Biologicals 46 (2017) 148–152

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

An inhibition enzyme immuno assay exploring recombinant invariant surface glycoprotein and monoclonal antibodies for surveillance of surra in animals

G.R. Rudramurthy, P.P. Sengupta*, M. Ligi, H. Rahman

National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Ramagondanahalli, P.B.No. 6450, Yelahanka, Bengaluru 560064, Karnataka, India

ARTICLE INFO

Article history:
Received 9 September 2016
Received in revised form
17 January 2017
Accepted 6 February 2017
Available online 21 February 2017

Keywords:
Trypanosoma evansi
Inhibition ELISA
ISG
Monoclonal antibody
Recombinant ISG
Serodiagnosis

ABSTRACT

The present study is aimed at the development of inhibition ELISA (I-ELISA) exploring monoclonal antibodies (MAbs) and recombinant invariant surface glycoprotein. The extracellular domain (ED) of invariant surface glycoprotein (ISG-75) from *Trypanosoma evasni* has been heterologously expressed in *Pichia pastoris* (X-33). The recombinant ISG-75 (rISG-75ED) was characterized by immunoblot and ELISA, followed by the production of MAbs against rISG-75ED. The MAbs were characterized by immunoblot and then explored in the development of I-ELISA for the detection of surra. The diagnostic potential of the developed test has been evaluated using 1192 field sera sample including cattle, buffalo, donkey, horse and camel. The statistical analysis of the data showed optimum combination of diagnostic sensitivity and specificity at 98.8% and 99.2% respectively, with cut-off percentage inhibition (PI) value of >45. The Cohen's kappa coefficient of agreement was found to be 0.98. Hence, the diagnostic test developed in the present study can be exploited as a potential and reliable tool in the serodiagnosis and surveillance of surra in animals.

1. Introduction

Different species of trypanosomes causes a disease called trypanosomosis in animals and humans which is endemic throughout Asia, America (central and south), Africa and Europe. *Trypanosoma evansi* a haemoflagellate parasite and believed to be originated from *T. brucei* [1] is an etiological agent of a disease called surra in animals. Surra is considered as an important, chronic wasting disease of domestic and wild herbivores and carnivores in tropical and sub-tropical countries. Moreover, the trypanosomes which are considered as non-infective to humans have emerged as potentially pathogenic to humans. A typical human trypanosomosis caused by different species of trypanosomes including *T. evansi, T. lewisi*, *T. congolense* and many more, have been reported by several groups [2–4]. The clinical symptoms of infected animals include severe anemia, fever, weight loss, hypoglycaemia, poor body weight gain, poor drauhgtability, infertility, abortion and may also lead to death in the untreated condition. In the high risk areas of infection there has been a significant reduction in the calving rate in cows and fertility rate in infected bulls [5]. Meanwhile, the buffaloes [6] and cattle [7,8] infected with *T. evansi* have been reported with reproductive problems such as, stillbirth and abortions. Moreover, in lactating cows the significant decrease in milk production has also been observed during the sub clinical stage of the disease [9]. The most susceptible hosts for surra in south-east Asia are cattle, buffaloes, camels and horses [10]. Buffaloes and cattle act as the main parasite reservoir hosts for *T. evansi* [11]. Laha and Sasmal [12] reported a high percentage (12.74%) of *T. evansi* infection in horses from India. Surra is mainly transmitted mechanically by the tabanid flies, however in carnivores the disease transmission has been observed after feeding on infected meat [13].

The detection of the carrier status and subsequent treatment of animals lead to effective control of the disease, as well as better production. Several diagnostic tests (serological and parasitological) have been developed for detection of surra. However, only clinical stages of infections can be diagnosed satisfactorily by parasitological test, but not latent or chronic infection [14]. The serological test such as, ELISA is likely to precisely identify healthy

* Corresponding author. Parasitology Laboratory, National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Ramagondanahalli, P.B. No. 6450, Yelahanka, Bengaluru 560064, Karnataka, India.
E-mail address: pinakiprasad_s@rediffmail.com (P.P. Sengupta).

http://dx.doi.org/10.1016/j.biologicals.2017.02.004
1045-1056/

animals and qualifies as a universal test [15]. However, the development of molecular diagnostic tools have given a break through in the detection of parasitic diseases. The carrier status of the infection can be detected effectively through detection of parasite nucleic acid [16,17] or by the development of sensitive and specific serological tests. Several genes of trypanosomes have been expressed heterologously for many purposes for instance, variable surface glycoprotein (VSG) gene [18–20], beta-tubulin gene [21], actin gene [22] and ISG-75 gene [23,24].

The blood stream stage specific surface glycoproteins such as, ISGs are uniformly distributed over the entire surface of the trypanosomes [25]. The bloodstream form trypanosomes contain an estimated 5×10^4 ISG-75 molecules on the surface [26], which do not exhibit antigenic variation unlike VSG [27]. Several bloodstream forms of ISGs have been identified and characterized which include ISG- 75 [28], ISG-65 or ISG-70 [29], ISG-64 [30], and ISG 100 [25]. The ISG-75 is conserved among all the species and subspecies of the *Trypanozoon* subgenus including *T. evansi, T. b. gambiense, T. b. rhodesiense* and *T. equiperdum* [26]. Moreover, ISG-75 is found as multiple copies in trypanosomes [31].

The proteins which are invariant and not subjected to antigenic variation such as ISG-75, represent promising and perspective alternatives in the development of diagnostic tests [32]. Hence, in the present study, the monoclonal antibodies were produced against recombinant ISG-75ED and explored in the development of antibody detecting inhibition ELISA (I-ELISA) for the detection of surra. Further, the developed assay was compared with CATT/*T. evansi* using a panel of serum samples collected from different states of India.

