Short Communications

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A STUDY OF PREVALENCE OF MALARIA INFECTION IN URBAN AREAS OF DISTRICT QUETTA, PAKISTAN

Abstract.- This paper reports the prevalence of malarial parasites in the human population of urban areas of Quetta District. *Plasmodium falciparum* was observed to be with a higher incidence (16.31%) in the age group of 21 years and above. Mosquitoes of the genus *Culex* was found to be more prevalent (96%), genus *Anopheles* was observed to be 0.86% and genus *Aedes* was found to be less prevalent (0.43%).

Key words: Malaria, *Plasmodium falciparum, P. vivax*, Mosquitoes.

Pakistan is almost in the middle of the malarial belt around the globe encompassing tropical and subtropical countries. Some 270 million new cases of malaria occur every year of which 95% are reported from these areas (Anwar *et al*., *Pakistan Armed Forces med. J*., **44**: 1-3, 1994). Abbasi *et al*. (*Pakistan J. med. Res*., **35**: 129-132, 1996) reported cerebral malaria in hospitalized children from Larkana. Afridi *et al*. (*J. med. Sci*., **8**: 102-105, 1998) studied prevalence of malaria in an urban slum of Peshawar. Ahmed and Ahsan (*J. Coll. Phys. Surg. Pakistan*, **7**: 128-130, 1997) studied Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) in cases of cerebral malaria in Karachi. Ahmed and Zareen (*J. Coll. Phys. Surg. Pakistan*, **6**: 325-326, 1996, *J. Coll. Phys. Surg. Pakistan*, **13**: 169-172, 1997) diagnosed falciparum malaria in pregnancy cases in Manshera on bone marrow examination and relevant aspects of cerebral malaria. Khan (*J. Coll. Phys. Surg. Pakistan*, **7**: 41-42, 1997) pointed out the problems of multi-drug resistant malaria in Pakistan. Memon (*Pakistan J. med. Sci*., **13**: 245-248, 1997) investigated dominant malarial parasite species in hospitalized children in Nawabshah. Shah and Ahmad (*Pakistan J. med. Sci*., **11**: 265-270, 1995) studied twenty complicated cases of falciparum malaria admitted and managed in Medical Unit II, JPMC, Karachi. In Balochistan too, cerebral malaria is a major community problem. Durrani *et al*. (*J. Pakistan med. Assoc*., **47**: 213-215, 1997) studied epidemiology of cerebral malaria and its mortality in patients of Quetta city. Nawaz and Yasmin (*Prevalence of malaria in Afghan Refugees settled in urban areas of district* Quetta. *7th Pakistan Congr. Zool., Abstract,* p. 10) reported malaria infection in Afghan Refugees residing in Quetta and also from the students residing in the University Hostel. However, the present study was carried out about the prevalence of malarial parasites in human population residing in the urban areas of district Quetta.

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Materials and methods

A survey was conducted during December 2002 in the urban areas of district Quetta to record and screen species of malarial parasites from the blood of human patients suffering from malaria. Malaria cases were detected by adopting two ways. Passive case detection (PCD) technique wherein blood films were taken from the patients presenting themselves to a health station with symptoms or a history suggestive to malaria. The other technique is Active case detection (ACD) in which home visits were made to the persons with sign or symptoms of malaria and blood films were prepared. Slides were taken back to the laboratory where they were stained and examined. Seasonal preparation of blood slides both thick and thin were made twice a month in each area of study and in the health station. Staining of slides with Giemsa's stain were made following techniques described by Manson-Bahr and Bell (*Manson's tropical diseases*. 19th Ed. English Language Book Society / Bailliere Tindall, London, 1987). Identification of species of malarial parasites and mosquitoes were made from the keys of Cheng (*General Parasitology*, 2nd Ed. Academic Press College Division, New York, pp. 1-787, 1986), DuBose and Curtin (*J. med. Ent*., **1**: 349-355, 1965), Service (*Lecture notes on medical entomology*, Blackwell Scientific Publications, Oxford, 1986), Sood (*Haematology*. 3rd Ed. Jaypee Brothers, Med. Publishers, (Pvt.) Ltd. New Delhi, 1989) and White (Mosquitoes, In: *Manson's tropical diseases* (ed.

P.E.C. Manson-Bahr and D.R. Bell), 19th ed. pp. 1404-1435, English Language Book Society / Bailliere Tindall, London, 1987), respectively.

Results and discussion

A total of 875 blood smears were prepared from age groups ranging from 1 year to 21 years and above residing in eight different localities (Tables I and II). However, variations were observed among different localities having different environment and hygienic conditions. The commonest species observed was *Plasmodium falciparum* (Fig. 1) with a highest incidence of 16.31% in the age group of 21 years and above whereas, in the age group of 1 to 10 years, was observed to be 7.29% and the lower was found to be 5.39% in the age group of 11 to 20

Table I.- Age wise over all incidence of malaria infection in Quetta (Urban)

S.No.	Age (Years)	No. of slides examined	Total No. of +ve	Overall % Infection	Infection by *P. vivax*	Infection by *P. falciparum*
1.	1 – 10	370	53	14.32	26 (7.02%)	27 (7.29%)
2.	11 – 20	315	31	9.84	14 (4.44%)	17 (5.39%)
3.	21 – above	190	51	26.84	20 (10.52%)	31 (16.31%)
	Total	875	135	15.42	60 (6.85%)	75 (8.57%)

Table II.- Age and area wise incidence of malaria infection in Quetta (Urban)

S. No.	Area	Age (Years)	No. of slides examined	Total No. of +ve	No. of +ve species wise		% Infection
					P. vivax	*P. falciparum*	
1.	Jinnah Road	1 – 10	15	5	1	4	33.3
		11 – 20	10	0	0	0	0
		21 – Above	10	0	0	0	0
2.	Kawari Road	1 – 10	27	3	0	3	11.11
		11 – 20	24	4	1	3	16.66
		21 – Above	16	2	0	2	12.5
3.	Mission Road	1 – 10	24	3	2	1	12.5
		11 – 20	21	2	1	1	9.52
		21 – Above	10	2	0	2	20.0
4.	Cantt	1 – 10	47	4	3	1	8.51
		11 – 20	41	4	0	4	9.75
		21 – Above	27	4	1	3	14.81
5.	Pashtoonabad	1 – 10	53	10	7	3	18.86
		11 – 20	49	10	4	6	20.40
		21 – Above	33	11	5	6	33.33
6.	Marriabad	1 – 10	33	6	0	6	18.18
		11 – 20	27	2	1	1	7.40
		21 – Above	13	5	3	2	38.46
7.	Sirki Road	1 – 10	33	2	1	1	6.06
		11 – 20	35	1	1	0	2.85
		21 – Above	17	6	4	2	35.29
8.	Satellite Town	1 – 10	138	20	12	8	14.49
		11 – 20	108	8	6	2	7.40
		21 – Above	64	21	7	14	32.81

Fig. 1. Showing gametocyte of *Plasmodium falciparum* in blood smear (100x).

