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An overview on different aspects of hypodermosis: Current status and future prospects

Haroon Ahmed [a,b], Muhammad Sohail Afzal [c], Muhammad Mobeen [d], Sami Simsek [b,*]

[a] Department of Biosciences, COMSATS Institute of Information Technology (CIIT), Islamabad, Pakistan
[b] Department of Parasitology, Faculty of Veterinary Medicine, University of Firat, 23119, Elazig, Turkey
[c] Department of Chemistry, School of Science, University of Management and Technology (UMT), Lahore, Pakistan
[d] Department of Earth Sciences, University of Sargodha, Sargodha, Pakistan

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ABSTRACT

Livestock plays a vital role in economic development of a nation and is being used in agriculture for draft power, production of farmyard manure as well as milk and meat production. Bovine hypodermosis is the top culprit among all parasitic infections across the world. Hypodermosis is an endemic disease in the mountainous areas/plain areas and is regularly observed in the northern hemisphere of the globe affecting cattle, deer, yaks and buffaloes. There is a wide variation in geographical distribution of Hypoderma spp. during the years 1945–2015. The manuscript includes a geospatial study that tries to maps the global distribution of hypodermosis in different areas of the world in order to detect hotspots or endemic areas that may be a potential source for disease spread. This information's are very useful to predict the potential high risk areas that are prone to disease outbreak. The present review aims to evaluate the global distribution, molecular discrimination, diagnostics and vaccination of hypodermosis, focusing on its current status and future perspectives towards the management of the disease and its control strategies.

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* Corresponding author.
E-mail address: ssimsek@firat.edu.tr (S. Simsek).

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1. Background

During the recent decade, livestock production has increased significantly. However, still this sector has not yet reached its full potential to deliver adequate meat for the rising human population across the globe (Thornton, 2010). Among several restraining factors, parasitic diseases that severely affect livestock are the biggest culprit in decline of the hide industry. Ectoparasitic infestation is a serious veterinary health problem affecting livestock industry, worldwide (Hourrigan, 1979). *Hypoderma* and *Przhevalskiana* spp. (Diptera: Oestridae) causing subcutaneous myiasis are common parasites. It's well known that this myiasis affects both wild and domestic ruminants across the northern hemisphere (Boulard, 2002). This is an endemic disease of livestock (including goats, deer, buffaloes, yaks and cattle) resulting a severe decline of milk and meat production and depreciation in hide quality due to exit holes (Hall and Wall, 1995). In this manuscript we have presented an overview of the disease with major emphasis on disease molecular diagnostics, immunohistochemistry, vaccination and future recommendations for effective management of the disease.

2. Global distribution and potential risk zones

Hypodermosis is an endemic disease and regularly obsern many parts of Canada, Africa and Europe (Scholl, 1993). There is a wide variation in the prevalence of hypodermosis depending upon diagnostic methodologies ranging from 21 to 79% in Spain, 43% in Belgium, 85% in Italy (Puccini et al., 1991; Preston, 1984; Frangipane di Regalbono et al., 2003), while it was 44.2% in Greece (Papadopoulos, 2004). Similarly in Middle East the disease had significant occurrence ranging from 14.1% in Libya (Otify and Mansour, 1994) and 23% in Iraq (Abdul-Hak, 1973). Besides, the prevalence of WFI has been significantly higher in South Asian countries. It has been recorded as 80% in China (Yin et al., 2001) and 18.4% in Pakistan (Ahmed et al., 2012). The maps show the global distribution of *Hypoderma* spp. in different areas of the world at different time periods (Fig. 1a and b). The interpolation method Inverse Distance Weighted (IDW) has been used for the manifestation of data. Interpolation produced the surface for distribution of hypodermosis on the basis of limited point of data by using ArcGIS. We have mapped for the first time *Hypoderma* spp. global distribution as reported from different countries of the world (Fig. 1a and b). The cases of infestation represent the severity of the issue and warrants serious efforts in identification, diagnosis and medication of this disease. An overview of recent reports on disease prevalence and diagnostics are provided in Table 1. But in past few years many countries eradicated the hypodermosis due to their eradication projects.

There is a wide variation in geographical distribution and evolution of *Hypoderma* spp. from 1945 to 2015. In F years there were reports of WFI in 17 countries, E years (No: 09), D years (No: 06) in comparison with other areas. In the years A (No: 02), years B (No: 01) and years C (No: 03) it was endemic in a few countries but the actual situation was totally different. This menace was present in other parts of the world in the early 1950 but was not explored nor reported in that decade. In the late 1980 and 1990 when the rate of infestation was drastically increased in different countries then the eradication strategies were applied for its control to reduce the economic losses. These information's can be used to predict the potential risk areas (Table 2).

The point of prevalence shown in Fig. 1 are the regions where the disease is endemic and may be a potential source for disease spread, hence may be regarded as hotspots for hypodermosis. The gradient of color depicts the degree of influence around the study areas. So it can be predicted that these hotspot areas reflect the risk zones for hypodermosis outbreaks. These areas in the map are based in the published data from some specific part of that country of that year. In the past decade there are only few published reports are available reflecting the actual situation of this myiasis. But these studies predict that the hypodermosis has remained unreported or neglected for decades in many areas of the world.

Geographical Information System (GIS) is a computerized system that combines spatial and descriptive data for mapping and analysis. In recent years, the application of geographical information system (GIS) is widely used in mapping endemic diseases. It is being increasingly used to map and collate the available epidemiological information, to relate climatic and environmental factors to distribution of diseases together with time, people and other parameters of interest (Burrough and McDonnell, 1998). This advanced analytical tool has its application in monitoring, identification and serosurveillance of diseases from high-risk regions to prioritize areas. It provides an effective tool for visualization and spatial analysis of epidemiological and environmental data (Robinson, 1999; Moore and Carpenter 1999; Cromley and McLafferty 2002; Fun-Mun et al., 2008).

GIS was used to show the cluster analysis of hypodermosis varied within zones and across Pakistan. The regression analysis showed that the temperature in January, February, March, August, and November, and the precipitation in September and October had significant impact on disease susceptibility (Ahmed et al., 2012; Khan et al., 2015). Similarly, in Belgium it is reported that larger herds and higher daily rainfall are also important contributing factors. Furthermore lower minimum temperature appears to protect against disease (Haine et al., 2004).

