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Participatory Disease Surveillance of Transboundary Animal Diseases in South Azad Jammu and Kashmir

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Abstract.- Transboundary animals diseases (TADs) including rinderpest, foot and mouth disease (FMD) and peste-des-petits ruminants (PPR) along with other livestock diseases were searched in south Azad Jammu & Kashmir (AJK). All possible participatory tools of disease surveillance were applied in randomly selected villages of three districts Mirpur, Bhimber and Kotli (South AJK). Analysis of data indicated that FMD was the most prevalent and haemorrhagic septicaemia (HS) was the most important disease in the region. Other important livestock diseases recorded during PDS activity were PPR, enterotoxaemia, prolapse (uterine, vagino-cervical) and mastitis. There was no history of rinderpest in AJK since its independence. PPR was noted as an emerging disease of small ruminants causing heavy losses especially in Bhimber and Kotli.

Key words: Transboundary animals diseases; participatory disease surveillance; livestock diseases in AJK.

Participatory epidemiology is the application of participatory methods to epidemiological research and disease surveillance. It is a proven technique which overcomes many of the limitations of conventional epidemiological methods, and has been used to solve a number of animal health surveillance and research problems. The approach was developed in small scale, community animal health programs and then applied to major international disease control efforts (Jost *et al.*, 2008).

Because of severe resource and logistical constraints in large areas of Africa, disease surveillance system need to maximize the use of information provided by livestock keepers and make correct interpretations of indigenous livestock knowledge (Catley, 2006). The failure of formal data-collection methods to produce cost effective and reliable information for designing rural development projects in developing countries was first recognized in the early 1970's (Chambers, 1983). This problem was related to their capacity to understand the problems of the poorest and most marginalized people in rural communities. In response to this situation, alternative system of inquiry has since been developed. These system include rapid rural appraisal (RRA) and participatory rural appraisal (PRA) which, to varying degrees, enable local people to play a more active role in defining, analyzing and solving their own problems (Chambers, 1994). Participatory epidemiology relies on the widely accepted techniques of PRA, ethno-veterinary surveys and qualitative epidemiology (Schwabe, 1984). This information can be used to design better animal health projects and delivery systems, more successful surveillance and control strategies or as new perspectives for innovative research hypothesis in ecological epidemiology (Ali *et al*., 2006).

The PDS approach was developed in Africa as an accurate and rapid method to understand the distribution and dynamics of rinderpest (Mariner and Peter, 2003). During the year 2003 a project, "Support for Emergency Prevention and control of Transboundary Animal Diseases in Pakistan" was launched. Eleven teams of veterinarians throughout the country after providing training were given the responsibility of disease search. The information collected was utilized to get accreditation of freedom from rinderpest from OIE during 2006. This paper reflects the situation of TAD and other animal diseases in southern region of Azad Kashmir determined by participatory disease surveillance (PDS) work during 2004-2006.

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Materials and methods

PDS targets

The PDS team for south AJK region consisted of three experienced veterinarians working together as facilitator, observer/moderator and recorder during the meetings with farmers and other field activities during 2004-2006. A total of 444 villages in three districts were given target by the project management. The participatory epidemiological methods used were proportional piling, seasonal calendar, mapping, interview with key informants including local experts (Siana) veterinary health personnel, livestock traders, milkmen, nomads etc through open and close ended questions to collect information about TAD and other Livestock diseases in the area.

Conduction of farmer's meetings

The meeting place, time, weather, language and other ethics consideration were kept in mind before arranging a meeting with the assistance of local veterinary officers, veterinary assistants, and social workers. Efforts were made to include farmers of all age, social and political groups. The meetings started with introduction of the team members and purposes to create good meeting atmosphere. General questions about day-to-day life of the farmers, their problems related to livestock health encouraged them to participate fully in the meeting. In order to avoid bias, the PDS team did not mention about TAD during interview. Whenever a respondent indicated the occurrence of any TAD, he was asked to describe the disease as a part of verification process and other probing questions specifically designed to elicit detailed information. If the farmer failed to describe the disease accurately, the report was eliminated. More then one meeting in the same village were organized to cross check the data collected in the first meeting.

Proportional piling

To estimate the relative prevalence and importance of livestock diseases, proportional piling method was used. Hundred beans or pebbles were given to the farmers and were asked to make piles according to the relative incidence to five most prevalent diseases. Similar exercise was carried out to determine relative importance of those five diseases. The farmers were encouraged to discuss freely among themselves during these exercises.

Seasonal calendar

Seasonal calendar was used to describe the prevalence of different animal diseases in different seasons. Each season was represented on the white paper by an object placed along x-axis and sketch or photograph illustration of disease along y-axis. Again ten beans or pebbles were given to the farmers to place them in each season according to their mutual understanding. A calendar was made through this exercise showing seasonal prevalence of livestock diseases in the area.

Mapping

Farmers were requested to draw a map of the village on the ground or with the help of a marker on white paper if possible. This map helped us to understand the resources available, possible interaction with animals from outside and to locate the cattle markets, ponds, grazing area**s,** and high risk areas.

Interview with key informants

After the completion of the meeting the PDS team visited the nearby cattle market, veterinary centers, dairy farms, sheep and goat farms, grazing areas to observe the health status of the animals and interviews were conducted with key informants to get secondary data about animal diseases. These key informants were identified during meetings and general survey of the area. Samples were collected for laboratory diagnosis where a TAD is reported.

Results

Local knowledge of animal diseases

It was noticed that farmers have good knowledge of animal diseases and seasonal occurrence. Use of local names of different diseases encouraged them to explain health problems in a better way. Use of pictures of diseased animals showing symptoms of the disease were quite helpful. It was noted that farmers and animal health practitioners were confusing the PPR with contagious caprine pleuropneumonia or enterotoxaemia as this disease was not experienced. Local names of the diseases were recorded.

Table I.- Traditional names, prevalence and importance of main livestock diseases in south AJK as determined by proportional Piling.

Technical name	Traditional name	Prevalence (%)			Importance (%)		
		Mirpur	Bhimber	Kotli	Mirpur	Bhimber	Kotli
Foot and mouth disease	Munkhurr	24.79	11.85	11.08	12.37	6.1	5.85
Peste-des-pitits ruminants	Seend/Thadi	1.25	9.12	8.78.	3.31	15.9	13.72
Haemorrhagic septicaemia	Gal Ghutu	9.75	6.45	11.98	26.64	17.02	27.43
Enterotoxaemia	Phetta	11.20	13.6	12.32	13.28	12.35	12.97
Mastitis	Munsari	6.77	9.85	10.94	6.49	7.32	7.25
Prolapse	Pahar	4.04	18.52	11.36	4.73	17.17	10.19
Black quarter	Tangopara	4.74	2.57	4.4	4.73	6.25	6.4
Fasciolosis	Peela	3.02	4.6	2.84	1.89	3.32	1.95
Pleuropneumonia	Kanar	5.34	4.10	4.43	7.12	2.90	3.77

Proportional piling

Proportional piling was a useful exercise to determine the prevalence and importance of livestock diseases. Results of proportional piling in all three districts are summarized in Table I. Farmers agreed that FMD , HS and enterotoxaemia in district Mirpur, FMD, PPR, HS, prolapse, enterotoxaemia and mastitis in district Bhimber and Kotli were the serious health problems and need attention. Prolapse in district Bhimber and Kotli badly affecting the reproductive performance of the buffalo. FMD was reported as the most prevalent disease in the region, while HS was ranked as the most important disease of cattle and buffalo because of its high morbidity and mortality rates. Enterotoxaemia was recorded as one of the common problem of sheep and goat. There was no history of rinderpest in the area and it was not reported in any of 444 villages surveyed by the PDS team. PPR was noted as an emerging disease of sheep and goat spreading infection in south AJK rapidly. Serious efforts are needed to control the spread of disease in rest of the state of Jammu & Kashmir.

Seasonal calendar

Most of the time farmers divided the year into four seasons *i.e.*, Spring, Summer, Autumn and Winter. Only at few occasions the rainy season was described separately. The overall seasonal calendar in three district elaborated that FMD outbreaks were constant throughout the year. Although the outbreaks were severe in spring and summer. HS outbreaks occurred in the four seasons but very severe in summer and moderate in winter. PPR out breaks occurred mostly in winter and spring. Mastitis was also found throughout the year. Season wise occurrence of important livestock diseases is shown in Table II.

Table II. Seasonal prevalence of diseases in South AJK.

Name of disease	Spring	Summer	Autumn	Winter
FMD	25.1%	28.8%	11.5%	18%
PPR	13.7%	6.4%	1.7%	19.6%
HS	6.5%	57.6%	4.1%	23.4%
Enterotoxaemia	7.2%	13.9%	1.3%	--
Mastitis	11%	47.4%	18.5%	9.8%
Black quarter	1.3%	9.7%	--	--

Discussion

Azad Jammu & Kashmir is located at the foothill of Himalayas, north east of Pakistan and is bordered by Gilgit in the north NWFP in the west, Punjab province in the south west and line of control in the east forming a "C" shape extending from north to south. In fact it can be distinguished into two regions north and south. The two regions of the state are quite different topographically. The south AJK is closely associated with Punjab province of Pakistan. The animals brought form Rawalpindi, Jehlum and Gujrat districts may introduce new disease in comparatively susceptible local animals. Results of PDS activity performed in south AJK during 2004-2006 disclosed that FMD was the most prevalent disease of animals while HS

was the most important infection of cattle and buffalo due to its high mortality rates. PPR, enterotoxaemia, prolapse and mastitis are also important health problems of animals in the region. There was not history of rinderpest for last 10 years.

PPR was observed as a new disease. Most of the farmers have not seen it before, they were confusing it with pleuropneumonia and enterotoxaemia. After comparing the data from different sources and laboratory diagnosis of the samples collected, it was easy to reach at the conclusion. Pictures illustrating different signs of the disease were quite helpful in this regard. HS has been reported as the most important bacterial disease of cattle and Buffalo in Pakistan (Munir *et al*., 1994). Data collected during PDS elaborates further its importance as animal health hazard. Through PDS the management of veterinary services become aware that HS was a great concern to farmer's livelihood than the three target diseases of international concern.

PDS, an active type of disease surveillance, is the application of participatory techniques, like owner's knowledge about the clinical signs, gross pathology and epidemiology, to draw conclusions about disease through variety of questions from the key informants during the interview (Farooq *et al.*, 2007). Data collection by applying participatory techniques was a new approach. Farmers in most of the villages appreciated the approach after realizing the importance of their knowledge. In addition to the data that emerged during participatory research the process of sitting and listening to the people had value in itself. This exercise took the form of providing people an opportunity to express their views and helped to improve the community's relationship with the researches (Hussain *et al.*, 2005).

The farmers in AJK along with the veterinary professionals became familiar with the current situation of some important livestock health hazards and the importance of active disease reporting using participatory approach had trust in it now. It is because of PDS that the PPR was found future threat for sheep and goat and the department of Animal Husbandry AJK taking measures to meet this challenge.

The PDS program has greatly enhanced the sensitivity of active clinical Rinderpest surveillance and directly contributed to Pakistan's confidence in the decision to declare provisional freedom from Rinderpest to the OIE in January 2003. The valuable data accrued by PDS work has been appreciated by the project management and all livestock departments in the country (Hussain *et al*., 2005).

Acknowledgement

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Endohelminth Fauna Linked to Seasonal Changes and Host Fish Size of Pike (*Esox lucius* L.) from Lake Eber, Turkey

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Abstract.- In this study, endohelminth parasite fauna of 195 pike (*Esox lucius* L.) specimens from Lake Eber were investigated. Two parasite species were recorded: *Neascus bravicaudatus* **(**Nordmann, 1832) in eyes (20%; 2.7±3.1) and *Acanthocephalus lucii* (Müller, 1776) in intestine of the host fish specimens (9.7%; 1.8±1.2). Both of the parasite species are new record for the host fish species in Turkey. *N. bravicaudatus* infection prevalence was highest during winter and spring, and minimum during summer and autumn, with smaller host fish specimens having more parasites than bigger. The other parasite species, *A. lucii* went through distinct seasonal changes, peaking during the spring with bigger host fishes.

Keywords: *Acanthocephalus lucii*, *Neascus bravicaudatus*, *Esox lucius*

The study area is a natural eutrophic lake features. It's geographically located at 38º 40' N, 31º 12'E in central region of Turkey. It is shallow (mean depth ca. 4 m) and has a surface area of ca. 150 km^2, and an altitude of 967 m above sea level (Atay *et al*., 2002). The aim of the present study is to determine endohelminth parasite fauna of the host fish *Esox lucius* from Lake Eber. It was also aimed at determining changes in the intensity and prevalence level of the parasite species related to the season and age of the host fish.

Materials and methods

In this study, 10 to 35 host fish specimens were collected each time, either monthly or two-monthly from July 2002 to July 2003, by local fishermen using bow-nets. The fish were placed in plastic containers containing lake water and then transferred to the research laboratory. The pike were measured and their age determined from their scales. The fish sacrificed in the laboratory immediately prior to examination and then skin, fins, pharynx, esophagus, liver, gall-bladder, heart, stomach and intestine were dissected out and placed in separate Petri dishes with physiological solution. Parasites which were found in the host were removed using a preparation needle. The parasite specimens were fixed in formaldehyde or Bouin's fluid, stained with Mayer's haematoxylin, using the reference keys by Pritchart and Kruse (1982).

The host fish size was determined and the prevalence and intensity of infection was calculated for each parasite according to Margolis *et al*. (1982). Spearman's test was used to measure correlation between the intensity of each parasite species and host fish size. Kruskal-Wallis analysis of variance was applied to the data to determine the existence of any meaningful difference in mean intensity of the parasite species.

Results and discussion

Endoparasite fauna of pike

Two endohelminth species identified according to Bykhowskaya-Pavlovskaya *et al*. (1962) were *Neascus bravicaudatus* **(**Nordmann, 1832) in eyes (20%; 2.7±3.1) and *Acanthocephalus lucii* (Müller, 1776) in intestine of the host fish specimens (9.7%; 1.8±1.1).

Adult *N. bravicaudatus* are intestinal parasites of various piscivorous birds (Gaten, 1987). The metacercaria of the parasite was recorded from the lens of pike by Mishra and Chubb (1969); It was found in eye-lens of other teleost fish such as *Leuciscus cephalus*, *Gobio gobio*, *Alburnoides bipunctatus*, *Vimba vimba* by Moravec and Scholz (1991); in *Cyprinus carpio* by Pojmańska and Chabros (1993); *Stizostedion lucioperca*, *Abramis brama*, *Carassius auratus*, *Silurus glanis* by Molnar and Székely (1995). The other species, *A. lucii* is a typical parasite of pike, as well as a common endoparasite of many freshwater fish species (Bykhowskaya-Pavlovskaya *et al*., 1962; Moravec, 1979; Moravec and Scholz, 1991).

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It is known that parasite fauna on various fish species of different geographical localities, especially those with different limnologic properties, exhibit very little homogeneity (Poulin, 2007; Iqbal and Haroon, 2014). Halvorsen (1971) said that local conditions are the most important factors in determining parasite fauna composition in freshwater localities. Parallel to the agreement, the parasite fauna of pike in Lake Eber is different to that reported from other freshwater localities (Öztürk *et al*., 2000, 2002; Kır and Tekin-Ozan, 2005). As a result of the present data, a new locality and a new host fish species were added to geographical invasions of the parasite species.

Seasonal changes of the parasite species

Figure 1 shows that the water temperature of Lake Eber falls into four different period: 12.5°C in spring (March-May); 22.9°C in summer (June-August); 11.4°C in autumn (September-November); 3.4°C in winter (December-February).

From these parasites, 106 *N. brevicaudatus* were found in the eyes of 39 host fish specimens and captured in all seasons. When seasonally viewed, prevalence of the infection increased steadily from winter reached its maximum value (26.8%) in spring, and then decreased in the other period (Fig. 1). *A. lucii* was found in 19 host fishes in the study period. The infection prevalence and mean intensity were low. The intensity of the parasite changed from 1 to 5, and the highest infection rate was observed in spring (Fig.1).

It is understand from the data that presence or absence of the parasites depends on the seasonal changes of host fish size and various factors such as food of fish, intermediate host, temperature and pH (Moravec, 1985; Moravec and Scholz, 1991). Those findings can be linked to the differences in the limnological characteristics of the different research areas (Moravec and Scholz, 1991; Pojmańska, 1994).