Fig. 2. Showing gametocytes of *Plasmodium vivax* in blood smear (100x).

years. *Plasmodium vivax* (Fig. 2) was also observed to be present in our study but comparatively with a less prevalence ratio *viz*., 10.52% in the age group of 21 years and above, 7.02% in the age group of 1 to 10 years and 4.44% in the age group of 11 to 20 years. However, a negligible mixed infection of *Plasmodium falciparum* and *P. vivax* was also observed. Durrani *et al*. (*J. Pakistan med. Assoc*., **47**: 213-215, 1997) reported 64% incidence of *P. falciparum* in children and thirty six percent in adults of Quetta and found no significant difference in the prevalence of cerebral malaria, in Karachi and Quetta. Malaria Control Program (*District-wise epidemiological data of malaria control program Balochistan for the year 1998*. M.C.P. Balochistan, Pakistan, 1998, *District-wise epidemiological data of malaria control program Balochistan for the year 1999*. M.C.P. Balochistan, Pakistan, 1999, *District-wise epidemiological data of malaria control program Balochistan for the year 2000*. M.C.P. Balochistan, Pakistan, 2000) reported 329 +ve *P. vivax*, 29 +ve *P. falciparum* and 383 +ve *P. vivax* and 3 +ve *P. facliparum* and 609 + *P. vivax*, 44 +ve *P. falciparum* out of 10763, 18000 and 16400 blood slides of patients from Quetta district respectively. Abbasi *et al*. (*Pakistan J. med. Res*., **35**: 129-132, 1996) reported 53% of the study population cases of cerebral malaria in children of 1-5 years of age group admitted in Children Hospital, Larkana.

Statistical analysis: Types of infection

Age (Years)	A		B		Total
	(fo)	(fe)	(fo)	(fe)	
1-10	27	24.9	26	23.6	53
11-20	17	17.2	14	13.5	31
21 above	31	28.5	20	22.7	51
Total	75		60		135

$$X^2_{cal} = \Sigma \frac{(fo - fe)^2}{fe} = 1.0173$$

Table I (urban areas) was statistically analyzed to test whether there is any association between types of infection and age groups through X^2 at 5% level of significance. X^2 calculated as 1.0173 and compared with the table value of $X^2 = 5.991$. Since calculated value of X^2 is less than the table value so it is concluded that there is no association between types of infection and age groups. Therefore, it can be said that the incidence of any type of infection can happen to any age group person independently.

Amongst all the four species of malarial parasites, *P. vivax* and *P. falciparum* are more common in Indo-Pak subcontinent (White and Breman, In: *Harrison's principles of internal medicine* (eds. Isselbacher, Branwauld, Wilson, Martin, Fauci, Kasper), pp. 887-896, McGraw Hill Inc. New York, 1994). Infection with *P. falciparum* is more serious than other species because of high

Table III.- Area wise distribution of mosquito species in Quetta (Urban).

S.No.	Area	Total No. of mosquitoes collected	*Anopheles*	*Culex*	*Aedes*
1.	Jinnah Road	42	0	41	1
2.	Kawari Road	34	1	33	0
3.	Mission Road	23	0	23	0
4.	Cantt	31	1	29	1
5.	Pashtoonabad	43	2	41	0
6.	Marriabad	37	1	36	0
7.	Sirki road	41	1	39	1
8.	Satellite Town	39	2	36	1
	Total	**290**	**8**	**278**	**4**

Table IV.- Species wise distribution of *Anopheles mosquitoes* in Quetta (Urban).

S.No.	Area	Total No. of mosquito species	*Anopheles culicifacies*	*Anopheles stephensi*	*Anopheles pulcherrimus*	*Anopheles superpictus*
1.	Jinnah Road	0	0	0	0	0
2.	Kawari Road	1	1	0	0	0
3.	Mission Road	0	0	0	0	0
4.	Cantt	1	1	0	0	0
5.	Pashtoonabad	2	1	1	0	0
6.	Marriabad	1	1	0	0	0
7.	Sirki road	1	1	0	0	0
8.	Satellite Town	2	0	2	0	0
	Total	**8**	**5**	**3**	**0**	**0**

frequency of severe and fatal complication namely cerebral malaria.

A total of 290 mosquitoes comprising in 3 genera and two species were collected during the survey. Species wise distribution of mosquito fauna has been given in Tables III and IV. Genus *Culex* was found to be more abundant (forming 96% of the total), followed by genus *Anopheles* (0.86%) and genus *Aedes* (0.43%). *Anopheles culicifacies* was observed to be 62.5% and *An. stephensi* was found to be 37.5% of the total. Aslamkhan (*Mosq. Syst. News letter*, **3**: 147-159, 1971) reported prevalence of 134 species of mosquitoes in west Pakistan but species from Balochistan Province were few. *Pyretophorus nigrifasciatus* Theobald, 1907, [=*Anopheles* (*Cellia* Theobald) *multicolori,* Cambouliu, 1902] female from Pishin Balochistan, *P. nursei* Theobald, 1907, [=*A. (C.) superpictus* Grassi, 1899], 1 female from Quetta; *Culex (Neoculex) quettensis* Mattingly, 1955, male from Quetta; *An. habibi* Mulligan and Puri, 1936, female from the banks of Habib Nalah, Hudda village, Quetta were reported from Balochistan (Aslamkhan, *Mosq. Syst*., **4**: 98-102, 1972). Malaria Control Program (1999, 2000) reported the prevalence of *An. culicifacies, An. stephensi, An. pulcherrimus, An. subpictus, An. habibi, An. superpictus* in Balochistan. The most common species recorded in the present study were *An. culicifacies* and *An. stephensi*. Afridi *et al*. (*Pakistan J. Hlth*., **8**: 71-76, 1958) reported *An. stephensi* and Aslamkhan and Baker (*Pakistan J.* Zool., **1**: 1-7, 1969) recorded *An. subpictus* from Karachi.

In conclusion, it can be suggested that infection with *P. falciparum* was noted to be more prevalent in the present study whereas *P. vivax* was observed to be more common in the study conducted by Malaria Control Program (1998, 1999, 2000). The

results of present study show more prevalence of *An. culicifacies* and *An. stephensi* in Quetta district, and very much coincides with the results of Malaria Control Program (1999, 2000).