2. Materials and methods

2.1. rISG-75ED, immune serum and field serum samples

The ED region of ISG-75 from *T. evansi* expressed in *P. pastoris* [24] was used in the present study for the production of MAbs and development of I-ELISA. In brief, the ED region of ISG-75 from *T. evansi* encoding a polypeptide of 440 amino acids has been cloned into pPICZα(A) vector and expressed in eukaryotic host such as *P. pastoris* (X-33). The expressed protein (rISG-75ED) was purified and characterized by both immunoblot and ELISA. Further, the diagnostic potential of rISG-75ED in ELISA was determined by panel of field sera sample. The rISG-75ED with an apparent molecular weight of 70 kDa was found to be highly immunoreactive with panel of sera sample [24]. The diagnostic sensitivity and specificity of rISG-75ED in ELISA was found to be 98.47% and 99.70% respectively at >0.547 cut off OD value [24]. The control antigens purified from induced X-33, X-33 with pPICZα(A) and uninduced X-33 with pPICZα(A) ISG-75ED were also used in the characterization of rISG-75ED [24] and MAbs.

The immune serum produced against buffalo isolate of *T. evansi* [18] and available in our laboratory was used in the standardization of inhibition enzyme immuno assay. The field serum samples were collected from different states of India from different species of animals including cattle (417), buffalo (172), horse (98), donkey (87), and camel (418). All the serum samples were screened for surra by CATT/*T. evansi* and I-ELISA in duplicate and comparative analysis was done between the two tests (Table 1). The CATT/*T. evansi* kit containing freeze dried *T. evansi* VSG RoTat 1.2 [33,34] was procured from Koning Leopold Institute of Tropical Medicine, Antwerp, Belgium (OIE reference laboratory of surra) and was used as a standard reference test. The field serum samples were tested in CATT/*T. evansi* as per the protocol mentioned earlier by Rudramurthy et al. [23] and in I-ELISA as per the protocol mentioned in section 2.4.

2.2. Production of MAbs

Hybridoma clones producing ISG-75ED specific antibodies were developed by fusing splenocytes and B cell myeloma cells, SP2/O [35]. The splenocytes for fusion were isolated from BALB/c mice (8–12 weeks old), immunized with rISG-75ED. The peritoneal exudate enriched with macrophages was used as a feeder layer for the growth of hybridoma. The splenocytes, SP2/O and macrophages (feeder layer) were prepared by following the protocol described earlier [36]. The sera collected from the immunized and non-immunized BALB/c mice were used as positive and negative controls respectively.

The splenocytes and SP2/O cells were fused by following the protocol mentioned earlier [36]. In brief, for the production of hybridoma, 20 million splenocytes and 2 million SP2/O cells were subjected to fusion using polyethelene glycol (PEG) and dimethyl sulphoxide (DMSO) mixture (Sigma Aldrich). The fused cells were incubated in the CO_2 (5%) incubator for 1½h and then pelleted at 214 × g for 10 min at room temperature. The cell pellet was suspended in IMDM, containing 0.1 mM sodium hypoxanthine (H), 0.4 μM aminopterine (A) and 0.016 mM thymidine (T) (Gibco®Life technologies), 20% fetal calf serum (FCS) and 20 mM L-glutamate. The hybridoma clones were then screened by indirect ELISA using rISG-75ED (3μg/well). The monoclones were selected from the positive hybridoma clones by dilution cloning technique. The hybridoma cells were seeded in the microtiter plates at 10% plating efficiency to give rise to a statistical probability of one colony/well. The monoclones were screened by indirect ELISA, expanded further and preserved till further use. The MAb was then produced in larger scale in 250 mL culture flasks. The culture rich in MAbs (>1.2 OD in indirect ELISA) was centrifuged at 3000 rpm for 3 min to remove the cells and MAbs were preserved at −80 °C till further use. The control clones (SP2/O, spleen and macrophage) and positive and negative sera were used during the selection of positive hybridoma and monoclones.

2.3. Characterization of MAbs by immunoblot and isotyping

The immunoblot analysis was carried out to characterize the MAbs by following the standard protocol [37]. In brief, the electrophoresed proteins (rISG-75ED and control antigens) were transferred on to nitrocellulose membrane by following the standard protocol and then the membrane was treated with culture supernatant from monoclones (MAbs) and SP2/O clones (undiluted). The substrate solution containing diamino benzidine tetrahydrochloride (DAB) was added finally to develop the immunoblot.

Mouse MAbs produced in the present study were isotyped using mouse isotyping (IgG1, IgG2a, IgG2b, IgG3, IgA and IgM) antibodies (Sigma Aldrich) by antigen mediated ELISA by following the protocol mentioned earlier [36].

2.4. Development of I-ELISA

Microtiter plates (Maxisorp®, Nunc) were coated overnight at 4 °C with (100 μL/well) purified rISG-75ED (6μg/well) antigen in PBS (pH 7.2). After overnight incubation the microtiter plates were washed and blocked as per the standard protocol. The test serum samples (20 μL of test serum, 20 μL of blocking buffer and 60 μL of MAbs) were added in duplicate and incubated at 37 °C for 1h on ELISA shaker followed by washing. Negative, strongly positive (>90% inhibition), weakly positive (60–70% inhibition), MAb (0% inhibition) and conjugate control wells were also run simultaneously. MAb control wells (Cm) contained all the reagents except the test serum, while conjugate control contained only blocking buffer. Hundred microlitre of anti-mouse antibody horseradish