There are different approaches, were used now a days for spatial distribution of parasitic diseases. The feature that distinguishes between them is the basic underlying statistical model, and the assumptions that this makes regarding the spatial processes involved (Diggle, 1990). For instance, spatial statistics investigating continuous spatial dependency assume that the outcome occurs and is potentially measurable throughout space and, as such, spatial variation in the outcome can be modelled explicitly. In contrast, discrete spatial statistics investigate proximity and are used when data are only available at an aggregate area level. Here, spatial structure is modelled by considering dependency between neighboring discrete units by using the previously published data. Spatial point processes, on the other hand, concern the physical location of events distributed within a study region and are used to investigate either the general (i.e. global) propensity for points to cluster or the location of individual (i.e. local) spatial clusters of infection, disease or vector and intermediate host populations, relative to the underlying population. Below, we describe each of these three approaches.

The spatial distribution of parasitic diseases is governed by environmental factors that determine ecological niches for the parasite and their vectors and/or intermediate hosts (Bergquist and Rinaldi, 2010). Geospatial statistics have proven useful to study the distribution and underlying risk factors for parasitic diseases, with environmental factors found to be important determinants of para-

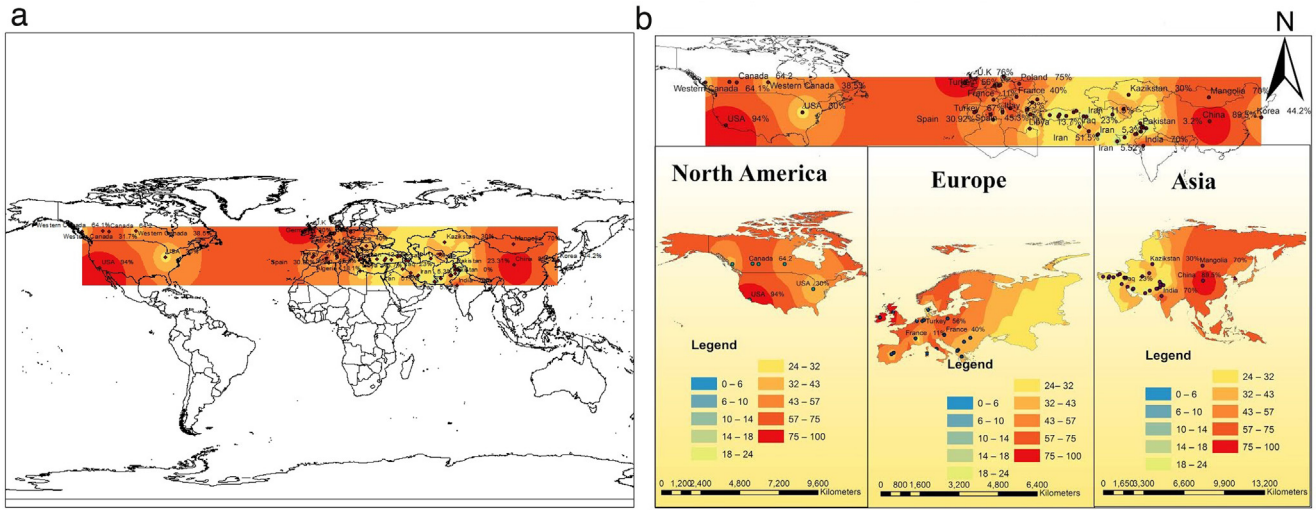

Fig. 1. (a) Global distribution of hypodermosis (1945–2015). (b) Spatial distribution of hypodermosis in different subcontinents.

Table 1
Diagnoses of *Hypoderma* spp. reported in last decade (2005–2015).

Region	Country	Year of study	Host	Species	Prevalence (%)	Diagnostic Method	Site of Incidence	References
Africa	Algeria	2009	Cattle	*Hypoderma* spp.	18.1	Palpation	Farm	Saidani et al. (2014)
America	Canada	2008	Calves	*Hypoderma* spp.	31.7	ELISA	Field	Colwell
		2009	Calves	*Hypoderma* spp.	64.1	ELISA	Field	(2013)
		2010	Calves	*Hypoderma* spp.	38.5	ELISA	Field	
Asia	China	2003	Cattle, Yaks	*H. bovis*, *H. lineatum*	98–100	Palpation	–	Otranto et al. (2005)
	India	2002–2004	Cattle	*H. lineatum*	9.30	Palpation	Field	Yadav et al. (2013)
	Iran	2014	Calves, Cow,	*Hypoderma* spp.	13.75	Palpation	Abattoir	Yagoob et al. (2014)
	Iran	2012	Goats, Cattle	*H .lineatum* *H. bovis*	61.4–12.6	Palpation	Abattoir	Dehghani et al. (2012)
	Pakistan	2009–2010	Cattle	*H. lineatum*	17.4	ELISA	Field	Ahmed et al. (2013a)
		2010–2011	Buffalo	*H. lineatum*	18.4	Palpation	Field	Ahmed et al. (2013b)
		2006	Cattle, Buffalo	*H. lineatum*	35.4	Palpation	Abattoir	Khan et al. (2006)
Europe	Romania	2011–2012	Deer	*H. diana*	58.6	Palpation	Necropsy	Ilie et al. (2012)
	Turkey	2005	Cattle	*Hypoderma* spp.	5.08	Palpation	Abattoir	Karatepe and Karatepe (2008)
		2005–2006	Cattle	*Hypoderma* spp.	23.3	ELISA	Abattoir	Simsek et al. (2008)
		2008–2009	Cattle	*Hypoderma* spp.	28.6	ELISA	Field	Balkaya et al. (2010)
		2008–2009	Cattle	*H. bovis*, *H. lineatum*	2.96	Palpation	Abattoir, Field	Cicek et al. (2011)
		2005	Cattle	*H. bovis*, *H. lineatum*	31.9	Palpation	Field	Kara et al. (2005)
	Albania	2002–2004	Cattle	*H. bovis*	38.66–41.28	Palpation ELISA	–	Otranto et al. (2005)

sitic infections (Zhou et al., 2009a,b; Zhan et al., 2010; Sithithaworn et al., 1997).