Department of Zoology, University of Balochistan, Quetta

M.I. YASINZAI
J.K. KAKARSULEMANKHEL

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SURVIVAL OF *TRICHOGRAMMA CHILONIS* ISHII (HYMENOPTERA: TRICHOGRAMMITIDAE) AFTER EXPOSURE TO DIFFERENT INSECTICIDES: LABORATORY STUDIES

Abstract.- Eight insecticides *viz.* diafenthiuron, buprofezin, thiodicarb, imidacloprid, carbosulfan, methamidophos, acetamiprid and thiamethoxam were tested for their toxicity against egg parasitoid *Trichogramma chilonis* through leaf dip bioassay method under laboratory conditions. Insecticides were applied at three levels of concentration *viz.,* low, recommended and high, in a completely randomized manner. The results revealed that all concentrations of imidacloprid, carbosulfan, methamidophos and thiodicarb were toxic to *T. chilonis*. Lower concentrations of acetamiprid and thiamethoxam were slightly harmful, while recommended and higher concentrations were found moderately harmful and harmful, respectively. All concentrations of buprofezin remained harmless. All insecticides were found toxic after 48 hours except buprofezin. Toxic insecticides are recommended for higher tire testing to evaluate their effectiveness in the field. Parasitism rate of *T. chilonis* was lowest (18%) in methamidophos and highest (100%) in buprofezin.

Key words: *Trichogramma chilonis*, insecticide toxicity, leaf dip bioassay.

Integrated Pest Management (IPM) programme is used worldwide including Pakistan for controlling agricultural insect pests. The use of natural enemies in combination with selective insecticides, which have no effect on them, is an effective method for depressing the population density of the insect pests. Assessment of the potential effects that pesticides have on the natural enemies will help the adjustment of timing of sprays, thus avoiding the most susceptible stage. Mass breeding and release of parasitoids for control of various lepidopteran pests is being adopted as commercial practice in many countries.

At the moment 18 species of *Trichogramma* are being used in many countries on crops such as corn, sugarcane, tomato, rice, cotton, sugarbeet, apple, plum, vineyard, pasture, cabbage, chestnut, sweet paper, pomergranate and forest (Hassan, In: *Pesticides and non-target invertebrates* (ed. P.C. Jepson), Intercept Wimborne, Dorset, pp. 18, 1989). However, the efficacy of the parasitoid is influenced a great deal by the insecticide spray schedule (Takada *et al*., *J. econ. Ent*., **94**: 1340-1443, 2001).

In Pakistan production of cotton relies heavily on the use of insecticide sprays. Most of the 10 to 12 sprays applied to cotton are for the control of *Helicoverpa* species (Fitt, *Ann. Rev. Ent*., **39**: 543-562, 1994). Some of these sprays contain additional insecticides for the control of other insect pests of cotton such as whitefly, jassid, thrips, aphids, spotted bollworms, pink bollworm, armyworms and mites. Associated with high doses of insecticides, problems like development of insecticide resistance, secondary pest outbreaks and pest resurgence have appeared. So there is a desire within cotton industry to integrate different control measures, in order to reduce the use of pesticides. The objective of this study was to determine the susceptibility of *T. chilonis* to insecticides and to evaluate its potential use in IPM programs in Pakistan.

Materials and methods

Insecticides

Formulated products of eight insecticides *viz*., diafenthiuron, buprofezin, thiodicarb, imidacloprid, carbosulfan, methamidophos, acetamiprid and thiamethoxam were selected to determine their toxicity against *T. chilonis* under laboratory conditions (Table I). Three concentrations of each insecticide *i.e.*, C1, C2 and C3 representing lower dose, recommended dose and higher dose, respectively, were prepared in tap water and used

throughout the experimentations. The results were classified according to the recommendation of IOBC/WPRS working group (Hassan, 1989 *op.cit.*) as under:
Harmless, less than 50% mortality; Slightly harmful, 50-79% mortality; Moderately harmful, 80-89% mortality; Harmful, more than 90% mortality.

Procedure adopted

Glass Petri dishes (2 inches diameter) were used for experimentations. Cotton leaves which have not been previously sprayed with insecticide were collected from the field and washed with tap water. Two inches diameter cotton leaf disks were cut, dipped into insecticide solution for 5 seconds and allowed to dry in open air. Untreated controls (T9) were dipped in tap water only. Moistened filter paper was placed beneath leaf disks to avoid the desiccation of leaves in the Petri plates.

Experiments were conducted in completely randomized design (CRD) with three replicates for each *T. chilonis* in the form of *Tricho*-cards (parasitized eggs of *Sitotroga cerealella* glued on cards) was obtained from IPM laboratory of University College of Agriculture, Bahauddin Zakariya University Multan. Fifty pupae of *T. chilonis* in the host eggs (near to emergence) glued on 0.5x0.5 cm paper card were placed in the center of Petri dish. Parasitization rate of treated *T. chilonis* was checked by placing a paper card (0.5x0.5 cm) having 50 fresh eggs of *S. cerealella* in Petri dish.

Results and discussion

Table II shows the results in terms of percent mortality of *T. chilonis* adults after 24 and 48 hour exposure to three concentrations of eight insecticides under laboratory conditions. All concentrations *i.e.* C1, C2 and C3 of imidachloprid, carbosulfan, methamidophos and thiodicarb were found harmful to the adults of *T. chilonis*. Carvalho (*An. Soc. Ent. Brasil*, **23**: 431-434, 1994) reported that methamidophos reduced the parasitization rate as well as its survival rate. Hassan (*IOBC Bull*., **21**: 89-92, 1998) reported that adults of *Trichogramma* showed higher susceptibility to several insecticides.

Lower concentration of acetamiprid was found slightly harmful while recommended and higher concentrations (C2 and C3) were found moderately harmful. On the other hand all concentrations of acetampirid were found harmful 48 hours after treatment. Lower concentration (C1) of thiamethoxam was found moderately harmful while recommended (C2) and higher concentration (C3) found harmful. Lawson *et al*. (*Proc. Beltw. Cott. Conf*., **2**: 1106-1109, 1999) reported that thiamethoxam is classified as slightly harmful to beneficial insects and harmless to predatory mites. It is often applied to the soil (*i.e.* infurrow, seed treatment, side dress through irrigation systems), where thiamethoxam systematically protects the plant without contacting beneficial species, which remain on the plant surface. Therefore, flexibility in the application of thiamethoxam with limited leaf surface residues resulted in excellent pest control without disrupting natural enemies. But our results showed that thiamethoxam was toxic when parasitoid was in direct contact with insecticide. It was previously reported by Schuld and Schmuck (*Ecotoxicology*, **9**: 197-205, 2000) that insecticides of neonicotinoid group were considered as harmless for immature stages of parasitoids but harmful for adults.

