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RESEARCH ARTICLE

Development of Indirect ELISA for the Diagnosis of Bovine Hypodermosis *(Hypoderma lineatum)* in the Cattle of Subtropical Region of Pakistan

Haroon AHMED [1] Rosario PANADERO-FONTÁN [2] Ceferino LÓPEZ SÁNDEZ [2]
Mobushir Riaz KHAN [3] Saira ASIF [4] Irfan MUSTAFA [5] Abdul GHANI [5]
Iftikhar AHMAD [5] Syed Muhammad Saqlan NAQVI [6] Mazhar QAYYUM [7]

[1] Department of Biosciences, COMSATS Institute of Information Technology, Park Road, Chakh Shazad, Islamabad, 46000, PAKISTAN
[2] Departamento de Patología Animal: Sanidad Animal, Facultad de Veterinaria de Lugo, Universidad de Santiago de Compostela, 27071 Lugo, SPAIN
[3] Department of Space Science, Institute of Space Technology (IST), Islamabad Highway, Near Rawat Toll Plaza, Islamabad, PAKISTAN
[4] Department of Botany, PMAS Arid Agriculture University, Rawalpindi, 46300, PAKISTAN
[5] Department of Biological Sciences, University of Sargodha, Sargodha, PAKISTAN
[6] Department of Biochemistry, PMAS Arid Agriculture University, Rawalpindi, 46300, PAKISTAN
[7] Department of Zoology, PMAS Arid Agriculture University, Rawalpindi, 46300, PAKISTAN

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Summary

The aim of this study was to develop an indirect ELISA for the detection of *Hypoderma lineatum* antibodies and to determine the influence of seroepidemiological factors on the seroprevalence of bovine hypodermosis in cattle of subtropical region of Pakistan. For this purpose a total of 1000 blood samples were taken from twenty eight villages of Rawalpindi, Attock, Chakwal and Jhelum districts. First instar larvae (L_1) of the warble fly were collected from the surrounding abattoirs to obtain the antigen (HyC) used for development of indirect ELISA. The seroprevalence was 17.4% (174/1000). The highest seroprevalence was recorded in the Rawalpindi district (28.81%), followed by Attock (21.51%), Jhelum (10%) and Chakwal (6.66%). In present study the sensitivity and specificity was 97.28% and 96.44%, respectively. The epidemiological factors showed that the village having hilly locations, local breed, young animals, water bodies, extensive grazing, primary exposed and non medicated were more seropositive as compare to others ($P<0.05$). In conclusion, indirect ELISA developed during this study is very useful tool for early detection of bovine hypodermosis in cattle grazing in subtropical region of Pakistan.

Keywords: *Hypodermosis, Hypoderma lineatum, Cattle, Indirect ELISA, Subtropical Region, Seroprevalence, Pakistan*

Pakistan'ın Subtropikal Bölgelerinde Sığır Hypodermozisinin *(Hypoderma lineatum)* Tespit Edilmesinde İndirek ELİSA Yönteminin Kullanılması

Özet

Bu çalışmanın amacı *Hypoderma lineatum*'a karşı üretilen antikorları tespit etmek amacıyla bir indirek ELİSA yöntemi geliştirmek ve Pakistan'ın subtropikal bölgelerinde sığır hypodermozisinin seroprevalansı üzerine seroepidemiyolojik faktörlerin etkisini belirlemektir. Bu amaçla Rawalpindi, Attock, Chakwal ve Jhelum bölgelerinde yirmi sekiz köyden toplam 1000 adet kan örneği alındı. Nokra sineğinin ilk instar larvaları (L_1) indirek ELİSA yöntemini geliştirmek maksadıyla kullanılacak antijeni (HyC) elde etmek amacıyla çevre kesimhanelerden toplandı. Seroprevalans %17/4 (174/1000) olarak belirlendi. En yüksek prevalans Rawalpindi (%28.81) bölgesinde tespit edilirken bunu Attock (21.51), Jhelum (%10) ve Chakwal (%6.66) takip etti. Bu çalışmada duyarlılık ve özgüllük sırasıyla %97.28 ve %96.44 olarak tespit edildi. Epidemiyoojik faktörlerden tepelik yerleşimli köyler, lokal ırklar, genç hayvanlar, su kaynakları, geniş meralar, primer maruz kalanlar ile ilaçlanmamış hayvanlarda diğerlerine göre seroprevalanslık daha yüksek olarak belirlendi ($P<0.05$). Sonuç olarak, bu çalışmada geliştirilen indirek ELİSA Pakistan'ın subtropikal bölgelerinde sığır hypodermozisinin erken tespitinde oldukça yararlıdır.

