Dot-ELISA based on recombinant Hypodermin C protein derived from Przhevalskiana silenus for field diagnosis of goat warb

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Abstract

Goat warble fly infestation (GWFI) is an economically important myiasis caused by larvae of *Przhevalskiana silenus* (Diptera, Oestridae), prevalent in countries of the Mediterranean Basin and Indian subcontinent. GWFI is characterized by the presence of subcutaneous warbles at the lumbar and sacral region of dorsum in the infested animal. The early larval instars (L1 and L2) remain inaccessible to physical detection due to their small size and subcutaneous presence thus causing prolonged economic loss to animal productivity. The early diagnostic intervention is needed during the disease monitoring and prophylactic management for effective control of the disease. The present study has developed an in-house dot-ELISA for the serodiagnosis of GWFI based on recombinant Hypodermin C (rHyC) antigen of *Przhevalskiana silenus,* expressed in *E. coli.* The purified protein was used for optimizing dot-ELISA in a checkerboard titration using goat warble fly infested serum as known positive. The optimized conditions require 188 ng of protein/dot, 1:800 dilution of serum sample, 1:4000 dilution of anti-goat IgG conjugate and 5% skim milk powder in phosphate buffer saline as blocking buffer. The assay was found to have a diagnostic sensitivity and specificity of 97.3% and 95.8%, respectively. The inter-rater reliability of dot ELISA with rHyC indirect ELISA was found to be almost perfect with a Cohen's kappa index of 0.973. Further testing at ambient temperature (18 °C) and shorter incubation steps (30 min) supported suitability of the assay for field diagnosis of GWFI. The rHyC protein based Dot-ELISA was evaluated using random field serum samples suspected for GWFI. The present study provides the first report of a sensitive and specific dot-ELISA for early diagnosis of GWFI which is rapid and cost effective. The test may provide an effective tool for sustainable control of GWFI.

Introduction

Goat warble fly infestation (GWFI) is a myiasis caused by larvae of*Przhevalskiana silenus (P. silenus)* which belongs to subfamily*Hypoderminae* of Oestridae family[1]. This disease is associated with huge economic losses by reduction in milk yield, meat and hide quality[2, 3], and it is characterized by the presence of subcutaneous warbles on dorso-lumbar region of affected goats and wild ruminants.[4] All three larval stages (L1, L2 and L3) cause subcutaneous infestation for 7-9 months and no internal migration is observed in contrast to other hypoderminae members such as *Hypoderma bovis* (*H. bovis*) and *Hypoderma lineatum* (*H. lineatum*).[5]

GWFI is widespread and commonly present in the Mediterranean Basin and Indian subcontinent that includes northern India, Pakistan, Iran, Turkey and southern Italy.[2,6-10] In India, the disease is most common in northwestern Himalayan area (Union Territory of Jammu and Kashmir), particularly in goats of the Bakerwali breed characterized by long hairy coat with a prevalence rate ranging between 13 to 56.5 % in Jammu region.[2]

Physicoclinical observation by palpation of warbles on infested animals is routinely used for diagnosis at

L2 and L3 stages which occurs about 3 months after infestation. Serological tests have advantages over physicoclinical observation in terms of early detection and high sample throughput along with wide diagnostic time window, as antibodies in the infested host may persist for about 16 weeks.[11,12] The serodiagnostic assays for hypoderminae insects have been explored with the key serine proteases namely Hypodermin A (HyA), Hypodermin B (HyB) and Hypodermin C (HyC). Among the three serine proteases, HyC is primarily produced in early stage of L1 that helps in the penetration of the skin due to its collagenolytic activity. HyC is characterized as a major immunodominant antigen. The conserved nature of HyC shows cross reactivity among Hypoderminae subfamily due to shared epitopes.[11, 13-18]