Distribution of the parasite species of host fish size

Detailed data related to size of the host fish are given in Figure 2. *N. brevicaudatus* was found in small sized fish. Its occurrence reached maximum in size group III, had maximum prevalence in the first group, and no infection in the group V. The other

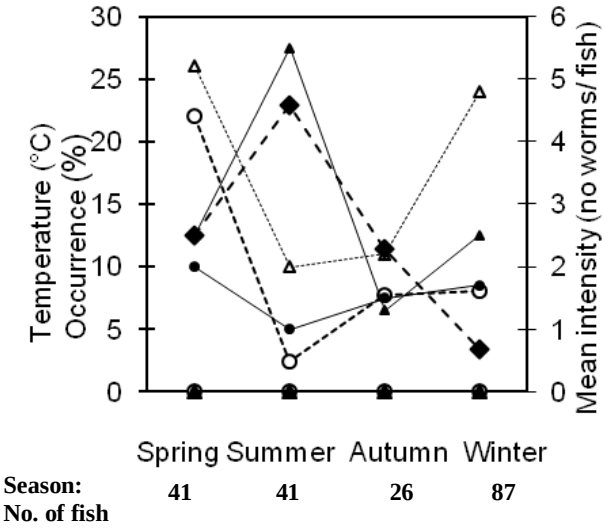

Fig. 1. Occurrence of *N. Brevicaudatus* (Δ), *A. lucii* (○) and mean intensity of *N. Brevicaudatus* (▲),: *A. lucii* (●) of *E. lucius* and water temperature (♦) of Lake Eber during the study period.

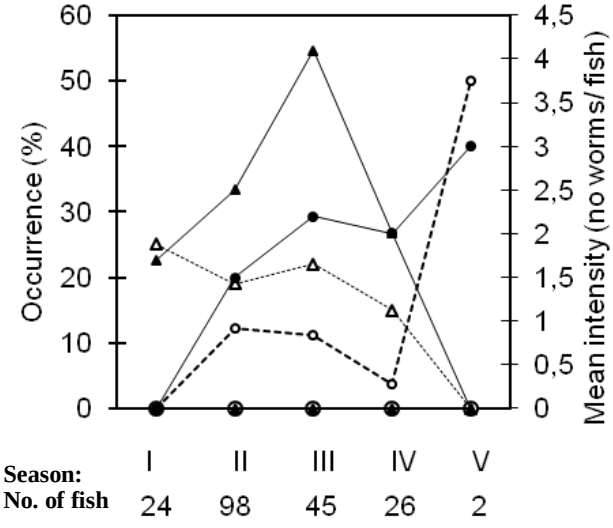

Fig. 2. Occurrence of *N. Brevicaudatus* (Δ),*A. Lucii* (○) and mean intensity of *N. Brevicaudatus* (▲) and :*A. Lucii* (●) in relation to size groups of *E. lucius* from Lake Eber.

A. lucii was found in the medium and big host fish specimens. The prevalence changed erratically from size to size, and it reaches the maximum intensity of 3.0±0.0 parasites for the biggest fish specimens (Fig.2).

Number of the parasites and their diversities on the basis of the host fish sizes and seasons showed significant variation. Other hand, details of the correlation coefficient and its significance value varies between (r) –0,220 and 0,430 of the each parasite species and fish size.

In the present study, prevalence of *N. brevicaudatus* changed erratically from size to size. And maximum value of mean intensity was recorded in medium fish specimens. For the other parasite species, *A. lucii*, the infection prevalence and intensity increase parallel with the age of the host fish. It may have an effect on compositions of parasite fauna depending on age of fish (Tieri *et al*., 2006). Geldiay and Balık (1999) support the aforementioned views stating that pike when it is young it eats organisms like infected insects, copepod, crustacea and finally when it becomes adult it eats small fish specimens.

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First Record of Genus *Protosticta* Selys, 1885 (Odonata: Zygoptera: Platystictidae) for Pakistan

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Abstract.- A new record to the Zygoptera fauna of Pakistan has been added by reporting *Protosticta hearseyi* from two different localities of the country. The genus also is a new record for Pakistan. *P. hearseyi* is a data deficient threatened species known earlier only from India. Detailed distribution and habitat description is provided to fill ecological information of the species. Diagnostic characters are also provided to facilitate identification for future taxonomists based on published description.

Key Words: *Protosticta hearseyi*, Oriental damselfly, Zygoptera.

Tropical family Platystictidae of sub order Zygoptera has diversified distribution with three reported subfamilies *i.e.,* Palaemnematinae, Platystictinae and Sinostictinae. Among these, Platystictinae is known from western parts of Oriental region to eastwards of Papuan region. Within Platystictinae, three genera (*Drepanosticta* Laidlaw, 1917; *Platysticta* De Selys, 1860 and *Protosticta* Selys, 1885) are known (Van Tol, 2000). Genus *Protosticta* first established by Selys during the year 1885 (Yu and Bu, 2009) is restricted to Oriental region (Wilson, 1997). It has been reported from China (Zhou, 1986), India (Fraser, 1933a), Laos (Fraser, 1933b), Burma and Thailand (Asahina, 1984; Hämäläinen and Pinratana, 1999), Hong Kong (Wilson, 1995; Yu and Bu, 2009), Malaysian peninsula and Borneo (Lieftinck, 1954), Celebes (Selys, 1885; Lieftinck, 1939), Vietnam (Asahina, 1997) and Philippines (Hämäläinen and Müller, 1997). Among neighboring countries to Pakistan, nine species of this genus are reported from India (Subramanian, 2009) and three are known from China (Yu and Bu, 2009). Genus *Protosticta* however remained unexplored in Pakistan earlier to this study.

Protosticta hearseyi is a poorly known species of the genus *Protosticta* with limited reported distribution. It is known only from the southern parts of India (Uttarakannad, Udupi, Nilgiris and Annamalai hills) with an elevation of 1000m approx. (Fraser, 1933a; Subramanian, 2013). It is a potentially threatened species with very limited information available for its habitat and population (Subramanian, 2013). Further surveys are therefore required to know more about its biology, population, habitat and distribution.

Materials and methods

During country wide surveys (2004 – 2010) carried out to explore Zygoptera fauna of Pakistan, nine specimens of *Protosticta hearseyi* were encountered in two small villages located at hilly belt separating Islamabad and Murree hills. Among these, Islamabad comes under Potohar plateau of Punjab province and represents an Oriental distribution of insect species with traces of Palearctic fauna. It lies at 33.43°N 73.04°E on the foot of Margalla hills with an elevation of 507m (1,663ft). However Murree stands on southern slopes of Western Himalayan foothills as they ascend northeast towards Kashmir. It holds high altitude *i.e.* 2,300m (7,500ft).

Material examined

L1: Chachal village (33°49'N, 73°29'E; 441m), 10. vii. 2005, 2♂ 1♀; 12. vii. 2005, 1♂ 1♀ (two pairs caught during mating), leg. Zia.

L2: Lehtarar (33°43'N, 73°27'E; 484m), 15. vii. 2005, 2♂ 2♀ leg. Zia.

Collected specimens were identified following Fraser (1933a). However due to some very minute and confusing differences among genus *Protosticta* and *Drepanosticta*, all specimens were further confirmed through Van Tol (2000) for generic characters. Diagnostic characters were also compared from Fraser (1933a) and are discussed as well. Both genus as well as species are new records for Pakistan.

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Results

Measurement of characters (in mm)

Male (♂): Abdomen, 33.50±2.04; forewing, 22.94±0.39; hindwing, 22.05±0.46.

Female (♀): Abdomen, 32.00; forewing, 22.83±0.57; hindwing, 21.83±0.76.

Diagnostic characters

The description of characters different from Fraser (1933a) is,

Male

Head: First antennal joint, vertex and occiput black. A broad turquoise blue stripe present between eyes. This stripe on one end touches occiput and on the other includes two ocelli in it. Prothorax: a broad black stripe on anterior of mesepimeron. Legs: femora on extensor surface without any black transverse lines. Wings: pterostigma dark brown, 13–14 post nodals to forewings, 12–13 in hind. Abdomen, segment 9 bluish black and a little more than twice the length of segment 10 which is itself having a very dull bluish spot at the mid basal dorsum.

Female

Wings: 14 post nodals to forewings and 13 in hindwings. Anal appendages: Brownish black.

Comments on habitat

Specimens were collected from two different sub-mountainous spots. L1 was a seasonal water flow passing by Chachal village. It was a smooth, narrow and slow water channel covered by dense shade of surrounding dwarf trees and tall grasses. Spiky bushes were bent over it with their tender stalks dipped into it thereby reducing speed of water flow. Lot of vegetables were also grown in close proximity to this water channel. In contrast to this L2 was a hilly spot with water flowing downwards from uphill in narrow twisted channels surrounded with dense wild flora and tall pine trees around it. Here spring water was also getting mixed with the seasonal flow. Contrary to expectations, the water in water way was clear and not dirty at all.

Remarks

Genus *Protosticta* along with its species *P. hearseyi* is being reported for the first time from Pakistan, thereby adding new geographic record to its distribution. Fraser (1933a) reported it from India where is a hill side with no human disturbance. However in the current study both localities were near roadsides with lot of human disturbance. Fraser (1933a) pointed out curious incidence of sexes in this species. Present study however shows normal trend reporting both the sexes from both localities in normal proportion.

According to Subramanian (2013) little information is available for this species and its habitat preferences and it is potentially on threat from agricultural pollution. Because of this lacuna about habitat details of *P. hearseyi*, detailed habitat information is provided to facilitate readers of this article. At L1 heavy pesticide spraying was also noticed to save vegetable fields from pest attacks, which is no doubt very harmful for this important and threatened species. Extensive surveys are recommended to know more about its biology and population so as to take necessary measures for its conservation. Odonata fauna of Pakistan should be further explored and measures should be taken to conserve many of the endangered species of the country that are getting at stake of extinction (Din *et al*., 2011; Zia *et al*., 2011a,b).

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Thawing/Holding Sahiwal Semen Straws in Ice Water for Artificial Insemination in Cattle – An Adaptive Experience

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Abstract.- This study investigated a practical method of carrying frozen semen for artificial insemination (AI) in small sized cattle herds. Conception rate of frozen semen of three Sahiwal bulls thawed either at 37°C for 45 sec (warm water thaw, WWT) or in ice water at 5°C for 35 min (ice water thaw, IWT) was compared. A total of 301 cows were inseminated in District Mianwali (Pakistan). Overall conception rate (determined by rectal palpation after 45 days of AI) with two thawing methods (WWT, 71.3%; IWT, 68.9%) did not differ ($P > 0.05$). There was no bull effect ($P > 0.05$) on the conception rate with WWT and IWT (Bull I, 66.0 and 76.0%; Bull II, 76.0 and 61.2%; Bull III, 71.7 and 69.2%). It is concluded that IWT of Sahiwal bull semen for 35 min did not seem to have adverse effects on cow conception.

Key words: Semen thawing, artificial insemination, conception, Sahiwal cattle.

Semen of elite bulls has been used extensively through artificial insemination (AI) for genetic improvement of dairy cows (Ansari *et al.*, 2010). Cryopreserved semen has to be thawed before insemination. The results from frozen-thawed bovine spermatozoa evaluations tend to indicate that thawing at 37°C for a minimum of 30 sec is an adequate method for obtaining superior post-thaw sperm quality and fertility in the field and same is being practiced now a day (Beran *et al.*, 2012). This means removing a straw from liquid nitrogen and placing immediately in 33–35°C water for a minimum of 40 sec before preparing the AI gun (Kaproth *et al.*, 2005). However, alternative thawing methods have been practiced in the field. They include pocket thaw, air thaw and thawing multiple straws of semen. As inseminations are aimed to achieve the best possible conception rate, extensive investigations were carried out for alternative thaw methods (Kaproth *et al.*, 2002, 2005; Oliveira, 2012). Due to shortage of liquid nitrogen tanks in developing countries of the tropics, semen straws have to be thawed at the collection site and kept in cold water until insemination (Hayashi, 2005). Similarly in Pakistan, the number of AI centers is limited and technicians have to manage a wide area with limited resources. It has been noticed that some AI technicians while performing inseminations at farmers' doorstep, carry semen in cold water (generally 1 to 5°C; hereafter called ice water) in a small insulated flask (hereafter called thermos). It is a practicable method for handling and using cryopreserved straws in regions where adequate equipment for holding straws at cow sides are not possible. The method also saves liquid nitrogen evaporation especially in the tropical areas, and evades damage to liquid nitrogen tank. Insemination is performed after thawing and holding straws in ice water for ≥ 30 min, as travel to insemination site may take half an hour or so. The present note reports on this adaptive experience of thawing/holding cryopreserved Sahiwal semen in ice water for AI in cattle.

Materials and methods

Source of semen

Frozen semen from Sahiwal bulls (n=3) kept at three different semen production units (Karaniwala, Qadirabad and Kloorkot) was used in this trial. A uniform one step extender (containing Tris, citric acid, fructose, egg yolk, glycerol) was used for processing semen into 0.5 mL straws and cryopreserved. Total sperm per straw prior to packaging ranged from 50 to 60 million, while live sperm number per straw was set at 40 million. The semen straws from each bull were prepared from the same ejaculate. Progressive motility of spermatozoa

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after thawing at 37°C for 45 sec was 45-50% in three bulls when the semen was received at the place of experiment. Post thaw semen motility after ice water thaw (IWT) for 35 min was 40 - 45%.

Thawing methods

Two thawing methods compared were warm water thaw (WWT) and IWT. For WWT a straw was retrieved from the liquid nitrogen container and placed in a water bath at 37°C for 30 sec. Exposure of straw to room temperature was not more than 2 to 3 sec and insemination was completed within 5 min after retrieval of the straw from liquid nitrogen. For IWT, water of 5°C was prepared by pouring ice cubes into a vacuum cooler flask. A semen straw was retrieved from liquid nitrogen container and immediately placed nearly vertically in the ~~flask~~ thermos containing enough ice cubes to maintain 5°C for the next 35 min. The straw was carried to the cow "in heat" by AI technician and insemination was performed 35 min after removal of semen straw from liquid nitrogen. The thaw method alternated with every two inseminations.

Study area

Inseminations were carried out in tehsil Mianwali, Pakistan (32.00°N and 71.50°E) over a period of nine months (from October to June). Month wise average ambient temperatures were: October 16–33°C, November 9–28°C, December 4–21°C, January 3–19°C, February 6–21°C, March 12–26°C, April 17–33°C, May 22–38°C and June 27–42°C.

Cows and inseminations

Inseminations were performed either in Sahiwal, Crossbred (Friesian x Sahiwal) or nondescript cows. Repeat breeder cows were not included in the trial. Parity of the cows ranged from 1 to 6 and service period ranged from 2 to 12 months. Natural heat was used for inseminating the cows at 12–24 h after start of standing heat. All the inseminations were performed by an experienced AI technician. The conventional rectovaginal insemination procedure was used during the trial. Half of the inseminations were made after WWT, while the other half after IWT. An effort was made to balance the number of inseminations per bull and thawing method over cow-breed.

Conception status

Conception status in each cow was determined by palpation per rectum after 45 days of insemination. Cows that showed heat and were served again before day 45 were considered as non pregnant. Conception rate (%) was calculated from No. of cows pregnant at 45 days x 100 / No. of cows inseminated.

Statistical analysis

Conception rate after two methods of thawing semen was compared using the chi square test.

Table I.- Conception rate of cows inseminated with frozen semen from three Sahiwal bulls thawed either in warm water (37°C for 45 sec; WWT) or ice water (5°C for 35 min; IWT).

Bull	Warm water thaw		Ice water thaw	
	No. of cows inseminated	Conception rate (%)	No. of cows inseminated	Conception rate (%)
Bull I	47	66.0	50	76.0
Bull II	50	76.0	49	61.2
Bull III	53	71.7	52	69.2
Overall	150	71.3	151	68.9
SD		5.0		7.4

Conception rate did not differ significantly between the two thawing methods (P> 0.05; bull-wise as well as overall).

