Parasitol Res (2005) 97: 302–308
DOI 10.1007/s00436-005-1431-6

ORIGINAL PAPER

**Dia Eldin A. Salih · Jabbar S. Ahmed
Mohammed A. Bakheit · Elzein B. Ali
Abdelrahim M. El. Hussein · Shawgi M. Hassan
Omer E. Shariff · M. Fadl · Frans Jongejan**

Validation of the indirect *Ta*SP enzyme-linked immunosorbent assay for diagnosis of *Theileria annulata* infection in cattle

Received: 30 May 2005 / Accepted: 2 June 2005 / Published online: 29 July 2005

Abstract An ELISA based on a recombinant Theileria annulata surface protein (TaSP) was evaluated for detection of antibodies in sera from cattle exposed to tropical theileriosis in Sudan. The reference positive samples, used in this study, were from Theileria-infected populations and consisted of 80 cattle from an endemic area in Khartoum State, with high antibody titers in the indirect fluorescent antibody test (IFAT). The reference negative samples were taken from non-exposed populations and consisted of 120 cattle maintained under strict tick control at a commercial farm in Sudan. The cut-off value determined by Two-Graph Receiver-Operating Characteristic (TG-ROC) curves was set at 31.6%, based on the positive reference samples. Further diagnostic validation was performed, which consisted of the measurement of the area under the ROC (AUC) and by valid range proportion (VRP), which was 0.97 and 0.98 for the cut-off, respectively. There were no cross-reactions with antibodies raised against Babesia spp. It is concluded that the TaSP ELISA is a useful test for the diagnosis of T. annulata infection in cattle under field conditions.

Omer E. Shariff Deceased.

D. E. A. Salih · E. B. Ali · A. M. E. Hussein · O. E. Shariff
Central Veterinary Research Laboratories,
Alamarat, P.O. Box 8067, Khartoum, Sudan

J. S. Ahmed (✉)
Forschungszentrum Borstel, Parkallee 22,
23845 Borstel, Germany
E-mail: jahmed@fz-borstel.de
Tel.: +49-45-37428
Fax: +49-45-37188627

M. A. Bakheit · S. M. Hassan · M. Fadl
Department of Parasitology, Faculty of Veterinary Medicine,
University of Khartoum, P.O. Box 32, Khartoum, Sudan

F. Jongejan
Division of Parasitology and Tropical Veterinary Medicine,
Faculty of Veterinary Medicine, Utrecht University,
P.O. Box 80.165, 3508 Utrecht, The Netherlands

Introduction

Theileria annulata is a protozoan parasite, which causes tropical theileriosis, a disease transmitted by several tick species of the genus *Hyalomma* (Dolan 1989). It is endemic in the area around the Mediterranean, the Middle East and reaches the southern parts of Asia (Brown 1990). Currently, the disease is kept under control by acaricides and attenuated vaccines (Boulter et al. 1998). Diagnosis of the disease depends on the detection of piroplasms and/or schizonts in blood smears and lymph node biopsy smears, respectively (Norval 1992). The parasite can also be detected in cattle by tick vector with species-specific PCR (d'Oliveira et al. 1995; De Kok et al. 1993) and the indirect fluorescent antibody test (IFAT) (Pipano and Cahana 1969). Both techniques are laborious and impractical for large-scale epidemiological surveys. Moreover, cross-reactions among several *Theileria* species have been observed with IFAT (Kiltz et al. 1986). In contrary, the enzyme-linked immunosorbent assay (ELISA) is easy to perform, inexpensive, reproducible and would be useful for monitoring tropical theileriosis and facilitating the study of its epidemiology. Previous efforts to develop an ELISA for *T. annulata* were based on purified schizont or piroplasms antigens (Manuja et al. 2000). It is difficult, however, to standardize antigens purified from parasite crude material, and there is also the requirement of experimental animals for parasite production. These problems have been circumvented by the use of several recombinant parasite antigens in ELISA, of which the merozoite rhoptry antigen Tams-1 and a sporozoite antigen SPAG-1 has been developed and used (Gubbels et al. 2000; Ilhan et al. 1998; Williamson et al. 1989). However, the sensitivity of SPAG-1 has not been determined, while the sensitivity and specificity of Tams-1 was determined using the two-graph receiver-operating characteristic (TG-ROC) (Gubbels et al. 2000). More recently, another protein, *T. annulata* surface protein (*Ta*SP), has been

