. Stain preparation and staining was done as below:

Stain Preparation (Shute and Maryon, 1957; Faust and Russel, 1953).

Following chemicals for Field stain were mixed with the help of electric mixer untill dissolved.

FIELD STAIN A

Methylene Blue	1.6 gm
Disodium hydrogen phosphate (Na_2HPO_4)	10.0 gm
Potassium dihydrogen phosphate (KH_2PO_4)	12.5 gm
Distilled water	1000.00 cc.

FIELD STAIN B

Eosin	2.0 gm
Disodium hydrogen phosphate (Na_2HPO_4)	10.0 gm
Potassium dihydrogen Phosphate (KH_2PO_4)	12.5 gm
Distilled water	1000.00 cc.

Staining

The slides were then stained in field stain as follows (Shute and Maryon, 1957, Faust and Russel, 1953).

1. Immersed in Field Stain A for 2 seconds.
2. Rinsed in distilled water until stain stop flowing from the film.
3. Immersed in Field Stain B for 2 seconds.
4. Rinsed in distilled water and dried.
5. The slides were then dried and observed under oil immersion objective.

RESULTS AND DISCUSSION

348 samples were examined during September-October 1988 and 327 during March-April 1989. The results are presented in table no.12. During September 4 Plasmodium vivax and one P. falciparum was detected. In P. vivax infections the trophozoites, gametocytes and schizonts were seen. One case had large number of trophozoites. The case of P. falciparum revealed large number of trophozoites and scarce gametocytes. It was concluded that the infections were fresh. During October 3 P. vivax and 2 P. falciparum cases were detected. P. vivax infections revealed trophozoites, schizonts and gametocytes P. falciparum revealed large number of trophozoites but gametocytes were also present.

During March two P. vivax and one P. falciparum case was observed. One P. vivax case had large number of trophozoites but the schizonts and gametocytes were also seen. In P. falciparum case, the infection was light and both the gametocytes and trophozoites were observed. During

April two P. vivax cases were detected and both the cases had trophozoites, schizonts and gametocytes.

Parasite rate was found as 3.08, 2.69, 2.03 and 1.11 during September, October, March and April respectively. Parasite rate during postmonsoon malaria transmission season (5.77) was found as higher than of spring season (3.14). The inhabitants of both the villages are frequent travellers and most of them daily go to Rawalpindi-Islamabad and other nearby areas for work and other business. Since both the areas under study are also tourist places, therefore, other travellers are also coming daily to these areas. Most of the malaria cases are therefore imported. Local transmission is also there because of the weather condition and presence of vector species in the area. The capital and its vicinities have not been sprayed since 1981 and therefore mosquito vector density remains high due to absence of mosquito control measures. Only the higher class of people in the area can protect themselves from mo mosquitos by using mosquito coils and other chemicals that are available in the local market but the middle and poor class inhabitants in and around Islamabad are facing serious problems from the bites of mosquitos.

BIOLOGICAL APPROACH FOR CONTROL OF MOSQUITOES

In almost all the developing and developed countries chemical insecticides are used for mosquito control programmes. Due to continuous and indiscriminate use of these chemicals the adverse toxic effects on fishes, aquatic life and on other biotic flora and fauna is always threatening and the mosquitoes are also getting resistant towards these insecticides (W.H.O., 1976). Lack of proper knowledge to the man who is actually spraying the insecticide and in the residents where insecticide is being used, results into human poisoning and mammalian toxicity. In 1976 alone 2500 poisoning cases were reported from Pakistan and 5 out of these were fatal (W.H.O., 1979) and therefore in the same bulletin of W.H.O. recommendations for safe use of pesticides were made. Due to world over increase in insecticide resistance and other related hazards to biotic flora and fauna, the W.H.O. expert committee on vector biology and control discussed and suggested that counter measures including the biological control of resistant strains of disease vectors should further be investigated and encouraged (W.H.O., 1980). When compared these biological agents are always non-toxic either to fishes, field crops or man and other mammals (Davidson and Sweeney, 1983;

Mulla et al., 1984; de Bariac et al., 1987). In the present studies Bacillus thuringiensis H-14, Bacillus sphaericus 1592, Neem Melia azadirachta L., Bakain Melia azedarach L., domestic ducks and larvivorous fish have been used and compared. The details of these studies have been dealt with separately.

BACILLUS THURINGIENSIS BERLINER

Bacillus thuringiensis (family-Bacillaceae) was described for the first time in 1915 (Anonymous, 1973). Ignofo (1961) studied the toxic effects of Bacillus thuringiensis israelensis on pink bollworh, Pectinophora gossypiella . In 1968 Bacillus thuringiensis strain BA 068 was isolated from dead larvae of Culex tarsalis (Anonymous, 1973). The spores sometimes germinate in the insects but further development is very rare. Bacillus thuringiensis crystals get dissolved in alkaline midgut of mosquitoes and other insects and endotoxins are released. Proteases attack these endotoxins and change them into toxic proteins and thus the epithelial gut cells get swollen and destroyed and the larvae die away (Forsberg, 1976). Bacillus thuringiensis serotype H-14 was isolated from soil samples from the breading place of mosquitoes from the Negev Desert of Israel in 1977 (Goldberg and Margalit, 1977; deBarjac, 1978a; 1978b; 1978c) and it was named as israelensis by de Barjac 1978. It has got subterminal endosphores and crystals. These spores and crystals has got heat labile delta endotoxin, a protoxin that is formed during sporulation and act as stomach poisons

for mosquitoes and other insects (Tyrell et al., 1979, Lacey and Lacey 1981, Armstrong et al., 1985). Bacilus thuringiensis H-14 has been used against different mosquito larvae (Garcia and Des rochers, 1979; Tyrell et al., 1979; Panbangred et al., 1979; Sun et al., 1980; Mulligen et al., 1980; Ramoska et al., 1982; Aly, 1983; Lacey et al., 1984; Mulla et al., 1984). The standard bioassay for the potency assessment of Bacillus thuringiensis H-14 against different mosquito larvae has also been developed (Mclaughlin et al., 1984; Hilmy and Merdan, 1985). Bacillus thuringiensis H-14 is commercially available in the Market under the trade names such as Bactimos, Vectobac, Sandoz SAN-402 and Abbott ABG-8108 (Ignofo et al., 1980). Majori and Ali (1984) compared the commercial preparations of Bacillus thuringiensis H-14 for their efficacy as mosquito larvicides. Serotype H-14 is toxic to a number of mosquitoes (Garcia and Desrochers, 1980; Van Essen and Hembree, 1980; Hudson, 1985). Weiser and Prasertphon (1984) detected five isolates (three H-14, one 5a5c and one 8a8d from Culex pipiens "autogenicus" larvae. By 1982 25 isolates were recorded from different parts of the world (W.H.O., 1982). Lot of work is being done on the production, formulation and its efficacy against different anopheline and culicine mosquitoes in foreign countries (Bulla et al., 1977; 1980; Chilcot et al., 1981; Klowden et al., 1983; McLaughlin and Vidrine, 1984; Laird, 1984; Davidson and Yamamoto, 1984; Aly, 1985; Lacey, 1985; Aly et al., 1987; Berry et al., 1987). Purification of its mosquitocidal and

cytolytic proteins has also been done.(Hurley et al., 1987, Brownbridge and Margalit, 1987; Pearson and Ward, 1988). Its settling rates has been worked out (Mullen and Hinkle, 1988) and mosquitocidal proteins were studied by Hofte and Whiteley (1989). Ahmed et al. (1988) worked on its effects against Bangladesh Culex mosquitoes. Lot of similar research work is being done in foreign countries on different aspects of this microbial larvicide whereas in Pakistan Bacillus thuringiensis H-14 has been tried only against Anopheles culicifacies and An. stephensi in the laboratory (Rathore et al. 1985).

BACILLUS SPHAERICUS NEIDE

It was isolated from mosquito larvae by Singer (1973). It is an endospore forming bacilli. Endospores are rod-shaped with swollen terminal position. Bacillus sphaericus needs amino acids for its carbon and nitrogen sources (Singer et al., 1966) and when the bacilli are ingested by the larvae, alongwith other gut flora they get digested in the peritrophic membrane, crystals-like bodies get dissolved, toxin is released which penetrates the trophic membrane and the larvae die and then bacilli invade host tissue, posterior gut gets swollen and deteriorated (Dadd, 1971). Earlier it was considered that the strains of Bacillus sphaericus do not produce parasporal crystals (Myers and Younsten, 1978; Mayers et al., 1979;

Davidson,1979) but Davidson and Myers,(1981) reported that highly toxic strains produce parasporal inculsions resembling the crystals of Bacillus thuringiensis. Bacillus sphaericus has got almost 30 strains. Strain SSII-I was used in early days (Singer,1974; 1977; Davidson,1977) but it was found as unstable and ultimately the present day dry and powder form strain is being produced on large scale i.e. 1593. Strain 2362 (Singer 1985) and 2297 (Wickremesinge and Mendis,1980) are also as active as 1593. The effects of mosquito larval feeding behaviour on its efficacy (Weiser and Prasertphon,1984), the factors that influence its activity (Mian and Mulla 1983), its efficacy and persistance against mosquito larvae in organically enriched habitats (Mulla et al.,1984), on its effects against Culex quinquefasciatus (Lacey et al.,1984), and on its efficacy and field evaluation against floodwater mosquitoes (Mulla et al.,1984) has been reported. The effect of oxygen on its growth, sporulation and mosquito larval toxin formation has been studied (Yousten et al.,1984). Davidson (1984) worked on the biological aspects important in field use and Desrochers and Garcia (1984) reported persistance and recycling. Lacey (1985) worked on the production and formulation. Berry et al. (1987) worked for its effect on Culex pipiens and flood water Aedes larvae. Mulla et al.(1986; 1987) on the laboratory and field studies of Bacillus sphaericus against mosquito larvae and Lacey et al.(1987) worked on its long-term effects on Culex quinquefasciatus. Slooff (1987) emphasised the need for new biologicals and environmental management in the context of health for all by the year 2000 global strategy. Lacey et al.(1988) worked on

its efficacy against different anopheline and culicine larvae. Lee(1988) and Gharib et al.(1989)worked on its isolation and evaluation, Mulla et al.(1988)on the efficacy of Bacillus sphaericus 2362 formulations against floodwater mosquitoes and Laojana et al.(1989) against Anopheles sundaicaus.

In Pakistan malaria vectors and other mosquitoes are being controlled by selective spraying of malathion 2g/sq.m. in the places where at least one Plasmodium falciparum or 3 Plasmodium vivax cases are confirmed positive microscopically. The resistance has already been reported in both the species of Anopheles culicifacies and Anopheles stephensi towards malathion (Rathore et al. 1980; 1983; Rathore and Toqir,1980).

In our country Bacillus sphaericus has so far not been tried against Pakistan mosquitoes except Anopheles culicifacies and Anopheles stephensi (Rathore and Toqir ,1987). Present study was therefore designed to see the efficacy of Bacillus thuringiensis serotype H-14 (ABG 6145) and Bacillus sphaericus 1593 in the laboratory against Anopheles fluviatilis, Anopheles maculatus, Anopheles pulcherrimus, Anopheles annularis, Culex tritaeniorhynchus and Culex fatigans. Both of these biological pesticides were also tested in small field trials to see their efficacy and residual longevity against all culicines and the anophelines present in the area under study. Bacillus thuringiensis has also been tested in combination with neem and bakain extracts. The specific objectives of this study were:

1. To see the effect of Bacillus thuringiensis and Bacillus sphaericus against those species that

have not been tried earlier.

2. To demonstrate the comparative reduction of malaria vector abundance through larval control by these microbial larvicides.
3. To find out an integrated biological control approach for the control of mosquito vectors.

MATERIALS AND METHODS

Preparation of bacterial suspensions and stains.

Two different concentrations each of Bacillus thuringiensis serotype H-14 and Bacillus sphaericus were used for this purpose. 1 ml of both the Bacillus thuringiensis serotype H-14 and Bacillus sphaericus 1593 were added separately into 1 liter of water each and stirring was done with magnetic stirrer for making suspensions. Now 1 ml from eacn of these suspensions was taken and added separately into another 1 liter ot water each for making 1 ppm suspensions.

Gram's stain was used for staining purposes during these studies and was prepared as follows:

1. Ammonium oxalate crystal violet.

   Solution A

   i. Crystal violet 2 gm.

   ii. 95 percent ethanol 20 ml

Solution B.

i.	Ammonium oxalate	0.8 gm
ii.	Distilled water	80.0 ml

Solutions A and B were mixed with the help of electric mixer, kept for 24 hours and then after filtering transfered to the bottle for later use.

2. Iodine solution (mordant).

i.	Iodine	1 gm
ii.	Potassium iodide	2 gm
iii.	Distilled water	300 ml

Iodine and Potassium iodide were mixed in distilled water in an electric mixer and then stored in a dark bottle for later use.

3. Counterstain

i.	Safranine (2.5% solution in 95% methanol)	10 ml
ii.	Distilled water	100 ml

LC50 and LC100 of Bacillus thuringiensis serotype H-14 and Bacillus sphaericus 1593.

Anopheles fluviatilis, An. annularis, An. pulcherrimus, An. maculatus, Culex tritaeniorhynchus and Cx. fatigans were used for determining the LC50 and LC100 of Bacillus thuringiensis H-14 and Bacillus sphaericus 1593 for their control. The mosquito species included in the present study are of medical

importance (Barnett, 1967; Burney and Munir, 1968; Rehman and Mutalib, 1967; McCarthy and Clyde, 1968; Ludlam, 1968; Barnet et al., 1968; Baker et al., 1968; Akhter et al., 1981; Reisen and Boreham, 1982; Reisen et al., 1982; Hayes et al., 1982; Igarashi, 1987; Kamimura et al., 1987). Anopheles fluviatilis, An. annularis, An. pulcherrimus, An. maculatus, Culex tritaeniorhynchus and Cx. fatigans adult collections were made and after identifications the females were kept at room temperature in medium size paper cartons (height 17 cm, diameter 17 cm) for egg laying and larval development (Ainsely, 1976). Water was supplied in small cups for egg laying and 10% sugar solution soaked cotton pad was kept over the carton for the feeding of adults. The cotton pad was replaced daily to avoid fungal growth. The eggs were removed next morning and panning was done for hatching. Some of the first instar larvae were separated for the LC50 and LC100 tests and after one day the remaining larvae were diluted in other pans with about 150 larvae in each enamel pan size 40 x 25 cm.

Dillutions from 1 ppm were made for use in the tests. 10 larvae were separated into small petri dish having 50 ml distilled water and different concentrations were added into each petri dish for running the bioassays. Larvae were exposed to these doses for 24 hours. There were three replicates and one control with each test. Liver powder was provided as food for the larvae.

Field Trials.

Study area for the field trials was selected by finding the availability of natural ditches serving as larval breeding places. Ideal places were found in Noor Pur Shahan wnere 2.5 x 4 sq.m. artificial plots with a depth of 8-10 cm were made around the stream. This slow moving stream having nearly clean water with gravel bed serves as good breeding place for both anopheline and culicine larvae throughout the year except severe winter and drought. Larvae along the sides of the slow moving water were entraped alongwith water in the artificial plots. Bacillus thuringiensis H-14 and Bacillus sphaericus 1593 were used at the rate of 1 and 2 ml/sq.m. (0.13 quart/acre). Each concentration was uniformly and separately sprayed in the plots by using small flit gun. Each concentration was replicated twice and a control was kept for comparison of the results. Observations were recorded 2,4,24,48 and 72 nours after in plots treated with both the microbial pesticides. After 72 hours observations with Bacillus sphaericus were recorded weekly. For taking observations larval dips were taken, the larvae were transfered to clean cloth, counted quickly and then again transfered back into the same plot. Observations for Bti were taken upto 1 week and for Bacillus sphaericus as seven weeks after treatment.

Slide Preparation and Staining.

For confirming the death of larvae due to microbes, 25 larvae from each ditch were picked randomnly and each of them was torn on a microscopic slide with the help of needle. The smear was demarked with a black marker, slides were dried and heat fixed. Then the slides stained as follows:

1. Smear was stained with crystal violet for 2-3 minutes.
2. Washed in distilled water.
3. 3-4 drops of iodine solution were added on the smear and left for 2-3 minutes.
4. Washed in distilled water.
5. Decolourized with a solution of equal parts of acetone and ethanol.
6. Counterstained with safranine for 10 seconds.
7. Washed in distilled water, dried and examined.

RESULTS:

Results of LC50 and LC100 tests of Bacillus thruingiensis H-14 and Bacillus sphaericus 1593 are shown in table No.12 and 13. The differences in the susceptibility of Anopheles fluviatilis, Anopheles annularis, Anopheles maculatus, Anopheles pulcherrimus, Culex tritaeniorhynchus and Culex fatigans to Bacillus thuringiensis H-14 are clearly shown as indicated in the lethal concentration values. Based on these values Anopheles fluviatilis and Anopheles maculatus (LC50 0.045-0.14, LC100 0.08-1.0 ppm) are more susceptible than An. pulcherrimus and An. annularis as shown by its lethal concentration values. Anopheles pulcherrimus appeared as less susceptible (LC50 0.055-0.16, LC100 0.09-1.0 ppm) than the other three anophelines tested. Both the species of Culex tritaeniorhynchus (LC50 0.03-0.09, LC100 0.07-0.18 ppm) and Culex fatigans (LC50 0.03-0.09, LC100 0.07-0.25) were less tolerant than the anophelines tested. The LC100 values obtained in 24 hours were clearly higher than the LC50 values.

The differences in the LC50 and LC100 of Bacillus sphaericus 1593 against Anopheles culicifacies, Anopheles stephensi, Anopheles fluviatilis, Anopheles annularis, Anopheles maculatus, Anopheles pulcherrimus, Culex tritaeniorhynchus and Culex fatigans are also clear as indicated in the lethal concentration values. Anopheles annularis (LC50 0.06-0.165, LC100 0.12-0.8 ppm) was found as highly susceptible, Anopheles fluviatilis (LC500.07-0.17, LC100 0.1-0.7 ppm) and Anopheles maculatus (LC50 0.07-0.2, LC100 0.15-1.00 ppm) were less susceptible than Anopheles pulcherrimus (LC50 0.08-0.18, LC100 0.14-0.85 ppm). Culex fatigans was less susceptible (LC50 0.025-0.075, LC100 0.05-0.16 ppm) than Culex tritaeniorhynchus (LC50 0.025-0.07, LC100 0.05 -0.165). However the anophelines were found as more tolerant than the culicines bioassayed.