Lower concentration (C1) of diafenthiuron after 24 hours treatment was found slightly harmful concentrations of diafenthiuron remained harmful after 48 hours treatment. Hassan *et al*. (*J. appl. Ent*., **103**: 92-107, 1987) obtained similar results and reported harmful effect of diafenthiuron on *T. chilonis* and reduced parasitism rate between 90-100% while direct spray of diafenthiuron was harmless to the parasitoid pupa with in the host.

Lower and recommended concentrations (C1 and C2) of buprofezin caused less than 50% mortality and were considered harmless but higher concentration (C3) was found slightly harmful. Gerling and Siani (*J. econ. Ent*., **87**: 842-846, 1994) and Jones *et al*. (*Biol. Cont.*, **11**: 70-76, 1998) supported our results. They studied the effect of buprofezin on parasitoids *viz. Eretmocerns* and *Encarsia* species and found that buprofezin had detrimental effect on immature stages of parasitoids but had no harmful effect on adults.

Table III shows the percent parasitization by *T. chilonis* to three concentrations of eight insecticides. Parasitism rate of *T. chilonis* was found high in lower and higher concentrations of imidachloprid (T1), carbosulfan (T2) and acetamiprid (T5). In T4,

Table I.- Common names, trade names and concentrations of eight insecticides used against *Trichogramma chilonis*.

Insecticides			Treatments	Concentration (%)		
Common name	Trade name	Group		C1	C2	C3
Imidacloprid	Confidor 20 SL	Neonicotinoid	T1	0.088	0.133	0.178
Carbosulfan	Advantage 20 EC	Carbamate	T2	0.25	0.3	0.35
Methamidophos	Tamaron 60 SL	Organophosphate	T3	0.75	0.9	1.05
Thiodicarb	Larvin 80 WP	Carbamate	T4	0.8	0.1	1.2
Acetamiprid	Raja 25 EC	Neonicotinoid	T5	0.5	0.1	0.15
Thiamethoxam	Actara 25 WG	Neonicotinoid	T6	0.005	0.008	0.01
Diafenthiuron	Polo 50 Sc	Thiourea	T7	0.25	0.375	0.5
Buprofezin	Sitara 25 WP	IGR	T8	0.406	0.469	0.531
Water			T9			

Table II.- Percentage mortality of *Trichogramma chilonis* after 24 and 48 hours exposure to three concentrations of eight insecticides.

Treatment	Percent mortality						Toxicity class					
	24 hours			48 hours			24 hours			48 hours		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
T1	100	100	100	-	-	-	4	4	4	-	-	-
T2	100	100	100	-	-	-	4	4	4	-	-	-
T3	100	100	100	-	-	-	4	4	4	-	-	-
T4	80	82.67	93.3	90	93	100	4	4	4	4	4	4
T5	68	82	88	100	100	100	2	3	3	4	4	4
T6	84	93	100	100	100	-	3	4	4	4	4	-
T7	78	93	100	100	100	-	2	4	4	4	-	-
T8	28	39	52	69	83	83	1	1	2	2	3	3
T9	0	0	0	0	0	0	1	1	1	1	1	1

the rate of parasitism was found increasing with the increase of concentration of thiodicarb. At lower concentrations of thiamethoxam (T6) and diafenthiuron (T7), the parasitism rate was 60% and 90%, respectively but had decreasing trend with the increase of the concentrations of the insecticides. Parasitism was 100% in all concentrations of buprofezin treatments (T8) as well as control (T9).

Hassan (1989, *op.cit.*) demonstrated that selectivity of the insecticides can be classified following the guidelines of IOBC / WPRS working group for beneficial in laboratory test for initial contact toxicity. A plant protection product (PPP) was considered harmless if mortality is less than 50% of the larvae treated in initial laboratory test and no further test in semi-field and field condition is recommended. According to general agreement, when PPP proved harmless in initial laboratory test for a particular beneficial insects then it is most likely to be harmless to the same organism in the field. Further testing (semi-field and field condition) is necessary when a pesticide is found to be harmful (class 3 and 4) that it caused more than 80%

Table III.- Percent parasitization by *Trichogramma chilonis* after exposure to three concentrations of eight insecticides.

Treatments	Percent parasitization		
	C1	C2	C3
T1	58	28	40
T2	36	24	28
T3	38	16	0
T4	30	40	48
T5	60	23	38
T6	60	52	24
T7	90	60	58
T8	100	100	100
T9	100	100	100

mortality to a beneficial in initial laboratory toxicity tests. However, the effect of insecticides on particular natural enemy involves numerous biotic and abiotic factors. Therefore it would be regrettable to exclude toxic compounds with out looking for their specific uses. Selection of a suitable insecticide in an IPM program not only depends on its toxicity level to beneficial insects but also on its efficacy against the target pest, weathering and persistency.

Department of Entomology, **A. NASREEN**
University College of Agriculture, **G.M. CHEEMA**
Bahauddin Zakariya University, **M. ASHFAQ**
Multan (AB, GMC, MAS) and **M.A. SALEEM**
Department of Entomology,
University of Agriculture,
Faisalabad (MA)

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EVALUATION AND DETECTION OF MYCOTOXINS IN FOOD AND THE FEED

Abstract.- Wheat flour (two samples), poultry feed, maize flour, gram meal and peanut were analyzed for the detection of mycotoxins and found to be heavily contaminated with *Penicillium* and *Fusarium* spp. *Aspergillus* was found only in one of the wheat flour samples. Thin layer chromatography of chloroform extracts of these samples showed that aflatoxin G_1 was detected only in wheat flour samples while T-2 toxin was found only in gram meal. The toxicity of food and feed extracts was also determined by using brine shrimp larvae where poultry feed was found to be highly toxic.

Key words: Poultry feed, maize flour, gram meal, peanut, mycotoxins.

The storage fungi produce the most potent mycotoxins known. These fungi can grow on a wide range of animal and human foods provided they contain sufficient moisture; and it is common experience that the growth of these fungi is not prevented by commercial and domestic refrigeration conditions. The mycoflora contamination in foods and feeds (Bragulat *et al*., *J. Sci. Fd. Agric*., **67**: 215-220, 1995; Forbito and Babsky, *Assoc. Off. Anal. Chem. J*., **68**: 941-944, 1985; Schollenberger, *Mycopathologia*, **147**: 49-57, 1999).

A number of rapid, economical analytical methods are available for determining mycotoxin levels in a wide range of agricultural food products. Among these methods thin layer chromatography (TLC) is relatively inexpensive and a number of samples can be analyzed simultaneously (Beaver and Wilson, *J. Agric. Fd. Chem*., **73**: 579-581, 1990; Scott *et al*., *Appl. Microbiol.*, **20**: 839-842, 1970). High performance liquid chromatography (HPLC) has been introduced for mycotoxin detection (Forbito and Babsky, *Assoc. Off. Anal. Chem. J*., **68**: 941-944, 1985; Rovira *et al*., *J. Agric. Fd. Chem*., **41**: 214-216, 1993) and it has the advantage of being easier, faster and giving more reproducible results than TLC and HPCL is also a more expensive procedure. A number of immunoassay methods are currently available including radioimmunoassay (RIA) an enzyme linked immunosorbent assay (ELISA) (Tanaka *et al*., *J. Agric. Fd. Chem*., **43**: 946-950, 1995).