Several enzyme immunoassays have been developed based on native and recombinant HyC derived from *H. bovis* and *H. lineatum*based indirect ELISAs have been developed for diagnosis of cattle hypodermosis.[19-23] Presently, recombinant protein based microtitre plate-ELISAs are widely used in routine diagnosis and seroepidemiological studies of hypodermosis.[6,18,23]However, the assay is expensive, time-consuming and requires equipment like ELISA reader and expertise to analyse the data. Diagnostic labs performing spectrophotometric ELISA are scarce in developing countries, especially at remote veterinary diagnostic centres. In contrast to such challenges, a simple, rapid and easy to perform test is required. Dot-ELISA is an inexpensive, sensitive and specific assay that provides utility to be performed at less equipped regional labs and provide visual readouts without spectrophotometry.[24]

Several dot-ELISA have been developed for various zoonotic and veterinary parasitic diseases such as Toxocariosis based on*Toxocara canis* Excretory –Secretory Antigens (TES), *Taenia solium* cysticercosis (neurocysticercosis) with partially purified antigen fraction (Cathepsin L like activity); Toxoplasmosis based on recombinant surface antigen (rSAG) and dense granule antigens (rGRA7), recombinant cathepsin L-cysteine proteinase for *Fasciola gigantica* , truncated recombinant merozoite antigen (rBgSA1) for*Babesia gibsoni* among others.[24-28]

The present study aimed to develop a dot-ELISA based on recombinant HyC derived from *P. silenus* for the serodiagnosis of GWFI in order to develop an economical, rapid and field applicable tool for serosurveillance of this myiasis during disease control programs.

Material & methods

Recombinant HyC antigen

The recombinant HyC protein was derived from *P. silenus* expressed in *Escherichia coli* (strain BL21 DE3 pLysS) transformed with the pET-32a(+) expression vector as described in Yadav et al.[18] The rHyC was obtained as fusion protein with thioredoxin tag and used as antigen in the present study. Briefly, a confirmed positive recombinant clone was cultured in LB broth containing ampicillin and chloramphenicol, incubated at 37°C till it attains an OD of 0.6. Subsequently it was induced by adding IPTG to a final concentration of 1 mM. The culture was allowed to grow at 37°C with shaking at 200 rpm for 6 h and purified under denaturing condition using 8 M urea dialyzed against gradient urea concentration (8 M to 0 M) and finally in Tris buffer pH 8.0. The homogeneity of purified protein was analyzed by 12% SDS-PAGE and quantified by Bradford reagent.

Reference Sera

Positive reference goat serum samples (n=109) were collected from farms and a municipal slaughterhouse from Union Territory of Jammu and Kashmir (North India), based on palpation of larvae in live animals and carcass examination. Negative goat sera (n=92) were procured from Punjab state of North India, where GWFI has never been reported. In order to rule out the cross reactivity of developed dot-ELISA, positive serum of several important parasitic and bacterial diseases of goats namely oestrosis, haemonchosis,fasciolosis, amphistomiosis, coenurosis, cryptosporidiosis, paratuberculosis, enterotoxaemia and brucellosis were assessed.

Optimization of rHyC based Dot-ELISA

Dot ELISA for simple and rapid diagnosis of GWFI was standardized on strips of Nitrocellulose membrane

(NCM) with pore size of 0.45 µm using positive and negative reference serum samples for GWFI. NCM were cut into 0.8 cm x 2.5 cm strips and rHyC protein was dotted at three different places on to membrane maintaining the gap of 0.5 cm to allow separation between the dots. The working concentration of recombinant antigen, serum antibodies and conjugate for dot-ELISA were determined by standard checkerboard titration. Two different concentrations of purified rHyC antigen 94 ng/dot (1 µL) and

188 ng/dot (2 µL) were titrated against three dilutions of serum (1:400, 1:800 and 1:1200) and two dilutions (1:4000 and 1:8000) of anti-goat IgG HRP conjugate in dot ELISA format using 5% skimmed milk powder (SMP) in PBS as blocking buffer. The antigen concentration, serum and conjugate dilution that showed the development of well-defined dots on the membrane with positive serum and none with negative serum were selected. The optimal conditions included NCM strips dotted with rHyC proteins 2 µL dots. The dotted strips were air dried for 15 min at room temperature and blocked with 5% SMP in PBS (pH 7.4). After incubation at 37 °C for 1 h, strips were washed two times with PBS-T 0.075% and one time with PBS. After washing, strips were soaked in 1 mL of known positive and negative serum sample diluted in PBS (1:800) and incubated at 37 °C for 1 h. After wash, the strips were incubated with 1 mL of anti-goat IgG HRP-conjugate (Sigma Aldrich, USA) at 1:4000 dilution in PBS (pH 7.4) at 37 °C for 1 h. Following three washing, ELISA dots were developed by incubating the strips with DAB substrate (VWR-Amresco Life Science, 5 mg/10 mL) solution for 5 min at room temp in dark. The reaction was stopped by washing the strips in distilled water. The development of brown dots on strips indicated that serum samples were positive for GWFI while negative sera showed no dots. All the strips were handled with forceps.