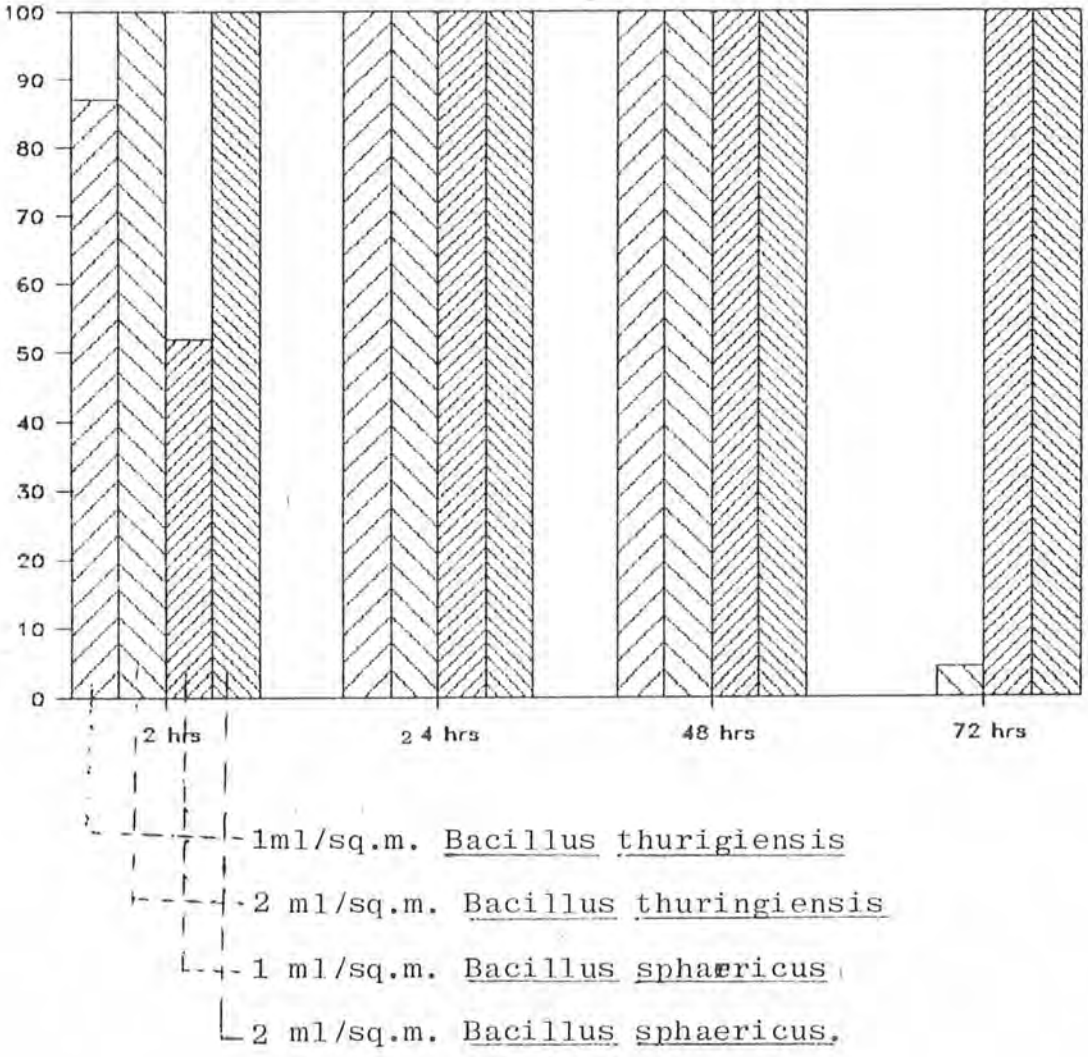
B. Field trials

Bacillus thuringiensis H-14

Before spraying the microbial pesticides the average larval dip count in 1 ml per sq.m. Bacillus thuringiensis gave 45.5 larvae per dip and in comparison control the larval density was 44.8 larvae per dip (Table 14, 15, Fig. 7). After 2 hours of Bti application larval count was found to be 6 larvae per dip in the treated site while in control the larval count was

FIG: 7

PERCENT REDUCTION IN LARVAL DENSITY AFTER EXPOSURE TO BACILLUS THURINGIENSIS H-14 AND BACILLUS SPHAERICUS 1593.



45.4 larvae per dip. Larval density reduced by 86.78 percent. After 24 hours of application the larval density in treated site was found as zero larvae per dip and in control the desntiy was 43.6 larvae per dip. The larval population reduced by 100 percent. After 48 hours of treatment there were no larvae in the treated sites but after 72 hours density in treated site was 50.4 larvae per dip and in control site as 49.1 larvae per dip count. Then the observations taken after one week showed larval density in treated and control sites as 52.5 and 48.4 larvae per dip.

In the site for 2 ml per sq.m. the average larval density before spray was 48.9 and 44.8 larvae per dip in the treated and control sites respectively (table 14, 15). After 2 hours of application the larval density site dropped to zero larvae per dip count in the treated site and it was 45.4 larvae per dip count in the control site. 100 percent reduction in larval density was observed. After 24 hours of application larval density in treated site was again found as zero larvae per dip count while in the control site larval density was observed as 43.6 larvae per dip count. 100 percent reduction in larval density was observed. Similarly no larvae were seen in treated sites after 48 hours of treatement but after 72 hours of treatment the larval density again increased and it was found as 47.6 larvae per dip count in the treated site. In control site the

desnity was found as 49.1 larvae per dip.

Bacillus sphaericus:

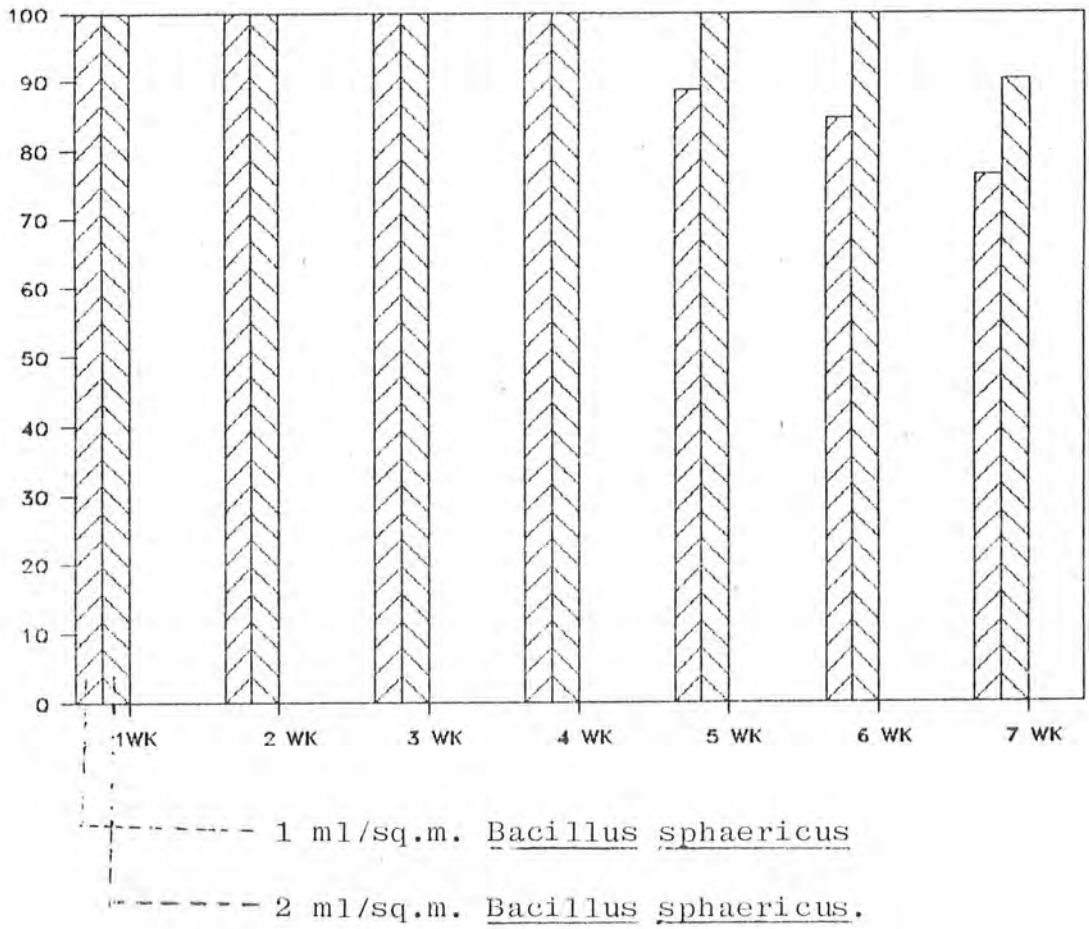
Before spraying Bacillus sphaericus as 1 ml per sq.m. the average larvae count in treated site was observed as 52.1 larvae per dip and in the control site larval count was found as 44.8 larvae per dip (table 14 and 16, Fig. 7). After 2 hours of microbial pesticide application larval density in the treated site was found as 21.8 larvae per dip and in the control site as 45.4 larvae per dip count. Reduction in population density was 51.98 percent. After 24 hours of application larval density in the treated site was zero larvae per dip count while 43.6 larvae per dip count in the control site. The reduction in larval density was by 100 percent.

From 1 to 4 weeks after treatment larval density in treated sites remained as zero. After 5 to 7 weeks it was found as 5.8, 7.6 and 12 percent in the treated sites and 51, 49 and 50.8 larvae per dip count in the control site.

The site where 2 ml per sq.m. Bacillus sphaericus was applied gave 51.6 larvae per larval dip count before pesticide application. The larval density in control site was 44.8 larvae per dip count. 2 hours after treatment the larval

FIG- 8

PERCENT REDUCTION IN LARVAL DENSITY AFTER EXPOSURE TO BACILLUS SPHAERICUS 1593.



dip count was zero and 45.4 larvae in the treated and control sites respectively. (Table 14 and 16, Fig. 8). 100 percent larvae control was obtained 2 hours after treatment. After 24 hours of pesticide application the observations showed that average larval density in treated and control sites was zero and 43.6 larvae per dip counts respectively. After one week of application till 6 weeks to larval dip count in treated site remained as zero whereas in control site 49-52.8 larvae per dip count were present. Seven weeks after treatment an average of 5 larvae per dip count were observed in treated site control site as 50.8.

DISCUSSION

Anopheline larvae were found as more susceptible to Bacillus thuringiensis H-14 with slight differences in their LC50 and LC100 values when compared with the same values of Bacillus sphaericus. Anopheles fluviatilis and Anopheles maculatus were found as more susceptible to Bacillus thruingiensis when compared with Anopheles annualaris and Anopheles pulcherrimus. Anopheles fluviatilis, Anopheles annularis and Anopheles maculatus were found as more susceptible to Bacillus sphaericus when compared with Anopheles maculatus and Anopheles pulcherrimus. The results obtained are according to the findings of Rathore et al. (1985) who tested Anopheles culicifacies and Anopheles stephensi and Bacillus thuringiensis H-14 and with the findings of

Lee (1988) who tested Anopheles balabacensis and Anopheles maculatus and found susceptible to Bacillus sphaericus strain 1593, 2297 and 2362.

Based on LC50 and LC100 Culex tritaeniorhynchus and Culex fatigans was highly susceptible to both the microbial larvicides. Culex larvae appeared as more susceptible to Bacillus sphaericus. The results obtained are in accordance with the findings of Lee (1988) and Rathore et al. (1985). Anopheline larvae were found as less susceptible to both the microbial larvicides when compared with Culex tritaeniorhynchus and Culex fatigans. This could be due to the surface feeding habits of the anophelines that could not ingest large number of bacterial spores from the bottom of water. The Culex tritaeniorhynchus and Culex fatigans larvae are bottom feeders and thus fed more vigorously on the bacterial spores. The results obtained are in accordance with the reports of Hudson (1985) who has reported that most probably the parasporal crystals of Bti settle down at the bottom and that better results could be obtained by developing floating and slow release formulations of Bt. H-14.

Bacillus thuringiensis H-14 showed good initial toxicity but short residual effect whereas Bacillus sphaericus lasted longer. William et al.(1987) has reported 12 to 76 percent reduction in larval population by using Bacillus thuringiensis H-14 but they also did not observe good residual effect. According to them the larvae of Culex pipiens and Aedes reinfested the breeding places after 48 hours

of exposure with Bt. H-14. During the present studies Bacillus thuringiensis H-14 has been used in combination with neem and bakain tree powders that has been mentioned in next chapter. From the present findings it has therefore appeared that the residual effect of Bacillus thuringiensis H-14 is short lived and is particularly effective for surface feeding anopheline larvae. Bacillus sphaericus has long residual effect and is particularly effective for bottom feeding culicine larvae. Both of these microbial larvicides can be applied for reducing the larval sources in mosquito breeding places, however Bacillus thuringiensis will have to be applied in every week. Both of these microbial pesticides will further be compared and discussed with the other larval control approaches in the coming chapters.

INSECTICIDAL EFFECT OF NEEM TREE, MELIA AZADIRACHTA L. AND BAKAIN TREE, MELIA AZEDARACH L. (FAMILY MELIACEAE)

INTRODUCTION

Neem Tree Melia azadirachta L. and Bakain Tree Melia azedarach L. family meliaceae are found in almost all over Pakistan. Neem tree is large and evergreen. Its flowering takes place in the months of March to May. Fruits ripe during July-August. This tree is mostly used as for shade or firewood in rural areas of our country.

The seeds/fruit and leaves of neem tree are used in indigenous medicine on very small scale by some people. Melia azadirachta contains a highly oxidized terpenoid, azadirachtin in the seeds (Butterworth and Morgan, 1968). Neem seed oil contains nimbin, nimbinin and nimbidnin (Zaman and Khan , 1972). The blossoms contain glucoside nimbosterin and a highly pungent essential oil nimbosterol, nimbecetin and fatty acids. Flowers contain a bitter substance and irritant bitter oil. Fruit contians bitter bakayanin and trunk yields nimbin, nimbinin and as essential oil. Bark is bitter and is used as tonic, leaves are antiseptic and are used in ulcers, eczyma and as poultice to boils. Gum is used as tonic in catarrhal affections. Oil is stimulant, antiseptic and is used in rheumatism and in skin diseases. The berries are used as purgatives and as anthelmintics (Ecyclopaedia Britanica, 1968; Butterworth and Morgan, 1968; Henderson et al., 1964; Connally et al., 1968). Occurance of triterpenoids and tetranorterpenoids (Selanin, Meldanin and Nimbidinin) has also been reported in neem seed oil (Henderson et al., 1964; Connally et al., 1968). Neem leaves can also be used against variety of forms for external

application against ulcers and skin diseases (Chopra et al., 1958). Effect of leaves extract of Melia azadirachta has also been studied against fourteen different species of pathogenic and nonpathogenic bacterial species and the bacterial extract has been found as active against Sarcinia lutia, all six strains of Staphylococcus aureus used in the studies, Bacillus cereus and Bacillus subtilis (Sheikh and Vahidy, 1983). Small fresh branches of neem tree are also used as tooth brushes (miswak) by people and for the past many years experience it has appeared that these chewing sticks or tooth brushes have not shown any visible toxic effect on man: Chewing sticks have variable antimicrobial activity against Bacillus subtilis, B. thuringiensis, B. megaterium, E. coli, Serratia maroesoens, Pseudomonas aeroginosa, Proteus vulgaris, Staphyllococcus aureus and the fungi Aspergillus niger and A. flavus (Khan et al., 1985)

Bakain or Persian Lilac Melia azedarach L. family meliaceae is a deciduous tree having dark grey bark. Its local names are drek or bakain. Leaves are 9-18 inches long and bi or occasionally tri-pinnate. Leaflets are 3-11, opposite or nearly so, ovate or lanceolate, glabrous, acuminate, serrate and some times lobed. Flowers are 3 inches long and fragrant. Drupe is 5 inches diameter, globose, 5-celled and 5 seeded or fewer. It ripens in March-May and the fruit ripens in cold season. Its leaves, bark and fruit mesocarp has got bitter taste. The tree grows at differents soils and situations all over Pakistan and is said to be indigenous to Jhelum Valley (Parker, 1918).

It has been reported that the plants of family meliaceae contain insecticidal effect (Chopra, 1928; Chopra et al., 1958). The extracts of Melia azadirachta L. and Melia azedarach L. has been used as a phagorepellent against locusts (Butterworth and Morgan, 1968; Lavie et al., 1967).

Neem seed extracts has been used against Aedes aegypti with only 2.6% fourth instar larvae mortality with 20 ppm extract in methanol (Zebitz, 1984). It has also bee reported that Ae. togi (theobald) and An. stephensi Liston mosquito species are comparatively more susceptible to neem seed extract when compared with Aedes aegypti (Zebitz, 1984; 1986). Neem leaves extract has been used against the larval stages of Culex fatigans (Chavan et al., 1979). Melia volkensii seed extract has been used against Aedes aegypti and it was found to have more toxic effects (Mwangi and Rembold, 1987; 1988) than the neem seed extracts in ethanol (Zebitz, 1984; 1986). Mwangi and Mukiama (1988) used the extract from Melia volkensii against Anopheles arabiensis and obtaining an LC50 of 5.4ug/ml in 48 hour exposure period. So far no research work has been done on the larvicidal value of either the Neem, Melia azadirachta L. or Bakain, Melia azedarach L. against Pakistan mosquito species. These trees are naturally available almost all over Pakistan. Therefore in the present research programme, laboratory studies and small scale field trials were designed to see the mosquito larvacidal effect of these trees. Extract has been obtained by different

methods and powder has been prepared by macerating and grinding the leaves and fruit. These preparations were used and then and compared to see the larvicidal effect of both the neem and bakain tree against Pakistan mosquito species. Specific objectives of this study were:

1. To see the effect of Neem and Bakain tree extract in reducing mosquito densities.
2. To see the combined effect of Bacillus thuringiensis H-14 plus neem tree.
3. To see the combined larval control effect of Bt. H-14 and Bakain tree.
4. To demonstrate the comparative reduction of malaria vector abundance through larval control by these biological vector control methods.
5. To select some of the suitable biological mosquito control approaches for the idea of shifting from the large scale use of chemicals towards biological approaches for integration into national mosquito control programmes.
6. To see the toxic effect, if any, of neem and bakain extracts on mammals.