identified (Schnittger et al. 2002). This gene is present as a single copy within the parasite genome and transcribed in the sporozoite and schizont stages. It codes for a protein of about 315 amino acids and has a predicted molecular weight of 36 kDa. Recently, Bakheit et al. (2004) applied this antigen in an indirect ELISA for the diagnosis of tropical theileriosis.

Comparison of the dichotomized test results with the true status of individual yields the operating characteristic sensitivity and specificity. It is well recognized that sensitivity and specificity are inversely related depending on the choice of the cut-off value (Martin 1984), which can be derived by different methods such as the Gaussian distribution method, the receiver-operating characteristic curves (ROC) (Greiner et al. 1995) and the TG-ROC (Greiner 1995) etc. The TG-ROC considers both sensitivity and specificity and allows the direct selection of the cut-off from the plot. The efficiency (Galen 1986), Youden's index (Youden 1950) and the likelihood ratio (LR) (Smith 1991) are used for further characterization of the cut-off value in TG-ROC.

This study aims at calculating the cut-off values for the *Ta*SP ELISA by the TG-ROC for diagnosis of tropical theileriosis under field conditions, comparing these values with those determined by conventional methods (Gaussian method and ROC) and evaluating the performance on the *Ta*SP ELISA for the diagnosis of the cattle naturally infected with *T. annulata* under Sudan field conditions.

Materials and methods

Study design

The validation of *Ta*SP ELISA was carried out using IFAT as a reference test. One hundred and twenty serum samples were collected from animals reared under closed zero grazing system in the Arab Company, Sudan. The sera yielded negative test results in the IFAT and were used as the negative reference in the validation study. Eighty serum samples with IFAT positive results were used as a positive reference. These sera were collected from cattle in Kuku area, Sudan, where the disease is known to be endemic (Elhaj and Hamid 2003).

IFAT

The procedures that were essentially followed in materials preparation and running of the test were described previously (FAO 1984). *T. annulata* schizont antigen slides were prepared from culture at low passage (< 20), which was initiated from infected blood from calf#382 which had been experimentally infected by application of infective *H. anatolicum* ticks collected from an endemic area in northern Sudan (EL Gahli and El Hussein 1995). The sera were serially twofold diluted from 1:10 to 1:10,240 in PBS and incubated for 30 min at 37°C after application onto slides. The slides were washed once quickly in PBS, followed by two washes in PBS of 15 min each on rocking platform. Fluorescein isothiocynate-conjugated rabbit anti-bovine total IgG (Nordic) was diluted 1:100 in PBS, added onto the slides, incubated for 30 min at 37°C and washed as described above. The slides were examined using a fluorescence microscope (Olympus BH-2, Japan).

ELISA

The recombinant *T. annulata* surface protein "*Ta*SP" (Schnittger el al. 2002) was used in the test. It was diluted (1 μg/ml) in carbonate–bicarbonate coating buffer and immobilized onto 96-well ELISA plates (Nunc, Denmark) by incubation for 1 h at 37°C. The plates were then washed and incubated for 1 h at 37°C with blocking buffer (PBS pH 7.2, supplemented with Tween-20 (0.05%) and skimmed milk (1%) (PBSTM). The plates were washed three times with PBS supplemented with Tween-20 (BPST). Sera were added at the dilution of 1:500 in PBSTM and incubated for 1 h at 37°C. All samples were tested in duplicates in the same plate. Plates were washed three times with PBST and the conjugate (rabbit-anti-bovine antibodies conjugated with horseradish peroxidase) diluted at 1:10,000 in PBSTM was added and the plates were again incubated for 1 h at 37°C. The plates were washed three times with PBST. Thereafter, a substrate/chromogene (hydrogen peroxide (H_2O_2)/tetramethyl benzidine (TMB) solution was added to each well. Color development was allowed for 20 min after which the reaction was stopped using 1 M phosphoric acid. Absorbance was measured at 450 nm using an ELISA reader (Labsystem, Multiskan, RC). Each plate contained one positive and one negative reference serum sample, and each one was applied into four wells of the plate. The median OD values of the control sera as well as the mean OD of the duplicate test sera were calculated and the results were expressed as a percentage positive (PP) value of the reference positive control.