3. Discrimination of Hypoderma species

3.1. Traditional methods or morphological identification

3.1.1. Stereo microscopy

There is a wide range of differences in *H. lineatum* and *Hypoderma bovis* based on their identification approach to cattle, size of egg and egg laying behavior (Scholl, 1993). *H. bovis* fly lays its egg singly over hocks of infested cattle. Adults *H. bovis* and *H. lineatum* are covered with hair but differ in body coloration i.e. *H. bovis* is greenish to reddish yellow and *H. lineatum* is yellowish white (Kettle, 1990). Adult flies lack functional mouth parts and life span is very short ranging from 3 to 5 days (Khan et al., 1991). This generation emerges from March to the end of May, June to mid-September, respectively (Tarry, 1980). The third instar larvae of *H. bovis* and *H. linatum* are identified on the basis of the presence or absence of spinulation pattern on tenth larvae segment (James, 1947) and on the structure of its peritreme (Zumpt, 1965). *Hypoderma lineatum* and *Hypoderma bovis* larvae have different routes of migration within the host body. *Hypoderma lineatum* larvae reach the back side of the host after resting in the sub-mucosal tissues, while the *H. bovis* larvae migrates to the spinal canal along nerve paths and reaches the epidural fat which serves as a resting site (Scholl, 1993).

3.1.2. Electron microscopy (EM)

Colwell et al. (1998) determined the accurate structural morphological identification of four *Hypoderma* spp. (*H. diana, H. bovis, H. lineatum,* and *H. actaeon*) in Yak China by using scanning electron microscopy. The cephalic segment contains mouths and opercular sutures with spinal bands in between in cattle. In yaks, among these three species, no morphological difference in shape of grubs heads have been reported. In *H. bovis* the spiracular plate is concave and contains many spines at the opening rims while in *H. lineatum* the spiracular plates are flat and contains no spines. On the other hand

Table 2
Showing the evolution of hypodermosis reported in different areas. These are the different studies which have been reported during the ranged years (1945–2015). This can be correlated to the sporadic nature if disease with the reported data.

S. No	Continent	Ranged of Years	Labels	Name of Country	No of Countries
1	Asia	1945–1955	A	Turkey, India	02
2	Europe	1956–1965	B	Italy	01
3	Asia, Europe,	1966–1975	C	France, Albania, Iraq	03
4	Asia, America, Europe	1976–1985	D	Italy, Turkey, Pakistan, USA, Germany, Turkmenistan	06
5	Asia, Africa, Europe	1986–1995	E	Denmark, Poland, Romania, Libya, Spain, France, Korea, Tunisia	08
6	Asia, America, Europe	1996–2005	F	Greece, Netherland, Ireland, Pakistan, Spain, UK, Korea, Algeria, Canada, Turkey, Italy, Kazakhistan, Uzbekistan, Mongolia, China, Albania, Turkey	17
7	Asia, America, Europe	2006–2015	H	Romania, Canada, India, Pakistan, Algeria, Iran, Korea, Canada, Italy, Kazakhistan, Uzbekistan, Mongolia, China, Albania	14

the spiracular plate of *H. sinense* is flat like that of *H. lineatum* but it contains small and fewer spines. The spinal band is absent in the tenth segment of the warble body of *H. bovis,* but *H. lineatum* has a single band at the posterior border on the tenth ventral segment, while *H. sinense* has two spines bands at both the anterior and posterior border of its tenth segment. Scanning electron microscopy has been used to identify *H. lineatum* on the basis of general morphology, antennae of males/females and size, type, distribution, ultra structure of sensilla. Meanwhile they provide protection to fragile antennal sensilla from mechanical irritation or damage (Li et al., 2015).

3.2. Molecular discrimination

In recent years, cytochrome oxidase I (COI) of the mitochondrial DNA (mt-DNA) has been used as a target gene for a number of molecular phylogenetic and identification studies. Its size (~1500 bp) and the presence of both highly conserved and variable regions with a range of closely associated mutational rates (Lunt et al., 1996) make COI ideal for such purposes; moreover, its utility as a global molecular clock gene has recently been demonstrated by Gaunt and Miles (2002).

An RFLP assay (*TaqI, RsaI, HpaII, HaeIII, TruII, HinfI* enzymes) of COI gene of most common Italian species of Oestridae (i.e. *Gasterophilus intestinalis*, *Oestrus ovis, Przhevalskiana silenus, H. bovis*, *H. lineatum*) has demonstrated a clear genetic difference between these species. However there is no interspecific variation in RFLPs between two species of *Hypoderma* (Otranto et al., 2000).

A reliable differentiation of both the *Hypoderma* spp. is essential to accurately assess its presence in a given area. It is a big challenge for farmers to treat animals on proper time. Furthermore, the differentiation of *H. bovis and H. lineatum* is important because both these species have different migrating patterns. So the problem could be arising from drug administration, when first *Hypoderma* instars are in migration phase inside the host. This is a major threat to *H. bovis* (paralysis of the hindquarters) as compared to *H. lineatum* (Losson et al., 1998). The molecular differentiation of *H. bovis* (Otranto et al., 2000; Boulard et al., 1996a) and *H. lineatum* (Colwell et al., 1998) (Diptera, Oestridae) was carried out by the amplification of variable region of cytochrome oxidase I (COI) gene by PCR and the sequenced analysed the resulting amplicons. The differential profile generated by restriction digestion using *BfaI*, *HinfI* and *TaqI*, was used to demarcate *H. bovis* and *H. lineatum* due to their different interspecific variation rates (Otranto et al., 2003).

Similarly, mitochondrial COI genes were used as a probe to identify species by PCR-RFLP in eastern Turkey. *Hypoderma bovis* and *Hypoderma lineatum* third instar larvae (L3) were differentiated by 438 and 250 bp bands and 488 and 200 bp bands, respectively while only *TaqI* enzyme was used in this study as compared to previous studies (*BfaI, HinfI* and *TaqB)* (Balkaya et al., 2010). Thus the COI gene region examined was useful for the differentiation of *H. bovis* and *H. lineatum* and that a PCR-RFLP assay is a practical tool for their identification, offering additional diagnostic and epidemiological information for the study of cattle grub infestation.