MATERIALS AND METHODS

Laboratory Trial

A. Extraction in simple water: 250 grams each of Neem and Bakain tree leaves were boiled separately in 1 liter of tap water each until 100 ml extract was left. The solution was cooled and

then used to see its effect on 3rd and 4th instar larvae of different anophelines and culicines collected from Noor Pur Shahan. Mosquito fauna present in the area is Anopheles culicifacies, An. stephensi, An. splendidus, Culex tritaeniorhyachus. Culex fatigans, Aedes albopictus, Aedes culicinus, Ae. walbus and other anopheline and culicine complex found in the area in small numbers(Sholdz et al., 1988). Ground Neem seeds (fruit) were also tried for determining their toxic effect if any on mosquito larvae. Mature dried fruit were ground in mortar with pestle and were used as powder and as powder extract at the rate of 70 and 140 ul/ml for trials against mosquito larvae. For getting powder extract 50 grams of neem seed powder was boild in 500 ml of water until about 100 ml was left. This extract was used to see if it gives better results than the powder itself. 70 and 140 ul/ml of neem tree leaves and fruit extract and 50 gm fruit powder was used separately against 20 field collected 3rd and 4th instar anopheline and culicine larvae in petri dish and the experiments were replicated three times. Controls were also kept for comparison and the observations were recorded

6 and then 12 hours after exposure with the larvae and daily after exposure of unidentified mosquito pupae. Liver powder was provided as larval food during the experiments with mosquito larvae. For pupae collections were made from the field and 50 unidentified pupae were exposed to 140 ul/ml of the extract in small sized cups and these cups were then placed in paper cartons for adult emergence.

B. Extraction with Soxhlet Apparatus Method (Vogel,1964): Fresh neem and bakain leaves/fruit were collected and dried separately at room temperature. After macerating 10 grams of the leaves were weighed and wrapped in a cotton cloth. These leaves were then in the Soxehlet apparatus. 125 ml of Acetone, Hexane and distilled water (53: 44: 3) was also added in this apparatus in the flask. The Soxhlet Apparatus was then placed in a water bath with the help of a stand. The water bath was turned on until the temperature reached 80 ± 4°C. centrigrade. The apparatus was cooled with the help of cold water connected from the water tap to save the apparatus from overheating.

. Ten cycles for the extraction of principles from leaves were done for this purpose. At the end of extraction the apparatus was cooled by connecting the water bath with water tap, adding cold water and removing hot water from the water bath. The extract was then transfered into separating funnel and the funnel fixed in the stand to separate acetone-hexane from the crude leaves extract. Acetone-hexane formed an upper layer and the extract at the lower in the funnel. The extract was removed by opening the tape of the funnel, transfered to a separate bottle and stored in the feezer for bioassay tests against mosquitoes. 20 larvae each of Anopheles eulicifacies, Anopheles stephensi and Culex tritaeniorhynchus were tried during these assays. There were three replications and one comparison control.

C. Extraction in Ethanol, Methanol and Acetone:

250 gram of neem and bakain free fresh leaves and fruit were weighed separately and cleaned with tap water. These leaves and fruit were macerated and were then soaked in 250 ml of ethyl alcohol, methyl alcohol and acetone separately in a 600 ml beaker covered with paper lid and were kept at room temperature for overnight.

The extract was then filtered to separate from leaves, fruit or its coarse particles and kept in the incubator at 50

degree centrigrade for five days untill about 25 ml crude extract was left at the bottom of beaker. The incubator had a small opening at the top for expelling the vapours of ethyl alcohol, methyl alcohol and acetone. This crude extract was then stored at 4°C for later use.

Since the mortality observed with tap water extract after 12 hours of exposure was nearly the same for both the species of Anopheles culicifacies and Anopheles stephensi, therefore twenty 3rd plus 4th instar larvae of Anopheles culicifacies and Anopheles stephensi mixed in 1:1 were used into a small sized petri dish during this experiment. 50 ml tap water was added into this petri dish and liver powder was also provided as food to these larvae. The Crude Extract from neem leaves and fruit at the rate of 0.8 ul and 1.0 ul/ml was added into 50 ml of tap water in the petri dish having larvae with the help of precision microliter pipette.

Observations were recorded after one hour interval till death of larvae or then the final readings were taken after 24 hours of starting the experiment. Experiments were replicated thrice and three controls were also kept for comparison of the results.

Both Anopheles culicifacies and Anopheles stephensi pupae mixed in 1:1 ratio were also exposed to 0.8 and 1.0 ul/ml of tap water for determining the growth inhibiting effect of

the crude extracts. Obssrvations for this purpose were recorded after 24 hours of exposure. Experiment was replicated thrice and a control was kept for comparison of the findings.

D. Laboratory bioassays for lethal concentration (LC50 and LC100) of Melia azadirachta L. and Melia azedarach L.

Bioassays for determining LC50 and LC100 values of Melia azadirachta L. and Melia azedarach L. against larvae of Anopheles culicifacies, An. fluviatilis, An. stephensi, An. annularis, An. pulcherrimus, An. maculatus, Culex tritaeniorhynchus and Cx. fatigans were done similarly as with those of Bacillus thuringiensis and Bacillus sphaericus. 250 grams of leaves and fruit of both the neem and bakain tree were macerated and soaked in 250 ml methanol separately. Next morning the extract was filtered and was kept in the incubator at 50°C for the evaporation of methanol untill 2 ml extract was left. This extract was then assayed against larvae of different mosquito species. Batches of 10 larvae of 1st to 4th instar were separately exposed to different concentrations of the extracts for 24 hours. There was three replications and one control for comparison with each concentration.

Field Trials:

After getting results from laboratory trials, both the leaves extract and fruit powder were tried in field conditions for determining their effect on mortality of different anopheline and culicine mosquito larvae present in the area under study. Extract and powder preparations were done similarly as described for laboratory trials but this time whole quantity (250 gm) of the leaves extract and powder prepared were added separately into 1 sq. m. ditches prepated for this purpose along the stream in village Noor Pur Shahan. Most of the larvae in the area were those of culicine mosquitoes therefore anopheline larval collections were made from other breeding places in nearby places and these larvae were then added into the ditches for trials. The depth of water was 9 to 11 centimeter. About half the bucket of water was daily added into these ditches. Total number of replications were three and a control was also kept for comparison of the results. Observations were made by taking dips by using a cup 500 ml capacity. Observations were taken before and then daily afterwards from start of the experiment.

Combination of Neem and Bakain Leaf/Fruit Powder with Bacillus thuringiensis H-14.

Considering the larvicidal effect of both the neem and bakain extract and the short residual effect of Bacillus thuringiensis H-14, the extracts (powders) were tried in combination with Bacillus thuringiensis H-14 to see if these combinations give better results for the control of mosquito vector species. Since Bacillus sphaericus has long residual effect it was therefore not tried in combination with these powders. Leaves and fruit of these tree were thoroughly dried separately at room temperature and were finely ground into powder with the help of electric grinder. 2.5 x 4 sq.m. artificial plots having 8-10 cm depth were prepared around Noor Pur Shahan stream. Plots were treated with 2 ml/sq.m. Bacillus thuringiensis H-14 and 250 gm/sq.m. leaf/fruit powder used separately and broadcasted in the plots. Since the anopheline density in the plots made for this purpose was very low, Anopheles culicifacies, Anopheles fluviatilis and Anopheles stephensi adult collections were therefore done and after egg laying and raising larvae in the laboratory, these were rleased in the field plots. Lots of these larvae were released into the plots only at the start of the bioassay trials. There were three replications for each treatment. There was one comparison control for each of the culicine and anopheline group. Larval density was observed before and after treatments by taking larval dip counts with the help of standard 500 ml capacity dip. Observations were recorded 2,4,6,8 hours after treatments and then daily till the effect of treatment was over.

SAFETY OF NEEM AND BAKAIN TO MAMMALS

People use small branches of both the Neem and Bakain tree as tooth brushes (miswak) in our country. It has been observed that the constituents of these trees does not show toxic effects on the people who use these tooth brushes. Considering the insecticidal properties of these trees, the extract from both the leaves and fruit of these trees was given orally and subcutaneously to albino rats, Ratus norvegicus for determining the toxic effects on the these animals.

20 days old female albino rats were selected and separated into the cages. 26 Groups of 6 females each were kept in separate cages and observed for the abnormalities if any already present in their health. Only healthy and active females were selected. 0.1, 0.2 and 0.3 cc of the neem leaves, neem fruit, bakain leaves and bakain fruit extracted separately in methanol as described earlier was given orally to 12 groups. 12 groups were injected the same quantity of extract subcutaneously. One group was kept as control with nothing but ordinary feed and the other with 0.3 cc methanol for comparison of the results. Feed for these rats was prepared by mixing and then drying the following ingredients.

Wheat flour	2 kg
Poultry feed	5 kg
Fish meal	1 kg
Mollasses	100 g
Water	3 liters

Weight of body and internal organs

The females were weighed 20 days after giving the extract. Rats were also observed daily for the visible pathological abnormalities, if any. After 20 days the females were dissected to see the pathological abnormalities and their liver, lungs, spleen and kidney removed and weighed. Since blood sample was taken from the heart, the weight of heart was therefore not taken for observations.

Haematological Parameters

Blood was collected from the heart in heparinized vials. Serum was separated and estimation of haemoglobin (Hb) according to Van-Kampen and Zijlstra (1961), pached cell volume (PCV) according to Strumia et al. (1954), total leukocyte count (TLC) and total erythrocyte count (TEC) was examined to calculate the mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) according to Dacie and Lewis (1977).

RESULTS WITH NEEM

A. Extraction in Simple Water: The 3rd and 4th instar anopheline larvae when treated with 70 ul/ml leaves extract gave only negligible mortality of 3.3% within 6 hours after exposure but when the observations were taken 12 hours after exposure all larvae in three different replicates died except 2 larvae in 3rd replication and thus an average of 98% mortality was observed 12 hours after exposure. with 140 ul/ml of the neem leaves extract all larvae died within 6 hours of exposure giving 100 percent mortality

When 70 ul/ml Neem leaves extract was used against field collected 3rd plus 4th instar culicine larvae, it killed all the larvae within 12 hours after exposure when treated with 140 ul/ml of the same extract all culicines died within 6 hours after exposure. In control experiment all culicine larvae were still alive(table 20). There was significant difference in the mortality due to 70 ul/ml and 140 ul/ml neem leaves extract (X^2=10.526, df=1, $p<0.05$) after 12 hours of exposure but there was non-significant difference between the mortality of anopheline and culicine mosquitoes due to neem leaves extract (X^2 = 2.020, df=1, $p<0.05$).

In case of experiment with Neem fruit powder on 30 field collected anopheline larvae both the doses of 3 and 6 gm per 50 ml of water were effective in killing these larvae during exposure period whereas in comparison control all 30 larvae were alive (table- 20).

With 70 ul/ml Neem fruit powder extract when observations were taken 12 hours after exposure average anopheline larval reduction was observed as 51% and after 24 hours of exposure an average of 90 percent reduction was observed. In control experiment only one larva died during this period. 140 ul/ml Neem fruit extract revealed 87 percent mortality after 12 hours of exposure and after 24 hours the mortality was observed as 100%. In control experiment all larvae were still alive (Table- 20).

With 140 ul./ml leaves and fruit extract adults should have emerged one day after starting the experiment with pupae but no emergence was observed one day after starting the experiment. Pupae started dying and 77.3 percent pupal mortality was observed within 3 days of exposure. In control experiment only 2% mortality was recorded. Neem fruit powder extract revealed 100 percent pupal mortality after 1 day of exposure (Table- 17 - 19)

B. Extraction with Soxhlet Apparatus Method (Vogel 1964): The extract obtained with this technique did not give kill in either species of the mosquitoes. The highest concentration of 10 ppm neem seed extract gave an average of 15 and 10 percent mortality of Anopheles culicifacies and Culex tritaeniorhynchus respectively. The extract did not kill the larvae of Anopheles stephensi during 24 hours exposure period. These results could be due to the differences in the boiling points of the solvents used and the seeds and leaves of neem and bakain trees.

C. Extraction in Ethanol, Methanol and Acetone: Laboratory reared 3rd plus 4th instar larvae of Anopheles culicifacies and Anopheles stephensi when exposed to 0.8 and 1.0 ul/ml of Neem leaves extract in ethanol only a non-significant difference of 13.3 and 16.7 percent respectively was observed after 24 hours of exposure period (Table-21). With Neem leaves extract in methanol 46 and 55 percent respective mortality was observed after 8 hours of treatment with 0.8 and 1.0 ul/ml extract and 100 percent after 24 hours with all the treatments. 0.8 and 1.0 ul/ml of leaves extract in acetone revealed 51.7 and 53.3 percent mortality respectively.

Both 0.8 and 1.0 ul/ml of fruit extracts with ethanol and methanol revealed 100 percent mortality after 24 and 7 hours of exposure respectively. With acetone extraction 0.8 ul/ml extract revealed a mortality of 8.3% whereas with 1.0 ul/ml of acetone extract mortality was 78.3% (Table-22).

The fruit extract revealed better kill with methanol extraction when compared with ethanol and acetone (X^2=84.878). The extraction with ethanol also revealed better anopheline larval mortality than acetone (X^2=16.338). It appeared that there was no significant difference between different concentrations of ethanol extracts (X^2=0.261). There was non-significant difference between different concentrations of the extracts with methanol and acetone (X^2=00,0.033, df=1). Significant difference between the seed and leaf extract in ethanol was observed (X^2=8.571). Fruit extract revealed 100 percent mortality after 24 hours of exposure.

There was non significant different between leaf and seed extract in methanol (X^2=100) and both the extracts revealed 100% mortality after 24 hours of exposure. When the leaf and fruit extract in acetone was compared it appeared that fruit extract significantly revealed higher mortality after 24 hours of exposure (X^2=21.539, df=1, P 0.05).

D. As regards the LC50 and LC100 values, with neem tree leaves extract Anopheles culicifacies, Anopheles fluviatilis and Anopheles maculatus we more susceptible showing LC50 as 0.35 and 0.3 ul per ml respectively. Anopheles stephensi (LC50 0.25-0.45), Anopheles annularis (LC50 0.3-0.45) and Anopheles pulcherrimus (LC50 0.3-0.5) were found as less susceptible when compared with An. culicifacies, An. fluviatilis and An. maculatus. Results are shown in table-23. All the anophelines tested were found to be less susceptible when compared with Culex tritaeniorhynchus and Culex fatigans. With neem fruit extract Anopheles culici facies(LC50 0.2-0.4 ul per ml) and Anopheles stephensi (LC50 0.2-0.4 ul/ml) were found as more susceptible than Anopheles fluviatilis (0.3-0.45 ul/ml), Anopheles maculatus (0.3-0.45 ul/ml), Anopheles annularis (LC50 0.2-0.45 ul/ml) and Anopheles pulcherrimus (LC50 0.25-0.45). Results are shown in table-24. There was little difference in the susceptibility of either the anophelines or culicines to the extract.

RESULTS WITH BAKAIN

A. Extraction in Tap Water: With 70 ul/ml Bakain leaves extract anopheline motality was observed as 85.8% after 6 hours and as 100% after 12 hours of treatment. With 140 ul/ml treatment 100% mortality was obtained after 6 hours of exposure. With culicine larvae 70% mortality was obtained after 6 hours and 100% after 12 hours with 70 ul/ml. With 140 ul/ml treatment 100% mortality was obtained after 6 hrs of exposure. The effect of 70 ul/ml Bakain fruit extract revealed 70% mortality after 6 hours exposure, 95% after 12 hours and 140 ul/ml extract revealed 73.3% after 6 hours and as 100% after 12 hrs exposure. With culicine larvae 70 ul/ml fruit extract gave 59% and mortality after 6 hours of exposure and as 87% after 12 hours. 140 ul/ml treatment gave 97.5% mortality of culicines after 6 hours and as 100% after 12 hours of exposure (Table-25). There was significant difference in the mortality due to 70 ul/ml and 140 ul/ml bakain leaves extract ($X^2=5.128$, df=1, $P < 0.05$) after 12 hours of exposure but there was non-significant difference between the mortality of anopheline and culicine mosquitoes due to bakain leaves extract ($X^2=0.00$, df=1, $P < 0.05$).

When unidentified mosquito pupae were exposed to a dose of 140 ul/ml bakain leaves extract the adult emergence was delayed by 1 day. Leaves extract revealed 94.6% and fruit extract as 63.3% pupal mortality 2 days after exposure (Table- 26, 27).

B. Results with Extraction by Soxhlet Apparatus: The extract with this method did not reveal any larval control except Anopheles culicifacies and Culex tritaeniorhynchus that revealed 10 and 5 percent respective mortality. The mortality with Anopheles stephensi was found as nil.

C. Results with Ethanol, Methanol and Acetone: With 0.8 and 1.0 ul/ml leaf extract in ethanol percent mortality observed was 88.3 and 95 respectively after 24 hours. With 0.8 and 1.0 ul/ml extract in methanol 88.3 and 90 percent respective mortality was observed after 8 hours of exposure and as 100 percent after 24 hours. With 0.8 and 1.0 ul/ml of the extract with acetone a respective mortality of 45 and 46.6 percent was observed when the observations were taken 24 hours after exposure (Table-29).