Evaluation of rHyC based dot-ELISA

The dot ELISA was performed with rHyC under optimized conditions to determine the assay specificity and cross reactivity using known positive sera against different important parasitic and bacterial diseases of goats. The diagnostic specificity (DSp) and diagnostic sensitivity (DSn) of the dot ELISA were determined by, using a two-sided contingency table **(Table 1.)** . Known positive serum samples with brown spots compared to nil in negative serum were reported as positive. The sensitivity and specificity were calculated by using the following formula: Sensitivity = (TP/TP+FN) × 100; Specificity = (TN/TN+FP) × 100; where, TP-true positive; TN-true negative; FP-false positive and FN-false negative. The positive predictive value (PPV) and negative predictive value (NPV) were also calculated based on the optimized assay as shown in **Table 1** .

Also, the inter-rater reliability of dot-ELISA was evaluated with Cohen's Kappa test, taking the rHyC based microtitre plate indirect-ELISA as reference standard.[18, 29] A set of 149 sera samples (69 positive and 80 negative) from ICAR-NF laboratory were tested for evaluating the kappa index between both assays for the diagnosis of GWFI.

In a separate experiment, the optimized dot ELISA was tested with neat samples (blood, plasma and serum) and lower dilutions at lower incubation temperature (18 °C) and time (30 min) to check the suitability of the test at field conditions under sub-tropical and temperate climates of GWFI prevalent regions. Samples of a known GWFI positive goat were used for blood and plasma whereas a GWFI negative goat was used to derive negative control samples of blood, plasma and serum. The positive samples of GWFI (blood, plasma and weak positive serum) were tested as neat, 1:2 and 1:10 dilutions and control negative samples for neat blood, plasma and serum. A weak positive serum was taken as positive control to ensure reactivity of weak titre at lower time and temperature of incubation.

Serum samples (n = 274) from different regions of Union territory of Jammu and Kashmir that were available in the ICAR-NF laboratory were tested with the optimized dot-ELISA.

Stability of developed Dot-ELISA strips

The stability of antigen dotted NCM strips was assessed at different time and temperature combinations with a panel of GWFI positive and negative sera. Strips were dotted with a fixed concentration of rHyC antigen (188 ng/dot), blocked with 5% SMP and stored in sealed 15 mL falcon tubes to avoid moisture at 4 °C and 37 °C, respectively for total six months. Stored strips were re-tested at every one month interval for

their reactivity with reference serum samples.

Results

Production of rHyC antigen

The expression of rHyC protein was induced using 1 mM IPTG and culture was allowed to grow at 37 °C with shaking at 200 rpm for 6 hr. Protein was purified under denaturing condition using 8 M urea in phosphate buffer. The homogeneity of purified fusion protein with molecular weight of about 45 kDa was analysed in SDS-PAGE (**Fig. 1**). The estimated concentration of purified rHyC protein was 94 µg/mL of elutes after dialysis.

Optimization of Dot-ELISA

Upon optimization, checkerboard titration revealed the optimal antigen concentration, serum and conjugate dilution at 188 ng/dot, 1:800 and 1:4000 respectively for clear distinction between positive and negative serum samples (**Fig. 2**). The serum and conjugate dilution were selected to attain an acceptable well defined dots with less background/noise signal on strips at the minimum concentration of rHyC antigen. The antigen volume was selected at 2 µL to obtain appreciable size of dot for better visual observation of results.