Cut-off determination

The cut-off was determined for the *Ta*SP ELISA using the Gaussian method, the conventional ROC and TG-ROC analysis. The Computational Methods for Diagnostic Tests (CMDT) software (http://www1.vetmed.fu-berlin.de/~mgreiner/) was used to determine the cut-off using TG-ROC. In this method, the specificity and sensitivity were plotted against the cut-off and the point of intersection of the two curves was taken as the value of the cut-off where both sensitivity and specificity have equal values. Two alternative cut-off values, which were the lower and the upper limits of the intermediate range (IR) were defined at an accuracy level of 90 and 95%. In the Gaussian method, the

ELISA cut-off value was calculated as the mean plus two standard deviation of the negative reference sample. As a measure of the discriminatory power of the test, the area under the ROC (AUC) was calculated using both the non-parametric and parametric approaches. The former is based on the non-parametric Mann–Whitney test and the latter utilizes the parameters of symmetry B and separation A. Both parameters were estimated using iterative and maximum likelihood in ROCKit software. For further optimization of the determined cut-off using the TG-ROC approach, Youden's index, LR index and valid range proportion (VRP) were calculated. The cut-off was calculated for both the crude optical density (OD) values and for the percentage positivity (PP) values.

Results

The overlap between positive and negative reference samples was explored using scatter plot (Fig. 1) and the data were screened for normality using D–Kolmogorov–Smirnov test. The deviation from normality was indicated by both D–komolograv test and the TG-ROC software ($P > 0.05$). A descriptive statistical summary of reference samples used in the validation is shown in Table 1 and Fig. 2. The cut-off and relevant sensitivity and specificity of *Ta*SP derived using the Gaussian method and the non-parametric approach in the TG-ROC for both OD and PP values are shown in Table 2. In comparison to the ROC, a high cut-off (PP = 36.5) and specificity were obtained with the Gaussian method. However, modality in the reference samples lead to the violation of the assumption underlying the implementation of the Gaussian method.

The deviation of the data from normality was taken seriously and only the non-parametric and semi-parametric approaches were used for selection of the cut-off in the conventional ROC. High estimates of the AUC as calculated by the non-parametric and the semi-parametric approaches were obtained (data not shown). They indicate a good performance of *Ta*SP, provided that the AUC of a perfect test that fully discriminate between positive and negative samples equal one.

According to the Gaussian method the cut-off value using PP values was found to be 36.5 with 98.3 specificity and 92.5 sensitivity. Using the crude OD values the cut-off was found to be 0.53 with 98.3 specificity and 92.5 sensitivity.

According to TG-ROC, the cut-off was found to be 31.6 (Fig. 3) and both the sensitivity and the specificity were 93.5 using PP values (Table 3). The cut-off using OD values was found to be 0.46 and the specificity and sensitivity were 93.5. Since a probable deviation from normality was indicated by TG-ROC, only the non-parametric approach was considered in the cut-off determination. However, when the cut-off was determined in the conventional ROC using the ROCKit software, normality of the data was assumed.