Materials and methods

Food and feed sources

Two wheat flour samples, one from heavily contaminated wheat (S_1) and the second from good quality wheat (S_2), poultry feed, maize flour, gram meal and peanut collected from different localities of Faisalabad, Pakistan.

Isolation, identification and purification of fungi

Nutrient broth agar (2% nutrient broth, 2% dextrose and 2% agar agar), Potato Dextrose Agar, PDA (2% potato starch and 2% agar agar) and Czapek Dox Agar (CDA) medium (6g Sucrose, 0.6g $NaNO_3$, 0.2g KH_2PO_4, 0.1g $MgSO_4$, 0.1g KCl, 0.0001g $FeSO_4$, 4g agar agar and 0.001 g/l Streptomycin) were used for the growth of mycoflora.

Finely ground samples (1.0g) were dissolved in 10 ml of distilled water to prepare the stock solutions. Further, $1/10^{th}$ and $1/100^{th}$ dilutions for each of the samples were prepared.

Media were inoculated with 100 µl of each dilution and then incubated at 25°C for ten days. The colonies were counted and single spore purified

by streak method on CDA medium were transferred to gram meal slants (2% gram meal, 2% dextrose and 2% agar agar) and then identified by examining morphology of the colony, type of pigmentation of microscopic appearance of the fruiting heads and spores from mould colonies (Pineiro *et al*., *Nat. Toxins*, **4**: 242-245, 1996).

Toxin extraction and identification

Test sample (50g) were dissolved in 200 ml of Methanol-H_2O (85:15) and then shaken for 30-45 minutes on shaker at 100 rpm. The mixture was filtered through Whatmann filter paper 1 and then 10% NaCl solution equal to the filtrate was added. Using chloroform drained lower organic phase. N-hexane was used only for peanut and maize flour for the extraction of oils.

The following solvent systems were used for better resolution of mycotoxins on plates of silica gel 60GF254 (Merck) which were activated for 1 hour at 110°C toluene: ethyl acetate: formic acid (6:3:1); benzene: methanol: acetic (24:2:1); chloroform; benzene: acetone: acetic acid (64:12:0.8); diethyl ether: methanol: water (96:3:1) and chloroform:acetone (9:1).

Chloroform extracts of food/feed samples were dissolved in methanol and spotted on chrormatographic plates of 0.5 mm thickness along with standard mycotoxins. After developing in an appropriate solvent system, plates were examined in visible and UV light (254 and 366 nm) before and after spraying the plate with a freshly prepared mixture of 0.5 ml of *p*-anisaldeyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5.0 ml of conc. H_2SO_4 heating at 130°C for 8 to 20 minutes. Colors of mycotoxins were visualized in visible and UV light and Rf values were calculated.

Brine shrimp toxicity assay

The bioassay was done by the method as described by Leistner *et al*. (*Nachweis, Bildung and Wirkung von Mycotoxinen,* Kulmbach, Germany, 1977). The brine solutiion for the hatching of *Artemia salina* larvae was prepared by dissolving 38 g commercial sodium chloride in one litre distilled water, filtered and then autoclaved. In hatching flasks (containing autoclaved brine solution) 50 mg of brine shrimp eggs were added. The flasks were then incubated at 27°C on a shaker for 30 hrs. The brine shrimp larvae were collected with the help of Pasteur pipette. The concentration was adjusted upto 10 larvae/ml. Test samples (250 µl dissolved in ethanol) were taken in scintillation vials (Cap. 20 ml) and then 5 ml of brine solution containing larvae were poured in each vial in three replicates. The vials were incubated at room temperature for 24 hrs under illumination. The live and dead larvae were counted using stereomicroscope and finally the percentage mortality was calculated. In control treatments 250 µl ethanol was used.

Results and discussion

Among the three media used Czpek dox agar was found to be the most suitable for mycofloral growth because thin mycelial growth was observed on PDA, while nutrient broth agar medium was full of bacterial growth. The feed and food samples were found highly contaminated with *Penicillium* and *Fusarium* spp. except peanut in which only *Penicillium* spp. was observed at $10/10^{th}$ dilution, while *Aspergillus* spp. was only found to contaminate wheat flour S1. The *Fusarium* colonies seemed to produce rose pink and purple pigments with thick walled and spiny conidia (4-9 µm). The colonies of *Penicillium* spp. were usually blue green and velvety. The conidia of this genus (2.5-5.0 µm) were round and born on metulae bearing phialides. *Aspergillus* mostly produced bluish green and black velvety colonies. In microscopic view, the conidiophores appeared unbranched and chains of round conidia (2.5 µm) were produced from phialides.

The poultry feed was highly contaminated with *Fusarium* and *Penicillium* spp. with an incidence of $1.1x10^4$ and $1.3x10^4$ colonies/g respectively, while peanut was found to be the least contaminated sample (Table I).

The chloroform extracts from food and feed samples showed best separation on silica gel TLC plates when these were developed in TEF, BMA and CM solvent system. Extracts of wheat flour samples and poultry feed were found to contain less polar compounds (contaminations) in high concentrations, which produced tailing on the TLC plates. The Rf values of fluorescence of these mycotoxins are shown in Table II. Aflatoxin G1 was

Table I.- Fungal species isolated from food and feed samples.

Food and feed samples	Species isolated (colonies /gm)								
	Penicillium spp.			*Fusarium* spp.			*Aspergillus* spp.		
	R_1	R_2	Mean	R_1	R_2	Mean	R_1	R_2	Mean
Wheat flour S_1	$1.5x10^3$	$5.0x10^2$	$1.0x10^3$	$5.0x10^3$	$7.0x10^3$	$6.0x10^3$	$1.0x10^3$	$1.0x10^3$	$1.0x10^3$
Poultry feed	$9.5x10^3$	$1.6x10^4$	$1.3x10^4$	$9.0x10^3$	$9.5x10^3$	$1.1x10^4$	-	-	-
Maize flour	$5.0x10^2$	$1.5x10^3$	$1.0x10^3$	$1.0x10^3$	$2.5x10^3$	$2.0x10^3$	-	-	-
Gram meal	$1.5x10^3$	$3.5x10^3$	$2.5x10^3$	$2.5x10^3$	$4.5x10^3$	$3.5x10^3$	-	-	-
Peanut	$1.5x10^3$	$5.0x10^2$	$1.0x10^3$	-	-	-	-	-	-
Wheat flour S_2	$5.0x10^2$	$2.0x10^3$	$2.0x10^2$	$1.0x10^3$	$3.0x10^3$	$2.0x10^3$	-	-	-

Table II.- Thin layer chromatography of mycotoxins.