3.3. Diagnosis

In an infested area, the planning of a program for treatment and the eradication of bovine hypodermosis is based on timely diagnosis (Boulard et al., 1996b; O'Brien, 1998; Otranto, 2001). This approach is widely used to monitor the efficiency of eradication campaign. It is suggested for examination due to the low infestation levels the clinical diagnosis becomes impractical (Iqbal, 1994; Webster et al., 1997; Colwell, 2000).

3.3.1. Conventional procedure

The traditional method for detection of hypodermosis is called palpation method. Generally, the bovine hypodermosis is diagnosed by the inspection of nodules in infested cattle during summer and spring season (depending on the region). The aim of this method to monitor the intensity of infestation on regular basis throughout the warble emergence period. This period lasts from three to four months (Argente et al., 1998).

3.3.2. Serological techniques

3.3.2.1. Proteolytic enzymatic secretions. The *Hypoderma* maggot secretes three types of proteolytic enzymatic secretions (Hypodermin A, Hypodermin B and Hypodermin C) (Lecroisey et al., 1979; Boulard, 1989). The first instar larvae move from conjunctive tissues and molts to second stage larvae once they reach subcutaneous tissues on the back soon after L2 molts to L3. Another family of secreted enzymes, i.e. trypsin, has two important biochemical properties (HyA and HyB) (Khaznadji et al., 2003). HyA protein belongs to serine protease, extracted from *H. lineatum* larvae. On the other hand sequence analysis of HyC shows that it belongs to the chymotrypsin family (Sandeman and Wikel, 1996; Lecroisey et al., 1979) which has a collagenolytic activity and is involved in tissue penetration. HyC is secreted during the migration phase by

1st instar larvae. After molting from 1st instar to 2nd instar, the production of HyC apparently ceases (Moiré et al., 1997) and the larvae become encysted. Therefore, this enzyme has been widely used in serodiagnosis of bovine hypodermosis for detection of 1st instar by serological tests. In order to master the identification skills and survey the proper symptoms pertaining to warble fly infestation, knowledge of larval feeding habits, physiological procedures facilitating parasitization and animal responses to the infections is a requisite. It is quite obvious that the hide of animals serves as a foremost mechanical barrier. To gain entry, the larvae use proteolytic secretions full of enzyme complex at the base of the hair follicle. After hatching, the complete entrance in skin takes place in six hours (López et al., 2005).

3.3.2.2. ELISA bioassays. Serodiagnosis, may result in early detection of infection and has been used in European countries (Boulard et al., 1996b; Argente et al., 1998). One of the traditional diagnostic approach is based on the palpation of warbles appeared at last stage of infection. It is an old technique to probe the grubs on the dorsal side of the infected animals. The hypodermosis detected at the final stage of infection has already inflicted damage to the hosts. Early and accurate detection techniques are prerequisite for avoiding economic losses connected to hypodermosis. It signifies one the foremost tasks in control of WFI, since the diagnosis of *Hypoderma* L_1 instars in initial migration phase permits treatment in a systemic way, preventing harm triggered by *Hypoderma* in the host body tissues (Panadero et al., 2007).

3.3.2.2.1. Indirect ELISA. The ELISA test is commonly used for diagnosis of WFI. It is commonly carried out using ELISA *via* detection of circulating antibodies in sera of infested cattle (Colwell and Baron, 1990; Boulard et al., 1996b; Otranto, 2001; Quintero-Martínez et al., 2007). Antibodies against HyC are usually found at a low level during early phase of the infestation and antibodies reach maximum levels in late stages of the lifecycle i.e during the permanence of warbles on the back. Meanwhile no association among numbers of cattle nodule on the back of the animal and antigen or antibody levels has been observed (Panadero et al., 2007). An antigen capture assay for the detection of hypodermin C was developed for diagnosis of hypodermosis in Spain having the sensitivity (96.4%) and specificity (95.6%). (Panadero et al., 2002).

In eastern and north-eastern Poland the highest level of antibodies were recorded during winter-spring season (March-May) indicating the best time for diagnosis of hypodermosis. In these areas the prevalence rate ranges from 10 to 86% (Cencek and Ziomko, 2001). A serological survey based on ELISA was conducted in Vicenza province (northeastern Italy). The survey was conducted in fifteen farms, only thirteen farms were positive by manual palpation method. Meanwhile seroprevalence was reported to be 43.3% (Regalbono et al., 2003). Herd-level seroprevalence in all herd types (mixed, dairy and beef) was reported to be 48.7% in the north sea coast and south part of the country (Haine et al., 2004).

In France, incidence of WFI in cattle by direct visual examination and milk based ELISA was performed. The results showed a higher sensitivity (92.2%) and specificity (98.1%) (Charbon et al., 1995). The major drawback of antibody detection *via* ELISA for surveillance and diagnostics is the fact that antibodies may persist in absence of active infestation. It is expected that the antibodies persist after all mature instars have existed in primary infestations and after the death of *Hypoderma* larvae by use of macrocyclic lactone (Colwell and Baron, 1990). ELISA based detection is considered a gold standard for the detection of *H. bovis* in infested animals due to its high specificity and sensitivity (Cencek and Ziomko, 2001).

Guan et al. (2005) investigated the sero-epidemiological surveillance of hypodermosis in north China from cattle and yaks. Similarly, the sero-prevalence of hypodermosis was reported in cattle from north western Spain (Panadero et al., 2007). Another sero-epidemiological study on WFI was conducted in Elazig, Malatya and Diyarbakir provinces (East and Southeast region) in Turkey. The results of indirect ELISA based on HyC shows that 23.3% (148/634) animals were positive for anti-hypoderma antibodies. The seroprevalence was higher in Elazig followed by Malatya and Diyarbakir provinces (Simsek et al., 2008). Therefore ELISA proves to be best diagnostic technique to date for the study of larval maturation inside the body of animal. An antigen capture or sandwich ELISA (sELISA) was devolved for the diagnosis of *Hypoderma lineatum* in cattle under field conditions in northwestern Spain to determine the kinetics of circulating hypodermin C (HyC) (Panadero et al., 2007).

3.3.2.2.2. Competitive ELISA. Webster et al. (1997) reported a competitive ELISA for diagnosis of *Hypoderma* spp. in cattle. They used clinically positive sera samples (n = 40) from cattle and negative sera samples (n = 200) from clinically unexposed animals. The sensitivity was 100% and specificity was 92% respectively. They conclude that competitive ELISA is less useful as compared to sandwich ELISA due to the non-specific binding properties.