Results and discussion

Conception rate of cows inseminated with semen thawed either in warm water (37°C) for 45 sec or in ice water (5°C) for 35 min is presented in Table I. An overall conception rate of 71.3% was observed for 150 inseminations performed with semen from three bulls after WWT. A similar conception rate (69.6%) was observed in cows by Andrabi *et al.* (2001) using Sahiwal bull semen cryopreserved in Tris-citric acid based extender and thawed with conventional WWT (37°C for 30 sec). Conception rate after WWT (71.3%) and IWT (68.9%) did not differ significantly (P>0.05) in the present study. These observations are supported by the findings of Anwar *et al.* (2008) who used a holding time of 30-60 min in ice water as compared to 35 min in the present study. The results indicate that Sahiwal bull semen frozen in Tris-citric acid based extender can be placed in ice water after

removal from liquid nitrogen and conception rate is comparable with the control method (WWT) if inseminations after IWT are made within 35 min of the removal. The method is user friendly as technicians have not to carry liquid nitrogen tanks on rough terrains to the insemination site saving the expensive tanks from possible damage. At the same time it saves excessive evaporation of liquid nitrogen due to exposure to sun and constant movement during travel.

A deviation from the standard insemination procedure during IWT was the prolonged period of time between removing the semen from liquid nitrogen tank and depositing it into the cow (35 min). It has been recommended that a dose of semen should be used promptly after it was thawed; an absolute maximum of 15 min should be aimed for (Ball and Peters, 2004). However Kaproth *et al.* (2002) reported that there was no fall in conception rate for up to 20 min after thawing when a highly competent technician performed the insemination. In the present study a semen straw was placed in ice water after retrieval from a liquid nitrogen tank, and the straw remained dipped in ice water for 35 min before it was loaded into the AI gun. There seemed no adverse effect of a period of 35 min incubation in ice water on conception in cows. There are observations that in practice, the rate of thawing is rarely critical. Perhaps more importance is that the temperature of thawed semen must be controlled and it should not be allowed to fluctuate during the thawing otherwise substantial sperm-losses can occur (Parkinson, 2009). So the semen should be saved from adverse environmental effects after thawing and the inseminators must thermally protect "semen", "thawing equipment", "sheath" and "AI gun" until the semen is in the cow. Kaproth *et al.* (2002) observed that for semen processed with procedures that permit flexible-thawing, thaw method (pocket thaw versus WWT) did not affect conception rate under commercial conditions using routine semen handling methods. It is concluded that thawing and carrying Sahiwal bull semen in ice water for 35 min did not adversely affect cow conception. This is an alternative to carrying semen in liquid nitrogen tanks and is a probable solution for distance and terrain compounded by the small herd size in countries like Pakistan.

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Molecular Diagnosis of *Streptococcus equi* subsp. *equi* along with Carrier Potential in Clinically Affected Horses

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Abstract.- *Streptococcus equi* subsp. *equi* is highly contagious equine-specific disease characterized by abscessation of the neck and head lymph nodes. The prevalence and carrier potential of *S. equi* was investigated using 194 nasal swabs and 56 pus samples collected from clinically infected horses and examined by PCR. *S. equi was* detected in 122 (48.8%) horses. Of these, 20 (10 <2 year and 10 between 2 and 5 years old) remained positive after one week of recovery, and were monitored for 12 weeks to study their carrier status using PCR. It is concluded that PCR is rapid and sensitive diagnostic method for the identification of *S. equi* and the recovered horses should be quarantined at least for 9 weeks because they remained carriers and potential source of infection for susceptible equines.

Key words: Horses, PCR, *Streptococcus equi*, carrier.

Strangles which is caused by gram-positive coccus *Streptococcus equi* subspecies *equi* is an acute upper respiratory infection in horses and characterized by fever, lethargy, purulent nasal discharge and regional lymph node abscessation (Sweeney *et al.,* 2005). *Streptococcus equi* subsp. *equi* (causative agent of equine strangles) is shed in nasal discharges and is found in pus samples from lymph nodes of affected horses. Routine laboratory detection of the organism is based on culture of nasal swabs and pus from abscesses but is often difficult because of background contamination (Timoney and Artiushin, 1997). The traditional laboratory diagnosis of strangles by isolation and biochemical analysis normally takes more than 2 days and is considered the gold standard for confirmation of a clinical diagnosis. On the other hand the presence of other group C β-haemolytic streptococci in samples can complicate a laboratory diagnosis as it can be difficult to differentiate colonies of *S. equi* subsp. *equi* from those of *S. equi* subsp. *zooepidemicus* and *Streptococcus dysgalactiae* subsp. *equisimilis*. A further impediment of strangles is that about 10% of infected horses may develop into chronic carriers with intermittent shedding of *S. equi* from the guttural pouch (Patty and Cursons, 2014).

When laboratory tests are unable to detect the presence of *S. equi*, the confirmation of clinical diagnosis of strangles is hampered. In suspected strangles cases, reliable, sensitive and quick diagnostic methods are required to identify the presence of *S. equi*. Notably, this lack of laboratory confirmation may decline attention of stable owners to keep up biosecurity and quarantine measures during outbreaks (Lindahl *et al.,* 2013).

The rapidity, sensitivity and specificity of polymerase chain reaction (PCR) techniques have amplified their use to detect bacterial diseases of equines (Anzai *et al*., 2002). Timoney and Artiushin (1997) also reported that PCR is approximately three times more sensitive than the culture in detecting *S. equi*. A PCR method targeting the *S. equi* SeM gene known to be a major virulence factor and protective antigen of *S. equi* (Meehan *et al.*, 2000a,b) has been established and its benefit for the diagnosis of strangles reported (Newton *et al.,* 2000; Verheyen *et al.,* 2000).

Once infected, the majority of animals recover and *S. equi* are eliminated over a period of 4–6 weeks. However, in 10% of clinically recovered animals, *S. equi* may continue to shed intermittently for prolonged periods (Sweeney *et al.,* 2005). This carrier status is probably caused by incomplete drainage of exudate from the guttural pouches (empyema) and sinuses following rupture of abscesses formed in the retropharyngeal lymph

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nodes (Newton *et al.*, 1997). This is a preliminary report of molecular identification of *S. equi* and its continued presence in the carrier horses in Pakistan.

Materials and methods

In this study 250 horses that were tentatively diagnosed positive for strangles (*S. equi*) on the basis of clinical signs at Lahore and Sargodha districts of Punjab province during January–December 2009 are included in the present study. Nasal discharges and pus samples were collected from the horses using aseptic sterile disposable syringes and cotton swabs. These samples were processed in Microbiology Laboratory, University of Veterinary and Animal Sciences, Lahore. Out of these, 20 horses (10 <2 year and 10 of 2-5 years) infected with strangles were treated with procaine penicillin at dose rate of 22000 IU/kg b.i.d. and monitored weekly, during 12 weeks to study the carrier status using microbiological culture and PCR techniques.

Isolation of S. equi and *DNA extraction*

Samples were cultured on blood agar plates and incubated at 37ºC for 24h under anaerobic conditions (Jorm, 1990). Typical β hemolytic *streptococci* colonies were detected on blood agar and identified on the basis of colony morphology, gram staining and biochemical tests (Quinn *et al.*, 1994). DNA was extracted from *S. equi* samples with a Genomic purification kit (Fermentas* #K0512) and stored at -20°C till further processing.

Polymerase chain reaction

The forward primer (5/ AAA GTG TGC CCA TAA CGG GTA) and reverse primer (5/ CGG CTA TTG TCC ATT GGG GAA) were used to amplify 1812 bp from IGSzM gene using PCR Jet Thermocycler (Megabase Research Products, Lincoln, NE). PCR was performed as follows, 94°C for five min, followed by 30 cycles, each of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and 72°C for 10 min. PCR product was stored at 4°C. After PCR, the correct amplicon size was evaluated on ethidium bromide agarose gel electrophoresis.

Statistical analysis

Data on prevalence of the disease was analyzed by Chi-square test using SPSS (Statistical Package for Social Sciences).

Results and discussion

Figure 1 shows colony characteristics of *S. equi*. Figure 2 shows PCR amplication of DNAs from *S. equi* isolates of horses.

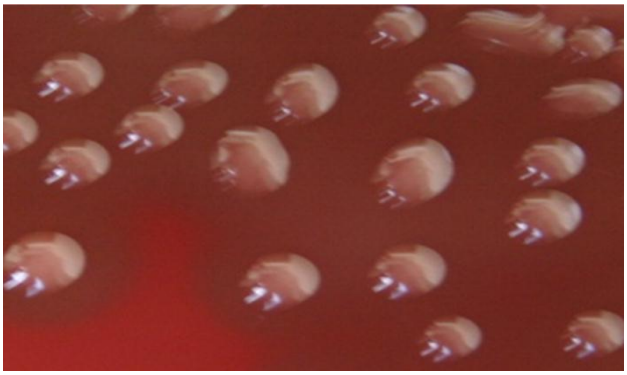

Fig. 1. Colony characteristics of *S. equi*.

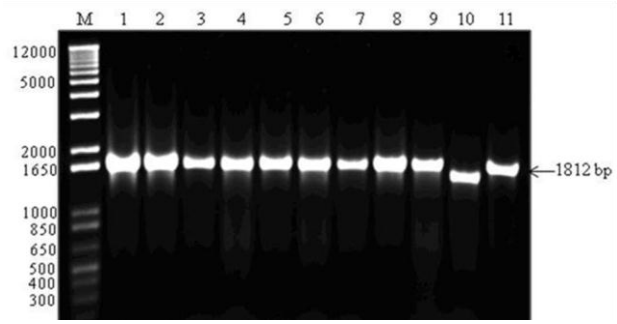

Fig. 2. PCR amplification of DNAs from *S. equi* isolates of horses with IGSzM primer. Lanes 1-9 shows the PCR products of *S. equi* with sized 1812 bp. Lane 10 and 11 serve as –ve control of (W 60, *S. zooepidemicus*, USA) and +ve control (CF32, *S. equi*, USA), respectively.

Data regarding seasonal prevalence of Strangles (*Streptococcus equi*) in various age groups of horses is shown in Table I. Of 250 horses, 122(48.8%) horses tested positive in the PCR for *S. equi.* The highest prevalence of strangles was observed in the group of horses having less than 1 year of age than 1-2 years and decreased with increase in age as reported by Ijaz *et al.* (2010, 2011, 2012), that younger equines having age less than 2 years are more prone to this disease in

Table I.- Prevalence of *Streptococcus equi* isolated from pus and nasal discharge of horses.

Species/sex/age	No. of animal	Positive		Negative	95% CI	Odd Ratio/P value
		N	%			
Overall *Streptococcus equi*						
Male	160	77	48.12	83	40.45-55.87	0.93 [reciprocal = 1.08]
Female	90	45	50.00	45	39.75- 60.25	
Total	250	122	48.80	128	42.64-54.99	-
Age						
< 1year	50	46	92.00	4	81.82- 97.41	P = 0.001
1-2 year	50	43	86.00	7	74.26 to 93.67	
2-3 year	50	17	34.00	33	21.93 to 47.88	
3-4 year	50	10	20.00	40	10.63 to 32.76	
4-5 year	50	6	12.00	44	5.01 to 23.29	
Frequency of *Streptococcus equi* isolated from Nasal Discharge						
Male	124	47	37.90	77	29.69-46.68	0.81 [reciprocal = 1.23]
Female	70	30	42.86	40	31.67-54.63	
Age						
< 1year	33	30	90.90	3	77.22-97.64	P = 0.001
1-2 year	37	31	83.78	6	69.29-93.15	
2-3 year	35	7	20.00	28	9.19-35.60	
3-4 year	45	6	13.33	39	5.59-25.68	
4-5 year	44	3	6.81	41	1.76-17.44	
Frequency of *Streptococcus equi* isolated from Pus						
Male	36	30	83.33	6	68.52- 92.96	1.67 [reciprocal = 0.60]
Female	20	15	75.00	5	52.98-90.21	
Age						
< 1year	17	16	94.11	1	74.25- 99.71	P = 0.005
1-2 year	13	12	92.30	1	67.52-99.62	
2-3 year	15	10	66.66	5	40.79-86.62	
3-4 year	5	4	80.00	1	33.44-99.00	
4-5 year	6	3	50.00	3	14.66-85.34	

Table II.- Identification of carrier of *S. equi* in naturally infected horses ≤ 5 years of age through PCR repeated measure.

Age of horse	N	Number positive for *S. equi* post week infection						*P-value*	95% Cl	
		2nd	**4th**	**6th**	**8th**	**10th**	**12th**		**Lower**	**Upper**
< 2 Year	10	10	10	05	02	00	00	.389	−.256	.658
2-5 Year	10	10	10	07	03	00	00			

Pakistan. The findings are also supported by the work of Timoney (1993) who reported that strangles may be prevalent in all ages of horses but younger horses are more susceptible to this disease. Strangles is mostly introduced in breeding farms by the entry of new animal incubating this infection or still shedding during convalescent phase. It was also observed that the incidence of strangles was greater from end of January to the start of May (2.6%) in foals ranging from 9 months to 2 years (Walker and

Timoney, 1998). In the present study, higher numbers of cases were observed in spring months (March-April) followed by winter (November-February) and summer (May-August) months while no case was observed during fall (September-October). The differences among prevalence during different seasons of year were found to be significant ($P<0.05$). These results are in agreement with those of Manzoor *et al.* (2008) who recorded 54% infection in younger horses in Punjab during the spring season. Data on identification of carrier of *S. equi* through culture and PCR in naturally infected horses ≤ 5 years of age is shown in Table II. Results were also broadly correlated with the findings of Kahn (2005) who reported that horses may shed and spread the *S. equi* up to one month following recovery. Three consecutive negative nasopharyngeal samples should be obtained at one week interval, prior to release from quarantine and a minimum isolation period should be one month. Similar findings were also observed by Anzai *et al.* (2005) who found that strangles organism have been shown to emerge over a short period. Newton *et al.* (2000) and Sweeney *et al.* (2005) reported that after subsidence of the clinical signs, the equine may remain carrier up to 8 months and the most principal site was the guttural pouches for *S. equi*, this also supports the findings of our study. Evidences have been found that significant number of horses remain infected by *S. equi* for many weeks after extinction of apparent clinical signs. Thus a convalescent horse can develop latent infection and may be a source of infection for other animals at least 6 weeks after recovery. Even though epidemics may begin by the introduction of asymptomatic carrier horses into a herd, this ailment may turn out to be enzootic on surroundings causing sporadic outbreaks when the number of vulnerable equines rises.

Therefore, considering the results reported here, spring months are very important regarding strangles outbreaks in Pakistan once the highest prevalence were recorded during this season of the year. The reason might be the maximum exposure and movement of horses during the months of spring as equestrian activities are at the peak during this period. It is also concluded that it is not appropriate to mix recently recovered equines from strangles with healthy equines at least for 9 weeks because the recovered equines remain carriers for prolonged period of time (6-9 weeks). Periodic shedding of *S equi* can be a source of infection for susceptible equines.

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Determination of Aflatoxin M1 in Raw Milk for Human Consumption in Peshawar, Pakistan

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Abstract.- The aim of this study was to evaluate the quantity of aflatoxin M1 in fresh milk being used for human consumption. To determine the aflatoxin M1 in milk, a total of 40 number of milk samples were collected in the months of June and July, 2013 from the local milk shops/sellers in Peshawar city. These samples were brought to the Laboratory of Center of Animal Nutrition and were analyzed using Vicam fluorometer (series 4EX) for aflatoxin M1. All the milk samples were found contaminated with aflatoxin M1, out of which, 55% of samples were found to contain aflatoxin M1 in higher quantities and 45% in acceptable quantities (Table I), as recommended by Food and Drug Administration (FDA), USA. The minimum quantity of aflatoxin M1 was 0.37 (ppb), maximum 1.2 (ppb) and average was found to be 0.715 (ppb) much greater than recommended level (0.5 ppb) and further confirmed by statistical testing. This level of aflatoxin M1 in milk consumed by human beings is alarming and requires improving the feed/ration storage conditions especially for dairy animals.

Key words: Aflatoxin M1, raw milk, human consumption.