Mycotoxin	Rf			Color			Color after spray	
	Solvent system			Visible	Ultraviolet		Visible light	Long wave ultraviolet light
	TEF	BMA	CM		Long-wave	Short-wave		
Aflatoxin G1	0.17	0.13			Green	Faint green		Blue
Aflatoxin B1	0.31	0.23			Blue	Faint blue		Pink
T-2 toxin	0.36	0.28	0.63				Grey pink	Blue
Zearalenone	0.78	0.42	0.60	`	Faint blue	Blue green	Faint brown	Faint yellow
Zearalenol	0.62	0.26	0.43					

Table III.- Detection of mycotoxins in chloroform extracts of food and feed samples using thin layer chromatographic technique.

Food and feed samples	Mycotoxin detected			
	Zearalenol	Zearalenone	T-2 toxin	Aflatoxin G1
Wheat flour S_1	+	-	-	+
Poultry feed	++	-	-	-
Gram meal	-	-	+	-
Maize flour	-	-	-	-
Wheat flour S_2 (white)	-	-	-	+
Peanut	-	-	-	-

detected in both wheat flour samples. In wheat flour sample S1 and poultry feed, α-zearalenol was detected and its concentration was high in poultry feed. T-2 toxin was only present in gram meal and none of the four toxins were detected in peanut (Table III).

The percentage mortality of chloroform extracts of food and feed samples are shown in Table IV. The chloroform extract from the poultry feed showed maximum toxicity to *Artemia salina* that was 97% when 250 µL of methanol extract was used. Least toxicity was observed in chloroform extract of wheat flour S_2 in which only 2.6% mortality was observed. Wheat S_1 (low grade) was also toxic to *Artemia salina* larvae in which 61% mortality was observed. Maize flour sample was also toxic to brine shrimps showing mortality of

Table IV.- Toxicity of chloroform extracts of food and feed samples to brine shrimp larvae.

	% Mortality		
	R1	R2	Mean
Wheat flour S_1	62	59	61.0
Poultry feed	95.5	97.5	97.0
Maize flour	13.8	15.2	14.0
Gram meal	49.6	53.4	51.6
Peanut	12	8	10.0
Wheat flour S_2	2.3	2.9	2.60

51%. The chloroform extracts of the rest of the feed samples were not significantly toxic with peanut 10% and gram meal 14% toxicity. A good correlation was found among toxin detection, microbiological studies and brine shrimp assay.

The food and feed samples collected from different localities of Faisalabad were highly contaminated with *Fusarium* and *Penicillium* spp. The other major storage fungus was the *Aspergillus* spp. found only in wheat flour S1 and this is probably due to the fact that the second grade wheat contained more amount of bran which is the primary site of infection for most of the fungi. Poultry feed was found to be the most infected feed because of rotten ingredients in it. Poultry feed has already been reported to be highly contaminated with *Penicillium* spp., *Fusarium* spp. and *Aspergillus* spp. (Bragulat *et al*., *J. Sci. Fd. Agric*., **67**: 215-220, 1995) but in our samples *Penicillium* and *Fusarium* had higher incidence. The multiple numbers of species of the same genus were purified from the same sample on the basis of colony morphology and pigment production.

The chloroform extracts of food and feed showing significantly different toxicity to brine shrimp larvae revealed the highest toxicity of poultry feed. Pineiro *et al*. (*Nat. Toxins*, **4**: 242-245, 1996) have also reported the highest values for all mycotoxins in poultry feed. The mycotoxin problem always exists mainly due to conditions of temperature and humidity that are favourable to the growth of toxigenic moulds (Sabino *et al*., *Rov. Inst. Adolfo Lutz*, **58**: 53-57, 1999).

In view of public health hazard, the food should be regularly assayed not only for the common food poisoning bacteria and associated toxins, but also for mycotoxins and the contaminating mycoflora.

Department of Zoology, GC University, Faisalabad, (AN, SM, TY), **A. NOREEN, S. MAHBOOB**
Nuclear Institute for Agriculture and Biology, P.O. Box 128, Jhang Road Faisalabad (JAK, IAK, SN) **J.A. KHAN, T. YASMIN, I.A. KHAN, S. NADEEM**

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PREDICTION OF THE ROLE OF POST-TRANSLATIONAL MODIFICATIONS IN PROCESSING OF TRANSFORMING GROWTH FACTOR B 1 IN *MUS MUSCULUS*

Abstract.- This communication describes modifications that can take place in the primary structure of Transforming Growth Factor β 1 in *Mus musculus*. These modifications in hydroxylated amino acids, serine and threonine, by phosphate group and *O*-glycosylated sugar residues are predicted based on neural network studies. These modifications induce structural changes and biological functions of the growth factor. The possibilities of these modifications in vivo are discussed in view of multifunctions of proteins.

Keyword: TGF β *O*-glycosylation, cytokines, Phosphorylation of protein.

Transforming growth factor β1 (TGF- β1) is the prototypic member of a big family of structurally related pleiotropic-secreted cytokines that play a critical role in the control of differentiation, proliferation, and state of activation of many different cell types including immune cells (Moustakas *et al., Immunol. Lett.*, **82**: 85-91, 2002). TGF- β1 is a 55 kDa, precursor protein of 390 amino acids in case of *Mus musculus.* The preproprotein that consists of a 23 aa signal sequence, a 255 amino acid pro-region and a 112 amino acid mature segment (Dubois *et al. J. biol. Chem*., **260**:10618, 1995). The pro-region is characterized by the presence of three potential *N*-linked glycosylation sites, while the mature segment contains nine cysteines and no *N*-linked glycosylation sites. Prior to secretion, the pro-region is cleaved at an RxxR site with a furin-like protease. Bioactive TGFβ1 is a 25-kDa homodimeric protein with potent effects on cell growth and differentiation (Sporn and Roberts *J. Cell Biol.*, **119***:* 1017-1021, 1992). With almost similar bioactivities three other isoforms (TGFβ1, TGFβ2, and TGFβ3) have also been recognized in mammalian tissues and TGFβ1 is the most comprehensively characterized isoform. A number of proteins more

distantly related to TGFβs such as actvins, inhibins, Müllerian inhibitory substance, bone morphogenic proteins, products of the *nodal* gene in mice as well as products of the decapentaplegic complex of *Drosophila* and the Vg1 gene from *Xenopus laevis* appear to play an important role in cellular differentiation (Kingsley, *Genes Dev*. **8**: 133-146, 1994).