3.3.2.2.3. Recombinant Hypodermin C based ELISA. Casais et al. (1998) reported the ELISA test based on recombinant HyC and very useful for the detection of *Hypoderma* antibodies. The cDNA encoding the entire mature hypodermin C (HC) was cloned and expressed in *Escherichia coli* as a glutathione S-transferase fusion protein using pGEX vector. The recombinant HC protein (rHC) was tested by Western blotting to detect antibodies to *H. lineatum* in cattle. It was shown *via* immunoblotting that the rHC antigen clearly differentiated between *H. lineatum*-infested cattle sera and normal cattle sera. By using western blotting forty six out of forty eight serum samples tested positive for hypodermosis in Central Mongolia whereas in Japan (Hokkaido). Similar investigations reported that Western blotting with rHC expressed in *E. coli*, might be a useful tool for the diagnosis of bovine hypodermosis in cattle (Boldbaatar et al., 2001). An antigen capture ELISA, using a murine monoclonal antibody recognising recombinant hypodermin C (rHyC), was used to evaluate the influence of early treatment with eprinomectin (Eprinex) or fenthion (Spotton) on the kinetics of circulating hypodermin C in calves naturally infested with *Hypoderma lineatum* (Colwell et al., 2003).

An indirect ELISA test was standardized in Spain to determine the *Hypoderma* specific antibodies from cattle sera by using rHyC and natural HyC. Excellent results were achieved using an approach with sensitivity and a specificity of 95.8% and 95.7%, respectively with the rHyC antigen compared to nHyC based ELISA where the sensitivity and specificity was 98.2%, 98.2%, respectively (Panadero et al., 2000).

3.4. Immunological responses

Colwell (2011) reported that previous contact with *Hypoderma* did not confer protective immunity in experimental infestations where a subcutaneous injection of larvae was administered. Study by Gingrich (1980) demonstrated that a huge number of *Hypoderma* instar might die in the resistant hosts unable to move to the oesophagus (i.e. after their entry in host), they would die in short time duration reflecting no impact in early phase of *Hypoderma* infection (Panadero et al., 2009).

Soluble fractions of *H. lineatum* third instar fat body, haemocytes and haemolymph were formulated with Quil A and used to immunize the different groups of calves in many combinations. These younger animals were exposed to fifty new hatched instar larvae of *H. lineatum*. The Penetration was permitted after two weeks of the final injection. They concluded that animals in all non-immunized and adjuvant injected groups were positive. While the animal groups immunized with fat body (100%), haemocyte (33%), and haemolymph components (33%) adjuvant or control, higher mor-

tality rate of first instar was observed, i.e; 99.3%, 95.1%, 95.8%, 83.9% and 80.4% mortality rates respectively, while the mortality rates of the 2nd and 3rd instars was higher in the immunized animals for fat body (100.0%), haemocyte (91.7%) and haemolymph (91.7%) compared to only adjuvant (14.0%) and unvaccinated (33.3%) animals. It was demonstrated that there are several common proteins among all three tissue extracts. All animal groups tested positive for *Hypoderma* antibodies, although the antibodies were transient in some immunized calves (Colwell, 2011).

Many researchers have explored the different sero-diagnostic tools for early diagnosis of hypodermosis (Sinclair and Wassall, 1983; Boulard, 1985; Pruett and Barrett, 1985; Panadero et al., 1997) or immunogens (Panadero et al., 2002; Colwell and Leggett, 2004). However, there is no association between the number of mature *Hypoderma* larvae and level of circulating antibodies has been established (Pruett and Barrett, 1985; Panadero, 1996).

HyA, a serine protease secreted plays a crucial role in induced immunosuppression during hypodermosis. Khaznadji et al. (2003) reported that the rHyA in Schneider 2 cells of *Drosophila melanogaster* share the same biological and biochemical properties with native HyA. They investigated the presence of two putative glycosylation sites that carry the glycan residues after tunicamycin treatment. While unglycosylated rHyA has the same enzymatic activity as the fully glycosylated protein, indicating that glycosylation does not play a role in the protease activity of HyA (Khaznadji et al., 2003).

3.4.1. Local and systemic cytokine responses during hypodermosis

López et al. (2005) studied the composition and dynamics of the infiltrate surrounding penetrating larvae and have observed that B cells, IgG plasma cells and CD3+ infiltrates were more abundant in previously infested animals. However, apart from these few reports, knowledge of the phenotype of cellular infiltrates at the site of larval penetration and the identity of the cytokines regulating this process remains scarce. The local and systemic cytokine-responses were investigated in three cattle groups with four animals per group. They were experimentally infested with 1st instar of *H. lineatum*. These groups were given a primary infestation (G-1), a 2nd group with secondary infestation (G-2) and the 3rd group was infested for three consecutive years (G-3). A total of twenty five 1st instars were transferred to cattle skin. Blood and skin samples were collected at 0, 6, 12, 48, 96 and 144 h post-infestation (hpi). Immunohistochemistry and sandwich ELISA was used to determine the production of interleukin 10, interleukin 4 and interferon gamma (IFN-γ). IL-4+ cells showed a significant increase at 6 hpi in both reinfested groups (G-2 and G-3) when compared with G-1. The penetration of *H. lineatum* in skin did not have any significant effect on IFN-γ serum concentrations and, except for IL-10 there was no correlation between local production and serum concentrations of cytokines. The increase in both Th1 (IFN-γ) and Th2-type cytokines (IL-4 and IL-10) reflects that bovine T-cell response during the first phases of the infestation by *H. lineatum* is apparently a Th0 response (Dacal et al., 2009).