Anahtar sözcükler: *Hypodermozis, Hypoderma lineatum, Sığır, İndirek ELİSA, Subtropikal Bölge, Seroprevalans; Pakistan*

İletişim (Correspondence)
+09251 9049150
haroonahmad12@yahoo.com

INTRODUCTION

Hypodermosis is caused by a parasite which belongs to genus *Hypoderma*. Bovine hypodermosis has a worldwide distribution pattern in the northern hemisphere of the world, where its larvae induce myiasis [1]. Previous studies showed that the animals were mostly infested by *Hypoderma bovis* and *H. lineatum* on worldwide basis [2]. In USA the economic losses attributed to hypodermosis were more than $ 600 million [3,4]. Hypodermosis causes economic losses due to meat trim at slaughter and also cause detrimental effects on hides [5].

Hypodermosis is an important endoparasitic infection of cattle and goats prevalent in hilly and semi-hilly areas of Pakistan [6]. In Pakistan, *H. lineatum* causes one of the most common parasitic problems reported in large ruminants in Potowar region [7]. The incidence of hypodermosis is reported to be 29% in cattle in Dera Ghazi Khan Hypodermosis, in cattle is resulted in more economic burden especially for the leather industry by lowering down the market value of hides. On the basis of warbled and warble-free hides, the economic losses were estimated as 22.8 million Pakistani rupees per annum. Economic losses due to warble fly infestation were reported to be 20.6 and 2.2 million Pakistani rupees in cattle and buffaloes in Dera Ghazi Khan and Rajan Pur districts, respectively [8].

There are number of diagnostic tools designed to diagnose the disease including grub monitoring procedure. By using traditional method of grub monitoring (back palpation), the hypodermosis is diagnosed at very late stages of infestation when a great extent of damage has already been done, so there dire need to diagnose the infestation at proper time to minimize the economic loss. So there should be a need of reliable diagnostic technique that would help us because the detection of L_1 at the beginning of the migratory phase allows systemic treatment, avoiding the damage provoked by the parasite on the host tissues [9]. The serodiagnosis is another method that is used for early diagnosis of hypodermosis. For this purpose, Enzyme Linked Immunosorbent Assay (ELISA) can be used for diagnosis of hypodermosis at early stage. This serological technique has been used to monitor levels of hypodermosis in Britain for a number of years [10]. In France hypodermosis was diagnosed by direct examinations of animals and also by using the ELISA test in milk samples with a highly satisfactory degree of sensitivity (92%) and specificity (98.1%) [11]. In Pakistan the best time to collect blood samples to perform an immunodiagnosis is from July to September.

Keeping in view the importance of ELISA for early detection of hypodermosis in cattle, the present study was designed to study the seroprevalence of hypodermosis *(Hypoderma lineatum)* in the cattle from a subtropical region (Pakistan) by using indirect (HyC) ELISA.

MATERIAL and METHODS

Study Area

The present study was carried out in subtropical region of Punjab Province, Pakistan.

Study Plan

The present study comprises into the following two phases; Firstly, the development of an indirect ELISA test and to determine the seroprevalence of *Hypoderma* spp. *(Hypoderma lineatum)* based on different epidemiological factors.

ELISA Development

The following steps were carried out in the ELISA development and validation:

- Larvae Collection and Storage

In present study the L_1 were collected in fresh state from esophagus of naturally infected cattle from local abattoirs. The first instar larvae of *H. lineatum* were dissected from the oesophageal tissue of affected animals from August to October and all visible tissue adhesions were removed. They were washed several times in 0.01M Phosphate buffer saline solution (PBS), pH 7.4 to remove mucosal debris and stored in PBS and were freezed at -20°C.

- Isolation of Antigen

In present study the raw or total extract antigen (crude extract) was used for ELISA, the larvae were homogenized with a tissue grinder Polytron (Kinematica AG) in Tris-HCl 0.1 M, pH 7.5, at the rate of 10 larvae per 5 ml buffer, using 5 cycles of 1 min at 14.000 rpm. To avoid temperature rises that could activate the enzymatic processes or denature the proteins, between each cycle, the mixture was kept in an ice bath. Subsequently, the homogenates were centrifuged during 5 min at 10.000 rpm to facilitate separation of the soluble fraction. The supernatant (crude extract) was distributed in aliquots and frozen at -30°C until use. The supernatant obtained in was fractionated by an ion-exchange chromatography, using as DEAE anion exchanger (Di-Ethyl-Amino-Ethyl) bound to cellulose (DEAE cellulose, Whatman) as described by Panadero [12]. The protein concentration of each aliquot by using the Pierce BCA technique method. The protein fraction called hypodermin C (HyC) was separated similar as previously made several authors. In the present study the confirmation of purity of the HyC was analyzed by Polyacrylamide Gel Electrophoresis under reducing conditions (SDS-PAGE) and non reducing (PAGE), following the classical discontinuous system described by Laemmli [13].