Last season fruit extract in ethanol at 0.8 and 1.0 ul/ml gave 46.6 and 55 percent anopheline larval mortality after 24 hours of exposure. The extract in methanol at the same concentrations gave 35 and 58.3 percent mortality after 8 hours exposure and 100 percent after 24 hours exposure. The extract in acetone revealed 93.3 and 95 percent mortality after 8 hours exposure whereas 100 percent after 24 hours exposure at 0.8 and 1.0 ul/ml crude extract respectively (Table-51). With the fresh bakain fruit extract 98.3, 100,100,100,51, and 62.6 percent respective mortality was observed with 0.8 and 1.0 ul/ml of the extract in ethanol, methanol and acetone after 24 hours of exposure (Table- 30).

D. With bakain tree leaves extract Anopheles culicifacies (LC50 0.35-0.6 and LC100 as 0.45-0.8 ul/ml) and An. maculatus (LC50 as 0.4-0.60 and LC100 as 0.5-0.65 ul/ml) were found to be more susceptible than the other anopheline species i.e. Anopheles fluviatilis (LC50 0.4-0.6 and LC100 as 0.45-0.75 ul/ml), An. stephensi(LC50 as 0.35-0.6 and LC100 as 0.4-0.75 ul/ml), Anopheles annularis LC50 as 0.45-0.55 and LC100 as 0.55-0.80 ul/ml), An. pulcherrimus (LC50 o.3-0.55 and LC100 as 0.45-0.75 ul/ml). Culex tritaeniorhynchus (LC50 0.3-0.55 and LC100 0.35-0.65) and Culex fatigans (LC50 0.35 to 0.55 and LC100 0.45-0.55) were almost equally usceptible (Table - 31).

With bakain tree fruit extract An. maculatus (LC50 as 0.35-0.65 and LC100 as 0.45-0.8 ul/ml) was found as more susceptible than Anopheles culicifacies (LC50 0.45-0.7 and LC100 as 0.55-0.9 ul/ml), Anopheles fluviatilis (LC50 0.5-0.7 and LC100 as 0.6-0.95 ul/ml), An. stephensi (LC50 as 0.45-0.75 and LC100 as 0.5-0.85 ul/ml), Anopheles annularis(LC 50 0.45-0.55 and LC100 as 0.55-0.08 ul/ml) and An. pulcherrimus (LC50 0.45-0.7 and LC100 and 0.55-.085). There was negligible difference in the susceptibility of these anopheline species from the larvae of Culex tritaeniorhynchus(LC50 0.35-0.58 and LC100 as 0.5-0.8) and Culex fatigans (LC50 as 0.35-0.65 and LC100 as 0.45-0.75 ul/ml). The results are depicted in table-32.There was a little difference in the mortality of 1st and 2nd instar Culex tritaeniorhynchus with bakain leaves extract. Both the anopheline and culicine larvae were more susceptible to neem fruit extract as compared to neem leaves. Neem extract on the whole was found as more insecticidal when compared with the mortalities with bakain extract.

FIELD TRIALS

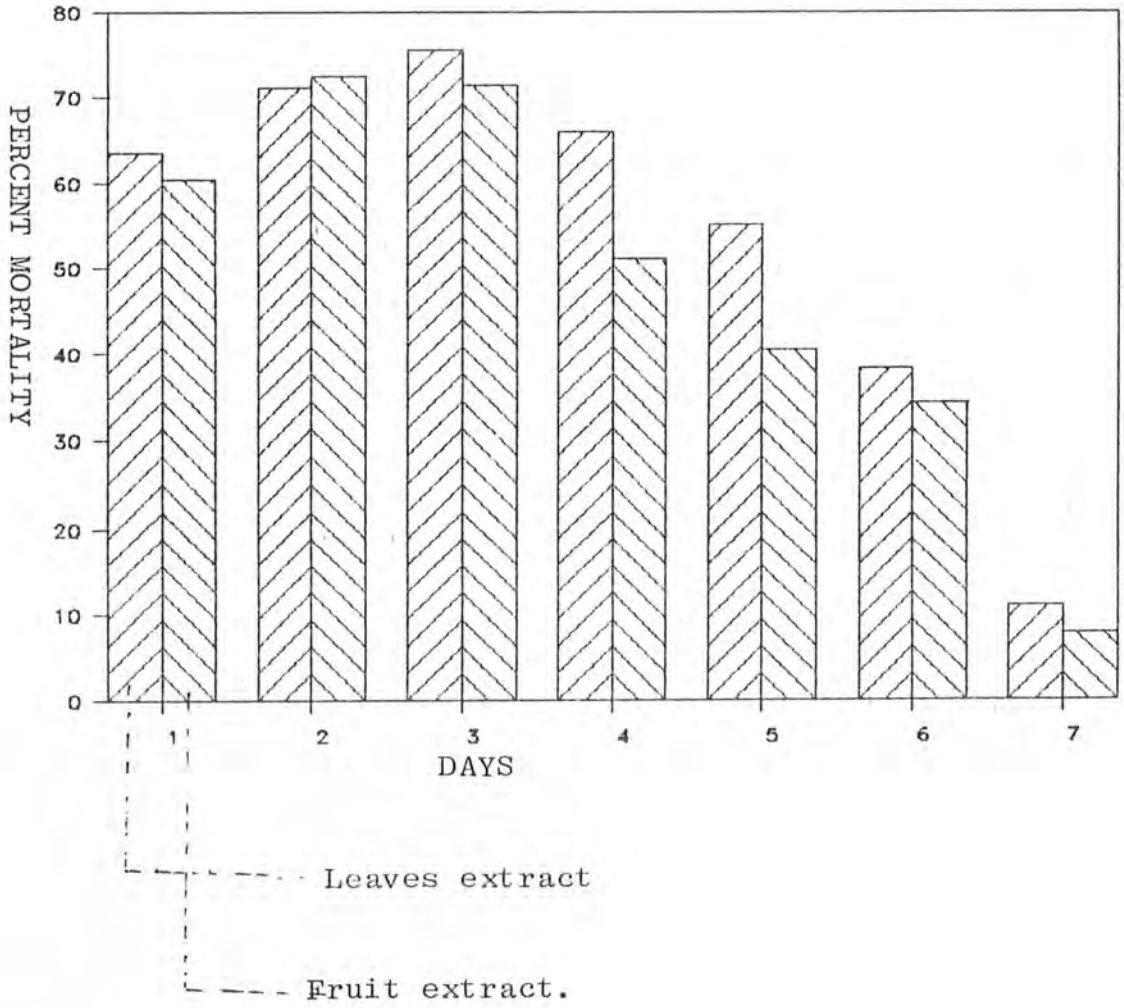
In field trials with Neem leaves extract average larval density (table-33, 34) in three replications was 34.3 larvae before starting the experiment. After one day of exposure average larval density in treated sites dropped to an average of 13.5 larvae, after two days to 10.7 larvae, after three days to 9.7 larvae, after four days to 11.9 larvae, after five days to 16.6 larvae, after six days to 25.3 larvae and after seven days the density observed was an average of 33.8 larvae. Percent larval density reduction after one day of exposure was 63.5, after two days 71, after three days 75.5, after four days larval density again started increasing and the percent reduction went down to 66.00 percent, after five days it was further down as 55.1 percent, after six days 38.1 percent and after seven days percent reduction in larval density was nonsignificant when compared with the control. In control experiment average larval density seven days after start of the experiment was 38 larvae per dip. Neem seed powder treatment revealed (table-35,36) an average of 36.9 larvae per dip count before treatment in three of the replications. One day after treatment average larval density was down to 14.6, two days after to 10.2, three days after to 10.9, four days after to 17.1, five days after to 22, six days after to 27 and seven days after to an average of 35 larvae per dip count. After one day of the treatment percent reduction in larval density was 60.5, after two days as 61.6, after three days

as 71.3 but on fourth day after treatment the larval density again started increasing and percent reduction went down to 51.1, on fifth day to 40.5, on sixth day to 34.4 and on seventh day it went down to negligible difference of 7.9 percent. Mortality due to neem leaves extract (X^2=4.792), df=1, $p < 0.05$) and neem fruit extract (X^2=6.037, df=1, $p < 0.05$) differed significantly when compared with the mortality in comparison control (Fig. 9).

Larval density in the plots treated with Bakain leaves extract remained as low upto 5 days after treatment but on fifth day the larval density stated increasing. The results are presented in tables 37 and 39. Average larval density in plots where treatments of leaves extract were given was found as 35.7 larvae per dip count before starting the experiment. In control average larval density was recorded as 34.8 larvae per dip count and there was non-significant difference between treated and control plots. When the observations were taken 1 day after treatment average larval density in treated plots dropped to 8.5 while in control it was an average of 35.8 larvae per dip count. Percent reduction in larval density observed after 1 day of exposure was 76.2%. After 2 days of exposure average larval density in treated plots was found as 5 larvae per dip count

FIG- 9

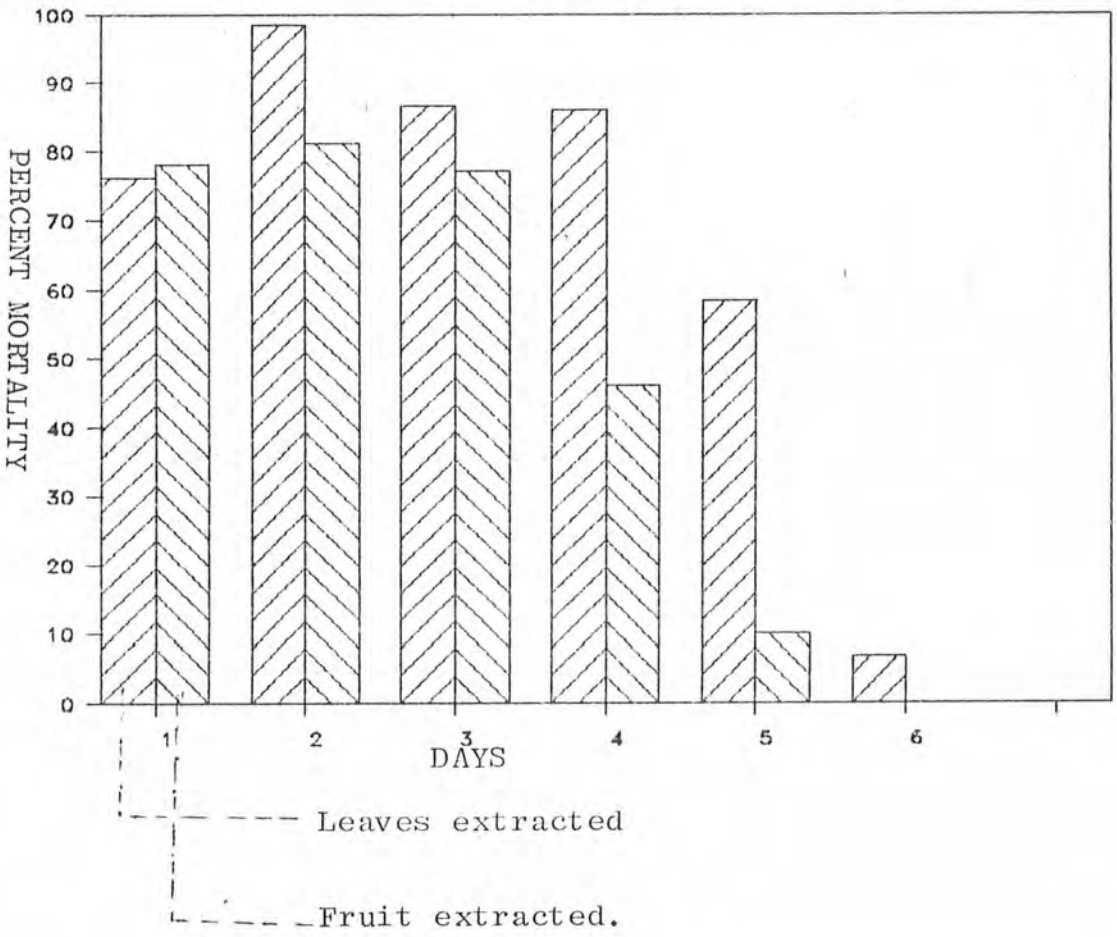
PERCENT REDUCTION OF MOSQUITO LARVAL IN FIELD TRIALS AFTER EXPOSURE TO NEEM LEAVES AND FRUIT POWDER IN WATER



in three of the replications. In control average larval density was observed as 35.8 larvae per dip count. 98.6 percent reduction in larval density was observed after 2 days of exposure with Bakain leaves extract. Average larval density 3 days after treatment was observed as 4.6 larvae per dip count showing 86.6% reduction, 5.6 larvae per dip count after 4 days showing 85.9% reduction, 15.5 larvae per dip count showing 58.5% reduction after 5 days of treatment(Fig.10).Then onwards the density again started increasing and reached to non-significant level with mortality in comparison control on 6th day after treatment. A reduction of only 6.7% was observed after 6 days of treatment. Average larval density in control plot remained in the range of 34 to 39 larvae per dip count. On 7th day after treatment average larval density in treated plots was 34 larvae and in control it was 36 larvae per dip count respectively showing larval reduction only to a non-significant level as 5.6. On 8th day after treatment it was 34 larvae per dip count in treated plots and in control it was 40 larvae per dip count thus showing non-significant difference in control and treated plots on the 6th, 7th, and 8th day after treatment. Mortality due to bakain leaves extract ($X^2=6.723$, df=1, $P < 0.05$) and bakain fruit extract ($X^2=4.037$, df=1, $P < 0.05$) differed significantly when compared with the mortality in comparison control.

FIG-10

PERCENT REDUCTION OF MOSQUITO LARVAE IN FIELD TRIALS AFTER EXPOSURE TO BAKAIN LARVAE AND FRUIT EXTRACT IN WATER.



Bakain fruit extract killed the larvae and reduced their density upto 3 days after treatment (table-38-39) but on 4th day after treatment density again started increasing and reached to non-significant level to that of comparison control. Before treatment it was observed as an average of 38.2 larvae per dip count in the plots for treatment and as 34.8 larvae per dip count in control site. 1 day after treatment average larval density dropped to 7.9 larvae per dip count in treated plot while in control it was 35.8 larvae per dip count and thus 78% reduction in larval density was recorded. After 2 days of treatment average larval density in the treated site was 6.8 larvae per dip count whereas in control it was 36.2 larvae per dip count and a reduction in population density was recorded as 81.2%. On 3rd day after treatment larval density in treated plot was 7.9 larvae per dip count whereas in control plot it was 34.4 larvae per dip count and 77.2% reduction in population density was recorded. 4th day after treatment the density started increasing again and reached to an average of 21.4 larvae per dip count in the treated plot and 39.6 larvae per dip count in control plot showing larval reduction of only 46.6%. On 5th day onwards larval density in treated and control plots showed non-significant difference.

Combination of Neem and Bakain Leaves/Fruit Powder with Bacillus thuringiensis H-14.

When neem leaves powder was applied in combination with Bacillus thuringiensis H-14 hundred percent of anopheline and 87.8 percent reduction in culicine larval density was recorded after two hours of application. Considering the average larval stage, the experiment was conducted upto seven days after treatment and no larvae were observed in the treated plots during this period (table-40, 44).100 percent larval reduction was observed with both the culicine and anopheline larvae in the plots treated with neem fruit powder plus Bacillus thringiensis H-14 from two hours till seven days after treatment (table-41, 44) Similarly 100 percent larval control was achieved after two hours of treatment till seven days after treatment in the plots treated with the combination of bakain leaves powder and Bacillus thuringiensis H-14 (table-42, 44) whereas in the plots treated with bakain fruit powder and Bacillus thuringiensis H-14 81.8 and 77.6 percent respective control was achieved after two hours of treatment in the plots of anopheline and culicine larvae. After four hours of this treatment hundred percent larval mosquito control was achieved and it remained so till four days after treatment. On fifth, sixth and seventh day after treatment with bakain fruit powder plus Bacillus thuringiensis H-14 larval density started slowly increasing. Percent reduction in the larval density observed on fifth, sixth and seventh day after treatment was observed as 69.3 and 80.2, 37.4 and 68.6 and 33.4 and 67.5 percent with anopheline and culicine larvae

respectively (table-43, 45). Bakain fruit treatment appeared as comparatively less active than the leaves. With anopheline larvae when the bakain fruit powder plus Bacillus thuringiensis was compared with either of the bakain leaves/ neem leaves or fruit powder plus Bacillus thuringiensis H-14, there was insignificant difference (X^2=0.084) between this and the other treatments. There was no difference from 4 hours after treatment till four days after treatment of anopheline larvae with bakain fruit treatment plus Bacillus thuringiensis H-14 but from fifth day onwards significant difference was observed (X = 45.31) between fruit and other treatments. Three of the other treatments remained effective upto seven days after treatment. With culicine larvae significant difference (X^2 = 32.50) was observed in the mortality with bakain fruit powder plus Bacillus thuringiensis H-14 and the other three extracts plus Bacillus thuringiensis H-14. From four hours till four days after treatment all treatments gave complete control of the culicine larvae but from fifth day onwards significant difference (X^2=21.97) was observed in the mortality due to Bakain fruit powder plus Bacillus thuringiensis H-14 and the other three treatments. In comparison controls for both the anophelines and culicines, larvae were still present with increasing number of pupae seven days after start of the experiment.

SAFETY OF NEEM AND BAKAIN TO MAMMALS

The weights of whole body, liver, lungs, spleen and kidneys after exposure to 0.1, 0.2 and 0.3 cc each of the leaves and fruit extract of both the neem and bakain trees are shown in table- 46. The weight of whole body, liver lungs spleen and kidneys in 40 days old rats kept as methanol and normal controls was observed as 47 - 47.03, 2.7 - 2.4, 0.45 - 0.5, 0.2 - 0.15 and 0.5 - 0.55 grams respectively. After 20 days of oral feedings and subcutaneous injection of 0.1, 0.2 and 0.3 cc each of the leaves and fruit of both the neem and bakain tree extracts in methanol did not show any significant changes in the weights of whole body, liver, lungs, spleen and kidneys.