The performance of the test as measured by the AUC of the ROC plots was 0.98 (Fig. 4). The VRP was approximately 1.0 (0.97) and the IR was four using the upper limit, 95th percentile of the PP of the infected population, and zero using the lower limit, 5th (Table 3). The efficiency of the test, measured by efficiency and Youden's index, which are displayed as functions of the cut-off value is shown in Fig. 5. An overlay of the parametric vectors Se^* and Sp^* to the observed cumulative frequency distributions (CFDs) is shown in Fig. 6 and the scatter plot of the non-parametric (Se and

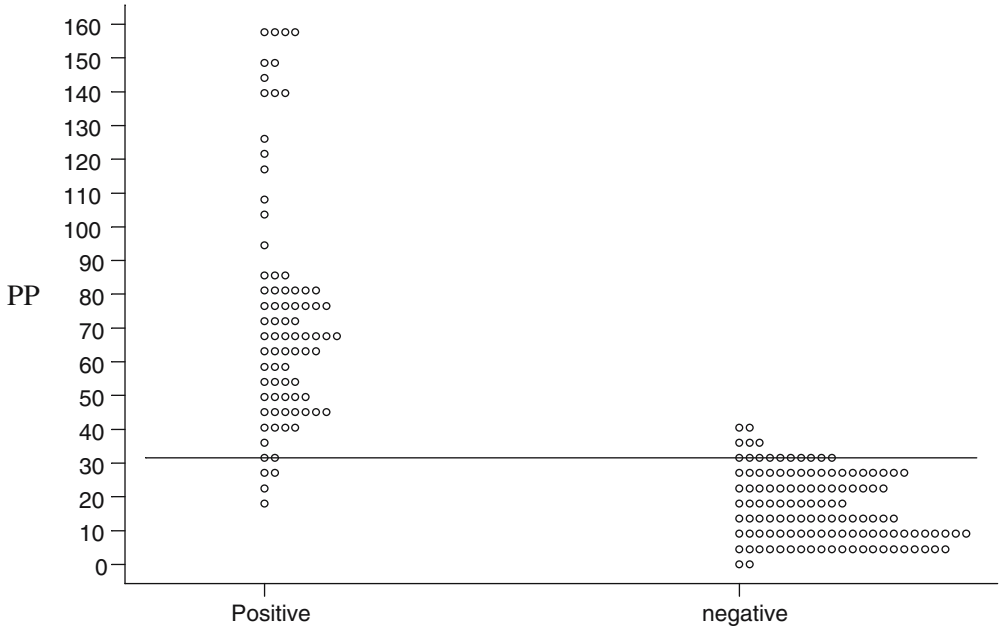

Fig. 1 Scatter plot of ELISA percentage positivity (*PP*) test results illustrating the overlap between positive and negative populations. A total of 80 and 120 sera obtained from animals exposed and unexposed to *T. annulata*, respectively, were tested

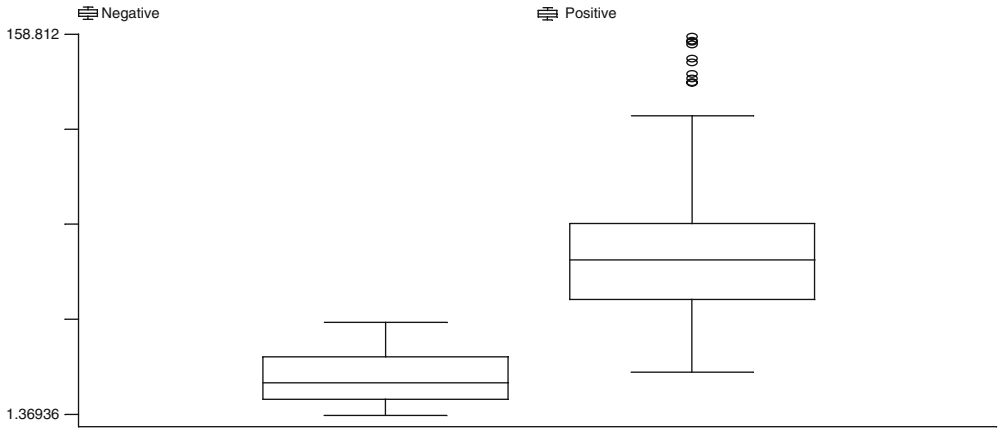

Fig. 2 Descriptive indices for the results of *Ta*SP ELISA percentage positivity (*PP values*) for positive and negative reference population of cattle. The *left graph* shows the frequency of PP for negative sera and *right graph* shows PP for positive sera, while the box and whisker reflect the dispersion of PP for negative and positive sera