Mycotoxins are fungal secondary metabolites that if ingested can cause a variety of adverse effects on both humans and animals (Hampikyan *et al*., 2010). More than 3500 mycotoxins with different toxicity levels have been discovered so far. Animals fed on aflatoxin-contaminated feed metabolize in their liver aflatoxin B1 into its hydroxylated product such as aflatoxin M1 that is excreted in milk (Van Egmond, 1989; Guerre *et al.*, 2000), feces and urine (Guerre *et al.*, 2000). About 1-3% ingested aflatoxin B1 is converted into aflatoxin M1 (Ali *et al.*, 1999; Barbieri *et al.*, 1994). Consumption of such milk may be the principle way for entrance of aflatoxin M1 into the human body (Galvano *et al.*, 2001). Aflatoxins are a group of structurally-related toxic compounds produced by certain strains of the fungi *Aspergillus flavus* and *A. parasiticus* (Baskaya *et al.*, 2006; Chen *et al.*, 2005). *A. parasiticus* produces four major aflatoxins: B1, B2, G1 and G2, while aflatoxin B1 is the most toxic in the group and the toxicity is in the order of B1>G1>B2>G2 (Abbas *et al.*, 2004). The aflatoxins do not have flavor, scent, they are fluorescent under the ultraviolet light and are resistant to high temperatures, more than 320ºC without fragmenting; to boil, to sew, to ferment or to pasteurize the foods, it doesn't exterminate them (Early, 2000). As both aflatoxins B1 and M1 may cause cancer in humans, the action level of 0.5 parts per billion of aflatoxin M1 in milk is strictly enforced by the United States Food and Drug Administration (FDA, 2011).

Rothschild (1992) has classified aflatoxin B1 and aflatoxin M1 as class 1 (carcinogen) and B2 (probable carcinogen) human carcinogens, respectively. Lafont *et al.* (1989) also have observed that aflatoxin M1 has high genotoxic activity, although aflatoxin M1 has been found to be about 10 times less carcinogenic than aflatoxin B1.

The exposure of infants to aflatoxin M1 is something to worry about because milk is the main nutrient for children and children are considered more susceptible to the adverse effects of toxins (Rastogi *et al.*, 2004). This is because the capacity for biotransformation of carcinogens is generally slower in children than in adults.

Keeping in view the above mentioned facts, the present study was designed to evaluate the status of local market milk in Peshawar for human consumption focusing the quantity of aflatoxin M1 in it.

Materials and methods

Sample collection

A total of 40 milk samples were randomly collected from milk shops/sellers in different parts of Peshawar. The samples were collected in separate

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polythene bags by the staff of the Center of Animal Nutrition and brought to the laboratory. The particulars of the milk shops/sellers were entered properly. Analysis was made immediately after the reception of the samples at the laboratory. All the samples were analyzed on the Vicam fluorometer (Series 4EX).

Analysis procedure

The fluorometer was calibrated as prescribed in the Vicam Aflatest Instruction Manual.

Salt (1g) was added to 50 ml of fluid milk, mixed and centrifuged at 2000g for 10 min. The skim portion (bottom layer) was carefully removed for analysis without disturbing the top layer. The skim sample was then filtered through 1.5µm glass microfiber filter.

Filtered skim milk (1.5 ml) was passed completely through Aflatest column at the rate of 1-2 drops/second until air came through the column. The column was then removed from the loading syringe barrel and the headspace of column was filled with methanol:water (10:90) solution. The column was then placed on a clean glass syringe barrel. The glass syringe barrel was filled with 10 ml methanol:water (10:90) solution. Now 10ml of methanol:water (10:90) solution was passed through the column at the rate of 2 drops/second. This step was repeated once again and then the glass cuvette (VICAM # 34000) was placed under column and 1 ml of methanol:water (80:20) was added in the glass syringe barrel. The column was eluated at the rate of 1 drop/second by passing the methanol:water through the column. All of the sample eluate was collected in glass cuvette. Now 1 ml of Aflatest Developer was added to eluate in the cuvette and mixed properly. The cuvette was placed in the calibrated fluorometer. The reading of aflatoxin concentration was taken after 60 seconds.

Statistical analysis

The data was analyzed using Z-statistics, Right tailed test at 5% level of significance by using Minitab 14.0 statistical software.

Results and discussion

Out of the forty milk samples analyzed for aflatoxin M1, 22 (55%) were found to contain aflatoxin M1 more than the recommended level, while 18 samples (45%) contained acceptable level of aflatoxin M1. The overall value was 0.72±0.28 (Mean±SD) with a range of 0.37-1.2ppb. Later on the mean of aflatoxin M1 was compared with standard concentration (0.5 ppb) of aflatoxin M1 in milk through Z-statistics by using Minitab 14.0 statistical software the results were found highly significant.

Different countries have different maximum tolerable limits for aflatoxin M1 in milk and milk products as described by (Lin *et al.,* 2004) and shown in (Table I).

Table I.- Maximum limits for aflatoxin M1 in milk and milk products in various countries.

Country	Maximum limit (µg/kg or µg/l)
France	0.05 Adult's milk
	0.03 Children's milk
Turkey	0.05 Milk and products
	0.25 Cheese
Czech Republic	0.1 Children's milk
	0.5 Adult's milk
Belgium	0.050 Milk
USA	0.50 Milk
Switzerland	0.050 Milk and milk products
	0.250 Cheese
Netherlands	0.020 Butter
	0.200 Cheese
Germany	0.050 Milk
Australia	0.050 Milk

According to the observations, levels of contamination of milk by aflatoxin M1 seem to vary in many studies. These variations may be related to different reasons such as milk manufacturing procedures, type of milk, conditions of milk ripening, geographical region, the country, the season and the analytical methods employed (Filazi *et al.,* 2010).

In agreement to our results, Celik *et al.* (2005) found 88.23% of milk samples contaminated with aflatoxin M1 and 64% of these were found to exceed the legal level of aflatoxin M1 in milk according to the Turkish Food Codex and Codex Alimentarius limit (50 ng/kg). This is in support with results obtained later by Rokhi *et al.* (2013) who reported 65.55% of milk samples found to be contaminated with aflatoxin M1 and contamination levels were between 2.1–131 ng/l. Also Kim *et al.* (2000) determined the incidence of aflatoxin M1 in pasteurized milk as 76% in Korea, with a mean concentration of 18 pg/g. Dashti *et al.* (2009) reported 28% of milk samples contaminated with

aflatoxin M1 in Kuwait. Henry *et al.* (2001) found high levels of aflatoxin M1 in Indonesia, Philipines and Thailand. Unusan (2006) conducted a study in Turkey and stated that 47% of the 129 analyzed samples contained aflatoxin M1 levels exceeding the European Union accepted limit. In India, the incidence of contamination of aflatoxin M1 in infant milk, milk based cereal weaning food and liquid milk samples was almost in the magnitude of 87% (Rastogi *et al.*, 2004), with 99% of contaminated samples exceeding the EU/Codex recommended limits. These results are in accordance with El-Sayed *et al.* (2000), Salem (2002) and Elgerbi *et al.* (2004) who reported high levels of aflatoxin M1 in bovine raw milk. In a study conducted by Khushi *et al.* (2010) in Lahore, Pakistan, it has been found that eighty one percent of raw milk samples contained aflatoxin M1 in levels exceeding the American and European tolerance limits.

In contrast to our results, Hussain and Anwar (2008) analyzed milk samples from dairy animals at Punjab level and found 96.4 % samples having toxin level below the US tolerance limit. Only 3% samples were having the levels higher than the tolerance limits. Lopez *et al.* (2003) analyzed 77 milk samples for aflatoxin M1. They found 23% samples positive for aflatoxin M1, however the positive samples were below the maximum tolerable levels. In the rest of samples aflatoxin M1 was not detected.

The contamination of milk and milk products with aflatoxin M1 display variations according to geography, country and season. The pollution level of aflatoxin M1 is differentiated further by hot and cold seasons, due to the fact that grass, pasture, weed and rough feeds are found more commonly in spring and summer than in winter (Azizollahi Aliabadi *et al*., 2012).

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Functional Annotation of Copy Number Variants in Pakistani Population

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Abstract.- Copy number variations (CNVs) have recently presented a dynamic aspect to the apparently static human genome. This study was conducted to analyze the CNVs ≥ 1000 bp to sub-microscopic sizes in 39 healthy individuals from Pakistani population. Genotypic data was taken from publically available online HGDP dataset. Common CNV regions and their frequency rates in the Pakistani population were examined to gain deep insights of CNVs. Total 2,016 CNVRs (60.2 on average in a sample) were discovered, 96% of these regions were found reported in Database of Genomic Variants (DGV). More than two thousand genes were observed fully or partially overlapped these regions. Genes in the novel CNVRs from the Pakistani samples were enriched for genes involved in regulation and development processes. We successfully discovered novel CNVRs in Pakistani population. Moreover, a pipeline for CNV analysis and its annotation was also developed. The results obtained will be a valuable resource for the estimation of effective level of CNVs in the Pakistani population.

Key words: CNV, PennCNV, HGDPGWAS**,** copy number variation, genomic variants.

Copy Number Variations (CNVs) are a form of structural variation in a genome, where large scale parts of DNA have variations in copy number between individual organisms of the same species (Feuk *et al*., 2006). The increased and decreased number of segment is called duplication and deletion, respectively. Small insertion and deletion (indel) are not included in CNVs, while CNVs represents large form of variants in the human genome (Collins *et al*., 2003). CNVs are an important source of human genetic diversity that has association with phenotypic differences. More specifically they relate to an individual's susceptibility to disease. The CNV data from patients will be soon become a tool for diagnostic studies in clinics.

The structural genomics variants that can be observed via karyotyping are known to be linked with highly penetrated genetic disorders. After the completion of first human genome project in 2003, it was believed that human genome sequences from two individuals are 99.9% virtually similar, except for single nucleotide variants (SNV) or short tandem repeats (Ames *et al*., 2008). But after discovering structural variants including CNVs and indels, this theory has been recently challenged by many researchers (Skipper, 2007). The two pioneer groups have investigated a large number of CNVs in healthy individuals using whole genome scanning technology (Iafrate *et al*., 2004; Sebat *et al*., 2004). A significant number of studies have validated the almost 11K CNVs in the complete genome, which suggests that human genome is more diverse than has ever been known (Costanzo *et al*., 2009). In comparison to the extent of SNPs in human genome (approximately 600 Mb comprising ~12% of human genome), not only the coverage of CNVs has exceeded in the human genome but also increased continuously. These large-scale structural variants, in addition to SNPs, will serve as powerful source to enhance our in-depth understanding of effects of genetic variation in human genome in future. Moreover, the differences in disease susceptibility for various diseases can be analyzed by CNVs. For instance copy variants are observed in several complex diseases including Charcot-Marie Tooth type 1 A (CMT1A), autism spectrum disorder (ASD), Parkinson, schizophrenia, HIV/AIDS and cancer. CMT1A is caused by 1.5-Mb tandem duplication on chromosome 17 resulting in three copies of the PMP22 gene (Lupski *et al*., 1991; Pinto *et al*., 2010; Xu *et al*., 2008; Singleton *et al*., 2003; Gonzalez *et al*., 2005; Campbell *et al*., 2008).

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The CNV regions (CNVRs) are enriched in functional categories such as cell adhesion, sensory perception of smell and chemical stimulus, and neurophysiological processes.

In the current study, PennCNV program was employed to analyze the data retrieved from Human Genome Diversity Panel (HGDP). PennCNV has effective algorithm to detect CN-altered regions from SNP genotyping array data with low false-positive rate (FPR) and false-negative rate (FNR) (Wang *et al*., 2007). Out of 2,020 CNVRs, 4% has not been previously reported in the Database of Genomic Variants. This study was conducted with the aim to discover reliable, common CNVRs and their frequency particularly in the Pakistani population. We believe these newly identified and previously reported CNVRs in the human genome will guide us to explore the functions of any particular gene in normal and disease condition in future studies. To the best of our knowledge, this study is the first attempt to examine CNVs in Pakistani population using computational tools.

Materials and methods

The CNVs data was retrieved from Human Genome Diversity Panel (HGDP) containing a set of 1,064 individuals sampled from 51 different ethnic groups from the world (Cann *et al*., 2002). A subset of the HGDP (n = 485) has been previously analyzed for CNVs (Itsara *et al*., 2009; Jakobsson *et al*., 2008). This study particularly presents the analysis of Pakistani cohort samples (n = 39), Accession Number GSE10331. SNP genotyping data was generated at the Stanford University using Illumina 650 arrays. Perl scripts were used to develop a pipeline for studying CNVs (Fig. 1). Copy number variations were called using PennCNV 2.0 (Wang *et al*., 2007) and was further annotated for functional classification using ANNOVAR (Wang *et al*., 2010) and PLINK (Purcell *et al*., 2007). CNV gene content and its genomic position were determined with UCSC RefSeq (hg19) gene annotation. To confirm the most probable overlapped regions, the identified CNVs were compared with the data available in the Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/) (MacDonald *et al*., 2014).

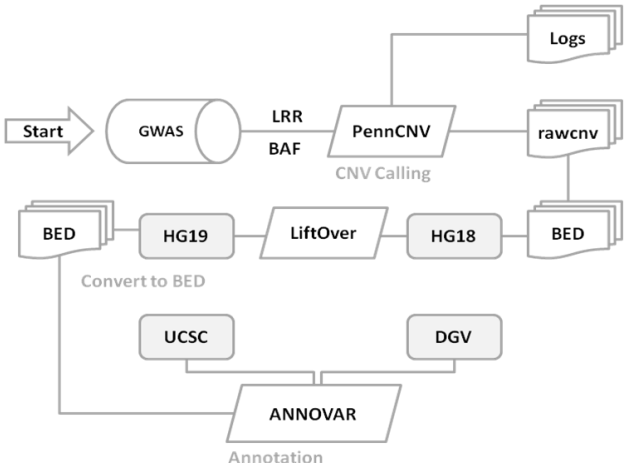

Fig. 1: Pipeline for detailed analysis of copy number variation regions from GWAS data.

Results and discussion

Stemming from our interest in genome analysis of Pakistani population [Publication in progress], herein we report the identification of CNVRs and their distribution in the genome of Pakistani Population. For this purpose, a set of 39 samples was retrieved from HGDP dataset publically available on NCBI GEO (Barrett *et al*., 2009), to call CNVRs distribution in the said population. We identified a total of 2,016 CNVRs, among which some CNVRs (3.9%) were novel and not reported in the Database of Genomic Variants before (Table I). Among the total number of CNV regions 190 were observed as duplicated and 1,826 were deleted, affecting about 2,180 overlapped genes. More than 1,000 of the total number of CNV regions had previously been described in the DGV. The sizes limit of the CNVRs ranged from one kb to 4 megabases. The smallest CNVR was 1046 bp, and the largest 3, 536, 635 bp. The most frequent CNVRs were detected in nine individuals (23%) out of the study subjects (n=39) in the combined CNV analysis (Table II). More than 80% of CNVRs from the Pakistani population were rare found once among the 39 individuals. The most frequent CNVRs were localized on chromosome 4, 5, 11, 8 and 19 with higher occurrence eight and nine individuals (Table II). Among all autosomal chromosomes, CNVs were detected most frequently on chromosome 19 (Fig.2).

Table I.- Summary of the observed CNVRs in Pakistani individuals.

Total CNVRs	Reported in DGV	Novel CNVRs	Exonic	Intronic	Intergenic	5' UTR	3' UTR
2,016	1,940	80	1,187	158	559	29	7

Table II.- Frequent regions included are those that were present in eight or more individuals.

Chr	Start	End	Size	Susceptible type	Freq.	Gene
19	20,422,200	20,473,895	51,695	Loss	20.5	ZNF486 MIR1270-1
5	46,227,977	46,384,240	156,263	Gain	20.5	HCN1
8	43,689,385	43,910,848	221,463	Gain	20.5	POTEA
4	64,381,774	64,392,223	10,449	Loss	23.1	LPHN3
11	50,343,409	51,171,349	827,940	Gain	23.1	TECRL LOC646813

Table III.- Reported CNV regions with associated diseases found in Pakistani individuals.