- Indirect ELISA Protocol

Simple ELISA plates (Delta Lab, Hot Bottom) were coated with natural hypodermin C (1:500): 20 µl HC + 10

ml PBS was added in 100µl/well and incubated at 37°C for 30 min. Wells were blocked for 30 min at 37°C with 200 µl of dilution buffer PTL (60 ml PBS-Tween +1.2 g skim milk powder) in each well. Then they were washed one time with 200 µl and three times with 100 µl PT. Serum samples were diluted in duplicate (1:10) (and 100 µl were added in each well. Both positive and negative control sera were prepared at the same dilution and were used as standards in each plate. Following the addition of sera, plates were incubated for 1 h at 37°C. Then they were washed once with 200 µl of PT and 3 times with 100 µl of PT. After incubation, Immuno-Conjugate (Horseradishperoxidase conjugated rabbit Antibovine Ig G (H + L) Geneway: GWB-C158CO) was used 50 µl in each well by dilution (1:800). The plate was incubated for 30 minutes at 37°C and them was washed once with 200 µl and twice with 100 ul PT and finally twice with 100 µl PBS. Finally, 100 µl of substrate consisting of 11 ml citrate buffer (pH 5) and 10 mg (0.01 g) of OPD (Ortho Phenylene Diamine), 4 µl of 30% H_2O_2 was added to each well. After 5-20 min in the dark the reaction was stopped by the addition of 100 µl of 3N sulfuric acid in each well. After 2-5 min the absorbance was measured at 492 nm read using a spectrophotometer Labsytem Multiskan™ spectrophotometer. The samples having greater value than the cut off value were considered as positive while below that value were considered as negative. In the present study positive and negative control sera were obtained from a clinically infested animal and a non-infested cow from a warble free area and these standards were provided from abroad [14]. In the present study the true positive animals were confirmed after the clinical examination of animals by Palpation Method and cutoff point to define a positive or negative serum sample was established using ELISA with a positive sera population obtained from cows that afterwards had grubs in their back and with negative sera from native animals that had never grazed.

The diagnostic sensitivity (DSN) and diagnostic specificity (DSP) was determined by the following formula:

Diagnostic Sero-negativity = True Positive/(True Positive + False Negative)

Diagnostic Sero-positivity = True Negative/(True Negative + False Positive)

The cutoff values were calculated by using the following formula:

Cut off value = Mean Negative Control Panel ± 2SD

Seroepidemiological Factors

One thousand random blood samples were collected from different herds in August to September from Attock (n=172), Chawkal (n=225), Rawalpindi (n=354) and Jhelum (n=249) districts. Different factors (district, village, age, sex, location, breed, management, presence of water bodies, medication, previous exposure and grazing pattern) were recorded in a questionnaire in order to study their influence on the warble fly seroprevalence. The animal's clinical examination was carried out on monthly basis.

Statistical Analysis

The statistical analysis was performed by using SPSS software (18 Version) and Chi square Analysis.

RESULTS

In the present study the L_1 larvae were used for antigen preparation and protein estimation was carried by BCA method and HyC was purified from crude protein by ion exchange chromatography *(Fig. 1)*. The HyC was used in development of indirect ELISA. In the present study, the sensitivity and specificity of HyC antigen based ELISA were 97.28% and 96.44%, respectively. The indirect ELISA was first time developed in this region. In Pakistan the life cycle of *Hypoderma* starts from February to December. So during this time the disease can be diagnosed by ELISA method, because the animals having higher titre of antibodies in their blood during the migration of L_1 larvae.