The haematological values are depicted in the table 47 . No significant difference between the normal and those treated with the extracts were observed during these studies. Highest and lowest haematological values after administration of different concentrations of neem leaves and fruit extract were observed as 13.45 - 13.98 g/dl haemoglobin, 6.52 - 7.02 $X10^6$ total erythrocyte count, 5.52 - 6.28 $X10^3$/ul total leukocyte count, 41.14 - 42.95% packed cell volume, 61-66 - 66.08 fl mean corpuscular volume, 20.33-20.94 g/dl mean corpuscular haemoglobin and 30.70 - 31.52 g/dl mean corpuscular haemoglobin concentration.

With the treatment of different concentrations of bakain leaves and fruit extract the highest and lowest haematological parameters were observed as 13.62 - 13.96 g/dl haemoglobin, 6.48 - 8.01 $X10^6$/ul total erythrocyte count, 5.75 - 6.22 $X10^3$/ul total leukocyte count, 41.14 - 43.12% packed cell volume, 61.80 - 64.31 fl mean corpuscular volume, 20.36-20.86 g/dl mean corpuscular haemoglobin and 30.76 - 31.46 g/dl mean corpuscular haemoglobin concentration.

With normal control these values were observed as 13.69 g/dl haemoglobin 6.58 $X10^6$/ul total erythrocyte count, 5.78 $X10^3$/ul total leukocyte count, 42.76% packed cell volume, 61.92 fl mean corpuscular volume, 20.34 g/dl mean corpuscular haemoglobin and 30.98 g/dl mean corpuscular haemoglobin concentration.

The weight and haematological parameters with the treated and untreated albino rats were found within normal range and no abnormal pathological effects were observed during these studies. The extracts did not reveal any adverse effects during handling as well and it has appeared that these extracts are non-toxic and safe to man and other mammals.

DISCUSSION

From laboratory trials with both the bakain and neem tree extracts it has been observed that the extract has got insecticidal action on mosquito larvae and pupae. The extract formed thin film on water surface in laboratory as well as in the field experiments which stopped breathing of immature mosqui stages and then resulted into their death. Mortality could be both due to the action of thin surface film as well as from bitter constituents of the extracts. The extracts of this tree are not only toxic to larvae but these are equally destructive to pupal stages as well. When the extracts of neem and bakain trees were compared, both the leaves and fruit of neem tree revealed better kill of anophelines and culicines tested. Methanol extract both from the leaves and fruit of neem tree exhibited comparatively better anopheline larval mortality than the extracts with ethanol and acetone. It exhibited 100 percent mortality after 24 hours of exposure with 0.8 and 1.0 ul/ml leaf extract. The extract by soxehlet apparatus from both the trees did not reveal good larval control. This could be due to the differences in the boiling points of the solvents used and the leaves/seeds of the trees.

When compared Bakain leaves extract revealed better kill than the fruit extract in simple water. It has also appeare that as observed with neem, the bakain leaves and seed extract in methanol also exhibited good larval mortality than other extractions with ethanol and acetone. When the extracts were tried in small field trials, the residual effect of the extracts lasted only upto nearly 5 days with neem leaves and four days with neem seed powder. Bakain extracts lasted only upto 5 days with leaves and 3 days with fruit. It has thus appeared that the quantity of extracts

used during these trials was low and thus the adults layed their eggs somewhere 2 to 3 days earlier. When the leaves and fruit were used in combination with Bacillus thuringiensis H-14 in small field trials, Neem leaves plus Bti, Neem fruit plus Bti, Bakain leaves plus Bti and Bakain fruit plus Bti revealed comparatively better control for 7,7,7 and 5 days respectively. It has also been observed that the extract from both the trees is completely non-toxic to man and other mammals. The extracts did not show any abnormal effects on albino rats.

Since 1976 malathian 2 gm/sq.m. is being used as indoor residual spraying for the control of malaria vectors in our country. Presently it is being used selectively in only those areas which are positive for malaria cases and have atleast 2 Plasmodium falciparum or 6 Plasmodium vivax cases. Mosquito vectors are gradually getting resistant to malathion. Since these trees are commonly available in our country and are not being used for any purpose except shadow from hot sun during summers and as firewood the products can therefore be utilized in unwanted water collections serving as breeding places for mosquitoes. It is suggested that leaves and fruit of both the neem and bakain trees may be used in rice fields. These leaves can be either beaten into small pieces or ground for easy broadcasting in rice fields. This practice can further be aided with the closing of scattered breeding places. This will not only help in controlling the mosquito vectors but these leaves and fruit will also serve as compost manure for the crops. The leaves and fruit of both the trees was also studied in combination with a local fish species that will be discussed in the chapter for larvivorous fish.

DOMESTIC DUCKS

INTRODUCTION

Domestic ducks, Anas platyrhynchos are well known aquatic birds. They belong to family anatidae and subfamily anatinae. Domestic ducks are kept for egg and meat production in the villages in our country. In England and Holand these are commercially used for egg production and in U.S.A. for meat production. They feed on small molluscs, crustaceans, frog tadpoles, water insects and other small animals. As far as the available information and literature is concerned, the ducks have not yet been tried to see their larvivorous habits. Therefore considering the aquatic feeding habits of domestic ducks, these aquatic birds were tried to see if they feed on mosquito larvae and if these animals could be of any help in reducing the mosquito populations in the country.

MATERIALS AND METHODS

For this purpose six domestic ducks were purchased from Aabpara local market and were then kept in a closed room where they were provided with bread pieces in a con-

tainer almost half filled with water.

Approximately 1600 to 2000 mosquito larvae were collected from the field with the help of a cup and a strainer. Most of these larvae were culicines. These were transfered into a tin having small quantity of water and were then brought into the room where ducks were kept. Other parasitic fauna was also present alongwith mosquito larvae.

Larvae were approximately divided into 3 lots. All of these larvae were then transfered into a pan having water in it. Another container with bread pieces in water was also provided to the ducks for the comparison of results.

Observations were recorded if the ducks were reducing larvae. These observations were made after every 30 minutes upto four hours.

After 4 to 6 hours ducks were slaughtered to see the presence of larvae in the gut.

The experiment was replicated three times.

RESULTS AND DISCUSSION

In one replicate ducks devoured all mosquito larvae and other parasitic fauna within two hours. The ducks were seen as picking larvae from and under water surface with the help of its bill. The water became comparatively cleaner after duck feeding. They did not even touch the container having bread. The intestines upon opening revealed dead larvae. Gizzard revealed only sand particles.

The idea for confirming if the ducks had eaten larvae or not was to see the presence of larvae in the container and then to slaughter the ducks but after about 4 hours when the larvae were eaten up, ducks started expelling larvae in their droppings. Droppings were seen as containing lots of dead larvae.

In second replicate the ducks were slaughtered after four and a half hours. About 50 larvae were still present in the container. Bread pieces in other container were also eaten up by the ducks. Dead larvae were seen in the droppingsand upon opening the intestines dead larvae were seen in them. Gizzard revealed sand and other food particles but no mosquito larvae.

In third replicate the ducks had eaten larvae within three hours and the bread container was lying as such without being eaten. Dead larvae were again seen in the droppings and in the opened intestine.

Domestic ducks were found as prefering mosquito larvae and other small parasitic animals when compared with bread pieces in water where as on other occasions they regularly ate bread pieces dropped in water. Since they feed on water insects and other small animals in water and are also a good source for egg and meat production, these animals should be tried in malaria control programmes initially as small projects separately in all the four provinces including Azad Kashmir to see the results and then the large scale rearing of ducks on government farms and providing at comparatively cheaper price to the low income dwellers at the peripheries of the cities and in the villages will be of help in reducing mosquito larvae.

W.H.O. (1980) has also considered the worldwide increase in insecticide resistance and other related hazards to biotic flora and fauna and thus the expert committee on vector biology and control discussed and suggested that counter measures should further be investigated and encouraged. It is therefore concluded that if the people are

once encouraged and motivated for keeping ducks for the control of mosquitoes, this will not only help in reducing mosquito borne diseases and in increasing egg and meat production but will also be of help in reducing other vector borne diseases in the country.

LARVIVOROUS FISH

INTRODUCTION

Mosquito larvae are closely associated with fish in their feeding habits. Larvivorous fish have been used for mosquito control in many foreign countries. Mosquito fish Gambusia affinis (Baird and Girad) has been shown by several workers as effective for the control of mosquito larval stages (Craven and Steelman, 1968; Hoy and Reed,1970). Davey et al. (1974) used various fish species for the control of dark rice field mosquito in Arkansas State of America. Legner and Fisher (1980) used Tilapia zillii against mosquitoes in Lower Colorado Desert Irrigation Cannals. The ecological studies of Gambusia affinis has been done earlier (Hirose et al.,1980; Farley,1980; Robinson et al., 1982; Bence and Murdoch,1983). Lot of other information on Larvivorous fish is available, however considering the volume of the work for the present studies important information is presented here. Gambusia affinis has been applied in combination with Temphos for the control of Culex tritaeniorhynchus (Mathur et al., 1982), for mosquito control in Casuarina rice fields (Shumkov et al., 1982; Zainiev and Muminov,1983; Stewart et al.,1983) and for mosquito control in underground drains (Mulligan et al.,1983). Different species of carp family have been tried against mosquitoes (Shumkov,1983). It has been observed that the monomolecular layer of ISA-20E

had no deterimental effects on fresh water fish (Webber and Cochrane,1984). These larvivorous fish have been used against different mosquito species (Wongsiri and Andre,1984; Miura et al., 1984; Meish,1985). The econological studies of Gambusia affinis has been studied by Lightenberg and Getz (1985). Kramer et al., (1987) did not find any reduction in larval population due to Gambusia affinis. Combs (1986) reported that mosquito control could be achieved by releasing mosquito fish at a higher rate of 3.4 kg per hectare. World Head Organization Published information on Tilapine fish for its use against disease vectors (WHO 1987). The inland Silverside, Menidia beryllina and Gambusia affinis have been used in wild rice fields (Kramer et al.,1987) and for the control of mosquitoes in crawfish ponds (Holck et al., 1988). Ecology of Gambusia affinis has also been studied by Sato (1989).

In Pakistan local fish species are available but no work has been done on their efficacy for the control of disease vectors. Different species of fish were therefore tried in the laboratory as well as in small field trials to see their larvivorous habits for reducing larval populations in mosquito breeding places. Four species of fish, Mori (Cirrinus mrigala), Tilapia (Tilapia mossambica), Silver Carp (Hypophthalmichthes militrix) and Green Carp (Ctenopharyngodon idellus) are the imported ones and have been localized in Pakistan. These four species were obtained from government fisheries department and

the other five i.e. Singhi (Heteropheusteus fossilis), Rohu (Labio rohita), Daula (Channa striatus), Kangi (Colisa fasciata) and Chidu Fish (Punctius ticto) were collected from the field. These fish species have been initially tried in the laboratory. Small sized Chidu fish, a surface feeder that breeds in clean as well as polluted water was particularly selected after laboratory studies and was then tried in small field trials. The specific objectives of this study were:-

1. To see the efficacy of each of these fish species as larvivorous.
2. To select some of the suitable larvivorous fish for mosquito control.
3. To see the efficacy of chidu fish in combination with neem and bakain.
4. To see the toxic effects if any of neem and bakain on the larvivorous fish.
5. Effects of deweeding in mosquito control programmes.

MATERIALS AND METHODS

Laboratory Trials

Four fish species were obtained from government hatchery and the other five were collected from the field. These

fish were kept in 48 x 12 cm plastic containers. Laboratory reared anopheline larvae were provided as food to these fish.

Lots of four hundred laboratory reared Anopheles culcifacies and Anopheles stephensi were counted separately with the help of rubber pipette and were then transferred to small sized plastic containers 17.5 x 13.5 x 8.5 cm in size. Plastic containers used for the study were washed with detergent and distilled water before adding the fish. Water collected from the breeding places of the fish was provided in these containers. Natural water with and without weeds was tried during these studies. Four to five centimeter long two fish of each species were released into each of these containers and the number of alive larvae were counted after 24 hours.

Initially fourth instar larvae were used for the trials but later on second and third instar were also tried. The fish was also dissected at the end of the experiment to see the presence of larvae in their gut. The experiment was replicated five times and control was kept for comparison of the results.

Field Trials

For field trials the experimental plots were 4 x 1.5 x 2 sq.m. water collection ditches. Both of anopheline and culicine

larvae were available in these natural breeding places but the density was very low in the range of about 1 to 6 larvae per 500 ml dip count. Therefore, laboratory reared 2nd, 3rd and 4th instar mixed larvae of Anopheles culicifacies and Anopheles stephensi were introduced two days before taking the observation on the larvivorous effect of this fish for increasing larval density for better comparison of results. For this purpose laboratory reared larvae were first of all introduced into natural water to see if the laboratory reared larvae remain alive in natural breeding places or not. After confirming the life of laboratory reared larvae in natural breeding places approximately equal number of about 5000 larvae were introduced into these experimental plots. Small size(upto about 5 centimeter) Chidu fish which was already tried in the laboratory to see its larvivorous habits was collected from the field and this fish was released at the rate of 1 fish per square meter, i.e. 12 fish per plot. The observations were taken by using standard 500 ml dip cups before and after release of fish in the plots. Fish from each plot were also dissected during the trials to see the presence of larvae in their guts. The experiment was replicated thrice. Control plot without fishes was kept for comparison of the results. Since Anopheles culicifacies, Anopheles fluviatilis and Anopheles stephensi are the major malaria vectors, therefore, attention has been paid for the control of these species in particular and other mosquito species in general. In natural breeding places larvae of

Anopheles culicifacies, Anopheles stephensi, Anopheles subpictus, Culex fatigans and Culex tritaeniorhynchus were present in small numbers at the time of release of laboratory reared larvae.

Combination of Chidu Fish with Neem and Bakain.

This experiment was done to see the combined larval control effect of Chidu fish with neem leaves, neem fruit, bakain leaves and bakain fruit. 250 grams of leaves and fruit of both the trees were separately combined with one chidu fish per square meter. Each treatment was replicated thrice and a control without treatments was kept for comparison of the results. After the completion of this study fish were collected and kept in the laboratory under observation for toxic effects if any due to the leaves and fruit of these trees to the fish. The fish were observed upto 15 days for abnormalities if any.

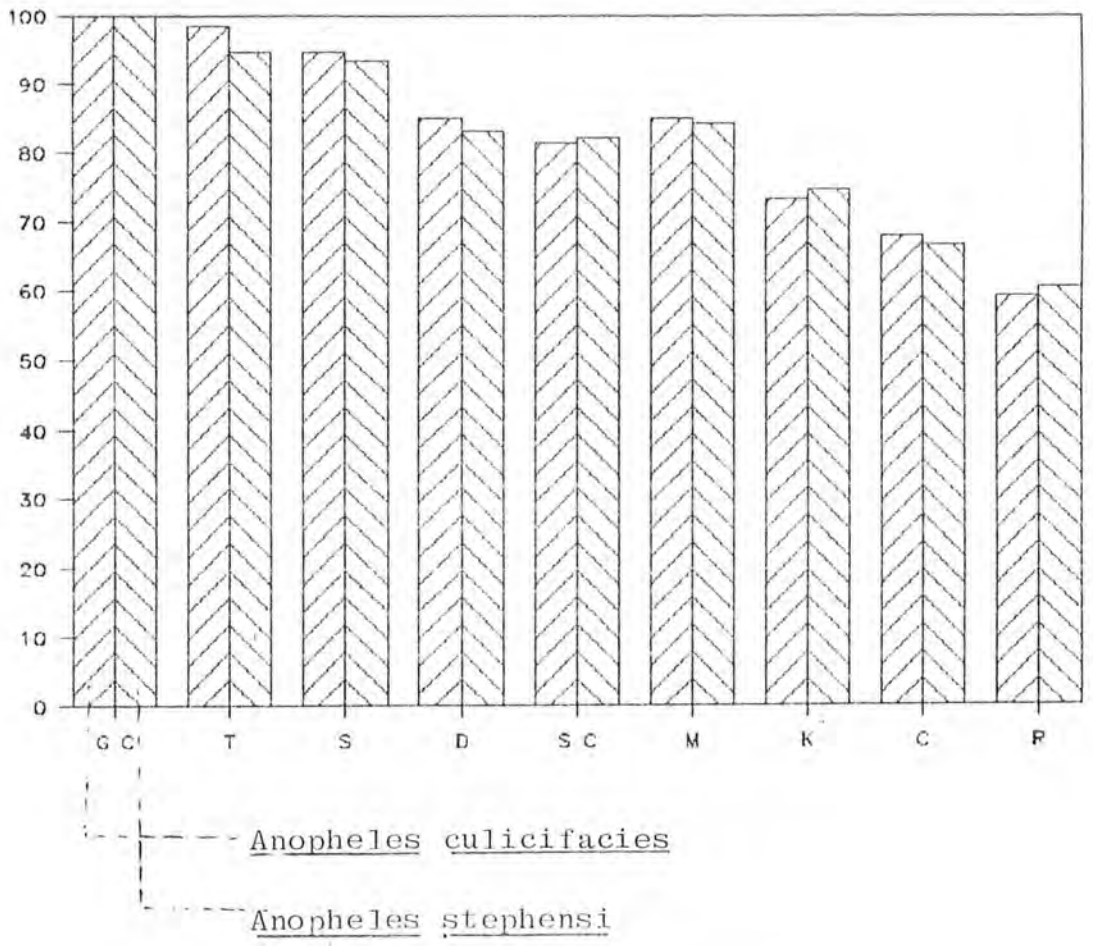
RESULTS

Laboratory Trials

Nine species of fish were tried in the laboratory to see their larvivorous effect on the laboratory reared Anopheles culcifacies and Anopheles stephensi larvae. Green Carp revealed hundred percent with all the 2nd, 3rd and 4th instar larvae.

FIG- 11.

PERCENT CONTROL OF ANOPHELES CULICIFACIES AND ANOPHELES STEPHENSI LARVAE BY DIFFERENT FISH WITHOUT WEEDS UNDER LABORATORY CONDITIONS.