Chr	Start	End	Size	Type	Gene	Phenotype	Reference
22	22,865,229	22,973,609	108,380	Loss	PRAME	Tumor suppressor gene-Mantle cell lymphoma	Beà *et al*. (2009)
22	22,865,229	22,948,331	83,102	Loss			
22	22,865,229	22,962,403	97,174	Loss			
22	21,606,454	21,807,970	201,516	Loss	HIC2	Heart defects	Fernández *et al*. (2009)
15	29,271,979	29,324,788	52,809	Loss	APBA2	Schizophrenia	Kirov (2010)
15	29,294,328	29,344,873	50,545	Loss			
2	49,387,002	49,391,299	4,297	Loss	NRXN1	Schizophrenia	Kirov *et al*. (2009)
2	50,882,166	50,913,340	31,174	Loss			
2	51,751,780	51,781,546	29,766	Loss			
2	51,780,103	51,781,546	1,443	Loss			
2	50,539,877	50,541,382	1,505	Loss			

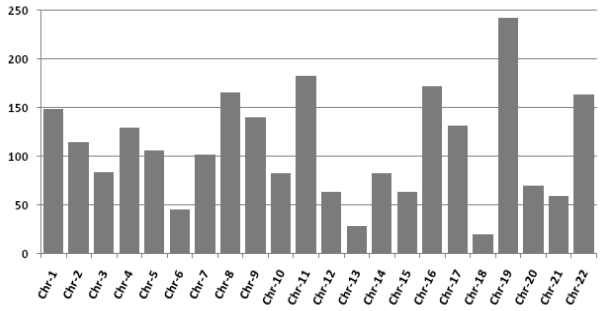

Fig. 2. Frequency distribution of copy number variation regions among each autosomal chromosome.

All the discovered regions were then further investigated for associated diseases. ANNOVAR was used to annotate the CNVRs and we found that diabetes, heart and brain related problems are frequently present in associated CNV genes in Pakistani individuals. Among the 80 novel CNVRs observed in the Pakistani samples, three CNVRs contained a total of six genes. The total length of the novel CNVRs was 30, 209, 276 bp; 16.8% of the total length of identified CNVRs.

We found extensive differences in the CNVR profiles among Pakistani individuals. Interestingly, many of these copy number loci overlap with known disease-associated genes and pharmacogenetic-related genes. Another CNV of interest is a 20-kb deletion that overlaps with TOMM40 (Loci responsible for the control of lipid levels and risk of coronary heart disease in 16 European population

cohorts) (Aulchenko *et al*., 2009). Duplication of the CHRNA7 gene was also found to be associated with an increased risk of Schizophrenia (Tam *et al*., 2009), however the analysis reflected a deleted region of a CNV locus which overlapped with the NRXN1 gene that has the association with Schizophrenia (Rujescu *et al*., 2009). Tobacco is usually used by schizophrenic patients in order to alleviate the neurophysiological deficits associated with Schizophrenia (Adler *et al*., 1993; Gray *et al*., 1996). Schizophrenia is associated with the up-regulation of α 7 nicotinic acetylcholine receptor (CHRNA7) (Stevens *et al*., 1998; Abdullah *et al*., 2014). More than five deleted CNV regions have been observed overlapped with genes (INS-IGF2, CLEC16A, MBL2, RASGRP1, SLC2A4, CAPN10, ZMAT4, CEL, CAMK1D) associated with diabetes and heart problems. Although a direct association between the CNVs and phenotypic differences has not been established in our studies, collectively our results suggest that CNV distributions are substantially different between populations and thus, may account for phenotypic or disease differences between them. As such, the potential implication of CNVs in clinical and public health practice is promising, however further studies are needed to establish their significance. A population screening program could be implemented in a high-risk group harboring multiple cancer predisposing CNVs of large effect sizes for early detection and treatment. Genetic information of CNVs overlapping with pharmacogenetic-related genes could also be beneficial for clinical drug trials, where it could be used to identify the population most likely to respond favorably to the drug.

CNVs of Pakistani populations has not been thoroughly studied as those of European, African and East Asian populations which results in the identification of novel CNVs in this diverse population. It is therefore suggested to study more individuals to fully represent the pattern of CNVs among Pakistani population.

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Investigation on Monofilament Gill Net Selectivity for Vimba (*Vimba vimba* Linnaeus, 1758) in Eğirdir Lake, Turkey

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Abstract.-This study was conducted to determine the selectivity properties of monofilament gill nets for vimba (*Vimba vimba* Linnaeus, 1758) in Eğirdir Lake. SELECT method was used to determine the selectivity parameters. The research area was carried out in three different stations of Eğirdir Lake, in a monthly period from January-2009 to December-2009 and with a total of 36 catching trials. In trials, 335 vimba species the length ranges between 12.2-44.0 cm were caught. According to the Bi- modal model which gave the lowest deviation for nets, the optimum lengths were determined as 12.93, 16.16, 20.20, 24.24, 28.28, 32.32 and 36.36 cm for 32, 40, 50, 60, 70, 80 and 90 mm mesh sizes (stretched) in gill net, respectively. As a result, the mesh size of monofilament gill nets should not be less than 50 mm for vimba catching in Eğirdir Lake at first maturity.

Key Words: Monofilament, selectivity, SELECT method, *Vimba vimba.*

Vimba (*Vimba vimba* Linnaeus, 1758) is known as a benthopelagic species distributed in the inland waters and lagoons of Europe, the Russian Federation, Serbia Montenegro, Slovakia, Croatia, Kazakhstan, Slovenia, Ukraine, and Turkey (Geldiay and Balik, 1988; Pecl, 1990; Froese and Pauly, 2008; Okgerman *et al*., 2009). This species was zoogeographically introduced to Turkey from western Thrace, and primarily spread to north and northwest Anatolia (Geldiay and Balik, 1988; Okgerman *et al*., 2009). In some places this species has a great economic importance. Catches commonly attain lengths of 20-30 cm and weight 250-500 g (Okgerman *et al*., 2009).

Vimba species is one of the commercial fish species in Eğirdir and it is eaten by local people as fresh and pickle. This is a natural fish species and subsisted in the lake since 1952. Nevertheless recently significant decrease in production of vimba has been reported (Yerli *et al*., 2013). The annual production was 25 tons between 1961-1964 which decreased to 0.8 tons recently (Yerli *et al*., 2013). This could be attributed to over fishing, uncontrolled fishing, fish introduction (such as pikeperch that is extremely carnivorous for other fish species), invasive species, pollution and habitat destruction.

Gill nets are used widely for vimba fishing in Eğirdir Lake because of ease of use, low cost, durable and efficient characteristics. Gillnets are simple fishing gears that are widely used in aquatic environment all over the world. They are comparatively less damaging to the environment due to their selectivity characteristics (Brandt, 1984; Kınacıgil *et al*., 2000; Yuksel *et al*., 2014). From this perspective the results of selectivity studies are very valuable for legal implications of fish stock sustainability. Alam *et al.* (1994) reported that using nets of unrestricted mesh size could deplete the stocks of smaller cyprinids.

Chaichi *et al*. (2011) reported the first maturity length (L_{50}) of 17 cm (fork length) for the *Vimba vimba persa* in Caspian Sea. Another study carried out by Becer and İkiz (2001) for *Vimba vimba tenella* in Karacaören-I Dam Lake Burdur-Turkey reported that the length of the smallest female vimba that reached sexual maturity was 17.4 cm (fork length).

In this study, selectivity properties of monofilament gill nets which have 32, 40, 50, 60, 70, 80 and 90 mm mesh size has been used for managing of decreased vimba population in Eğirdir Lake.

Materials and methods

This study was done at three different stations of Eğirdir Lake, on monthly basis from January to December 2009 with a total of 36 catching trials (Fig. 1). Monofilament gill nets with 32, 40, 50, 60, 70, 80, 90 mm mesh size (stretched) were used. All of them were made up of 0.20 mm thick rope and

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were 100 m long with 0.50 hanging ratio and depth of 50 mesh as vertical. All nets were set at the bottom of an area in the afternoon and was hauled the following day before noon. Fish catches were classified according to the nets and total lengths determined by 1 mm precision measurement board and weights with 1g precision digital scale.

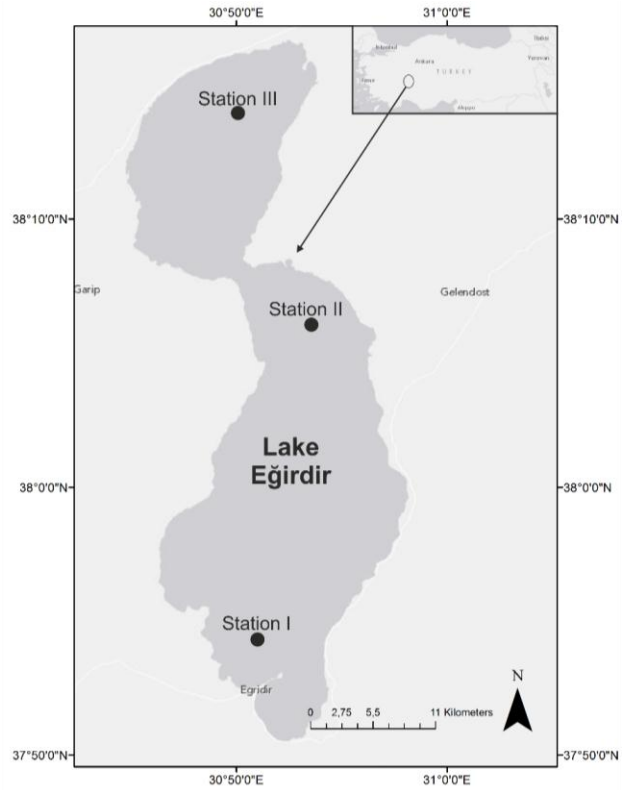

Fig. 1. Study area and sampling station in Lake Eğirdir.

The SELECT (Share Each Length class Catch Total) method was used to determine the selectivity (Millar, 1992; Millar and Fryer, 1999; Millar and Holst, 1997). This method assumes that the number of fish of length *l* caught with a mesh size with *j* size has a nl_j Poisson distribution, and is defined by the following equation (Acarlı *et al*., 2013):

$$n_{lj} \approx n_{lj} \approx \text{Pois}(p_j \lambda_l r_j(l))$$

where λ_l is the abundance of fish of size *l* caught in net; $p_j\,(l)$ is relative fishing intensity (the relative abundance of fish of size *l* that j mesh size can catch). The Poisson distribution of the number of fish of size*l*caught by fishing gear with *J* mesh size is defined as $p_j(l)\lambda_l.r_j(l)$ is the selectivity curve for *j* mesh size (Acarlı *et al*., 2013).

$$\sum_l \sum_j \{n_l \log[\, p_j\, \lambda_l\, r_j\,(l)] - p_j\, \lambda_l\, r_j\,(l)\}$$

The data obtained from field studies were analyzed by PASGEAR version 2.5 (Kolding, 1999) computer program. The program calculates parameters of 5 different model (normal location, normal scale, log-normal, gamma, and bi-modal) based on SELECT (Millar, 1992; Millar and Fryer, 1999; Millar and Holst, 1997) method.

Standard deviation of all models evaluated when selecting the most suitable model for calculations. The model that has greater standard deviation shows that the model in question is not appropriate for the obtained data (Park *et al*., 2004; Akamca *et al*., 2010). The most suitable model was chosen taking into account the lowest deviation value. Bi-modal equations of SELECT method as follows:

$$\exp\left(-\frac{\left(L-k_1.m_j\right)^2}{2k_2^2.m_j^2}\right)+c.\exp\left(-\frac{\left(L-k_3.m_j\right)^2}{2k_4^2.m_j^2}\right)$$

Kolmogorov-Smirnov (K-S) test was used to determine differences between size frequency distributions of fish caught by nets that have varying mesh size (Siegel and Castellan, 1988; Karakulak and Erk, 2008; Acarlı *et al*., 2013).

Results

As a result of 36 catching operations, a total of 335 vimba lengths (range 12.2-44.0 cm) were caught, while the most productive net was 50 mm mesh size with 30.1% catching capacity of total catch; 90 mm mesh size net was unproductive with 6.0% catching of total catch. The distribution of caught fish according to the nets is shown in Table I. Average length (±SD) for 32, 40, 50, 60, 70, 80, 90 mm mesh size gill nets were determined as 14.3±1.7, 17.3±1.4, 20.9±1.9, 22.7±2.1, 28.1±3.0, 30.5±3.0, 32.3±1.4, respectively (Table I).

The total length–frequency distribution for fish caught using different mesh size is shown in Figure 2.

With the PASGEAR computer program, parameters of normal location, normal scale, lognormal, gamma and bi-modal models calculated separately and the results have been shown in Table II. As a result of comparing model deviance it was determined that the most appropriate model was bi-modal for nets.

Table I.- Catch composition data of trial nets.

Length of mesh size (mm)	Number (%) of fish caught	Average length ±SD (cm)	Minimum (cm)
32	27 (8.1%)	14.3±1.7	12.2 (18.6%)
40	66 (19.7%)	17.3±1.4	15.4 (22.1%)
50	101 (30.1%)	20.9±1.9	18.2 (31.5%)
60	47 (14.0%)	22.7±2.1	20.1 (33.1%)
70	41 (12.2%)	28.1±3.0	24.3 (38.5%)
80	33 (9.9%)	30.5±3.0	27.0 (44.0%)
90	20 (6.0%)	32.3±1.4	29.4 (34.3%)

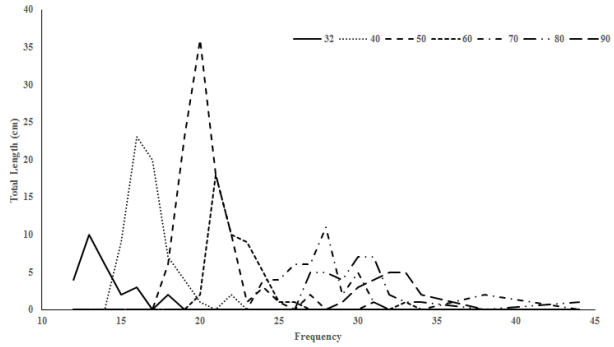

Fig. 2. Total length frequency distribution of fish caught using different mesh sizes.

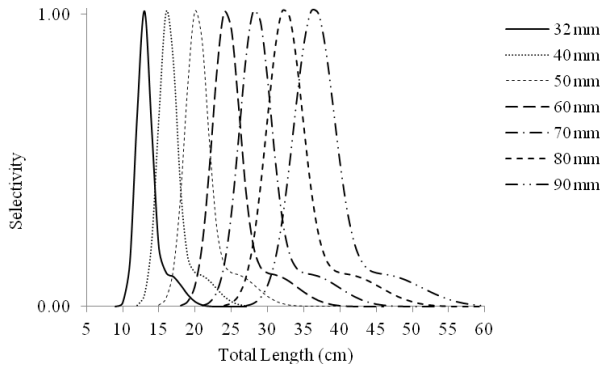

Fig. 3. Selectivity curves of vimba.

Table II.- Selectivity parameter values of vimba.

Model	Parameters	Model deviance	p-value	Degree of freedom (d.f.)
Normal location	(k, σ)= (0.403, 2.573)	178.377	0.000000	53
Normal scale	(k1, k2) = (0.422, 0.046)	151.975	0.000000	53
Log normal	(μ_1, σ) = (2.594, 0.108)	125.620	0.000000	53
Gamma	(k, α) = (0.005, 85.640)	131.291	0.000000	53
Bi-modal*	(k_1, k_2, k_3, k_4, w) (0.404, 0.031, 0.510, 0.059, 0.109)	96.841	0.0000080	50

*Parameters of appropriate model

The selectivity curves that were drafted by bi-modal parameters have been showed in Figure 3. The optimum length and spread values calculated in regard to the bi-model for each net panels that have different mesh size are given in Table III.

According to the results of K-S test that were applied for query difference of length frequency distributions of fish caught by nets, differences were founded in all nets (Table IV).

Discussion

In this study, the selectivity properties of gillnets that used widely for vimba fishing in Eğirdir Lake was determined. These results are first reports for this species both for Eğirdir Lake and Turkey. Unfortunately there are no more studies in gillnet selectivity on vimba fishing in the world. Petriki *et al*. (2014) studied the gillnet selectivity properties of some fish species in three lentic freshwater systems in Northern Greece according to Kirkwood and Walker (1986). The results are similar to those of our studies. For example, optimum length was calculated as 16.16 and 24.24 for 40 and 60 mm mesh size respectively in our study and reported as 15.9 and 23.3 for *Vimba melanops*. There are less

Table III.- Optimum length and spread values of vimba according to the bi-modal.

Mesh size	Model length (cm)	Spread value (cm)
32	12.93	0.99
40	16.16	1.24
50	20.20	1.55
60	24.24	1.86
70	28.28	2.17
80	32.32	2.48
90	36.36	2.79

Table IV.- Result of the K-S test used to compare length frequency distributions of catch.