The results of present study were summarized in *Table 1*. The overall seroprevalence was 17.4% (174/1000). The results showed that the conditions in hilly and semihilly areas were more favorable for the life cycle of *Hypoderma* as compared to the plane locations. The type of management (extensive and intensive) was considered. The animals were in extensive system were significantly more infested (P<0.05) then intensive system. The presence of water

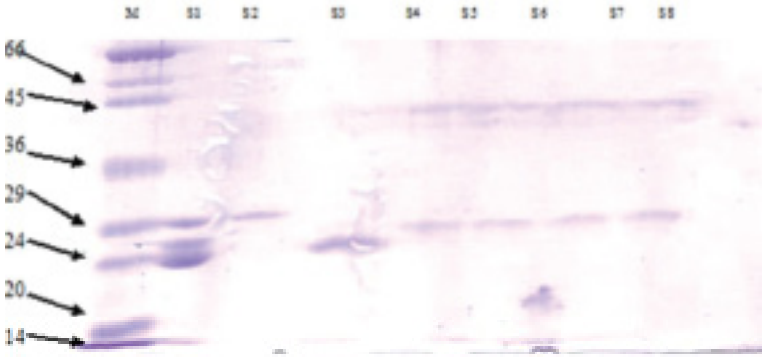

Fig 1. SDS-PAGE (12%) showing the different proteins of H. lineatum {HyC (24) kDa, HyA (31) kDa and HyB (23) kDa. lane 1 molecular weight markers}

Şekil 1. SDS-PAGE (%12) H. lineatum'un farklı proteinlerinin görünümü {HYC (24) kDa Hya (31) kDa ve Hyb (23) kD. Sütun 1 moleküler ağırlık işaretleyicisi}

Table 1. *Potential risk/indicator factors for animal-level bovine hypodermosis sero-positivity. The p-values were determined based on a Chi square (χ^2 Test) analysis with infested and non infested animals of each factor values and levels*

Tablo 1. *Hayvan düzey sığır hypodermozis sero-pozitifliği için potansiyel risk/göstergesi faktörler. P-değerleri enfekte olan ve olmayan hayvanlarda her bir değer faktörleri ve düzeyleri için ki-kare (χ^2 Testi) kullanılarak belirlendi*

S. No	Factor Values	Levels	Examined Animal	Indirect (HyC) ELISA test			
				Negative	Positive	Seroprevalence (%)	P-value
1	Districts	Jhelum	249	225	25	10	F = 60, df = 3, P<0.05)
		Rawalpindi	354	252	102	28.81	
		Chakwal	225	210	15	6.66	
		Attock	172	135	37	21.51	
2	Water Bodies	Water bodies present	437	324	113	25.85	F=43.16 df = 1, P<0.05
		Water bodies Absent	563	506	57	10.12	
3	Grazing Pattern	Tired at Home	378	374	4	1.05	F=109 df = 1 P<0.05
		Field	622	456	166	26.68	
4	Locations	Plane	484	445	39	8.05	F=61.16 df = 2 P<0.05
		Hilly	175	142	33	18.85	
		Semi hilly	341	243	98	28.73	
5	Management	Intensive	313	300	13	4.15	F =53.48 df = 1 P<0.05
		Extensive	687	530	157	22.85	
6	Breed	Local Breed	651	495	156	23.96	F = 69.91 df = 6 P<0.05
		Cross Breed	181	180	1	0.55	
		Sahiwal	59	57	2	3.38	
		Australian	29	28	1	3.44	
		Neelibar	3	2	1	33.3	
		Jarsi	6	5	1	16.66	
		Lohani	71	63	8	11.26	
7	Age	1-3 Years	380	289	91	23.94	F = 58 df = 3 P<0.05
		4-6 Years	324	272	52	16.04	
		7-9 Years	198	179	19	9.59	
		10< Years	98	90	8	8.16	
8	Sex	Male	133	121	12	9.02	F =6.91 df = 1 P<0.05
		Female	867	709	158	18.22	
9	Medication	Medicated	191	182	9	4.71	F =65.57 df = 1
		Non Medicated	809	648	161	19.9	P<0.05
10	Previous Exposure	Reinfested	22	13	9	40.9	F = 9.11 df = 1 P<0.05
		Prim Infested Exposed	978	817	161	16.46	

bodies (present, 25.85% and absent, 10.12%) is considered as an important seroepidemiological factor having a significant difference (P<0.05). The seroprevalence in the animal having previous infestation was significantly higher (P<0.05) than those without previous infestation. It is evident from the results that the older animals have lower prevalence rate then the younger one. The medication (ivermectin) based sero-epidemiological information's were taken in this study. The results showed a significant difference (P<0.05) between non-medicated (19.9%) as compared to medicated (4.71%) animals. The seroprevalence in cattle raised in extensive system was higher than in those kept at intensive system. The Chi square analysis showed that there is significant difference (P<0.05) between these two groups. The seroprevalence in the female (18.22%) was higher as compared to male (9.02%) and having a significant difference (P<0.05) *(Table 1)*.