Table 1 Descriptive indices for the results of *Ta*SP ELISA for infected and non-infected reference populations of cattle[a]

Measurement	Percent of positive cattle sera	
	Infected (80)	Non-infected (120)
Mean	74.38	16.76
Median	66.13	14.94
SD	35.42	9.85
Minimum	19.44	1.36
Maximum	158.81	40.07

[a] Results are expressed as percentage of an internal positive control. The number of animals in each group is indicated in *parentheses*

Table 2 Cut-off values determined by different methods with their corresponding sensitivity and specificity

Results	Gaussian method		TG-ROC	
	OD	PP	OD	PP
cut-off	0.53	36.5	0.46	31.6
Sensitivity	92.5	92.5	93.3	93.5
Specificity	98.3	98.3	93.3	93.5

Sp) versus the parametric (Se^* and Sp^*) parameters is demonstrated in Fig. 7.

Discussion

The main aim of this study was firstly to determine cut-off values by TG-ROC analysis. Secondly, to compare them with those obtained by previously used methods and lastly to evaluate the performance of *Ta*SP ELISA for the diagnosis of *T. annulata* infection in the cattle naturally infected under Sudanese field conditions.

There is a number of critical points that have to be put into consideration when it is planned to establish a cut-off value. The selection of a cut-off value is not an estimation in the statistical sense (for example, no confidence interval is given for the cut-off). However,

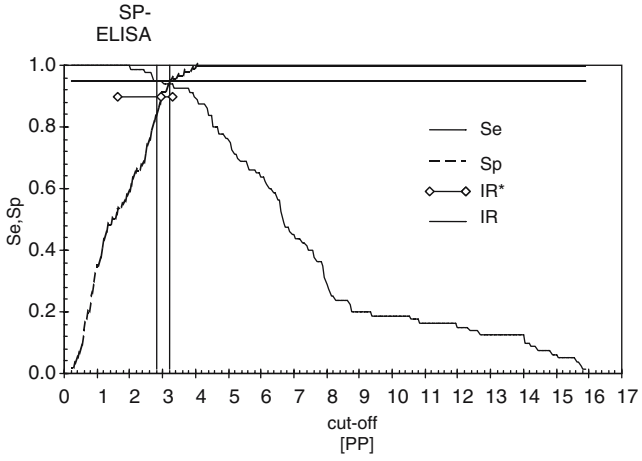

Fig. 3 TG-ROC analysis of the SP. *Ta* ELISA results for cattle sera naturally infected with *T. annulata*. The intermediate range (*IR*) is determined by the cut-off values at 95% sensitivity (*Se*) and 95% specificity (*Sp*). * The accuracy level is shown as *horizontal line*

similar considerations as in parameter estimation apply. The sample size has to be large enough to minimize the stochastic uncertainty in the cut-off selection (Metz 1998). Moreover, the reference population has to be representative of the target population (Greiner et al. 1994). We conducted this study with the minimal number of positive and negative samples required for a meaningful analysis. The latter precondition is very

Table 3 Analysis of the TG-ROC results at 95 and 90% confidence interval (CI)

Measure	Non-parametric	
	95% CI	90% CI
Theta 0	0.935	0.935
D_0	32 (29, 36)	32 (29, 36)
IR	4 (0, 19)	0
Upper limit	32 (30, 93)	30 (28, 32)
Lower limit	28 (19, 39)	39 (26, 43)
VRP	0.973	1.0