Net 1	Net 2	K-S Test	Decision
32	40	0,7407> 0,3081	H_0 Reject
32	50	0,9429>0,2935	H_0 Reject
32	60	1,0000>0,3237	H_0 Reject
32	70	1,0000>0,3311	H_0 Reject
32	80	1,0000>0,3442	H_0 Reject
32	90	1,0000>0,3815	H_0 Reject
40	50	0,8368>0,2136	H_0 Reject
40	60	0,9545>0,2536	H_0 Reject
40	70	1,0000>0,2629	H_0 Reject
40	80	1,0000>0,2793	H_0 Reject
40	90	1,0000>0,3242	H_0 Reject
50	60	0,6043>0,2336	H_0 Reject
50	70	0,9307>0,2438	H_0 Reject
50	80	0,9703>0,2613	H_0 Reject
50	90	0,9901>0,3088	H_0 Reject
60	70	0,8473>0,2836	H_0 Reject
60	80	0,9787>0,2989	H_0 Reject
60	90	0,9787>0,3412	H_0 Reject
70	80	0,4858>0,3084	H_0 Reject
70	90	0,7632>0,3495	H_0 Reject
80	90	0,5152>0,3648	H_0 Reject

H_o: There are no significant differences in the length frequency distributions (α=0.05; k=1.36)

differences between two studies, it's thought to arise from using selectivity method, net product material and habitat differences. Balık and Çubuk (2001) reported that gill net's selectivity may show differences for different fish species and also for the same fish species in different habitats. Therefore, the gill net selectivity must be determined for each fish species separately (Sümer *et al.*, 2010). The K-S test shows that there are differences in length frequency distribution of fishes for all net panels, so monofilament gill nets have very well length selectivity for vimba fishing.

There are no regulations regarding minimum landing size for vimba fishing in Turkey. The fisherman can market every length of vimba. For the sustainability of Vimba in the Lake, its catching should not be done unless it reaches the first maturity length.

Chaichi *et al*. (2011) reported that first maturity length (L_{50}) for the *Vimba vimba persa* in Caspian Sea 17 cm (fork length). When the fork lengths are converted to the total length by Gaygusuz *et al*. (2006), it was 19.3 cm. So the vimba which have less than 19.3 cm length should not be caught in Eğirdir Lake. For providing this, the nets that have less than 50 mm mesh size should not be used for vimba fishing according to length reported in Table III.

The reproductive properties show differences by location, so first maturity length and maturity period of vimba should be determined by studying reproductive properties of fish in Eğirdir Lake.

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Evidence of Transmission of Dengue Virus by Different Developmental Stages of Mosquitoes, *Aedes aegypti* and *Aedes albopictus*

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Abstract.- *Aedes aegypti* and *Aedes albopictus* are well known vectors for dengue virus. Larvae act as reservoir of dengue virus during inter-epidemic period. To determine the role of developmental stages of *Aedes* in dengue infection, 523 larvae were collected from different localities of Lahore from March to mid of June, 2013. Laboratory reared adults of *Aedes aegypti* (60) and *Aedes albopictus* (45) were collected from Institute of Public Health, Lahore. Larvae and adult were divided into 47 pools and each containing 1-15 larvae. Molecular analysis was done to detect the dengue virus. Of the total of 40 pools of larvae, 5 were found positive for dengue. Only one pool of laboratory reared adult mosquitoes was found positive. Detection of dengue virus from field caught larvae and laboratory reared adult mosquitoes confirm the existence of vertical transmission of dengue virus in nature.

Key words: *Aedes* larvae, Dengue virus, vertical transmission.

Mosquitoes are medically important arthropods because they transmit various pathogens hence cause major public health problems (Kovendan and Munigan, 2011). It is estimated that dengue affects 100 million people and cause more than 20,000 deaths per year (Gubler, 1998). *Aedes aegypti* is known as a vector for dengue which is endemic in many countries especially in tropical and sub tropical areas which include South Asia, Africa, Americas and Pacific Island area (WHO, 2011). *Aedes aegypti* inhabits urban and pre domestic environment (Gubler, 1998). *Aedes aegypti* is an anthropophilic and nervous feeder *i.e.* it bites more than one host to complete one blood meal. It also requires more than one feed for the completion of gonotropic cycle and this feeding behavior results in spreading of virus on a large scale which leads to multiple cases of dengue (WHO, 2011).

Dengue is a viral disease caused by a virus that belongs to the family Flaviviridae. There are four serotypes of dengue virus which are known as DENV-1, DENV-2, DENV-3 and DENV-4. Each of these can cause dengue fever, dengue haemorrhagic fever and dengue shock syndrome in humans. Dengue fever spreads through bite of infected *Aedes aegypti* (Kovendan *et al*., 2011). Dengue virus is very small in size *i.e.*, 50nm. Its genome comprises single stranded RNA of 11 kb (Gubler, 1998). Fatima *et al*. (2011) reported that only two serotypes *i.e.*, DENV-2 and DENV-3 have been reported from Pakistan.

Control of different developmental stages of vector *i.e*. adult and larvae are important to control dengue. The larvae of *Aedes* act as reservoir of dengue virus during inter-epidemic seasons. Dengue virus multiply unchecked in these larvae and could be a source of dengue outbreak (Rohani *et al*., 2008). Dengue virus is maintained in nature by *Aedes aegypti* as natural vertical transmission is reported in certain adverse environmental conditions (Arunachalam *et al*., 2008).

The purpose of current study was to make a comparison of vertical transmission of dengue virus in field caught larvae and laboratory reared adult mosquitoes. In field caught larvae, the possibility of occurrence of dengue virus was higher because they be infected with dengue virus during the previous blood meal. The laboratory reared adult mosquitoes have less chances of being exposed to dengue virus. So the rate of vertical transmission is low.

Materials and methods

Collection of larvae

Larvae were collected from different

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localities of urban Lahore during the months of March to mid of June, 2013.These localities included Wahga town, Ravi Town, Gulberg, Nishtar Town, Aziz Bhatti Town and Samanabad Town. Survey was conducted on weekly basis. Collection was done indoor as well as outdoor in man made such as air cooler tray, discarded receptacles and water storage tanks and natural containers such as mud pots, plastic pots, cement tanks, tyres, metal pots and tree holes (Saifur *et al.*, 2012).

Laboratory reared adult *Aedes* mosquitoes of unknown origin were obtained from National Institute of Malarial Research and Training, Institute of Public Health, Lahore.

Both field collected larvae and laboratory reared adult mosquitoes were identified according to Florida Manual for mosquitoes identification. After identification, head-thoraces to adult mosquitoes were excised and pooled. Larvae (15) were also placed in pooled head-thoraces and larvae were stored at -70˚C until further processing (Arunachalam *et al*., 2008).

Molecular detection of dengue virus

RNA extraction: RNA was extracted from each pool by using FAVORGEN total RNA extraction kit as per manufacturer's instructions (Fatima *et al*., 2011). RNA was extracted from 47 samples out of which 40 were larval samples and 7 samples were of laboratory reared adult mosquitoes.

cDNA preparation: Complementary DNA (cDNA) was prepared by reverse transcription. A 5 µl of extracted RNA was added in reaction mixture of 5 µl to make a total volume of reaction 10 µl. Reaction mixture of 5 µl contains, 5Xfirst strand buffer (FSB) 2 µl, 0.1 M dithiothreitol 0.25 µl,10 mM dNTPs 1 µl, 0.75 µl distilled water with 0.2 µl of RNase inhibitors**.** 0.5 µl of Moloney-Murine Leukemia virus (M-MLV) Reverse transcriptase (Invitrogen Biotechnologies USA) and 0.5 µl 2pM anti-sense primer (reverse primer D2) was added. Total volume of 10 µl was incubated at 37°C for 50 min followed by 2 minutes at 95°C and 2 min at 22°C. The sequences of primers used are described in Table I (Fatima *et al.*, 2011).

Nested PCR

Nested PCR was performed for serotype analysis. In the first round amplification of cDNA was done. PCR mix (8 µl) was used which contained 1 µl 10x PCR buffer (with ammonium sulphate), 1.2 µl $MgCl_2$, 0.5 µl 500 µMdNTPs, dH_2O 3.5 µl, 0.5 µl 20 pM forward and reverse primers each (Table I), 1 µl of 2U *Taq*-polymerase (National Centre of Advanced Molecular Biology) and 2 µl of cDNA was added. Thermal profile for the first round was initial denaturation at 94°C for 2 min followed by 35 cycles each of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, extension at 72°C for 2 min and final extension at 72°C for 10 min. In the second round serotype-specific sense and anti-sense primers were used. Chemicals used for second round were the same as used in the first round but sequences of forward and reverse primers were different from first round of PCR. Thermal profile for the second round was the same as for the first round only the annealing was carried out at 54°C for 45 seconds in 35 cycles. The amplified product was detected by agarose gel (2%) electrophoresis.

DNA sequencing

Nucleic acid extraction kit (Vinantis Technologies Malaysia) was used following the manufacturer's instructions. Sequencing PCR was done on automated genetic analyser as per manufacturer's instructions (Big Dye Deoxy Terminators; Applied Biosystems Weiterstadt, Germany). Briefly, 10 µl reaction mix was prepared using 0.6 µl of BigDye, 1.5 µl 5X sequencing buffer, 1.5 µl template, 1 µl sense or anti-sense primer (20 pM) and 5.4 µl of dH_2O. The amplification steps in thermal cycler were: initial denaturation at 96°C for one min followed by 35 cycles each of denaturation at 96°C for 15 seconds, annealing at 50°C for 10 seconds and extension at 60°C for 4 min. Final extension was given at 60°C for 4 min. Ethanol precipitation was carried out by adding 2 µl 3 M sodium acetate, 2 µl 125 mM ethylenediaminetetraacetic acid (EDTA) and 26 µl of absolute alcohol. It was left at room temperature for 15-20 min then centrifuged for 30 min at 3800 rpm at 4°C. 36 µl of 70% ethanol was added and centrifuged for 15 min at 3800 rpm. Finally, 12 µl of formamide was added to dried pellet and mixed well. It was followed by heat shock at 95°C for

Table I.- Sequences of primers used to amplify C-prM gene junction of dengue virus.

Primer name	5'-3' Sequence	Size of amplified product in base pairs	Use in PCR round
D1-D	TCAATATGCTGAAACGCGWGAGAAACCG	511 bp	Ist Round
D2-D	TTGCACCARCARTCWATGTCTTCWGGYTC		
TS1-F	AGGACCCATGAAATTGGTGA	411 bp	IInd Round
TS1-R	ACGTCATCTGGTTCCGTCTC		
TS2-F	AGAGAAACCGCGTGTCAACT	403 bp	IInd Round
TS2-R	ATGGCCATGAGGGTACACAT		
TS3-F	ACCGTGTGTCAACTGGATCA	453 bp	IInd Round
TS3-R	CAGTAATGAGGGGGCATTTG		
TS4-F	CCTCAAGGGTTGGTGAAGAG	401 bp	IInd Round

5 min and was loaded onto automated sequencer (Applied Biosystems, 3100 DNA Analyzer) for sequence analysis.

Results

A total of 523 larvae of *Aedes* were collected from different localities of Lahore (Ravi Town, 14; Gulberg Town, 7; WAPDA Town, 4; Nishter Town, 2; Aziz Bhatti Town, 10, Samanabad Town, 3). All the collected larvae were divided into 40 pools each having 1-15 larvae. After RNA extraction, cDNA preparation and molecular diagnosis by nested PCR, it was found that only five pools (12.5%) were positive for dengue virus. Among positive pools, one was collected from Gulberg town (14.28%), 1 from Nishtar town (50%) and 3 pools were positive from Aziz Bhatti town (30%).

Total pools of laboratory reared mosquitoes were 7 (*Aedes albopictus*, 3; *Aedes aegypti*, 4) out of which only one pool of *Aedes aegpypti* was found positive (14.29%).

Total pools for larvae and adults were 47, out of which six (five larval and one adult) were positive for dengue virus (12.76%). All positive pools of both larvae and adult were of serotype 2. No other serotype *i.e.* DENV1, DENV 3 and DENV 4 was found in this study.

Out of 6 positive samples, only one sample was sequenced and it was selected randomly. Length of amplified product was 403 bp. Sequences were submitted to Genbank, accession number assigned by Genbank for DENV2 is KF288930. Sequence of amplified portion of C-rpM gene junction of dengue virus showed 99 % homology with reported sequence with accession No. JQ396233.1

Discussion

Aedes aegypti are more efficient vectors of dengue because they are anthropophilic (Arunachalam *et al*., 2008). Mosquito gets infected with dengue virus by two possible ways, first, by taking a blood meal from infected person. The mosquito becomes infected for life time and will be able to continue human-vector cycle (Gubler, 1998). Second way is vertical transmission of virus in mosquito. In this way female mosquito can transfer dengue virus to offspring during development of egg and transfer of egg to oviduct (Lutomiah *et al*., 2007). Another way is reported that the male mosquito can transfer dengue virus to its offsprings via sperms during copulation with non infected female (Hutanai *et al*., 2007).

Vertical transmission of dengue virus in vector is better approach for the survival during inter epidemics. It was observed when no clinical cases were reported (Arunachalam *et al*., 2008). All these routes of dengue virus transmission show that probably virus is well adapted to live in mosquito host compared to human and other primates. If a mosquito feeds on infected person and lays eggs before arrival of summer (favorable conditions for development) the eggs will survive and emerging adults will have ability to continue inter epidemic cycle (Joshi *et al*., 2002). As there is no proper treatment for dengue fever with no vaccination, vector control is a very important step to eradicate dengue.

Detection of dengue virus from field collected larvae can be expressed through early warning for outbreak of disease in an area (Kow *et al*., 2001). Early warning regarding outbreak is helpful so that control could be taken before any outbreak could emerge (Rohani *et al*., 2008). In this study dengue virus was detected from larvae in order to describe the chances of outbreak.

The results of this study revealed that only DENV-2 was isolated from field collected *Aedes* larvae. No serotype DENV-3 was detected in this study, although DENV-2 and DENV-3 had been reported from Pakistan (Fatima *et al*., 2011). Current study showed that out of 40 pools of larvae only 5 were found positive for dengue virus, and out of 7 pools of laboratory reared adult mosquitoes only one was positive for dengue virus. First time, Ibanez-Bernal *et al*. (1997) detected dengue virus in field collected adult male mosquitoes by using RT-PCR. Presence of dengue virus in male mosquitoes indicates presence of vertical transmission in nature.

Current study concluded that the virus was found in very small proportion but could cause outbreak of dengue at any time. Presence of dengue virus in larvae of *Aedes* mosquitoes showed that virus is transmitted vertically but at very slow rate. Although these results showed that chances of any outbreak are very low but persistence of dengue virus in larvae is alarming. It could start an outbreak any time. Therefore, control of vector is very important before any outbreak might emerge.

Conclusion

Only 12.5% of field collected larvae were found positive for dengue virus serotype 2, which indicates vertical transmission of dengue virus in nature but may be at a very slow rate. Control of vector is important for eradication of dengue. Breeding sites must be identified and destroyed. This study indicates that dengue virus is maintained in nature and could cause outbreak in near future, if remained unchecked.

Conflict of interest declaration

We declare that we have no conflict of interest.

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Birth Seasonality, Infant Sex Ratio, and Interbirth Interval in Captive Northern Pig-Tailed Macaques (*Macaca leonina*): The First Evidence Based on an 11 Year Record

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Abstract.- Based on the record of 11 consecutive years, this study for the first time describes reproduction in the northern pig-tailed macaque (*Macaca leonina*). Totally 10 infants were born as singletons across the whole survey period. 60% of infants were males and sex ratio did not significantly deviate from 1:1, which to some extent supports local resource competition hypothesis. The birth date ranged from early February to late May, in which seventy percent of infants were born in March. The interbirth interval was 401±146 days on average. There was no significant difference between interbirth interval after the birth of male infants and that after the birth of female infants. All infants survived over the first year of their ages. The reproductive parameters in captive *M. leonina* were discussed in the context of other captive primate studies, and positive suggestions on zoo breeding and management have been proposed.

Key words: Reproductive parameters, *Macaca leonina*, local resource competition hypothesis, zoo management.

Zoos are well known as institutions exhibiting wildlife to the public and also have the conservation mission especially for endangered animal species (Rabb and Saunders, 2005; Fazal *et al*., 2014). Captive conditions are beneficial to reproduction of threatened species due to sufficient food supply and inexistent predation risk (Bercovitch and Ziegler, 1989). Research on captive endangered species which are difficult to access in the wild could improve our understanding on their natural history and further enhance the corresponding biological conservation (Snyder *et al*., 1996; Miller *et al*., 2004).