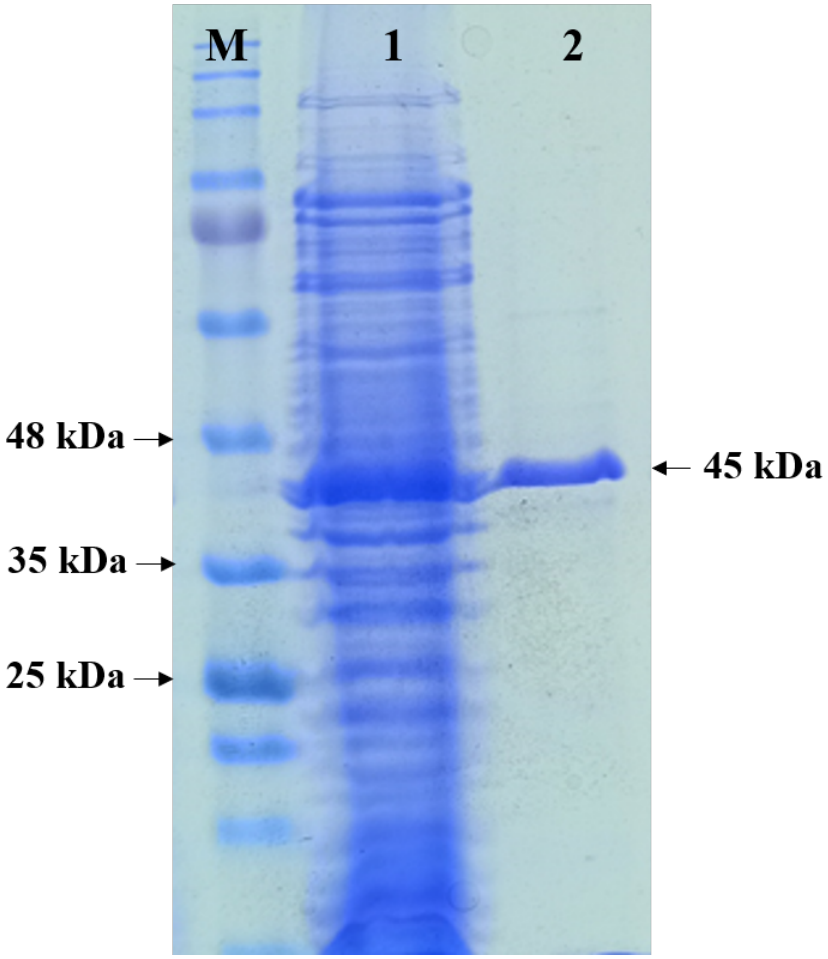

Fig. 1 SDS-PAGE analysis showing purified rHyC protein along with induced culture; Lane 1: 6 h post induction 8M Urea lysate; Lane 2: purified rHyC; Lane M: Prestained protein marker.