The isoforms of TGF β are produced by a wide range of normal and malignant cells and have been isolated from different tissues such as blood platelets, bone, and placenta (Assoian *et al., J. biol. Chem.*, **235**: 1357-1370, 1978; Childs *et al., Proc. natl. Acad. Sci. U. S. A.*, **79**: 5312-5316, 1982). It has also been shown that most of the cell types secrete TGF β1 in an inactive form (Lawrence *et al., Biochem. biophys. Res. Commun.*, **133**: 1026-1034, 1985; Pircher *et al., Biochem. biophys. Res. Commun.*, **136**: 30-37, 1986) which does not interact with specific TGF β cell surface receptors thus failing to educe TGF β-induced biological responses. Inactive TGF β may take multiple forms: for example, the 25-kDa mature TGF β may be complexed with specific binding proteins such as the TGF β latency-associated peptide (NH_2-terminal part of the precursor sequence) (Gentry and Nash *Biochemistry*, **29**: 6851-6857, 1990) and the latent TGF β-binding protein and thus becomes unable to bind to its complement receptors leading to an incomplete processing of TGF β 1 precursor (Kanzaki *et al., Cell*, **61**: 1051-1061, 1990). The presence of TGF β receptors majority of cell types (Tucker *et al., Proc. natl. Acad. Sci. U. S. A.*, **81**: 6757-6761, 1984) and the ubiquity of the TGF β molecule itself (Moses *et al., Cancer Cells* (*Cold Spring Harbor)*, **3**: 65-71, 1985) advocate that processing and activation of TGF β is an important step in the regulation of TGF β action.

The 25-kDa TGF β1 bioactive fragment consists of two identical disulfide-linked 12.5-kDa polypeptide chains (Assoian *et al., J. biol. Chem.*, **258**: 7155-7160, 1983). Cloning and primary structure (amino acid sequence) determination of precursor cDNA of TGF β1 revealed that the mature 112-amino-acid chain of TGF β1 is derived from the COOH terminus of a 390-amino-acid pre-pro-TGF β1 by proteolytic cleavage (Derynck *et al., J. biol. Chem.*, **261**: 4377-4379, 1986). This processing event is predicted to occur following an R-H-R-R sequence immediately preceding the NH_2-terminal Ala 279 residue of the mature growth factor. This suggests that processing of the growth factor involves an endoprotease which shows cleavage specificity toward pairs of basic amino acids (Derynck *et al., J. biol. Chem.*, **261**: 4377-4379, 1986).

Materials and methods

The sequence data

The sequence data used for predicting phosphorylation and glycosylation sites for TGF beta 1 of *Mus musculus* was retrieved from SWISSPROT sequence database with entry name TGF1_MOUSE and primary accession number P04202 and Entrez protein database (NCBI sequence viewer) with database number NP_035707. The sequence of the protein has been described (Derynck *et al., J. biol. Chem*., **261**: 4377-4379, 1986; Guron *et al., Gene*, **165**: 325-326, 1995; Chen *et al., Curr. Dir. Autoimmun*, **5**: 62-91, 2002) since eighties. On comparing the sequence data from two databases no difference was found with respect to number and sequence position of amino acids between the sequences retrieved from the two sources.

Prediction methods

Prediction methods used to predict potential glycosylation sites involved four methods; Three for potential *O*-linked glycosylation sites and one for predicting *N*-linked glycosylation sites. Methods used for predicting potential *O*-linked glycosylation sites include *NetOGlyc* 3.0 (http:// www.cbs.dtu.dk/services/NetOGlyc/), that precdicts *O*-glycosylation sites in mucin type proteins (*i.e.,* for *O*-GalNAc sites), *DictyOGlyc* 1.1 (http:// www.cbs.dtu.dk/services/DictyOGlyc/) predicts *O*-α-GlcNAc sites in eukaryotic proteins and YinOYang 1.2 (http://www.cbs.dtu.dk/services/ YinOYang/), that predicts *O*-β-GlcNAc sites in eukaryotic proteins. The *NetNGlyc* 1.0 (http:// www.cbs.dtu.dk/services/NetNGlyc/) was used for precdicting *N*-glycosylation sites. The above-mentioned four methods for predicting the glycosylation sites are neural network based. For prediction of phosphorylation sites in selectins

NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was used. The *NetPhos* 2.0 is also a neural network based program designed by training the neural networks through protein phosphorylation data from phosphobase 2.0.

Results

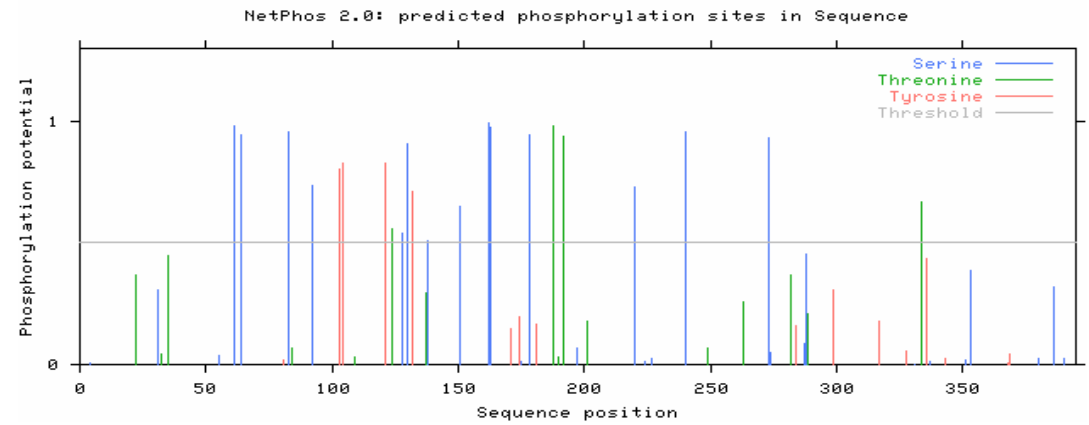
Prediction results from different methods depicts that transforming growth factor beta 1 shows the greatest potential for *O*-linked phosphorylation with 22 potential sites (Ser 14, Thr 4, Tyr 4) for phosphate addition (Fig. 1A). The data from glycosylation prediction methods shows that *O*-β-GlcNAc potential among all four types of glycosylation (*i.e. N*-linked GlcNAc modification, *O*-linked GalNAc and GlcNAc modification in alpha anomeric form, and *O*-linked GlcNAc modification in beta anomeric form) is most numerous (Fig. 1b). NetNGlyc 1.0 predicted only two potential sites for *N*-linked glycosylation whereas, NetOGlyc 3.0 predicted only one site potential for modification by α-GalNAc, DictyoGlyc 1.1 predicted three potential sites for modification by *O*-α-GlcNAc, and YinOYang predicted eight sites potential for modification by *O*-β-GlcNAc with two Yin Yang sites (Fig. 1B).