GC	Green carp.	C	Chidu
T	Tilapia	R	Rohu
S	Singhi		
D	Daula		
SC	Silver Carp.		
M	Mo ri		
K	Kangi		

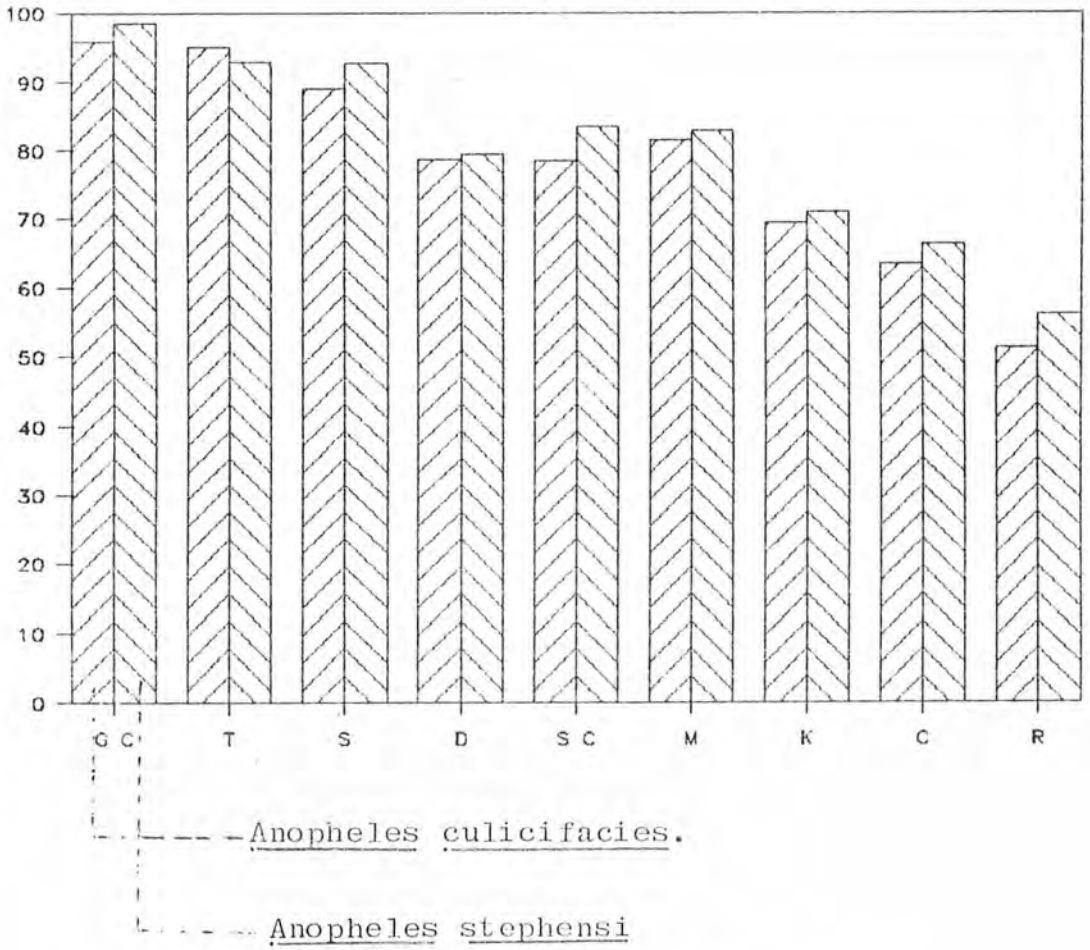
Average reduction of 4th instar Anopheles culicifacies larvae with fish species was found as 100, 98.35, 94.50, 85.00, 81.4, 85, 73.25, 67.98 and 59.00, percent and that of Anopheles stephensi as 100, 94.6, 93.2, 83, 82, 84.2, 74.6, 66.5 and 60.4 percent with Green Carp, Tilapia, Singhi, Daula, Silver Carp, Mori, Kangi, Rohu, and Chidu respectively in natural water having no weeds (Table- 48, Fig. 11).

When different weeds from the same natural water were also added into the pans, Anopheles culicifacies revealed 95.7, 95, 89.00, 78.69, 78.36, 81.57, 69.43, 63.5 and 51.37 percent mortaility and with Anopheles stephensi it was observed as 98.5, 92.9, 92.72,79.48, 83.41, 82.85, 71, 66.48 and 56.24 percent mortality with Green Carp, Tilapia, Singhi, Daula, Silver Carp, Mori, Kangi, Rohu and Chidu respectively (Table -49, Fig. 12).

Upon providing second and third instar larvae Green Carp devoured all of them within first two hours, Daula and Kangi devoured within three hours, Tilapia and Mori finished all of them in five hours, Silver Carp in six hours, Singhi in 24 hours and with that of Rohu only a reduction of 65.5 percent has been found. Chidu fish devoured 67.98% Anopheles culcifacies and 66.5% Anopheles stephensi larvae when no weeds were added and as 63.5 and 66.48 percent respectively when the weeds were also added alongwith anopheline larvae. There was nonsignificant difference in the mortality during first and second week ($t=10.77$, $P<0.005$).

FIG- 12

PERCENT REDUCTION OF ANOPHELES CULICIFACIES AND ANOPHELES STEPHENSI LARVAL DENSITY BY DIFFERENT FISH WITH WEEDS IN LABORATORY CONDITIONS.



GC	Green Carp.	C	Chidu
T	Tilapia	R	Rohu
S	Singhi		
D	Daula		
SC	Silver Carp.		
M	Mori		
K	Kangi		

Field Trials

In field trials 12 small size fish contributed the reduction in larval density by 91.8 percent when the observations were taken two days after release of larvae and fish in the plots. Similarly during the trial of second week the reduction in larval density by 92.2 percent was recorded when the observations were taken two days after release of larvae in the field plots (Table _ 50).

At the end of second week observations when four fish from each plot were recaptured and slaughtered to see the presence of larvae if any in the stomach of the fish, only five fish revealed the presence of larvae whereas seven fish did not reveal larvae upon dissections. It is, therefore, concluded that the fish having no larvae in their gut have already digested the larvae and have expelled the waste product.

Combination of fish with neem and bakain

Chidu fish in combination with neem leaves revealed 98.16 and 95.00 , Chidu plus neem fruit as 96.33 and 97.00 , Chidu plus bakain leaves as 96.66 and 95.66 and chidu plus bakain fruit as 96.86 and 93.16 percent respective reduction in the first & second week (table-51-53). No significance difference due to these combinations was observed during the present studies. When the fish

were then collected after completion of these experiments and kept under observation in the laboratory for two more weeks, no visible abnormalities were observed in the fish due to combination with the leaves and fruit of both of these trees.

DISCUSSION

It has been observed from this study that the fish devours larvae when it is still small in size. The size of chidu fish is small ranging from about 4-12 centimeters. When compared Greep Carp was found as eating and finishing the larvae earlier than other fish species. Daula, Tilapia and Mori took longer time in eating the larvae and Silver Carp and Singhi took considerably more time than the other fish species except Chidu and Rohu. Deweeding exposes larvae to the fish.

From these studies it has appeared that this small size Chidu fish have its role in reducing natural populations of both the anopheline and culicine larvae in the permanent breeding places. In our field studies an average of 91.8 and 92.2 percent reduction in larval density was observed from the trials during first and second week respectively. There were significantly greatly difference between the treated plots and the control. This small size fish that is available throughout the year in our country and it devours not only the mosquito larvae but

other aquatic insects as well. It is concluded that the large size fish i.e. Green Carp or Silver Carp etc. could be of value in protected places like government and private agencies owned ponds, canals and large size rain water collections whereas the collection of small size fish from unwanted places and their release in breeding places of malaria vector mosquitoes and other mosquitoes in the vicinity of 2 miles radius in both the peripheries of the cities and the villages will help in reducing mosquito population and malaria transmission. Chidu fish if reared in the local areas to be treated coupled with proper management, education and involvement of the local community of the area to be treated and then the integration of this small size larvivorous fish into other national malaria vector control programmes will contribute in the reduction of malaria and other mosquito vectors. The extracts from both the trees of neem and bakain was also found as non-toxic to the fish. The fish collected from the experimental plots did not show any visible abnormalities. This fish can therefore be applied in combination with the leaves and fruit powder of both of these trees.

GENERAL DISCUSSION

Malaria is a serious health problem in Pakistan. Anopheles culicifacies and Anopheles stephensi were earlier considered as transmitting malaria in Pakistan but from the present elisa studies Anopheles fluviatilis has also been observed as infected with the sporozoites. An. fluviatilis was earlier observed in the laboratory as having the malaria transmission capability (McCarthy and Clyde, 1968). It was also observed during the present study that Anopheles fluviatilis remains dominant species during March to July and An. culicifacies from August onwards till December. Infected mosquitoes were observed during postmoonsoon rains as well as during spring season.The human feeding rate in these malaria vectors has been observed as 9.78, 13.66 and 11.43 percent respectively. This high percentage of human feedings could be due to very close living habits of the people along-with the animals. During summers they sleep very close with the animals in a small yard and whenever there is need to sleep inside due to rains, hot sun etc. they sleep in the same room where cattle are tied. This gives an equal chance to the mosquitoes to feed on animals as well as on man. Since both the Plasmodium falciparum and Plasmodium vivax infections were also present in the local inhabitants and socio-economic factors favour the close contact between man and the mosquitoes. It is concluded that this much percentage

of human feedings by the vector species could transmit significant malaria under favourable climatic conditions. It has been suggested that Anopheles fluviatilis may thus be considered as one of the target species for focussing the control efforts.

Bacillus thuringiensis H-14 was used for the control of local mosquito species and was observed as having its effect for 36 to 48 hours and as particularly effective for surface feeding anopheline larvae. Bacillus sphaericus was observed as having long larvicidal effect and as particularly effective for bottom feeding culicine larvae. It was observed from the studies on both the neem and bakain trees that the constituents have insecticidal as well as growth retarding effect on mosquito larvae. It was concluded that the Pakistan species of these trees have got strong insecticidal values when compared with the studies of Zebitz (1984; 1986). The leaves and fruit from these trees were therefore used in combination with Bacillus thuringiensis H-14 and larval control was obtained upto one week.

The domestic ducks, Anas platyrhynchus L. when tried to see their efficacy in controlling mosquito larvae in a small experiment under controlled conditions it appeared that the ducks prefer mosquito larvae and other aquatic fauna

as their diet when compared to pieces of bread. It has thus appeared from this experiment that the ducks also have role in reducing mosquito populations.

Out of nine different fish species that were tried in the laboratory, chidu fish was selected for use in small field trials for its efficacy against mosquitoes in natural breeding places. This fish appeared as suitable for use in rice fields and other large size mosquito breeding places. It was also observed that deweeding exposes mosquito larvae to the fish. The extracts from both the trees of neem and bakain when used in combination with Chidu fish did not show any abnormal pathological effects to the fish and it thus appeared that the fish and the leaves and fruit of both the neem and bakain have a combined role in controlling larval stages of the mosquitoes. Extract from both the trees of neem and bakain are equally non-toxic to man and other mammals. 0.1, 0.2 and 0.3 cc extracts from the leaves and fruit of both of these trees were given orally and subcutaneously to Albino rats and it did not show any pathological or toxic effects to the rats. No change in the normal and treated rats were observed in their weight and haematological parameters.

Malaria situation is continuously worsening in our country. Zulueta et al. (1980) studied the trend of

malaria in Pakistan and described it as having long term periodicity. According to them the epidemics are regularly occuring in Punjab Province at an interval of approximately 8 years. The slide positivity rate of malarial parasite is again on the increase. During 1988 and 1989 it was 2.01 and 3.32 percent respectively. This reported malaria rate is always less than that usually present in the field. This less reporting is due to lack of proper microscopic diagnosis of the parasite, lack of proper diagnostic and case treatment facilities in rural areas and lack of proper education in the local community regarding malaria and the mosquitoes. Other major problems of malaria and mosquito control programmes are the development of malathion resistance in malaria vectors (Rathore and Toqir,1980; Rathore et al., 1985) and chloroquine resistance in Plasmodium falciparum (Shah and Yasin,1987) and lack of well trained specialists. If these obstacles are removed, there will be no more mosquito and malaria problem in the country. From the present studies it has been concluded that mosquito problem and the malaria incidence can be brought down with the shifting of the mosquito control strategy from the use of malathion towards biological approaches. After comparing different biological approaches the following suggestions have been made for the control of mosquitoes in our country.

1. Either the Neem or Bakain Tree Leaves or Fruit at the rate of 250 gm/sq.m. may be used in mosquito breeding places in the vicinity of 2 miles radius that is the approximate flight range of the mosquitoes.

2. Small sized surface feeder chidu fish that is available in our country and can breed both in clean as well as in dirty water has been recommended for use at the rate of 2 fish/sq.m. in rice fields and other permanent mosquito breeding places. The use of large size fish i.e. Green Carp or Silver Carp is suggested in protected places like government and private agencies owned canals, ponds and large size permanent rain water collections.

3. The highly zoophilic feeding pattern of the anophelines can be utilized in malaria and mosquito control programmes. For this purpose it is suggested that more cattle may be introduced at the perpheries of the cities and the villages for deviating the mosquitoes from human host towards these animals. Keeping of ducks at the peripheries of the cities and in the villages will be of added advantage

in reducing mosquito densities.

4. For an effective mosquito and malaria control programme the above three approaches may be supplemented with the environmental management by closing of the unwanted water collections that serve as mosquito breeding places, introducing of proper or covered drainage system and maintenance of these drains for continuous flow of water, the education of local community, assignment of malaria and mosquito control responsibilities to the well trained specialists and regular mosquito and malaria surveillance programmes.

SUMMARY

1. Anopheline surveys were conducted during June 1987 to May 1988 in Islamabad-Rawalpindi and its vicinities and fifteen different anopheline species were collected from the area under study. These include, Anopheles culicifacies, An. fluviatilis, An. stephensi, An. subpictus, An. gigas, An. annularis, An.nigerrimus, An. pulcherrimus, An. lindesayi, An. jamesi, An. sergenti, An. superpictus, An. maculatus, An. splendidus and An. barbirostris. It was observed that Anopheles fluviatilis remains dominant species during March to July and An. culicifacies from August to December.

2. For bloodmeal identification enzyme-linked immunosorbent assay was used. 2538 anophelines of different species were tested for their bloodmeal source. 9.78 percent Anopheles culicifacies were found as having human blood, 50.14% as bovine and the remaining 40.58 percent An. culicifacies were neither human or bovine. 13.65 percent An. fluviatilis were positive to human source, 61.87 percent to bovine and the remaining 23.62 percent were non-human non-bovine. Density of all other anophelines was found as low and therefore the number tested was also low.

3. Dipstick elisa technique was also tried for bloodmeal identifications. It took 40 to 50 minutes for identifying mosquito bloodmeal with this technique.

4. During salivery gland dissections none of the anophelines were found as sporozoite positive. The anopheline collections made round the year were assayed using elisa technique for sporozoite detection and the infections were detected during both the spring and postmonsoon season. Anopheles fluviatilis that was earlier considered as suspected malaria vector has been incriminated as malaria vector. Parasite survey was therefore conducted during the same months of September-October 1988 and March-April 1989 to determine the parasite situation in the area under study. Parasite rate during postmonsoon malaria transmission season (5.77%) was found as higher than that of spring season (3.14%).

5. LC50 and LC100 of Bacillus thuringiensis H-14 and Bacillus sphaericus 1593 were determined against some of Pakistan mosquito species. Bacillus thuringiensis H-14 was used and it was observed that this microbial larvicide lasts for 36-48 hours and is particularly effective for surface feeding anopheline larvae. Bacillus sphaericus has long residual effect and is particularly effective for bottom feeding culicine larvae.

6. Neem and Bakain Tree leaves and fruit extract has been separated in tap water, by soxehlet apparatus and in ethanol, methanol and acetone. It appeared from laboratory trials with both the Neem and Bakain Tree Extracts in simple water that the extract has got insecticidal action on mosquito

larvae. In field trials the residual effect of the extracts in simple water lasted only upto nearly 5 days with neem and Bakain leaves extract, 4 days with neem seed powder and 3 days with bakain fruit extract. The extract in methanol provided excellent kill of mosquito larvae, however the use of ground leaves or fruit of either the neem or bakain tree revealed good control of mosquito larvae and were found as economical and simple in application.

7. Considering the aquatic feeding habits of domestic ducks, these birds were tried to find out if these animals are of any help in reducing the mosquito populations. The ducks devoured mosquito larvae in almost 6 hours after release.

8. Nine different fish species were tried in the laboratory to select a suitable larvivorous fish for its efficacy against mosquitoes in natural breeding places. Small sized Chidu Fish was found as larvivorous and suitable for rice fields and other mosquito breeding places. The use of large size fish i.e. Green Carp or Silver Carp has been suggested in protected places like government and private agencies owned canals, ponds and large size permanent rain water collections. It also appeared that deweeding exposes mosquito larvae to the fish.

9. The extracts from both the trees of neem and bakain were used in combination with chidu fish. This combination did not show any significant increase in larval control. However it did not show any abnormal pathological effects to the fish.

10. Bacillus thuringiensis H-14 was combined with neem and bakain leaves/fruit powder and an increased in residual effectiveness upto one week was recorded. This effectiveness was due to the insecticidal properties of these trees.

11. When 0.1, 0.2 and 0.3 cc leaves and fruit extract of both the neem and bakain trees was given to the albino rats through oral and subcutaneous routes and compared with the normal rats, no change was observed in the haematological values and the weights of whole body, liver, lungs, spleen and kidneys after treatments with the extract from both of these trees. The extracts thus appeared as nontoxic to albino rats.

PUBLICATIONS FROM PRESENT STUDY

Following articles were written on different aspects of present study. Most of these articles have been published, some of these have been accepted for publication and the others are still under review.