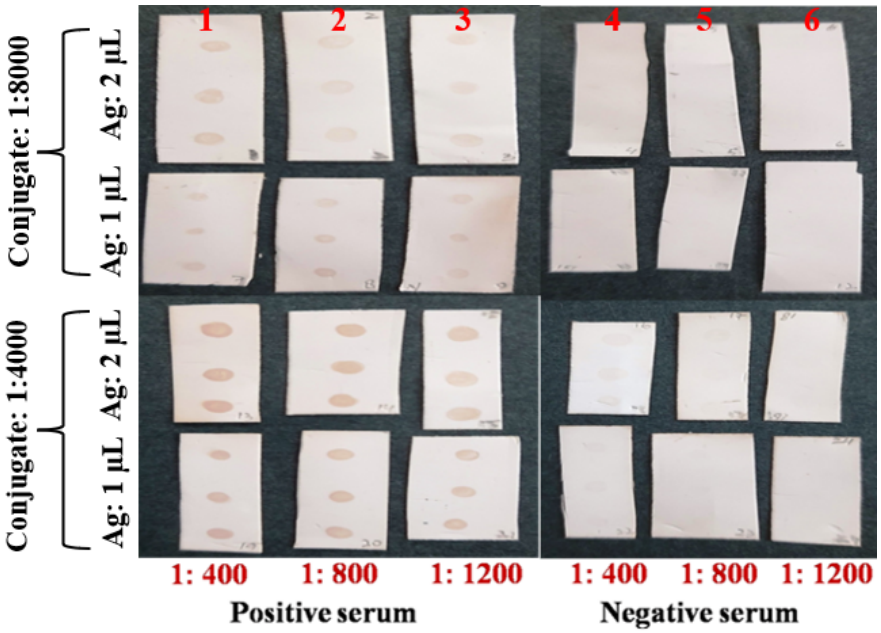

Fig. 2 Checkerboard titration to standardize rHyC based dot-ELISA. Antigen concentration (94 ng/1µL and 188 ng/2 µL); Serum (1:400, 1:800 and 1:1200) and conjugate (1:4000 and 1:8000) dilution.

Evaluation of Dot-ELISA

The diagnostic sensitivity and specificity of the assay was assessed with 109 known positive serum for goat warble fly disease and 92 true negative serum samples. The developed dot-ELISA provided good parameters with DSn of 97.3% and DSp of 95.8% **(Table 1).** The rHyC based dot-ELISA showed strong reactivity with positive serum of GWFI and no cross-reactivity was observed with positive sera of important parasitic and bacterial diseases of goats as listed earlier **(Fig 3).**

Table1. Diagnostic parameters of the optimized rHyC based dot-ELISA for GWFI.

		Physico-clinical Evaluation	**Physico-clinical Evaluation**	**Diagnostic Parameter**
rHyC Dot-ELISA	+	+	-	**Total**
		106	4	**110**
	-	3	88	**91**
Total	**Total**	**109**	**92**	**201**

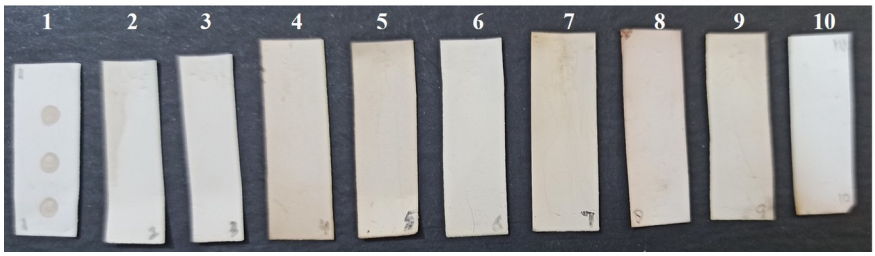

Fig. 3 Specificity and cross reactivity of developed dot-ELISA; Lane 1: Goat warble fly; Lane 2: Oestrosis; Lane 3: Haemonchosis; Lane 4: Fasciolosis; Lane 5: Amphistomosis; Lane 6: Coenurosis; Lane 7:

Cryptosporidiosis; Lane 8: Paratuberculosis; Lane 9: Enterotoxaemia; Lane 10: Brucellosis.

A Cohen's kappa index of 0.973 with Standard Error (SE) of 0.019 at very high significance level (P<0.001) was observed between the rHyC based dot-ELISA and microtitre plate indirect-ELISA with a percentage of agreement of 98.657% (**Table 2**).

Table 2. Evaluation of Cohen's kappa for inter rater reliability with rHyC based indirect ELISA as reference standard.

		rHyC Dot-ELISA	**rHyC Dot-ELISA**	**Inter-Rater Relaibility**	**Inter-Rater Relaibi**
rHyC iELISA	+	+	-	**Total**	**P A = 98.657%**
		68	1	**69**	**ὁηεν′ς ϰ = 0.973**
	-	1	79	**80**	**SE=0.019**
Total	**Total**	**69**	**80**	**149**	**P<0.001**

[Strength of agreement: ϰ index <0, poor; 0-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; 0.81-1.0, almost perfect; SE: Standard Error; PA: Percent Agreement]

The assessment of rHyC based dot-ELISA at lower incubation temperature (18 °C) and time (30 min) provided valid results suitable for field diagnosis of GWFI with dilutions below 1:10. The limited assessment of neat and lower dilutions of samples at lower temperature and incubation period were found to exhibit faint dots at neat and 1:2 dilution whereas clear distinction was observed at 1:10 dilution of known samples. Moreover, the positive control taken as a weak positive sample based on microtitre ELISA (OD=0.537) provided valid results at the above conditions.