3.4.2. Antigen-specific lymphocyte proliferative responses

Cattle infested with the common cattle grub, *H. lineatum* develop specific humoral antibodies and a cellular immune reaction, defined by delayed-type hypersensitivity, to purified *H. lineatum* proteins. This investigation was designed to study the antigen-specific bovine lymphocyte response to HyA, a serine protease of larval first-instar *H. lineatum*. The young calves were vaccinated with native or denatured HyA, and challenge-infested with the *H. lineatum*. The developmental pattern of immune response to HyA was measured during the time of vaccination and infestation. The HyA specific responses were highly weak and variable during these two phases. Similarly, HyA-specific lymphocyte blastogenic responses were measured. It was observed that no correlation exists between the magnitude of antigen-specific, peripheral lymphocyte proliferation and larval mortality. In striking contrast to responses observed during infestation phase, intense HyA-specific lymphocyte responses were noticed in three calves (six months' years old) after recovery (Fisher et al., 1991).

3.4.3. Cellular & humoral responses

Primary (G1), secondary (G2) and tertiary (G3) experimental infestation of *H. lineatum* 1st instar was investigated to determine the functional role of cutaneous T- and B-cell responses in three different groups. The pattern and number of CD3+ T infiltrating cells were similar in all three groups of cattle, representing a progressive increase until 96 hpi. A significant increase in number of CD4+ T helper cells was observed in G1, G2 and G3 at 96, 6 and 48 hpi respectively. The same concentration of CD8+ cytotoxic T-cell infiltration was reported in different cattle groups except at 48 hpi where a significant difference was observed between G1 and G3. The CD4/CD8 ratio reflected the predominance of CD4+ cells throughout the response. The number of previous infestations was directly proportional to the number of B cells having significant differences between G1 and G3 at 12 hpi. There was predominance of CD4+ cells during primary larval infestations, whereas in cattle sensitized by previous infestations, B cells were most abundant in the infiltrate. These findings indicate that humoral immunity might play a significant impact in immunity to *Hypoderma* infestation (Dacal et al., 2011).

Hypoderma instar larvae are tissue invading parasites that spend several months during migration phase within the host tissues before completing their developmental stages in sub-dermal tissues of the back. The macrophages and lymphocytes were the predominant cells in granulomas (type 2) formed in relation to remnants of the dead parasite, which may contain remains of the altered cuticle. Scars (type 3) were characterized by tissue granulation. Immunohistochemistry analysis showed that B lymphocytes and IgG+ cells were predominant in the lesions, as long as the cuticle of the larvae was intact. On the other hand, CD3+ lymphocytes increased once the cuticle is destroyed and a granuloma is formed. Macrophages, revealed by CD68+, MAC387+ and lysozyme immunolabeling, were detected in all types of lesions, but they were more abundant in type 2 and scarce in scars, isolated around the intact larvae or forming aggregates around its remains in the granuloma. Moreover, immunolabelling against MAC387 antibody was pronounced in the squamous epithelium covering the breathing pore. This finding may be associated with the expression of calprotectin, a molecule involved in the healing process of the skin after larvae emergence (Cabanelas et al., 2015). In conclusion, it may be suggested that the humoral response is predominantly inside the warble as long as the larvae are intact. Once they are destroyed, cellular response occurs, isolating and destroying the remains of the larvae until healing process completes and scars with few inflammatory cells appear.

3.4.4. Immunosuppression mechanism of HyA

Hypodermin A (HyA), secreted by 1st instar larvae, is able to escape the host immune responses and suppress lymphocyte proliferation (Moiré et al., 1997), and bovine IL-2 production *via* the prostaglandin pathway (Nicolas-Gaulard et al., 1995). It can also cleave the complement C3 component from bovine serum, block the complement pathway and reduce inflammation (Baron, 1990; Boulard, 1985; Boulard, 1989). The rejection of heterogenic transplantation is attributable to the activation of the host complement system where C3 assumes an important role (Pratt et al., 2003). HyA is able to inhibit C3-mediated cytotoxicity and cleave the C3 in rat and humans. That is why HyA shows enormous potential

to slow down the rejection associated with xenotransplantation (Malassagne et al., 2003).

HyA is associated with inflammatory and specific immune responses in cattle hosts. Association between recombinant HA and guinea-pig complement component 3 (C3) through a co-immunoprecipitation assay was explored by Chen and colleagues. Cos7 cells stably expressing HyA were generated, and were found to be more resistant to lysis by guinea-pig C3 than the controls. They DNA binding site of HA with guinea-pig C3 was detected by an electrophoretic mobility shift assay (EMSA). In contrast, after stable transfection, mHA was unable to reduce the amount of C3 or to inhibit its cytotoxicity, while HA could degrade guinea-pig C3 and inhibit the complement pathway. So it can be suggested that recombinant HyA could serve as an immunosuppressive agent against organ rejection after xenotransplantation (Chen et al., 2014).

Cattle are known to acquire immunological resistance to hypodermosis by repeated exposure to both species of cattle grubs, *H. lineatum* and *H. bovis*. Vaccination of cattle with purified proteins of *H. lineatum*, particularly HyA, is known to protect cattle against hypodermosis by this species. The development of a protective recombinant vaccine against both species using HyA isolated from *H. lineatum* would require that immunological epitopes be shared by complementary proteins in *H. bovis*. The researchers investigated the soluble proteins of *H. bovis* first-instars for shared epitopes with *H. lineatum*. Soluble *H. lineatum and H. bovis* first-instar larval proteins were resolved by non-denaturing polyacrylamide electrophoresis, blotted onto nitrocellulose membrane, and probed with selected polyclonal cow and polyclonal rabbit sera, as well as mouse monoclonal antisera. Considerable cross-reactivity was demonstrated by antibodies in the serum of an *H. lineatum*-infested cow as 6 of 10 resolved *H. bovis* proteins were bound by the antibodies. The most common shared epitope(s) were associated with HyC, a collagenolytic protease. HyA shared epitope(s) were noted on one prominent *H. bovis* band (HB1-2). Hypodermin B, a prominent protein in *H. lineatum*, did not appear to share epitopes with *H. bovis* proteins. Shared epitopes between *H. bovis* proteins and HyA and HyC of *H. lineatum* suggested that cross-protection of cattle against *H. bovis* can be expected by vaccination with recombinant proteins of *H. lineatum* (Pruett et al., 1990).

3.5. Putative antigenic epitopes for vaccination

3.5.1. Vaccination

Vaccination is another important strategy to confer immunity to cattle hosts against hypodermosis. The primary causes of morbidity and mortality in agricultural species are the myiasis flies and man has not diminished despite the existence of good control strategies. The development of vaccines against these fly i.e. *Hypoderma* has been relatively quiescent for more than 10 years despite the rapid development of genomic and proteomic analysis and of skills in data interpretation (Sandeman et al., 2014).