1. Aziz A, Qazi MH & Pal RA 1988. Anopheline species encountered from Islamabad/Rawalpindi area. Pakistan Journal of Medical Research 27(4):303-305.
2. Aziz A, Pal RA & Qazi MH 1988. Density of Anopheles culicifacies and Anopheles fluviatilis in Rawalpindi/ Islamabad and its vicinities. Pakistan Journal of Health 25:28-31.
3. Aziz A, Qazi MH & Pal RA 1989. Dipstick elisa for the identification of human bloodmeal in malaria vectors and other anopheline mosquitoes. Mosquito Borne Diseases Bulletin 6(1):5-6.
4. Aziz A, Pal RA & Qazi MH 1988. Application of enzyme-linked immunosorbent assay in the study of cattle bloodmeal of anopheline mosquitoes. Pakistan Journal of Health 25: 38-42.
5. Aziz A, Qazi MH & Pal RA 1989. Bacillus sphaericus as mosquito larvicide. Journal of Pakistan Medical Association 39(8):199-201.
6. Aziz A, Qazi MH & Pal RA 1989. A trial of Bacillus thuringiensis H-14 for mosquito control. Pakistan

Journal of Health 26 (1,2): 30-34.

7. Aziz A, Pal RA, Nadeem A & Awan MB 1989. Effect of Melia azadirachta L. leaves extract against Enterobius vermicularis L. Pakistan Journal of Health 26 (1,2): 60-63.

8. Aziz A & Shah IH 1988. A laboratory trial on the effect of Green Carp, a larvivorous fish in controlling malaria vector mosquitoes. Pakistan Journal of Health 25(3): 66-68.

9. Aziz A & Pal RA 1990. Domestic ducks prefer mosquito larvae as their diet. Journal of Pakistan Medical Assoication 40(2):42-43.

10. Aziz A & Pal RA 1990. Biological control of mosquitoes by larvivorous fish. Pakistan Journal of Scientific and Industrial Research (accepted for publication).

11. Aziz A, Pal RA & Qazi MH 1990. Application of elisa technique for human bloodmeal detection in anopheline mosquitoes. Pakistan Journal of Medical Research (accepted for publication).

12. Aziz A & Pal RA (1990). Survey of malarial parasite in Islamabad. Journal of Pakistan Medical Association (accepted for publication).

13. Aziz A & Pal RA (1990). Punctius ticto, Chidu Fish for Mosquito Control. Journal of Pakistan Medical Association (sent for publication).

14. Aziz A & Pal RA 1990. Mosquito larvicidal effect of Neem Tree, Melia azadirachta L. Journal of Pure and Applied Sciences (sent for publication).

15. Aziz A & Pal RA 1990. Insecticidal effect of the Bakain Tree Melia azedarach L. (Family-Meliaceae). Journal of Pharmacy (sent for publication).

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TABLE - 1

SLIDE POSITIVITY RATE OF PAKISTAN
1960 - 1989

Year	Percent Slide Positivity Rate
1960	15.57
1961	3.64
1962	0.91
1963	2.75
1964	1.63
1965	1.47
1966	0.36
1967	0.25
1968	0.36
1969	0.98
1970	2.81
1971	5.36
1972	14.58
1973	14.09
1974	9.82
1975	7.43
1976	4.28
1977	1.78
1978	0.62
1979	0.46
1980	0.59
1981	1.26
1982	1.71
1983	1.99
1984	2.27
1985	2.49
1986	3.13
1987	2.16
1988	2.01
1989	3.32

TABLE - 2

MAN-HOUR DENSITY OF ANOPHELES FLUVIATILIS AND AN. CULICIFACIES

Month	An. fluviatilis	An. culicifacies
February	9	5
March	21.1	19.6
April	45.2	16
May	23.8	4.6
June	11.3	3
July	26	13
August	15.7	34.3
September	19.8	54.6
October	16.3	41.2
November	4	19.5
December	3	8
January	00	00

TABLE- 3

WEATHER DATA

Month	Minimum Temp.	Maximum Temp.	R.H. 0800 hrs	R.H. 1700 hrs	Rain-fall
1987					
June	21.5	38.3	49	29.5	271
July	24.2	39.0	57	32	63.4
August	24.3	35.9	76	56	264.5
September	21.9	36.1	72	49	13
October	15.0	30.9	76	39	74.4
November	7.5	27.9	76	34	00
December	4.0	22.1	83	41	7
1988					
January	4.7	19.6	84	42	17.1
February	6.6	21.5	79	40	23.2
March	10.6	23.2	76	42	153.3
April	16.7	33.2	57	27	6.9
May	21.2	38.6	37	18	8.6

TABLE - 4

Human Blood Positive Mosquitoes

Species	Mosquitoes examined	Positive	Percentage
An. culicifacies	1370	134	9.78
An. fluviatilis	834	114	13.66
An. stephensi	175	20	11.43
An. subpictus	24	5	
An. nigerrimus	5	1	
An. superpictus	12	2	
An. maculatus	23	-	
An. annularis	19	2	
An. sergenti	14	1	
An. splendidus	62	5	
TOTAL	2538	285	

Average positive percentage of all anophelines = 11.3%

TABLE - 5

Bovine positive mosquitoes.

Species	Mosquitoes examined	Positive	Percentage
An. culicifacies	1370	687	50.14
An. fluviatilis	834	516	61.87
An. stephensi	175	87	49.71
An. subpictus	24	9	
An. nigerrimus	5	1	
An. superpictus	12	5	
An. maculatus	23	13	
An. annularis	19	13	
An. sergenti	14	5	
An. splendidus	62	28	45.16
TOTAL	2538	1364	

Average bovine positive percentage of all anophelines = 53.74%.

TABLE - 6

Non-human, Non-Bovine Mosquitoes

Species	Total	Non-H, Non-B	Percentage
An. culicifacies	1370	556	40.58
An. fluviatilis	834	197	23.62
An. stephensi	175	68	38.85
An. subpictus	24	10	
An. nigerrimus	5	3	
An. superpictus	12	5	
An. maculatus	23	9	
An. annularis	19	4	
An. sergenti	14	8	
An. splendidus	62	29	46.77

TABLE - 7

Results of Human dipstick elisa

	Number tested	Number Positive
An. culicifacies	21	3
An. fluviatilis	101	16
An. stephensi	29	4
An. subpictus	4	-
An. superpictus	3	-
An. splendidus	18	1

TABLE - 8

Results of Bovine dipstick elisa

	Number tested	Number positive
An. culicifacies	21	10
An. fluviatilis	101	58
An. stephensi	29	15
An. subpictus	4	2
An. superpictus	3	2
An. splendidus	18	8

TABLE- 9

AN CULICIFACIES SHOWING ELISA POSITIVE CSP.

Month	P. vivax	P. falciparum
August	2	1
September	3	2
October	2	2
March	1	0
TOTAL	8	5

TABLE- 10

AN. FLUVIATILIS SHOWING ELISA POSITIVE CSP.

Month	P. vivax	P. falciparum
August	1	Nil
September	2	Nil
October	1	1
March	1	Nil
May	1	-
TOTAL	6	1

TABLE- 11

SURVEY OF MALARIAL PARASTIE

Month	Number examined	Pv	Pf	PR
September	162	4	1	3.08
October	186	3	2	2.69
March	148	2	1	2.03
April	179	2	0	1.11

Pv= Plasmodium vivax, Pf=Plasmodium falciparum
PR= Parasite Rate.

TABLE-12

LC-50 AND LC-100 VALUES OF BACILLUS THURINGIENSIS H-14 (ABG 6145) AGAINST DIFFERENT MOSQUITO LARVAE

SPECIES	INSTAR	LC-50 (ppm)	LC-100 (ppm)
Anopheles fluviatilis	1st	0.045	0.08
	2nd	0.045	0.09
	3rd	0.07	0.14
	4th	0.14	1.00
Anopheles annularis	1st	0.045	0.08
	2nd	0.05	0.09
	3rd	0.08	0.15
	4th	0.15	1.00
Anopheles pulcherrimus	1st	0.055	0.09
	2nd	0.06	0.10
	3rd	0.08	0.16
	4th	0.16	1.00
Anopheles maculatus	1st	0.045	0.08
	2nd	0.045	0.09
	3rd	0.07	0.14
	4th	0.14	1.00
Culex tritaeniorhynchus	1st	0.03	0.07
	2nd	0.03	0.07
	3rd	0.07	0.15
	4th	0.09	0.18
Culex fatigans	1st	0.03	0.07
	2nd	0.04	0.07
	3rd	0.08	0.19
	4th	0.09	0.25

TABLE- 13

LC-50 AND LC-100 VALUES OF BACILLUS SPHAERICUS AGAINST DIFFERENT MOSQUITO LARVAE.

SPECIES	INSTAR	LC-50 (ppm)	LC-100 (ppm)
Anopheles fluviatilis	1st	0.07	0.10
	2nd	0.08	0.14
	3rd	0.15	0.45
	4th	0.17	0.70
Anopheles annularis	1st	0.06	0.12
	2nd	0.075	0.155
	3rd	0.15	0.60
	4th	0.165	0.80
Anopheles pulcherrimus	1st	0.08	0.14
	2nd	0.09	0.18
	3rd	0.15	0.60
	4th	0.018	0.85
Anopheles maculatus	1st	0.07	0.15
	2nd	0.08	0.18
	3rd	0.17	0.65
	4th	0.20	1.00
Culex tritaeniorhynchus	1st	0.025	0.055
	2nd	0.03	0.065
	3rd	0.060	0.11
	4th	0.07	0.165
Culex fatigans	1st	0.025	0.05
	2nd	0.03	0.06
	3rd	0.065	0.12
	4th	0.075	0.16

TABLE- 14

Average larval dip counts

	Bacillus thuringiensis		B. sphaericus		Control
	1 ml.	2 ml.	1 ml.	2 ml.	
Before spray	45.5	48.9	52.1	51.6	44.8
2 hours after	6	00	21.8	00	45.4
24 hours after	00	00	00	00	43.6
48 hours after	00	00	00	00	46
72 hours after	50.4	47.6	00	00	49.1
1 week after	52.5	48.4	00	00	51.2
2 weeks after	N/A	N/A	00	00	52.8
3 weeks after	N/A	N/A	00	00	50.8
4 weeks after	N/A	N/A	00	00	50.4
5 weeks after	N/A	N/A	5.8	00	51
6 weeks after	N/A	N/A	7.6	00	49
7 weeks after	N/A	N/A	12	5	50.8
8 weeks after	N/A	N/A	10.7	8.8	14.8

TABLE-15

Percent reduction in larval density

Bacillus thuringiensis

	Larval dip count.				
	Control	1 ml	%reduction	2 ml.	% reduction
Before spray	44.8	45.5	-	48.9	-
After 2 hours	45.4	6	86.78	00	100
After 24 hours	43.6	00	100	00	100
After 48 hours	46	00	100	00	100
After 72 hours	49.1	50.4	-2.64	47.6	3.05
After 1 week	51.2	52.5	-2.53	48.4	5.47

TABLE-16

Percent reduction in larval density

Bacillus sphaericus

Larval dip count

	Control	1 ml	% reduction	2 ml	% reduction
Before spray	44.8	52.1	-	51.6	-
2 hours after	45.4	21.8	51.98	00	100
24 hours after	43.6	00	100	00	100
48 hours after	46	00	100	00	100
72 hours after	49.1	00	100	00	100
After 1 week	51.2	00	100	00	100
After 2 weeks	52.8	00	100	00	100
After 3 weeks	50.8	00	100	00	100
After 4 weeks	50.4	00	100	00	100
After 5 weeks	51	5.8	88.62	00	100
After 6 weeks	49	7.6	84.48	00	100
After 7 weeks	50.8	12	76.38	5	90.15
After 8 weeks	14.8	10.7	27.21	8	40.55

TABLE-17

EFFECT OF 140 ul/ml NEEM LEAVES EXTRACT TO FIELD COLLECTED MOSQUITO PUPAE

	RI		RII		RIII		Control	
Exposed pupae	50		50		50		50	
Emergence after 1 day	M	F	M	F	M	F	M	F
	00	00	00	00	00	00	17	25
Mortality after 1 day	5		3					
Emergence after 2 days	00	6	00	3	00	5	3	5
Mortality after 2 days	22		17		45			
Emergence after 3 days	00	00	00	00				
Mortality after 3 days	44		27		45			

TABLE - 18

EFFECT OF NEEM FRUIT EXTRACT ON MOSQUITO PUPAE (140 ul/ml)

Replication	RI	RII	RIII	Control
Number of pupae	50	50	50	50
Emergence after 12 hrs	3d	1d	2d	43
Dead pupae after 12 hrs	47	49	48	1

TABLE- 19

PERCENT MORTALITY OF MOSQUITO PUPAE AFTER EXPOSURE TO 140 ul/ml NEEM LEAVES AND FRUIT EXTRACT IN WATER

	Leaves extract	Fruit extract	Control
After 1 day	5.3	100.0	2
After 2 days	56.0	-	2
After 3 days	77.3	-	2

TABLE-20

PERCENT MORTALITY OF 3RD PLUS 4TH INSTAR ANOPHELINE AND CULICINE LARVAE AFTER EXPOSURE TO NEEM LEAVES AND FRUIT EXTRACT IN WATER

Leaves Extract

	Anopheline		Culicine		
	70 ul/ml	140 ul/ml	70 ul/ml	140 ul/ml	Control
After 6 hrs	3.3	100.0	15.5	100.0	00
After 12 hrs	98.0		100.0		00

Fruit Powder

	Anopheline		Culicine		
	3 gm/50 ml	6 gm/50 ml	3 gm/50 ml	6 gm/50 ml	Control
After 6 hrs	0.0	45.5	12.6	64.5	00
After 12 hrs	100.0	100.0	100.0	100.0	00

Fruit Extract

	Anopheline		Culicine		
	70 ul/ml	140 ul/ml	70 ul/ml	140 ul/ml	Control
After 6 hrs	0.0	28.0	36.0	87.4	00
After 12 hrs	51.0	86.6	100.0	100.0	00

TABLE- 21

PERCENT MORTALITY OF 3RD PLUS 4TH INSTAR AN.CULICIFACIES AND AN. STEPHENSI LARVAE AFTER EXPOSURE TO NEEM LEAVES EXTRACT IN ETHANOL, METHANOL AND ACETONE

	Ethanol		Methanol		Acetone	
	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml
After 1 hr	0.0	0.0	0.0	0.0	0.0	0.0
After 2 hrs	1.6	0.0	0.0	0.0	5.0	1.7
After 4 hrs	6.6	6.6	0.0	5.0	5.0	5.0
After 6 hrs	6.6	6.6	31.7	41.7	18.3	13.3
After 8 hrs	13.3	10.0	46.7	55.0	30.0	16.7
After 24 hrs	13.3	16.7	100.0	100.0	51.7	53.7

TABLE- 22

PERCENT MORTALITY OF 3RD PLUS 4th INSTAR AN.CULICIFACIES AND AN. STEPHENSI LARVAE AFTER EXPOSURE TO NEEM FRUIT EXTRACT IN ETHANOL, METHANOL AND ACETONE

	Ethanol		Methanol		Acetone	
	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml
After 1 hr	1.8	1.8	55.0	65.0	0.0	3.3
After 2 hrs	8.3	6.6	78.3	71.6	0.0	3.3
After 4 hrs	8.3	10.0	81.6	76.6	1.6	3.3
After 6 hrs	25.0	35.0	86.6	78.3	1.6	3.3
After 8 hrs	33.3	56.6	100.0	100.0	10.0	11.6
After 24 hrs	100.0	100.0	(after 7 hrs).		78.3	78.3

TABLE - 23

LC-50 AND LC-100 VALUE (ul/ml) OF NEEM TREE, MELIA AZADIRACHTA L., LEAVES EXTRACT AGAINST LARVAE OF DIFFERENT MOSQUITO SPECIES

SPECIES	INSTAR	LC-50 (ul/ml)	LC-100 (ul/ml)
Anopheles culicifacies	1st	0.3	0.35
	2nd	0.35	0.40
	3rd	0.4	0.55
	4th	0.45	0.6
Anopheles fluviatilis	1st	0.35	0.4
	2nd	0.4	0.45
	3rd	0.45	0.55
	4th	0.5	0.65
Anopheles stephensi	1st	0.25	0.40
	2nd	0.3	0.45
	3rd	0.40	0.6
	4th	0.45	0.65
Anopheles annularis	1st	0.3	0.4
	2nd	0.35	0.45
	3rd	0.4	0.55
	4th	0.45	0.6
Anopheles pulcherrimus	1st	0.3	0.4
	2nd	0.35	0.5
	3rd	0.45	0.65
	4th	0.5	0.7

(Contd....p/)

Contd ...

Anopheles maculatus	1st	0.3	0.4
	2nd	0.4	0.45
	3rd	0.45	0.55
	4th	0.5	0.65
Culex tritaeniorhynchus	1st	0.25	0.35
	2nd	0.3	0.4
	3rd	0.35	0.45
	4th	0.4	0.50
Culex fatigans	1st	0.25	0.3
	2nd	0.28	0.35
	3rd	0.3	0.40
	4th	0.35	0.48

TABLE- 24

LC-50 AND LC-100 VALUES OF NEEM TREE MELIA AZADIRACHTA L., FRUIT EXTRACT AGAINST LARVAE OF DIFFERENT MOSQUITO SPECIES

SPECIES	INSTAR	LC-50 (ul/ml)	LC-100 (ul/ml)
Anopheles culicifacies	1st	0.2	0.35
	2nd	0.25	0.35
	3rd	0.35	0.4
	4th	0.4	0.45
Anopheles fluviatilis	1st	0.3	0.35
	2nd	0.35	0.4
	3rd	0.4	0.5
	4th	0.45	0.55
Anopheles stephensi	1st	0.2	0.35
	2nd	0.2	0.35
	3rd	0.35	0.45
	4th	0.4	0.50
Anopheles annularis	1st	0.2	0.35
	2nd	0.3	0.4
	3rd	0.4	0.55
	4th	0.45	0.60
Anopheles pulcherrimus	1st	0.25	0.35
	2nd	0.3	0.4
	3rd	0.40	0.50
	4th	0.45	0.55

(Contd...p/)

Contd...