Further for serosurveillance, the serum samples available in the ICAR-NF laboratory were tested, which could clearly differentiate between infested and uninfested animals. A total of 274 serum samples suspected of GWFI were tested, out of which 83 samples were found to be positive for GWFI.

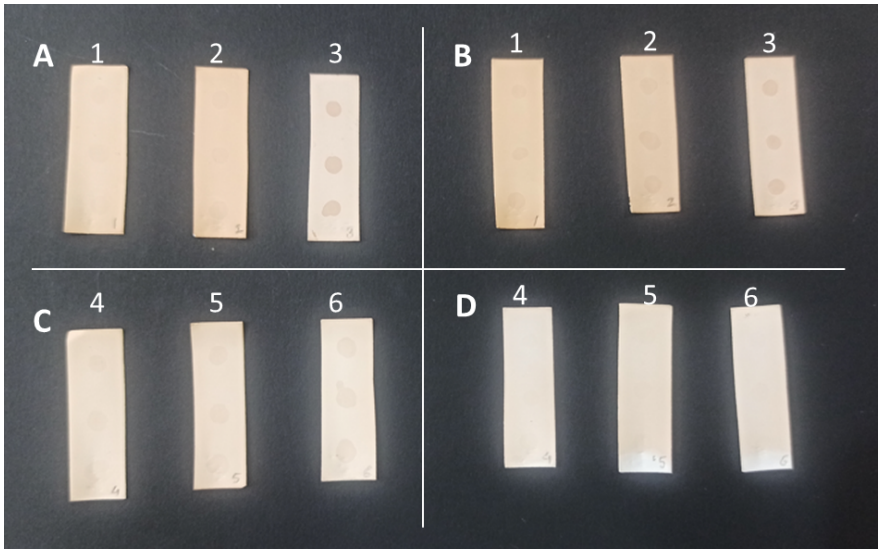

Fig. 4. Comparative evaluation with samples of blood (A1-3) and plasma(B1-3) with weak positive serum(C4-6) as positive control and negative controls for blood, plasma and serum (D 4-6) under incubation at 18 °C for 30 min.

Stability of Dot-ELISA strips

The stability of the dotted strip was assessed with a panel of three positive and three negative control sera after storage at 4 °C and 37 °C at 2, 4, 6 months of time period. The results showed clear differentiation of the dots at both 4 °C and 37 °C storage temperatures for up to 6 months without losing their reactivity to positive serum samples **(Fig 5).**

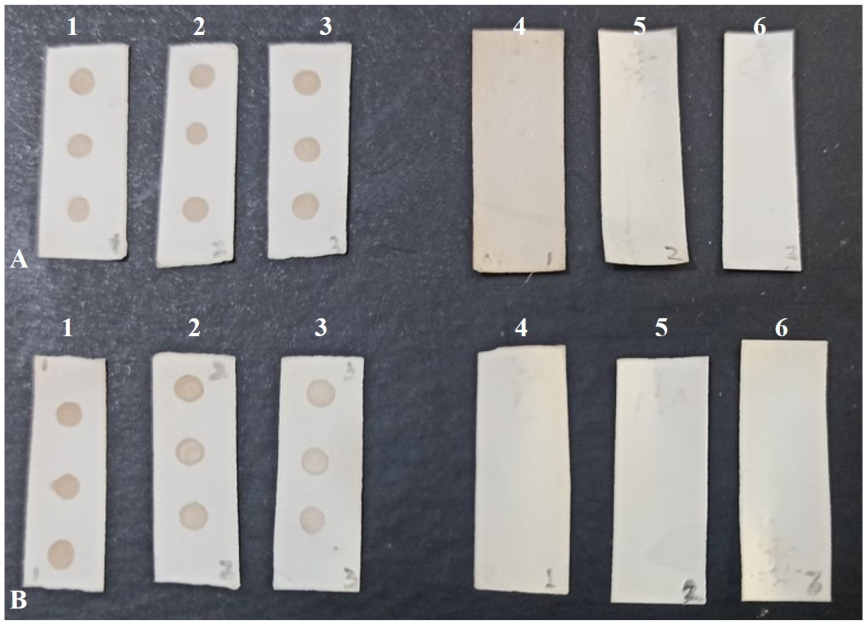

Fig. 5 Stability of dotted NCM strips after six months of storage at 4 °C and 37 °C. Panel A: Storage of strips at 4 °C; Lane 1-3: GWFI positive serum; Lane 4-6: Goat negative serum. Panel B: Storage of strips at 37 °C; Lane 1-3: GWFI positive serum; Lane 4-6: Goat negative serum.