The pig-tailed macaque is listed in the IUCN Red List and has been reclassified into two separated species, northern pig-tailed macaque (*Macaca leonina*) and Sundaland pig-tailed macaques (*M. nemestrina*) based on genetic data and distinction of sex skin swelling (Groves, 2001; Brandon-Jones *et al*., 2004). *M. leonina* occurs in southern China, eastern Bangladesh, Cambodia, India, Lao PDR, Myanmar, Thailand, and Vietnam. Its total population has declined by more than 30% over 30 years and such decreasing tendency may continue or be exacerbated in the recent future (Boonratana *et al*., 2008).

Nowadays information on the ecology and behavior of northern pig-tailed macaques is very limited (Whitehead and Jolly, 2000; Kitamura *et al*., 2002; Albert *et al*., 2011, 2013a,b; Zhao *et al*., 2015). Their reproductive characteristics remain unclear, regardless of captive or wild populations. The main purpose of this study was to provide basic information on distribution of birth seasons, interbirth intervals (IBIs), and infant sex ratio of *M. leonina* in captive conditions, compare results with related findings in other primate species, and provide suggestions on captive breeding management in this endangered species.

Materials and methods

The northern pig-tailed macaques were together housed in one indoor room (night room: 4.8m×3.2m×7m) and one outdoor room (day room: 7.1m×5.6m×3.8m) at Tianjin Zoo, China (Zhao *et al*., 2015). There is a hatch between the two rooms. Monkeys could freely shuttle back and forth. Visitors observe monkeys via the broad glass wall of both rooms. Generally the monkeys sleep in the indoor room every night. The animals' diet contains fruits, vegetables, nuts, and monkey chow. Food was presented two times per day. Water was provided *ad libitum*.

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The survey period ranged from January 2004 to June 2014. There were 3 males and 4 females at the initial stage, and there are 5 males and 4 females at the present time. During the whole study period, group membership changed due to individual births, deaths and transfer to other facilities. The mean group size was 6.2 members with 1.2 adult males, 1.6 adult females, 1.7 male immatures (older than one year), 0.8 female immatures, and 0.9 infants.

All focal individuals were born in captivity without artificial nursery. We obtained data on infant births, deaths, and gender from Tianjin Zoo's primate archives and calculated the IBI (days), infant sex ratio (%) and infant mortality rate (%). We used Chi-squared tests to estimate whether infant sex ratio deviated from 1:1. We used the Man-Whitney U test to analyze the difference between IBI after the birth of male infants and that after the birth of female infants. This study adhered to the Chinese legal and ethics requirements concerning animal research.

Results

In total 10 infants were born over 11 years. All births were singletons. Sixty percent of infants were males, but the infant sex ratio did not significantly deviate from 1:1 ($\chi^2 = 0.400$, df = 1, p = 0.527). One female (TWF003) gave birth to 2 infants and died at five years of age; the other female (TWF001) gave birth to 8 infants from 2008 to 2014 and became the sole fertile female.

Birth date ranged from early February to late May, with seventy percent of infants born in March (Fig. 1). The mean birth date was 22 March. The IBI was 401±146 days on average (N = 8, range from 284 to 751 days). There was no significant difference between IBI after the birth of male infants and that after the birth of female infants (U = 5.00, Z = -0.357, p = 0.857) (Fig. 2).

All infants (excluding the new infant born in 2014, who is still alive) survived beyond one year. Two of them were transferred to other facilities when they were more than 2 years old. One infant was born blind and died when it was 5 years old due to uncertain causes.

Discussion

Ecological and social conditions drive some mammal species to concentrate their reproductive events within a special time window every year giving maximum opportunity for infant survivorship (Di Bitetti and Janson, 2000; Brockman and van Schaik, 2005; Odhiambo *et al*., 2008). With regard to *M. leonina*, 90% of infant births fall within a three-month period (March to May), which is similar to that of the closely related Sundaland pig-tailed macaque (Fa and Lindburg, 1996). Birth seasonality has also been reported in other macaque species (*e.g.*, *M. thibetana*: Zhao and Deng, 1988; *M. mulatta*: Lehman *et al*., 1994; *M. m. tcheliensis*:

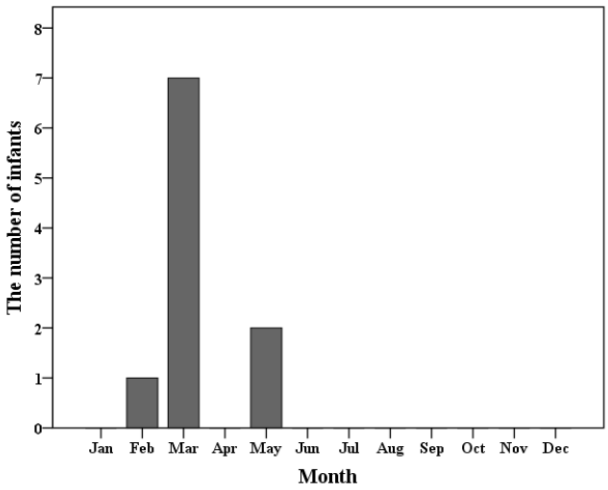

Fig. 1. Distribution on birth months in *M. leonine.*

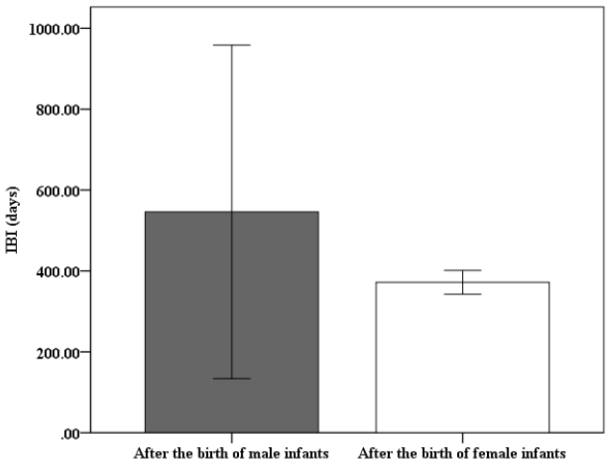

Fig. 2. IBIs after the birth of male/female infants in *M. leonine.*

Tian *et al*., 2013) as well as some snub-nosed monkey species (*e.g., Rhinopithecus roxellana*: Li and Zhao, 2007; *R. bieti*: Cui *et al*., 2006).

The IBI of macaque species ranges from 1.0 to 2.2 years due to various socioecological factors (Ross, 1992; Thierry, 2011). The mean IBI of *M. leonina* is about 13.3 months (*i.e.* 401 days), which is higher than that of *M. cyclopis* (12.1 months: Hsu and Lin, 2001) and *M. m. brevicaudatus* (11.9 months: Jiang *et al*., 1988), and is lower than that of *M. fuscata* (more than 24 months: Fooden and Aimi, 2003, 2005) and *M. maurus* (22.4 months: Okamoto *et al*., 2000). The IBI in *M. leonina* is within the range of variation of other macaque species.

The local resource competition hypothesis of sex allocation theory proposed that males (the dispersing sex) will be overproduced in species with female philopatry whereas females are overproduced in species with male philopatry (Fisher, 1930; Trivers and Willard, 1973; Clark, 1978; Faust and Thompson, 2000). For wild pig-tailed macaques, males appeared to leave the group when they were more than four years old (Oi, 1990); hence, it is expected that females will produce more male than female infants in this species. In captive conditions, we found that 60% of infants are males, although the sex ratio was not significantly deviate from 1:1. Such findings might to some extent lend support to the local resource competition hypothesis. In addition, we found no significant difference between IBIs after the birth of male infants and that after the birth of female infants, which suggests that maternal investment is independent of infant sex, in accordance with related studies in other captive primate species (*e.g. Trachypithecus cristatus*: Shelmidine *et al*., 2009). The proximate reason may be that the absence of predators as well as that of female competition (*i.e.* only one fertile female during the most of the survey period) would largely obviate maternal effort on differentail resource allocation.

In our study of northern pig-tailed macaques, all infants survived. Either greater food availability in captivity supplemented prenatal maternal nourishment or, simply, the way the mothers reared their infants is favourable to postnatal growth and survival (Worlein and Sackett, 1997; Sackett *et al*., 2002).

It should be emphasized that the male TWM002 and the female TWF001 produced offspring together for seven consecutive years, so that potential inbreeding and incest risks exist and are bound to increase with the sexual maturity of their offspring. Some management measures are urgently required to deal with this problem. For instance, it is feasible to split the current group to avoid the occurrence of sexual behavior between related individuals, and exchange individuals with other zoos so as to improve genetic diversity.

The present results should obviously be treated with caution due to limited sample size and the potential influence of photoperiod. It is well known that daily activities of wild *M. leonina* living in tropical forests follow the variation in photoperiod (Albert *et al*., 2011). Being housed at Tianjin Zoo, in the temperate zone, the disparity of photoperiod may influence reproductive behavior, as reported previously in other primate species (*e.g*., *M. fuscata*: Fooden and Aimi, 2003). It is necessary to study a larger sample size across longer periods for captive *M. leonina* and explore the impact of photoperiod on reproductive parameters. The reproductive knowledge gained from wild populations could be beneficial to make comprehensive analysis in this endangered species.

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Prevalence and Chemotherapy of Canine Diabetes Mellitus in and Around Lahore, Pakistan

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Abstract.- To study the canine diabetes mellitus, 500 blood samples were collected and blood glucose level (mg/dl) was measured. Out of 500 samples, 16 (3.2%) were positive for diabetes mellitus. Out of 16, 11 (68.8%) were female and 5 (31.2%) were male dogs. Clinical signs like hyperglycemia (95% CL= 82.93-100), polyuria (95% CL= 56.99-95.00), polydypsia (95% CL= 43.68-87.54) and anemia (95% CL= 31.95-78.46) were frequently observed while dehydration and cataract were less common in diabetic dogs. Among various breeds Yorkshire terrier, Pomuranian, Samoyed and Cross bred were at high risk for diabetes mellitus. The therapeutic trial conducted with Humulin-N (insulin) showed a significant decrease ($P<0.05$) in blood glucose level. In conclusion from the results of present study it can be suggested that canine diabetes mellitus is more prevalent in old and female dogs and insulin can be effectively used for management of canine diabetes.

Key words: Dog, diabetes mellitus, insulin, chemotherapy.

Diabetes mellitus is one of the most important diseases of endocrine glands in dogs and cats. In this disease, β cells of pancreas either fail to produce the insulin or have lost the ability to produce the sufficient quantity of insulin for the requirements of the body. Diabetes in veterinary species can be sub divided into either insulin-dependent diabetes mellitus or non-insulin dependent disease, although this is not particularly helpful in canine diabetes because virtually all diabetic dogs require insulin therapy. The cause of diabetes mellitus in canine is likely to be multi-factorial. Factors including obesity, feeding practices, exposure to toxic material or drugs, destruction of islet cells and secondary to pancreatitis may play an important role (Rucinsky *et al*., 2010). Some drugs also have antagonistic effects with insulin production and may lead to diabetes mellitus. The long term uses of Glucocorticoids and hormones, for heat control, are more likely to cause diabetes mellitus. Insulin therapy is supposed to have good efficacy against canine diabetes mellitus (Cook, 2011).

Diabetes mellitus is common in dogs with 5 to 12 years of age and is rare in less than 3 years of age (Davison *et al.*, 2005). Breeds like the Samoyed, Tibetan terrier and Cairn terrier are more prone to diabetes mellitus, while other breeds like Boxer and German shepherd are less susceptible to the diabetes mellitus (Catchpole *et al.*, 2005). Female dogs had more risk of diabetes mellitus as compared to male dogs (Fall *et al.*, 2010).

This disease shows various clinical signs, nearly same as in human diabetic patient (Catchpole *et al.*, 2005). The most commonly occurring disorders during diabetes mellitus in dogs are hyperadrenocorticism, urinary tract infection, dermatitis, otitis, acute pancreatitis, neoplasia and hypothyroidism (Hess *et al.*, 2000). In Pakistan, no documented evidence has been found about canine diabetes mellitus. So, a preliminary study was designed and executed to study the prevalence of diabetes mellitus in pet dogs, the effect of diabetes mellitus on various blood parameters and to evaluate the efficacy of insulin therapy in diabetic dogs in and around Lahore areas.

Materials and methods

The study was carried out through systematic random blood sampling technique from Lahore city and its peri-urban areas. Blood samples with EDTA

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@1mg/ml from 500 dogs having age ≥5 years were collected from various private and public pet clinics. Age was assessed by asking from the pet owner and later confirmed by teeth of dogs. Glucose level (mg/dl) was measured by Glucometer (Accu Chek® Active; Reference number, REF 05234441021). The dogs showing clinical signs and having glucose level >200 mg/dl in blood were considered as diabetic (Stein and Greco, 2002). Following clinical signs were recorded polyuria, polydipsia, anemia dehydration, polyphagia, cataract and jaundice in a questionnaire.

Chemotherapy

Sixteen diabetic dogs were selected and divided into two groups, A and B having eight dogs in each group. The dogs of group A were treated with Humulin-N @ 0.5 U/Kg, BID, SC for 7 days while the dogs in group B were kept as positive control and monitored by regular visits. The blood samples were collected from diabetic dogs every 2 hours interval for 12 hours after one week of treatment and measured the blood glucose level.

Statistical analysis

The data regarding the age and breed was analyzed by frequency analysis using chi-square test. The percentages and 95% confidence limit for some parameters were determined and where appropriate odds ratio was also computed. Data on chemotherapy was analyzed with *t*-test using statistical package for social sciences (SPSS) version 17.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Prevalence

The data regarding prevalence of canine diabetes mellitus in different age, sex and breeds is presented in Table I. In this study, 16 (3.2%) dogs were found positive for canine diabetes mellitus. Frequency analysis using chi-square test showed non-significant (P>0.115) difference among different age groups (Table I). The prevalence of canine diabetes mellitus was non-significantly higher in female dogs as compared to male. Previous studies by Fracassi *et al.* (2004) in Italy (1.33%) and Davison *et al.* (2005) in United Kingdom (0.64%) reported comparatively lower prevalence of canine diabetes mellitus. The comparatively higher prevalence in present study could be due to different geographical conditions and poor management. Moreover, the increase number of cases in female dogs can be due to the antagonistic action of progesterone on insulin. Further, this study was carried out during the season when more whelping occur and more predispositions of diabetes mellitus in female dogs could also be due to the onset of progesterone-induced diabetes mellitus initiated by increased estral activity associated with mammary gland derived growth hormone (Fall *et al.,* 2010). The prevalence of diabetes mellitus was also non-significantly (P>0.115) higher in dogs having age >9 years as compared to other age groups (5-7 years and 7-9 years). Davison *et al.* (2005) reported that diabetes mellitus is more prevalent in older dogs as compared to younger dogs. Controversies exist about the prevalence of diabetes mellitus in older age. The diabetes mellitus could be due to latent autoimmune diabetes in adults (LADA), or due to secondary to hyperadrenocorticism (Fall *et al.*, 2007) or it may also be due to aging process of insulin producing cells. Results revealed that out of 16 affected cases on the basis of various breeds the distribution of diabetes mellitus was non-significantly higher in Yorkshire terrier and cross breed followed by Pomuranian, Poodle and Samoyed, while Boxers, Labrador retriever, German shepherd and Pugs were least affected breeds. Different prevalence distribution of diabetes mellitus among various breeds suggests the involvement of genetic and dietary factors. As it is commonly observed that the toy breeds and the dogs having smaller size more like to take canned and table foods which have high fat and low carbohydrates. Because, this kind of food generate more proportion of calories from fat than to carbohydrates and that might lead to low glucose tolerance and decreased sensitivity to insulin. Moreover, different genetics among different breeds also change the resistant and susceptibility pattern in these breeds (Guptill *et al.,* 2003).

The results of this study showed that among various clinical signs the frequency of hyperglycemia (95% CL= 82.93-100), polyuria (95% CL= 56.99-95.00), polydypsia (95% CL= 43.68-87.54)

Table I.- Prevalence of canine diabetes mellitus in different breeds, age and sex groups.