Cattle develop resistance to *Hypoderma* spp. following repeated exposure to natural infestations (Gingrich, 1980; Baron and Weintraub, 1986) and artificial exposures (Pruett and Kunz, 1996; Colwell, 2001). Promoting resistance is one of the big challenges in vaccinated cattle with 1st instar secretary proteins (Baron and Weintraub, 1986; Baron and Colwell, 1991; Boulard, 2002). The immuno-reactive portions of bovine immunoglobulin survive exposure to extracorporeal larval enzymes, as well as transit of the mid gut, and pass through epithelial cells to enter the larval haemocoel (Colwell and Leggett, 2004).

Vaccines administration has advantages like complete and life-long resistance as well as less damage to the environment, over the use of chemical treatment. The efforts towards determining the mechanism of immune response of different *Hypoderma* species and vaccine development has been carried out in many countries (Scholl, 1993). Initially, crude extract of *Hypoderma* species larvae was used as vaccine (Colwell and Baron, 1990) later, systems approaches were adopted for this purpose. Recently in use vaccines are based on three enzymes (i.e. HyA, HyB and HyC). HyA is being used in its purified form (Pruett and Kunz, 1996) whereas; HyB and HyC are used in a variety of combinations (Colwell and Baron, 1990) (Table 3).

3.5.1.1. Types.

3.5.1.1.1. Natural antigens. The progress towards vaccine development started in 1950s (Sergent and Sergent, 1950) and 1960s (Khan et al., 1960; Khan, 1968). Vaccines against hypodermosis are usually composed of whole 3rd instar antigens. The animals are treated using this vaccine after natural infestation. Baron and Colwell (1991) investigated the use of native hypodermins as a component of vaccine along with the adjuvant component (Monophosphoryl lipid A). They reported that the cattle vaccinated with native HyA have higher grub mortality, but the mortality rate is higher in 2nd and 3rd instar stages in the subdermal tissues of host (Pruett, 1999). So use of this vaccine for disease control limits the degree of immediate damage to skins and carcasses. Panadero et al. (2009) reported the immuno-modulatory impact of three serine proteinases from first instars larvae of *H. lineatum*. They reported that lymphocyte proliferative responses were down regulated by HyA. Similar observations were reported by Nicolas-Gaulard et al. (1995). HyA has a very strong effect on down-regulation of cytokine responses; HyC had a much less significant effect, while HyB demonstrated intermediate effect.

3.5.1.1.2. Hidden antigens. The use of 'hidden antigens' within the larval haemocoel has potential for vaccine development against hypodermosis. The proteinases secreted by the larvae destroy the antibodies under *in vitro* conditions (Pruett, 1993), however, there is no direct evidence under *in vivo* conditions. The development of hidden antigen vaccine would be dependent on the rate of immunoglobulins (IgG) presented to the larvae during their migration phase.

Colwell (2011) reported the immunization efficiency of soluble fractions of *H. lineatum* 3rd *Hypoderma* instar fat body mixed with fraction Quil A. The resulting vaccine was used to immunize three different calve groups while 4th and 5th groups were adjuvant treated or untreated controls. In vaccinated cattle the mortality was 100%. A significant increase in the mortality rate of first instar larvae migration was noted as well as increase in mortality rate of 2nd and 3rd instars in vaccinated animals compared to untreated animals controls and adjuvant treated animals was observed. The candidate proteins with protein scores of >400 are the hexamerins/arylphorins (also known as larval serum proteins), which are the storage proteins of the haemocyanin family. They perform functions like an amino acid pools and are associated with insect metamorphosis and support egg production under some conditions (Cristiano et al., 2010). These proteins have also been identified by Roelfstra et al. (2009) in second and 3rd instars of the parasite, *Gasterophilus intestinalis*.

Glutathione-*S*-transferase belongs to a multifunctional family of enzymes. It protects the cells by preventing the damaging effects of oxygen and other free radicals. That's why they are commonly used in antiparasite vaccines (Parizi et al., 2011) *Schistosoma mansoni* (Lebens et al., 2003), *Psoropotes ovis* (Nisbet et al., 2008), *Necator americanus* (Zhan et al., 2010). Arginine kinase is another important enzyme that catalyses the transfer of phosphoryl groups from ATP to arginine in insects. That is why it could be an excellent candidate for the development of a drug or vaccine. It has also been implicated as a major human allergen (Ilg and Werr, 2012) and is suggested as a potential vaccine candidate in parasitic nematodes (Umair

Table 3
Potential immunogenic protein can be used as vaccines candidates for *Hypoderma* spp.

Year	Vaccine composition	Source	Outcomes	Reference
1970	Crude collagenase	First-instar *H. lineatum*	Resistant	Magat and Boulard (1970)
1991	Native or denatured HyA,	First-instar *H. lineatum*	HyA-specific responses were highly variable and weak	Fisher et al. (1991)
1991	HA antigen alone or with adjuvants	Hypoderma spp.	No significant protection	Chabaudie and Boulard (1991)
2011	Larvae Fat Body, haemocytes and haemolymph and Quil A as an Adjuvant	3rd Instar	100% Mortality	Colwell (2011)

et al., 2013). Phenol oxidase is one of the major constituent of the insect immune system. It plays a key role in encapsulation and wound healing through the formation of melanin (Eleftherianos and Revenis, 2011). So it is recommended to use such type of native proteins as a vaccine against *Hypoderma* spp.

3.5.1.1.3. Recombinant antigens. Three serine proteins secreted by first instar of *Hypoderma* spp. were sequenced in early 1990's (Temeyer et al., 1993), but work with recombinant versions of these enzymes pre-dated that description. The formulation and expression of these recombinant serine proteinases were carried out as inclusion bodies in *E. coli*. The primary component of the vaccine was HyA in combination with the adjuvant (alhydrogel/amphigen) (Moiré et al., 1997).