Discussion

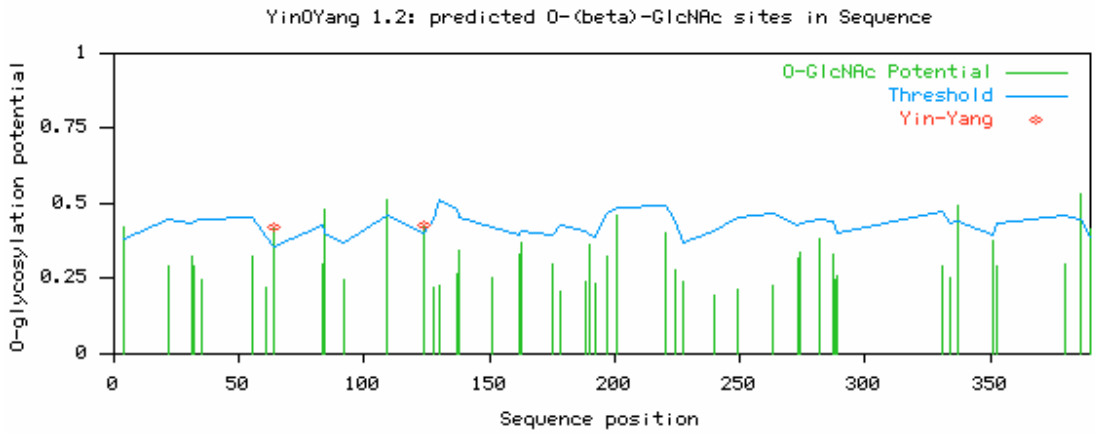
As described earlier TGF-beta is a multifunctional peptide that control proliferation, differentiation, and other functions in many cell types. Many cells synthesize TGF-beta and essentially all of them have specific receptors for this peptide. TGF-beta regulates the actions of many other peptide growth factors and determines a positive or negative direction of their effects. Play an important role in bone remodelling. It is a potent stimulator of osteoblastic bone formation, causing chemotaxis, proliferation and differentiation in committed osteoblasts.

Many proteins including polypeptide hormones, viral proteins, growth factors, and receptors are synthesized as large inactive precursor proteins that must be proteolytically processed in order to release the bioactive polypeptide (Sossin *et al., Neuron*, **2**: 1409, 1989). The most commonly occurring site of proteolysis is at the carboxyl-terminal side of basic amino acids residues found within the pro-protein (Sossin *et al., Neuron*, **2**: 1409, 1989). Recently, a family of mammalian processing enzymes called SPCs (subtilisin-like pro-protein convertases) has been characterized (Steiner *et al., J. biol. Chem.*, **267**: 23435, 1992). Up to six members of this family have been identified to date. These are Ca^{++}-dependent serine proteases that have been shown to cleave mostly at the R-R or K-R pairs of basic amino acids. Furin, the first SPC member to be extensively characterized, has been shown to process many proproteins including pro-β-NGF (Bresnahan *et al., J. Cell Biol.*, **111**: 2851, 1990), the insulin receptor (Robertson *et al., J. biol. Chem.*, **268**: 24274-24277, 1993) , and the HIV-1 glycoprotein gp160 (Hallenberger *et al., Nature*, **360**: 358-361, 1992) among others. Expression of the *fur* gene, which encodes furin, appears to be ubiquitously expressed in all tissues and cell types examined to date (Hosaka) *et al., J. biol. Chem.*, **266**: 12120-12130, 1991). Colocalization with TGN 38 and failure to redistribute to the endoplasmic reticulum in the presence of brefeldin A suggest that furin is mostly localized in the *trans*-Golgi network (Molloy *et al., EMBO J.*, **13**: 18-33, 1994). Substrate specificity studies have revealed that furin requires a R- *X*-*X*-R motif for cleavage while the R-*X*-K/R-R sequence provides an optimum-processing site (Molloy *et al., J. biol. Chem.*, **267**: 16396-16402, 1992). Upon inspection of the amino acid sequence of the TGF β1 precursor, such an optimum furin cleavage motif was revealed immediately upstream of the amino acids constituting the NH_2-terminal of the mature TGFβ. Colocalization of TGF β precursor with mannosidase II and its sensitivity to endoglycosidase H suggest that pro-TGFβ processing occurs in the Golgi complex, the subcellular site of furin location (Miyazona *et al., J. biol. Chem.*, **267**: 5668-5675, 1992). Therefore, the nearly ubiquitous expression of both furin and TGFβ, the correlation between their cellular localization, and the nature of the TGFβ-processing site make furin a good candidate for the physiological processing of TGFβ.

On the basis of prediction results in TGF β1 it is clear that potential of phosphorylation sites is the most frequent one. Whereas, among the *O*-linked predicted potential glycosylation types *O*-GlcNAc addition in beta anomeric conformation is most frequent. According to prediction results Ser 273



(A)



(B)

Fig. 1 Graphs showing the predicted potential for different residues for phosphorelation (a) and *O*-β-GlcNAc modification (b)

(SSRHRR) very close to RHRR sequence motif (Fig. 1a) described earlier (Dubois *et al. J. biol. Chem.*, **260**: 10618-10624, 1995) for the action of enzyme furin to cleave it into bioactive fragment is potential for phosphorylation. We propose here that phosphorylation at Ser 273 is able to create some conformational changes in the sequence motif RHRR making it suitable for the action of furin. Similarly, Ser 273 and 274 (Fig. 1B) are showing the potential very close to the threshold line and are likely to be modified by *O*- β-GlcNAc at the same site or region (Yin Yang sites). We speculate the role of *O*-β-GlcNAc addition in blocking the phosporylation site thus inhibiting the processing of TGF β1 precursor into its fragments that will become bioactive for carrying out its multi functions. But the blocking of phosphorylation of TGF β1 will lead to stop or abnormal processing resulting in performing abnormal processes.

These prediction results are in line with our assumption that proteins are capable of performing multifunction *in vivo* arising due to structural changes as a consequence of primary amino acid modifications. Currently, predicted substitution of hydroxyl group of serine and threonine by a sugar residue or phosphate induce functional change of the preotein molecule.

Institute of Molecular Sciences and Bioinformatics, Lahore (IA, MO,FU, ND)
HEJ Research Institute of Chemistry,University of Karachi, Karachi (MIC)
and School of Biological Science, Punjab University, Lahore (ARS)

I. AHMED
M. OVAIS
FAYYAZ UDDIN
M.I QBAL CH
A.R. SHAKOORI
NASIR-UD-DIN

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