Parameters	Positive		Negative	95% CL	MH Chi-sq P value	OR/ reciprocal
	n	%				
Age (Years)						
5-7	4	1.91	209	0.60-4.47	>0.115	-
7-9	7	3.70	182	1.63-7.19		
>9	5	5.10	93	1.89-10.94		
Sex						
Male	5	2.00	245	0.74-4.38	-	0.44/2.26
Female	11	4.40	239	2.34-7.52		
Breed						
Yorkshire Terrier	3	6.00	47	1.55-15.46	>0.930	-
Pomuranian	2	4.00	48	0.68-12.59		
Labrador/ Retriever	1	2.00	49	0.10-9.47		
German Shepherd	1	2.00	49	0.10-9.47		
Pugs	1	2.00	49	0.10-9.47		
Bull dogs	0	0.00	50	0.00-5.82		
Samoyed	2	4.00	48	0.68-12.59		
Boxer	1	2.00	49	0.10-9.47		
Poodle	2	4.00	48	0.68-12.59		
Cross Breed	3	6.00	47	1.55-15.46		
Overall	16	3.20	484	1.91-5.04		

and anemia (95% CL= 31.95-78.46) were more common whereas dehydration, polyphagia, cataract and jaundice were less prevalent in cases of canine diabetes mellitus as shown in Table II. Canine diabetic patients showed the signs of polyuria and polydipsia when concentration of blood glucose was beyond the renal tubular threshold for glucose filtration in to the urine (Rucinsky *et al*., 2010).

Table II.- Clinical signs observed in diabetic dogs (n=16).

Clinical Signs	Positive		95%CL
	n	%	
Hyperglycemia	16	100	82.93 - 100.00
Polyuria	13	81.3	56.99 - 95.00
Polydipsia	11	68.8	43.68 - 87.54
Anemia	9	56.3	31.95 - 78.46
Polyphagia	7	43.8	21.54- 68.05
Dehydration	7	43.8	21.54- 68.05
Cataract	3	18.8	5.00 - 43.01
Jaundice	3	18.8	5.00 - 43.01

Chemotherapy

The findings of present study indicated that the values of glucose (mg/dL) were decreased significantly after 4-10 h interval after one week of insulin therapy showing the useful effects of insulin. Insulin therapy seems a very effective approach for the treatment of canine diabetes mellitus along with other dietary management (Davison *et al.*, 2005; Rucinsky *et al*., 2010). In present study, glucometer was efficiently used to monitor the effectiveness of

Table III.- Effectiveness of insulin therapy after one week of therapy.

Time period (h)	Glucose level (mg/dl)	
	Group A	Group B
Before medication	267±20.3	256±16.3
One week after medication		
0	247±3.4^{a}	256±2.7^{a}
2	234±2.9^{a}	262±2.6^{a}
4	170±3.1^{b}	257±3.2^{a}
6	129±2.2^{b}	255±4.7^{a}
8	158±2.8^{b}	254±3.7^{a}
10	194±3.4^{b}	258±1.9^{a}
12	232±3.8^{a}	260±2.3^{a}

Two-Sample Independent *t*-test showed mean values in rows with different superscript indicate significant difference (P<0.05) while with same superscript indicate non-significant difference (P>0.05)

insulin therapy. Portable glucometer are extensively being used in human for the rapid diagnosis of blood glucose levels. Previously scanty information is available about the use of glucometer in veterinary practice in Pakistan. Therefore it can be suggested that such kind of portable glucometer can be used for the rapid diagnosis of diabetes mellitus for favorable management and treatment (Stein and Greco, 2002). Moreover, Lack of insulin, affects the metabolism of carbohydrate, protein and fat, and causes a significant disturbance of water and electrolyte homeostasis (Hassan *et al.,* 2010).

Conclusion

From the results of present study it was recorded that the canine diabetes mellitus is more prevalent in old dog as compared to young ones and female dogs as compared to male. The therapeutic trial conducted with insulin showed effective way for the management of diabetes.

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Insecticidal Action of Three Plants Extracts Against Cowpea Weevil, *Callosobruchus maculatus* (F) and Bean Weevil, *Acanthoscelides obtectus* Say

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Abstract.- The aim of this study was to determine the insecticidal and repellant activity of three plants *viz.* peppermint *(Mentha piperita),* sage *(Salvia officinalis)* and feverfew *(Tanacetum parthenium)* against two bruchid species *Acanthoscelides (A.) obtectus* Say and *Callosobruchus (C.) maculatus*. The results revealed that all of the tested materials had repellent and fatal effects against the pests compared to control. Sage showed maximum mortality in *A. obtectus,* whereas feverfew caused highest mortality in *C. maculatus*. Sage and feverfew exhibited maximum repellency against *Callosobruchus maculates* and *Acanthoscelides obtectus* respectively. It is accomplished that extracts of sage (S*alvia officinalis),* peppermint *(Mentha piperita)* and fever few (*Tanacetum parthenium)* have insecticidal and repellant activity and could be used to protect stored grains at farm level without hazardous effects.

Keywords: Botanical insecticide, stored grains, peppermint, sage, fever few, bean weevil, cowpea weevil.

Farmers used to store grain for the food and also as seed for the future cultivation. Storage of food items like grains, cereals, pulses have a problem of pests and insects for the farmers that causes serious economical losses. Farmers adopt different ways and means to cope up with this problem. Bruchids are the most important pests of pulse crops in Asia and Africa under storage conditions (Raja *et al.,* 2000; Tapondjou *et al.,* 2002). Bruchid can damage 100% of the stored grains after 3-5 months of storage (Singh, 1977). *Acanthoscelides (A.) obtectus* bean weevil (BW) and *Callosobruchus (C.) maculatus* are the most damaging legume bruchids (Kamga *et al.,* 1992). *C. maculatus* Cowpea weevil (CW) is one of the most destructive and common pests of the stored cereals and legumes (Demitry *et al.,* 2007). They reproduce very fast in storage, giving rise to a new generation every month (Ouedraogo *et al.,* 1998). The BW, *A. obtectus* (Say) (Coleoptera: Bruchidae) is one of the most destructive pests of the kidney bean. In the stored beans, these insects cause weight losses up to 30% (Pemonge *et al.,* 1997). It is estimated that the losses with a very wide range of 20–100% are recorded for grains due to the attacks caused by these bruchids (Schmale *et al.,* 2001). Therefore, it is indispensible to reduce such losses by controlling the stored grains pests (Tapondjou *et al.,* 2002).

In practice, the use of chemical insecticides has been preferred as the main method of grain protection against insect's attack, due to the fact that it is the simplest and most commercial way of dealing with the pests (Hidalgo *et al.,* 1998). However, there are several disadvantages of chemical agents used as preservatives, *viz.*, pest revival and resistance, the risk of user's contamination, residual effect, toxic effects on non target organisms, and environmental hazard (Tapondjou *et al.,* 2002). The use of botanical insecticides may not be as injurious as chemical insecticides for the environment. These insecticides are efficient against a limited number of organisms and are biodegradable, nontoxic products, are fit for use in integrated pest management (IPM) and could lead to the development of new means of safe pest control agents (Kim *et al.,* 2003). The farmers are practicing botanical agents for the protection of their products for long periods due to cost effectiveness, easy availability and processing (Belmain *et al.,* 2001). Besides in the previous study, Raja *et al.*

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(1998, 2001) have investigated many plant species with the objective to control the losses of the bruchids. Koona and Dorn (2005) evaluated hexane, acetone and ethanol extracts of *Tephrosia vogelii* to control three bruchid species BW (*A. obtectus)*, CW (*Callosobruchus maculatus)* and gram dhora *(Callosobruchus chinensis).*

The objective of the present research was to investigate the insecticidal activity of three plant extracts *Mentha piperita, Salvia officinalis* and *Tanacetum parthenium* against two bruchid species *A. obtectus* and *C. maculatus.*

Materials and methods

This study was carried out at the Stored Grain Research Laboratory Arid zone Research Centre, Quetta during 2010.

Preparation of plant extract

Fresh leaves of three plants, peppermint (*Mentha piperita*), sage (*Salvia officinalis*) and fever few (*Tanacetum parthenium)* were collected in March, 2010 from Arid zone Research Centre, Quetta. For the preparation of plant extract, the 20g of chopped leaves of each plant sample was soaked in 160 ml of methanol for 10 days at room temperature, with shaking after every at 12 h. Each plant extract was evaporated to dryness and the residue was weighed and re-dissolved in the methanol again (Jbilou *et al.*, 2006). After 10 days these extracts were filtered and stored in spray bottles in the refrigerator for experimental purpose. All of these extracts were used as 1ml per 50g of grains.

Rearing of insects

Cultures of the BW (*A. obtectus*) and CW (*C. maculate)* were reared in laboratory on disinfested lentil at ambient temperatures of 28ºC and relative humidity of 60%. Adults that emerged from this culture were used for the bioassay within 48h of emergence.

Bioassays

Two 500 ml plastic jars (A and B) disinfected with 90% alcohols were connected by a clear plastic pipe for each replication (Saljoqi *et al.*, 2006).

One of the jars (A) in all was sprayed with respective extracts, while the other jar (B) was not treated. Grain (50 g) was added in jar A and then twenty newly emerged adults of *C. maculatus*, collected from infested lentil were added. The mouth of each jar was covered with muslin cloth for ventilation and to avoid escaping of adult beetles. For control no extract was applied on grains. In total there were 4 treatments including control. Each treatment was replicated 3 times. Same method was used for *A. obtectus.*

The mortality and repellency data *i.e.,* dead (in jar A and B) and alive insects (in jar B) were recorded for 7 days at an interval of 24 h. The ones found alive in the plastic pipes were considered repelled insects.

Statistical analysis

The data for percent mortality and percent repellency was subjected to statistical analysis using RCB. Mean separation was performed by using LSD test (Steels and Torrie, 1960) at the 5% significance level. The package used for the calculations, was M-StatC.

Result and discussion

The result of mean mortality of *C. maculatus* and *A. obtectus* against plant extracts is given in Table I. The effect of peppermint and sage was observed significantly different from fever few. The data reflected obviously that all plant extracts exhibited mortality against *C. maculatus*. The highest mortality of 96.67% was estimated in the jars treated with sage and fever few on day 7. The average mortality was 52.62% and 46.43% in fever few and sage, respectively. The mortality recorded in peppermint extract ranged from 11.67% (day 1) to 91.67% (day 7) and the overall mortality was 49.05%. Derbalah and Ahmed (2010) investigated the *Mentha viridis* against *C. maculatus* and found *M. viridis* effective causing mortality of adults. The findings are in agreement with the present study. In control jar, the initial mortality 10% was noted on 3rd day of the experiment which gradually increased to 40% on day 7 with the overall average of 15.48%. In present study, all of three treatments resulted in mortality of insects earlier compared to the control group. Similar result was reported by Brisibe *et al.* (2011) for *Artemisia annua, Azadirachta indica* and *Ocimum gratissimum.*

Table I.- Mortality of *Callosobruchus maculatus* (cowpea weevil) and *Acanthoscelides obtectus* (bean weevil) against different treatments for different days.

Treatments	Mortality (%)							Mean
	Day1	Day2	Day3	Day4	Day5	Day6	Day7	
Callosobruchus maculatus								
Peppermint *(Mentha piperita)*	11.6^{a}	26.6^{a}	31.67^{a}	48.33^{a}	60.00^{b}	73.33^{b}	91.67^{a}	49.05
Sage (S*alvia officinalis)*	1.67^{b}	6.67^{c}	16.67 ^{b}	50.00^{a}	70.00^{a}	83.33^{a}	96.67^{a}	46.43
Fever few *(Tanacetum parthenium)*	15.00^{a}	21.67^{b}	38.33^{a}	48.33^{a}	65.00^{ab}	83.33^{a}	96.67^{a}	52.62
Control	0.00^{c}	0.00^{d}	10.0^{b}	15.00^{b}	20.00^{c}	23.33 ^{c}	40.00^{a}	15.48
Means	7.08	13.75	24.1	40.42	53.75	65.83	81.25	
Acanthoscelides obtectus								
Peppermint *(Mentha piperita)*	1.67 ^{a}	16.67^{a}	35.00 ^{a}	50.00 ^{a}	63.33^{a}	76.67^{a}	90.00^{a}	47.62
Sage (S*alvia officinalis)*	1.67 ^{a}	16.67^{a}	28.33 ^{ab}	50.00 ^{a}	65.00^{a}	80.00^{a}	93.33^{a}	47.86
Fever few *(Tanacetum parthenium)*	0.00 ^{b}	13.33^{b}	21.67^{b}	33.33^{b}	43.33^{b}	55.00^{b}	65.00^{c}	33.10
Control	0.00^{b}	0.00 ^{c}	6.67^{c}	8.33^{c}	13.33^{c}	16.67^{c}	20.00^{d}	9.29
Means	0.83	11.67	22.92^{e}	35.42	46.25	57.08	67.08	

*Means with different superscript indicate significant difference (P<0.05)

The effect of extracts on the mortality of *A.obtectus* was significant (P<0.05). Especially, the highest mortality of 93.33% was determined in the sage extracts treatment group after 7 days, and the mean mortality was 47.86%. Karaborklu *et al.* (2010) investigated the toxic impact of ten plant essential oils against *A. obtectus*, and reported that sage oils showed 60% lethal effect on the adult pest. The mortality in peppermint was 1.67% on day 1, which increased to 90% on day 7 with the average mortality of 47.62% in the treatment. In fever few the mortality was recorded 65% for day 7. Considering all treatments, it was determined that the mortality increased with increasing exposure time. Our findings supported those reported by Bittner *et al*. (2008), who tested different plants against adults of *Sitophilus zeamais* and *A. obtectus* and concluded that, mortality raised with increased exposure time.

The present study also demonstrated significant repellent effect of the plants on the pests. The highest repellency against *C. maculatus* was recorded in the extract of sage *i.e*., 4.76% (Table II). But, there was non-significant difference (P>0.05) between sage, fever few and peppermint. Sage and fever few observed repellent when compared to control. However, no significant difference between peppermint and control was found. Different plant extract was used against the same insect by Udo (2011), who investigated that methanol extracts of

Table II.- Comparison of mean repellency values of treatments against *Acanthoscelides obtectus* and *Callosobruchus maculatus* against different treatments.

Treatment	Mean repellency	
	A. obtectus	*C. maculates*
Peppermint *(Mentha piperita,)*	3.09^{bc}	3.33 ^{ab}
Sage (S*alvia officinalis)*	5.95 ^{ab}	4.76^{a}
Fever few *(Tanacetum parthenium)*	6.19 ^{a}	4.29 ^{a}
Control	2.86 ^{c}	1.43 ^{b}

*Means with different superscripts are significant (P< 0.05).

Z. xanthoxyloides evoked moderate repellent effect against *C. maculatus.* The maximum numbers of *A. obtectus* were repelled by extract of fever few *i.e.*, 6.19%. Earlier, Jovanovic *et al*. (2007) evaluated the repellent effect of five medicinal plants against *A. obtectus*, and reported that 100 and 30% concentrated extracts of *Urtica dioica* and *Taraxacum officinale* produced effective repellency. Fever few had a significant effect as compared to peppermint. Highest day wise repellency of *A. obtectus* and *C. maculatus* was noticed on day 2 8.75% and 5.83%, respectively (Table III). Rahman *et al.* (2013) suggested that the plant extract may be fractionated for better results or the change of insects may also provide species specific insecticidal action.

Table III.- Comparison of mean repellency values of *C. maculates* and *A. obtectus* on different days.

Days	Mean repellency	
	A. obtectus	*C. maculates*
Day 1	4.58^{bc}	5.00 ^{ab}
Day 2	8.75 ^{a}	5.83^{a}
Day 3	7.92 ^{ab}	5.00^{ab}
Day 4	7.08^{ab}	3.75 ^{abc}
Day5	2.92 ^{cd}	2.92^{bcd}
Day6	0.42^{d}	1.25^{cd}
Day7	0.00 ^{d}	0.42^{d}

*Means with different superscripts are significant (P< 0.05).

It is concluded that extracts of sage (S*alvia officinalis),* peppermint *(Mentha piperita)* and fever few (*Tanacetum parthenium)* have significant insecticidal effect and could be a potential grain protectant against stored grain pests. Results also revealed that sage displayed maximum mortality in *A. obtectus* however, fever few caused highest mortality in *C. maculatus.* Sage and fever few illustrated maximum repellency against *C. maculates* and *A. obtectus,* respectively.

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