Discussion

Goat warble fly infestation is an economically important disease of goats. In India, the disease is prevalent in Union Territory of Jammu and Kashmir (North India)[2] and causes huge economic losses in the region.[3] Globally, GWFI causes huge production losses to goat farming in the Mediterranean and Indian subcontinent with consistent reports from south Italy, Greece, Egypt, Jordan, Saudi Arabia, Iraq, Iran, Pakistan with variable prevalence rates over the years.[4] The development of a sensitive and specific technique to diagnose warble fly infestation is an important step towards promoting sustainable control of the disease.[30,31]

The diagnosis of GWFI is commonly based on the physicoclinical observation of subcutaneous warbles at the late stage of L2 and L3 which occur after 3-4 months of infestation. For early and confirmatory diagnosis, serological test has been developed for the detection of*Hypoderma* spp. specific antibodies. Many workers have developed crude and native HyC based indirect ELISA derived from *H. lineatum* with DSn 100%, DSp 92%;[21] DSn 98.2% and DSp 98.2%,[20] respectively. However, it is well known that crude or native HyC antigen preparation is challenging to standardize and availability of L1 larvae throughout the year is not feasible because of the seasonal pattern of the disease and variable prevalence in a geographical region. An alternative approach is the use of recombinant protein produced in heterologous system instead of native antigen. This provides the advantage of purified specific antigen and amenability to standardization of immunoassay with less background signal. Recombinant protein based immunoassays overcomes the limitations associated with available serodiagnostic tests. HyC is the major immunodominant protein that is present in all members of*Hypoderminae* . Indirect ELISA based on the rHyC of *H. lineatum* [22,20] and *H. bovis* [23]have been developed and used successfully for detecting hypoderma-specific antibodies from cattle serum.

A competitive ELISA based on crude lysate protein derived from*Przhevalskiana* spp. has been developed for the early detection of GWFI in goats (8). Microtitre plate ELISA has also been developed based on *P. silenus* derived recombinant HyC.[18]However, the serosurveillance based on microtitre plate ELISA should be performed by skilled personnel in spectrophotometry equipped laboratories which is impractical at field laboratory in the developing countries in much of the Indian subcontinent and Mediterranean Basin. Field applicability of diagnostic tests is a desirable attribute for livestock farmers with small holdings in developing countries which can provide sensitive and specific diagnosis. Upon testing the dot-ELISA within a limited trial at ambient temperature (18 °C) the results were found to be valid and supported the suitability of the test under field conditions for the diagnosis of GWFI under sub-tropical and temperate zones. Also, the time required to perform the present dot-ELISA after blocking of strips is about 1 h 15 min which further supports the field applicability. Several other studies on parasitic diagnosis based on dot-ELISA require a time period ranging between 1h 35 min to 3 h after blocking step in various assays for other parasitic diseases.[25,26,32] The shorter incubation time and temperature conditions makes the assay amenable to field application.

As described earlier, diversity of antigenic preparations have been used for parasite immunodiagnosis with dot-ELISA such as excretory-secretory antigens, crude lysate preparations, purified native antigens.[25,26] However, in the recent decades, recombinantly expressed protein antigens have been emphasized through various studies for their immunodiagnostic applications.[24,27,28,32]

In the present study, dot-ELISA was standardized based on rHyC derived from *P .silenus* for serodiagnosis of GWFI. The expression of rHyC fusion protein derived from *P. silenus* in prokaryotic expression system has been previously optimized.[18] The rHyC fusion protein was produced in bulk and purified under denaturing condition and predicted size of purified fusion protein ˜45 kDa was obtained. The yield of expressed protein after dialysis was about 3 mg/L of induced culture which is sufficient to screen approximately 5000 serum samples in triplicate dots.