Anopheles maculatus	1st	0.3	0.4
	2nd	0.4	0.45
	3rd	0.43	0.55
	4th	0.45	0.60
Culex tritaeniorhynchus	1st	0.25	0.30
	2nd	0.28	0.35
	3rd	0.30	0.40
	4th	0.35	0.45
Culex fatigans	1st	0.25	0.35
	2nd	0.30	0.4
	3rd	0.35	0.45
	4th	0.40	0.50

TABLE - 25

PERCENT MORTALITY OF 3RD AND 4TH INSTAR ANOPHELINE CULICINE LARVAE AFTER EXPOSURE TO BAKAIN LEAVES AND FRUIT EXTRACT IN WATER

Leaves Extract

	Anopheline		Culicine		
	70 ul/ml	140 ul/ml	70 ul/ml	140 ul/ml	Control
After 6 hrs	85.5	100.0	70.0	100.0	00
After 12 hrs	100.0	-	100.0	-	00

Fruit Extract

After 6 hrs	70.0	73.3	59.0	97.5	00
After 12 hrs	95.0	100.0	87.0	100.0	00

TABLE-26

EFFECT OF 140 ul/ml BAKAIN LEAVES EXTRACT ON MOSQUITO PUPAE

	RI	RII	RIII	Control	% Mortality
Exposed pupae	50	50	50	50	
Emergence after 1 day	00	00	00	39	
Mortality after 1 day	5	8	4	00	11.3
Emergence after 2 days	3	1	4	9	
Mortality after 2 days	47	49	46	2	94.6

TABLE-27

EFFECT ON 140 ul/ml BAKAIN FRUIT EXTRACT ON MOSQUITO PUPAE

	RI	RII	RIII	Control	% Mortality
Exposed pupae	50	50	50	50	
Emergence after 1 day	00	00	00	42	00
Mortality after 1 day	3	2	6	00	
Emergence after 2 days	17	8	12	8	
Mortality after 2 days	24	42	29	00	63.3
Emergence after 3 days	9	00	00	00	
Mortality after 3 days	24	42	38	00	69.3

TABLE- 28

PERCENT MORTALITY OF 3RD PLUS 4TH INSTAR AN. CULICIFACIES AND AN. STEPHENSI LARVAE AFTER EXPOSURE TO BAKAIN LEAVES EXTRACT IN ETHANOL, METHANOL AND ACETONE.

	Ethanol		Methanol		Acetone	
	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml
After 1 hr	00	00	00	00	00	3.0
After 2 hrs	00	00	00	00	00	6.6
After 4 hrs	00	00	35.0	35.0	10.0	11.6
After 6 hrs	20.0	11.6	75.0	61.0	10.0	21.6
After 8 hrs	33.3	30.0	88.3	90.0	15.0	30.0
After 24 hrs	88.3	95.0	100.0	100.0	45.0	46.6

TABLE - 29

PERCENT MORTALITY OF 3RD PLUS 4TH INSTAR AN. CULICIFACIES AND AN. STEPEHNSI LARVAE AFTER EXPOSURE TO BAKAIN FRUIT EXTRACT (LAST SEASON) IN ETHANOL, METHANOL AND ACETONE

	Ethanol		Methanol		Acetone	
	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml
After 1 hr	00	00	00	00	00	00
After 2 hrs	00	00	00	00	11.6	20.0
After 4 hrs	00	5.0	00	00	25.0	45.0
After 6 hrs	6.6	13.3	00	00	78.3	80.0
After 8 hrs	15.0	21.6	35.0	58.3	93.3	95.0
After 24 hrs	46.0	55.0	100.0	100.0	100.0	100.0

TABLE - 30

PERCENT MORTALITY OF 3RD PLUS 4TH INSTAR AN. CULICIFACIES AND AN. STEPEHNSI LARVAE AFTER EXPOSURE TO BAKAIN FRUIT EXTRACT (FRESH FRUIT) IN ETHANOL, METHANOL AND ACETONE.

	Ethanol		Methanol		Acetone	
	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml	0.8 ul/ml	1.0 ul/ml
After 1 hr	00	00	00	00	00	00
After 2 hrs	00	00	00	00	00	00
After 4 hrs	23.3	8.3	28.0	32.0	16.0	23.6
After 6 hrs	65.0	71.6	64.6	58.0	21.3	28.6
After 8 hrs	73.3	90.0	71.0	76.3	26.0	33.3
After 24 hrs	98.3	100.0	100.0	100.0	51.0	62.6

TABLE - 31

LC-50 AND LC-100 VALUES OF BAKAIN TREE, MELIA AZEDARACH L., LEAVES EXTRACT AGAINST LARVAE OF DIFFERENT MOSQUITO SPECIES

SPECIES	INSTAR	LC-50 (ul/ml)	LC-100 (ul/ml)
Anopheles culicifacies	1st	0.35	0.45
	2nd	0.4	0.45
	3rd	0.55	0.65
	4th	0.6	0.80
Anopheles fluviatilis	1st	0.4	0.45
	2nd	0.4	0.45
	3rd	0.55	0.70
	4th	0.6	0.75
Anopheles stephensi	1st	0.35	0.4
	2nd	0.35	0.45
	3rd	0.5	0.60
	4th	0.60	0.075
Anopheles annularis	1st	0.45	0.50
	2nd	0.45	0.55
	3rd	0.50	0.75
	4th	0.55	0.80
Anopheles pulcherrimus	1st	0.30	0.45
	2nd	0.35	0.50
	3rd	0.45	0.60
	4th	0.55	0.75

(Contd...p/)

Contd...

Anopheles maculatus	1st	0.4	0.5
	2nd	0.5	0.55
	3rd	0.55	0.6
	4th	0.60	0.65
Culex tritaeniorhynchus	1st	0.30	0.35
	2nd	0.36	0.40
	3rd	0.45	0.55
	4th	0.55	0.60
Culex fatigans	1st	0.35	0.45
	2nd	0.35	0.45
	3rd	0.45	0.50
	4th	0.55	0.55

TABLE - 32

LC-50 AND LC-100 VALUES OF BAKAIN TREE, MELIA AZEDARACH L., FRUIT EXTRACT AGAINST LARVAE OF DIFFERENT MOSQUITO SPECIES

SPECIES	INSTAR	LC-50 (ul/ml)	LC-100 (ul/ml)
Anopheles culicifacies	1st	0.45	0.55
	2nd	0.5	0.65
	3rd	0.65	0.85
	4th	0.70	0.90
Anopheles fluviatilis	1st	0.50	0.6
	2nd	0.55	0.6
	3rd	0.65	0.75
	4th	0.70	0.95
Anopheles stephensi	1st	0.45	0.5
	2nd	0.48	0.55
	3rd	0.50	0.75
	4th	0.75	0.85
Anopheles annularis	1st	0.50	0.60
	2nd	0.50	0.65
	3rd	0.65	0.95
	4th	0.80	1.05
Anopheles pulcherrimus	1st	0.45	0.55
	2nd	0.45	0.55
	3rd	0.60	0.75
	4th	0.70	0.85

(Contd....p/)

Contd....

Anopheles maculatus	1st	0.35	0.45
	2nd	0.40	0.50
	3rd	0.55	0.65
	4th	0.65	0.80
Culex tritaeniorhynchus	1st	0.35	0.5
	2nd	0.42	0.55
	3rd	0.55	0.75
	4th	0.58	0.8
Culex fatigans	1st	0.35	0.45
	2nd	0.42	0.54
	3rd	0.56	0.65
	4th	0.65	0.75

TABLE 33

AVERAGE LARVAL DENSITY WITH NEEM LEAVES EXTRACT TREATMENT

Replication	RI	RII	RIII	Average larval density
Larval dip count before exposure	31.5	36.4	35.0	34.3
After 1 day	10.5	13.8	16.2	13.5
After 2 days	10.8	10.0	10.2	10.7
After 3 days	10.0	8.4	10.8	9.7
After 4 days	10.2	12.5	13.0	11.9
After 5 days	15.4	14.1	20.5	16.6
After 6 days	22.6	23.6	29.6	25.3
After 7 days	30.3	33.5	37.8	33.8

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TABLE - 34

PERCENT LARVAL REDUCTION WITH NEEM LEAVES EXTRACT

	Average larval density	Average control	Reduction	% Reduction
Larval dip count before exposures	34.3	35	0.7	2.0
After 1 day	13.5	37	23.5	63.5
After 2 days	10.7	37	26.3	71.0
After 3 days	9.7	38	28.7	75.5
After 4 days	11.9	35	23.1	66.0
After 5 days	16.6	37	20.4	55.1
After 6 days	25.3	41	15.7	38.3
After 7 days	33.8	38	4.2	11.0

TABLE - 35

AVERAGE LARVAL DENSITY WITH NEEM FRUIT POWER TREATMENT

Replication	RI	RII	RIII	Average larval density
Larval dip count before exposure	35.8	36.4	38.6	36.9
After 1 day	9.8	15.2	17.8	14.6
After 2 days	9.2	10.8	10.6	10.2
After 3 days	9.0	11.0	12.6	10.9
After 4 days	12.8	19.6	19.0	17.1
After 5 days	19.2	22.4	24.4	22.0
After 6 days	25.0	27.4	29.8	27.0
After 7 days	33.4	34.4	37.2	35.0

TABLE - 36

PERCENT LARVAL REDUCTION WITH NEEM FRUIT POWDER

	Average larval density	Average control	Reduction	% Reduction
Larval dip count before exposure	36.9	35	-	-
After 1 day	14.6	37	22.4	60.5
After 2 days	10.2	37	22.8	72.4
After 3 days	10.9	38	27.1	71.3
After 4 days	17.1	35	17.9	51.1
After 5 days	22.0	37	15.0	40.5
After 6 days	27.0	41	14.0	34.4
After 7 days	35.0	38	3.0	7.9

TABLE- 37

PERCENT LARVAL REDUCTION WITH BAKAIN LEAVES EXTRACT

	Replications					
	RI	RII	RIII	Aver-age	Contr-ol	% Reduc-tion
Larval dip count before exposure	33.8	35.6	37.8	35.7	34.8	00
After 1 day	7	7.8	10.8	8.5	35.8	76.26
After 2 days	4.6	5.8	4.8	5	36.2	98.6
After 3 days	3.8	5	5.2	4.6	34.4	86.6
After 4 days	5.8	6.2	5.2	5.6	39.6	85.9
After 5 days	14.2	15.4	17	15.5	37.4	58.5
After 6 days	26	35.4	38.4	33.2	35.6	6.7
After 7 days	34.8	39.4	38	34	36	5.6
After 8 days	35	36.6	35.6	35.6	40.2	11.4

TABLE - 38

PERCENT LARVAL REDUCTION WITH BAKAIN FRUIT EXTRACT TREATMENT

	Replications					
	RI	RII	RIII	Total average	Control	% Reduction
Larval dip count before exposure	37	37.6	40.2	38.3	34.8	00
After 1 day	7.2	7.6	9	7.9	35.8	78
After 2 days	6	7.2	7.4	6.8	36.2	81.2
After 3 days	6.8	7.8	9.2	7.9	34.4	77.1
After 4 days	20	22.6	21.8	21.4	39.6	46
After 5 days	35	32.6	33.6	33.7	37.4	10
After 6 days	39.2	36.8	36.8	37.6	35.6	00

TABLE- 39

PERCENT MORTALITY OF MOSQUITO LARVAE IN FIELD TRIALS AFTER EXPOSURE TO BAKAIN LEAVES AND FRUIT EXTRACT IN WATER

	LEAVES EXTRACT	FRUIT EXTRACT
After 1 day	76.26	78.0
After 2 days	98.6	81.2
After 3 days	86.6	77.1
After 4 days	85.9	46.0
After 5 days	58.5	10.0
After 6 days	6.7	00.0

TABLE - 40

EFFECT OF 2 ml/sq.m. BACILLUS THURINGIENSIS H-14 AND 300 gm/sq.m. NEEM LEAVES POWDER ON THE DENSITY OF MOSQUITO LARVAE

	AVERAGE LARVAL DENSITY			
	Anophelines		Culicines	
	Treated	Control	Treated	Control
Before treatment	23	19	48	51
2 hours after	00	22	06	49
4 hours after	00	18	00	54
6 hours after	00	23	00	52
8 hours after	00	22	00	49
After one day	00	20	00	47
After two days	00	16	00	50
After three days	00	17	00	48
After four days	00	19	00	44
After five days	00	13	00	46
After six days	00	11	00	38
After seven days	00	9	00	43

TABLE- 41

EFFECT OF 2 ml/sq.m. BACILLUS THURINGIENSIS H-14 AND 250 gm/sq.m. NEEM FRUIT/SEED POWDER ON THE DENSITY OF MOSQUITO LARVAE

	AVERAGE LARVAL DENSITY			
	Anophelines		Culicine	
	Treated	Control	Treated	Control
Before treatment	17	19	59	43
2 hours after	00	22	00	51
4 hours after	00	19	00	49
6 hours after	00	23	00	54
8 hours after	00	22	00	49
After one day	00	20	00	47
After two days	00	16	00	50
After three days	00	17	00	48
After four days	00	19	00	44
After five days	00	13	00	46
After six days	00	11	00	38
After seven days	00	9	00	43

TABLE - 42

EFFECT OF 2 ml/sq.m. BACILLUS THURINGIENSIS H-14 AND 250 gm/sq.m. BAKAIN LEAVES POWDER ON THE DENSITY OF MOSQUITO LARVAE

	Anophelines		Culicines	
	Treated	Control	Treated	Control
Before treatment	16	19	45	51
2 hours after	00	22	00	49
4 hours after	00	18	00	54
6 hours after	00	23	00	52
8 hours after	00	22	00	49
After one day	00	20	00	47
After two days	00	16	00	50
After three days	00	17	00	48
After four days	00	19	00	44
After five days	00	13	00	46
After six days	00	11	00	38
After seven days	00	9	00	43

TABLE- 43

EFFECT OF 2ml/sq.m. BACILLUS THURINGIENSIS H-14 AND 250 gm/sq.m. BAKAIN FRUIT POWDER ON THE DENSITY OF MOSQUITO LARVAE

	Anophelines		Culicines	
	Treated	Control	Treated	Control
Before treatment	21	19	54	51
2 hours after	04	22	11	49
4 hours after	00	18	00	54
6 hours after	00	23	00	52
8 hours after	00	22	00	49
After one day	00	20	00	47
After two days	00	16	00	50
After three days	00	17	00	48
After four days	00	19	00	44
After five days	04	13	09	46
After six days	07	11	12	38
After seven days	06	9	14	43

TABLE -44

PERCENT ANOPHELINE LARVAL CONTROL AFTER TREATMENT WITH NEEM AND BAKAIN EXTRACT IN COMBINATION WITH BACILLUS THURINGIENSIS H-14

	Neem leaves plus Bti.	Neem fruit plus Bti.	Bakain leaves plus Bti.	Bakain fruit plus Bti.
After 2 hours	100	100	100	91.9
After 4 hours	100	100	100	100
After 6 hours	100	100	100	100
After 8 hours	100	100	100	100
After one day	100	100	100	100
After two days	100	100	100	100
After three days	100	100	100	100
After four days	100	100	100	100
After five days	100	100	100	69.3
After six days	100	100	100	37.4
After seven days	100	100	100	33.4

TABLE - 45

PERCENT CULICINE LARVAL CONTROL AFTER TREATMENT WITH NEEM AND BAKAIN EXTRACT IN COMBINATION WITH BACILLUS THURINGIENSIS H-14

	Neem leaves plus Bti.	Neem fruit plus Bti.	Bakain leaves plus Bti.	Bakain fruit plus Bti.
After 2 hours	87.8	100	100	77.6
After 4 hours	100	100	100	100
After 6 hours	100	100	100	100
After 8 hours	100	100	100	100
After one day	100	100	100	100
After two days	100	100	100	100
After three days	100	100	100	100
After four days	100	100	100	100
After five days	100	100	100	80.2
After six days	100	100	100	68.6
After seven days	100	100	100	67.5

TABLE-46

AVERAGE WEIGHT IN GRAMS OF ALBINO RATS AFTER 20 DAYS OF FEEDING/INJECTION WITH NEEM/BAKAIN, LEAVES/FRUIT EXTRACT

Control Normal	NEEM LEAVES						NEEM FRUIT						Control Normal
	Subcutaneous Injection			Oral Feeding			Subcutaneous Injection			Oral Feeding			
	0.1cc	0.2cc	0.3cc	0.1cc	0.2cc	0.3cc	0.1cc	0.2cc	0.3cc	0.1cc	0.2cc	0.3cc	
Body weight	47	47.06	46.8	46.6	48	47.06	46.7	47	48.4	47.03	47.03	47	47.03
Liver	2.5	2.2	2.4	2.3	2.5	2.1	2.3	2.5	2.3	2.5	2.3	2.1	2.4
Lungs	0.45	0.43	0.44	0.55	0.45	0.44	0.45	0.42	0.42	0.5	0.45	0.45	0.56
Kidney	0.56	0.49	0.38	0.5	0.45	0.38	0.45	0.39	0.45	0.44	0.44	0.45	0.55
Spleen	0.15	0.13	0.13	0.14	0.15	0.13	0.14	0.14	0.13	0.14	0.13	0.14	0.15

	BAKAIN LEAVES						BAKAIN FRUIT						Control Methanol
Body wieght	46.6	46.8	47.06	47	47.03	47	47	46.08	46.5	47.03	46.5	46.8	47
Liver	2.6	2.6	2.4	2.4	2.4	2.7	2.25	2.4	2.5	2.25	2.5	2.7	2.7
Lungs	0.49	0.45	0.34	0.48	0.48	0.35	0.45	0.45	0.45	0.5	0.46	0.5	0.45
Kidney	0.5	0.55	0.45	0.5	0.5	0.45	0.47	0.5	0.45	0.6	0.6	0.47	0.5
Spleen	0.15	0.16	0.15	0.15	0.17	0.15	0.15	0.15	0.15	0.18	0.15	0.18	0.2