DISCUSSION

The present study shows that hypodermosis is moderately distributed in Pakistan. Similarly, the prevalence of bovine hypodermosis in district Chakwal was 35.50% in field area [6], 21.62 to 23.8% in cattle [15], 29% in Dera Ghazi Khan, 26% in Rajanpur districts [8], in D. I. Khan, Kohat, Malakand and Abbotabad districts of NWFP [15] and D. G. Khan & Barkhan districts [7,16]. Warble fly infestation ranged from 25 to 90% in goats and 21 to 26% in cattle [15]. The infestation rate of hypodermosis varies within Pakistan [15-18] and many parts of globe [19-21] it might be due to the differences in climatic conditions. In Pakistan all the studies in the past were based on traditional method of grub monitoring that has very low sensitivity and specificity. Similarly, the prevalence of bovine hypo-dermosis is regularly observed in Canada, Europe and in Africa [21], Czech and Solvac Republic (80%), Italy (85%), Greece (49.2%), United Kingdom (40%) at the time of eradication and in Romania (32-43%) [18]. In the east and southeast region of Turkey seroprevalence was 22.1%, 22.3%, 26.3% from Diyarbakir, Malatya and Elazig provinces, respectively. Similarly, it was 47.8%, 38.9%, 33%, 41.6% and 41.9% in Southeast Anatolia, Central Anatolia, Mediterranean, Agean and East Anatolia [23]. In Spain the seroprevalence was 26-42% [20]. In Turkey, the seroprevalence in Thrace region was 3.56% [24] and 4% in Elazig province [23], while 43.3% in Vicenza province of North eastern Italy [25]. In north China, from Xinjiang, Inner Mongolia, Heilongjiang, Jiling and Gansu provinces obtaining percentages of 51.77%, 27.02%, 13%, 6.03% and 44.41% respectively in yaks and cattle by ELISA [26]. In Pakistan, so far there is no previous study on the seroprevalence of hypodermosis. In the present study, the seroepidemiological information was taken in the form of questionnaire based on the district, village, age, sex, breed, location, herd size, previous exposure, grazing pattern and medication. This variability might be due to the differences in the climatic factors that affects on the developmental stages of the larvae [27,28].

Palpation method is used for the monitoring the intensity of infestation on regular basis throughout the warble emergence period, the emergence period of fly lasts from 3-4 months [29]. The ELISA test is used to detect the circulating antibodies in the sera of infested animals and diagnose the cattle grub infestation [30-32]. The antibody detection ELISA test is based on the hypodermin C [14]. In France, Charbon reported the higher degree of sensitivity and specificity 92.2% and 98.1%, respectively in ELISA [11]. Our results are similar to hypodermin C ELISA having sensitivity and specificity 96.4% and 95.6%, respectively [12]. Webster *et al.*[4] reported a competitive ELISA for the detection of antibodies to *Hypoderma* species in cattle sera with a sensitivity and specificity 100% and 92%, respectively. During the last years, immunodiagnostic techniques for cattle hypodermosis have been improved so that their increased diagnostic sensitivity allows investigators to avoid: visual examinations, palpation of warbles or postmortem examination of the oesophagus *(H. lineatum)* [32]. In the present study, we isolate the HyC from the local *Hypoderma* strain and it is used for the development of indirect ELISA. The sensitivity and specificity of the ELISA test developed in this study was 97.28% and 96.44%, respectively.

The hypodermosis is endemic and reported in hilly region of Pakistan [33]. The young animals were more infested due to their softer skin, that's makes easier for 1st instar in to their penetration as reported earlier [34]. On the contrary, the destruction of the developing larvae by internal regulatory systems of the host and development of resistance by continuous exposure of animals to larvae could also explain the low prevalence in aged animals. The age-wise trend was determined [35]. The younger animals have higher infestation then the mature milking cows. Because the older animals has develop the immunity against larvae [36]. Previous insecticide treatments had also influence in the prevalence, but the grazing pattern was the most influencing factor, so that the prevalence of WFI was higher in those animals maintained in an extensive management system than in those kept at home. Otranto reported that free grazing practice is one of the major risk factor for hypodermosis herd's positivity [32]. On contrary, the prevalence was higher in males (26.50%) than in females (20.50%) in Chakwal districts [6]. The seroprevalence was 31% in female and 14.1% in male [37]. The desi breed (27.7%) has higher seroprevalence, crossbreed (26.8%) and pure breed (19.7%), respectively [23].

The present study concludes that the bovine *H. lineatum* infestation is a serious threat and it is moderately distributed in subtropical region, Pakistan. Because there might be a possibility of increasing its prevalence rate in future. So, there is a need of further studies on immunodiagnosis of WFI. Eradication strategies should be implemented because Pakistan is an agricultural country and livestock contributes a major part of the economy.

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