The isolation of cDNAs encoding proteins makes it possible to produce enough antigens to carry out extensive immunological assays. Lecroisey et al. (1983) and Moiré et al. (1997) sequenced the cDNA of HyC. Casais et al. (1998) performed a useful procedure for the rapid and efficient purification of recombinant mature HyC in an enzymatically active form. Similarly, it has been investigated that recombinant HyC appears to be a useful alternative to the natural parasite antigen for the serodiagnosis of *Hypoderma* spp. in cattle (Panadero et al., 2000).

3.5.1.1.4. Effects of adjuvants on bovine humoral and cellular responses to hypodermin. Hypodermin A, of the first-instar larva of the common cattle grub, *H. lineatum*, formulated with complete Freund's adjuvant and administered to native calves, elicits protective immunity defined by an increase in *in vivo* larval mortality. Pruett and Kunz (1995) and Pruett and Stromberg (1995) reported that there is increased mortality of larvae under *in vivo* conditions due to the protective immunity. They reported that alhydrogel and amphigen (alone and in combination) are acceptable adjuvants for HyA in veterinary vaccines. Due to the adverse reactions at injection site the freund's complete adjuvant (FCA) is not recommended for use. However the acceptable veterinary adjuvants are not as effective as FCA in inducing an antibody response as detected in the peripheral circulation. A mixture of adjuvants of alhydrogel and amphigen induce the highest serum antibody response to HyA. Although the mixture of alhydrogel and amphigen, when compared with FCA, did not elicit comparable levels of responsiveness in all parameters tested, the overall performance of the mixture suggests it to be worthy for further efficacy investigation in a vaccine formulation with hypodermin A (Pruett and Kunz, 1995; Pruett and Stromberg, 1995).

3.5.1.1.5. Key challenges for Hypoderma vaccine production. There are different key challenges in current vaccine technologies of myiasis. They are reviewed mainly in primary research genera of *Hypoderma* spp. These flies are one of the causes of morbidity and mortality in livestock sector and so far no control strategies exist. From the last decade the researchers are trying to develop vaccines against myiasis. Although there is a dire need of the rapid development in genomic and proteomic analysis, the alternative controls strategies are constantly evolving due to the drug resistance that has a strong impact on animal welfare (Colwell, 2013).

3.6. Control strategies

Eradication campaigns for WFI have been developed during the last decade and many strategies were used in the warble-infested country (Tarry et al., 1993). The overall control plan depends on environmental conditions and the country (Tarry, 1980; Jesperson, 1995). Several control programs have been launched for the control of hypodermosis in Netherlands, Czech Republic, UK, Irelands, Denmark and Switzerland due to which farmers were able to control the menace in these countries (Boulard, 2002) but still the disease persists in Europe, a few regions of United State of America and Africa (O'Brien, 1998), Canada (Scholl, 1998) and Italy (Otranto, 2001).

In United Kingdom and Denmark during the eradication program, over 40%, herds suffered from the infection. Therefore, pour-on organic phosphorous compound on affected cattle was recommended (Tarry, 1980; Boulard et al., 1996b). Controlling warble fly natural population can also limit the infection spread; for this purpose use of chemical compounds (insecticides) has been recommended (Drummond et al., 1981). In north China, the chemical control of the pest was practiced in early 1980s and prevalence decreased from 1980 to 2002 (91.84–28.45%) respectively (Guan et al., 2005). Similarly in Netherlands, Denmark and other islands, rotenone treatment was introduced in 1953, but eradication plan was based on systemic treatments. In Canada, use of organophosphate poisoning has been in practice for a long time (Afzal et al., 1997).

On the other hand macrocyclic lactones were used in a systemic way to treat the infested animals. The prevalence of *Hypoderma* spp. has decreased in these treated animals. These eradication strategies were implemented on a national level in many countries of world. Nevertheless there is a wide variation in the prevalence of *Hypoderma* infestation in certain areas and *Hypoderma* spp. have a higher capacity for population regeneration. This might be due to (i) incomplete control strategies (ii) re-introduction (iii) import of infested cattle (iv) fly immigration (O'Brien, 1998; Scholl, 1998; Colwell, 2002; Colebrook and Wall, 2004).

Second-generation avermectin is also known as Eprinomectin. It belongs to the class of macrocyclic lactones of parasiticides. Eprinomectin has endectocidal activity in a 0.5% formulation. It consists of natural oils for pour-on administration at 0.5 mg eprinomectin/kg body weight reported in many laboratory and field studies across the globe (Davey and George, 2002; Rehbein et al., 2005).

The accuracy and persistency of eprinomectin activity against the endoparasites has increased by standardizing the injectable formulation of this drug. It releases the active components over time in different concentrations. So it gives effective prevention against dif-

ferent bovine infections (Soll et al., 2013). In this extended-release injection (ERI) formulation, eprinomectin is released from a matrix formed with poly (D,L-lactide-*co*-glycolic) acid (PLGA). It is an as safe and effective as biodegradable material. It has been used as a drug delivery system for extended-release applications of various pharmaceutical compounds e.g. ivermectin (Lewis, 1990; Miller et al., 1999; Clark et al., 2004; Winzenburg et al., 2004).

3.7. Future recommendations

The guidelines described subsequently contribute to an optimal control program that can be adjusted based on individualized risk assessment according to animal lifestyle factors and local conditions.

1. Younger animal having age less than 3 years are required special attention. Because they are more susceptible to parasite infection and are more at risk for developing disease.
2. Traditional parasite control programs should be implanted that involve rotational treatment with insecticide drugs like ivermectin or doramectin, *etc.* at regular intervals.
3. The farmer should be educated about strategies to protect their animals from warble fly.
4. Regular monitoring by the livestock department in fly season should be ensured for eradication and control of disease.
5. The agriculture department should map the potential risk areas on yearly basis.
6. ELISA test is an effective diagnostic tool that should be used for early diagnosis of hypodermosis to ensure the proper treatment at right time.
7. There is a dire need to invest funding on research focusing on the development of a recombinant vaccine for *Hypoderma* spp.

4. Conclusion

Hypodermosis poses is a serious global threat for animal health and has a serious impact on economic loss. This menace has a worldwide distribution pattern and is prevalent in many countries. In many countries the disease is not explored yet and there is a dire need of research on hypodermosis in those countries. It is therefore imperative that timely diagnosis and effective control strategies be employed in order to limit the disease spread. We recommend enforcement of eradication and vaccination programs in developing countries for betterment of livestock sector.

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