Moreover, recombinant antigen source provides a sustainable and consistent source of antigen for diagnostic application all-round the year thus eliminating the need of larval origin antigens, apart from providing a diagnostic test with improved parameters. The purified rHyC protein reacted very well with GWFI positive serum in dot-ELISA format at an antigen concentration of 188 ng/dot and antibody and conjugate dilution 1:800 and 1:4000 respectively. The diagnostic sensitivity and diagnostic specificity of rHyC dot-ELISA were found to be 97.3 % and 95.8 %, respectively. The sensitivity and specificity of the developed dot-ELISA were found comparable as to those of rHyC based ELISA.[20,22,23] Recombinant antigen used as purified antigen helps in reduction of non-specific reaction.[33]The assessment at lower time and temperature conditions for incubation showed variable intensity of dots. This variation in dot color intensity might be attributed to the prozone phenomenon as neat samples might present disproportionately high amount of antibodies in the GWFI positive samples.[34] Samples with weaker signals were taken as positive and the dot ELISA was applied for qualitative assessment of 274 samples for GWFI. The dot-ELISA optimized in the present study provides a high serum dilution rate at 1:800 which permits the scope of using pooled serum samples for the assessment of herd screening for warble fly infestation.[31,35] The present dot ELISA provides a high sample dilution rate which improves specificity and may be used with dried blood sample on filter paper availed in field conditions with minimal resources for GWFI diagnosis (36). This provides an opportunity for a standardized simple diagnostic tool for mass surveillance of GWFI at the national eradication program.

The optimized dot-ELISA was found to be highly specific with GWFI serum and did not exhibit any visible reactivity with other important parasitic and bacterial diseases of goats. The rHyC based dot-ELISA was found to be non-reactive to *Oestrus ovis* positive sera of goats which is in accordance with the previous observation in western blotting and microtitre rHyC indirect-ELISA.[14,18] The optimized dot-ELISA can serve as economical, efficient, field applicable and thus forms a suitable tool for serosurveillance of GWFI during disease control program.

Moreover, blotting technique including dot-ELISA has multiple advantages over indirect ELISA since protein adsorption is higher in Nitrocellulose membranes,[37, 38] which permits the higher dilution of samples as observed in the optimized assay. The technique provides simple execution and interpretation with minimal

reagents. Also, it can be used for large volume tests or small number of samples.[39, 40]

The cross-reactivity of HyC due to its conserved nature and shared epitopes among Hypoderminae members has been evaluated in several studies.[11, 13, 14, 16, 41, 42] This suggests that the rHyC based dot-ELISA can be used for serodiagnosis of hypodermosis in cattle, yalk and cervids with other hypoderminae infestation such as*H. sinense, H. bovis, H. lineatum, H. diana* and *H. actaeon* . Antibody kinetics studies for hypoderminae infestation show that antibodies are observed as early as fourth week of L1 infestation and persists up to 16 to 20 weeks.[11] This provides an opportunity for early diagnosis based on antibody detection and intervention at L1 stage with prophylactic treatment to avoid production losses to milk, meat and hide.[11]

The antigen dotted membranes can be stored for up to 6 months at both 4 °C and 37 °C without any changes in reactivity.[27,36,43] This stability of NCM coated antigen dots makes it more suitable for field application with minimal storage specification at regional veterinary centers. Further thermostability studies of antigen dotted NCM strips would be required for longer time period of storage.

Conclusion

Dot-ELISA has several advantages over commercially available ELISA kits, including simplicity of the procedure, no requirement of highly precise equipment such as an ELISA reader, ability to interpret results visually, low cost and high sensitivity and specificity. The test is also desired to be amenable to sample pooling to cover herd level screening through bulk samples which facilitates mass surveillance. The results of the present study demonstrate that dot-ELISA may provide a suitable alternative to plate ELISA for the serodiagnosis of GWFI at regional centres. The present study reports the first dot-ELISA based on rHyC of *P. silenus* which can support mass surveillance programs at the field level to achieve sustainable control of GWFI.

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