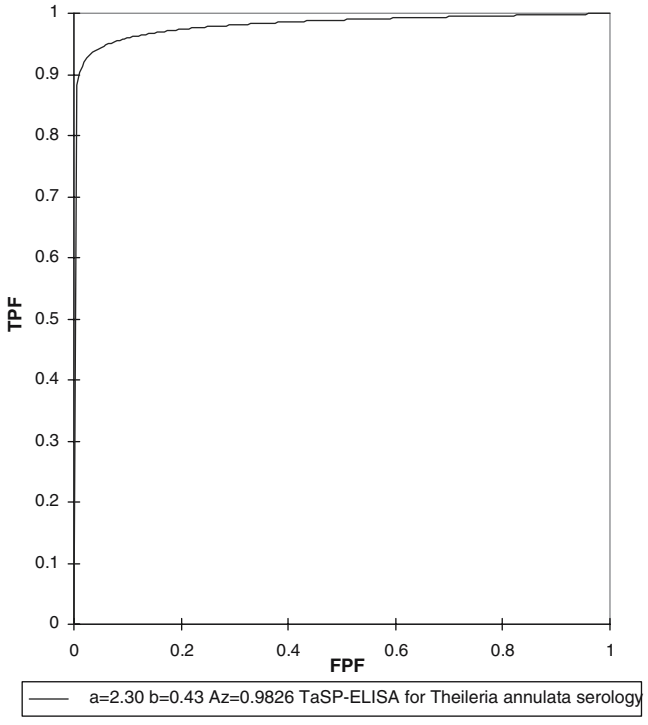

Fig. 4 Conventional binormal ROC curves

difficult to realize for ELISA tests designed for the screening of tropical infectious disease. Control sera from animals in regions where tropical theileriosis is not endemic are guaranteed to be disease free but might not be representative of the target population. On the other hand, negative sera from animals in regions where tropical theileriosis is endemic cannot be guaranteed to be disease free (Voller et al. 1977).

Central to any serological assay is the determination of the diagnostic cut-off value. The establishment of a reliable cut-off value is essential for a serological test to be useful in differentiating infected from non-infected animals (Mboloi et al. 1999). The assembly of the reference population used for the calculation of a cut-off value is a critical procedure. Jacobson (1996) suggested the use of at least 300 known positive and 1000 known negative samples in order to compensate for various factors that may influence the diagnostic sensitivity and specificity. These numbers are very difficult to obtain under practical conditions. The selection of IFA test positive and IFA negative samples as positive and negative controls, respectively, follows the logic of assuming IFA test as reference test (Ilhan et al. 1998). However, control sera from cattle exposed to *T. annulata* infection are guaranteed to have antibodies against *T. annulata*, and thus represent the target positive populations. The use of IFA test as a reference and gold standard test supported this assumption.

By using the Gaussian method (Wright et al. 1993), the cut-off was set at 36.5 and 0.53 using the PP values and crude OD values, respectively. This method is assumed to be leading to a specificity of 97.5% (Barajas-Rojias et al. 1993); however, this assumption is true only for normally distributed test variables (Greiner et al. 1994), since the ELISA results in the present study were

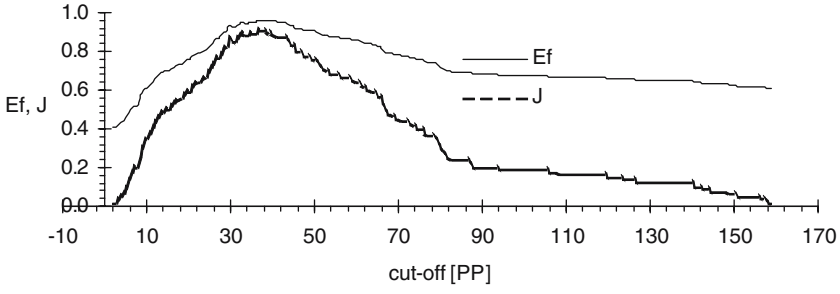

Fig. 5 Youden's index (*J*) and efficiency (*Ef*) of the SP. *Ta* ELISA as a function of the selected cut-off value for cattle sera naturally infected with *T. annulata*. (Ef and J are summary measures for Se and Sp; the latter being independent on prevalence)

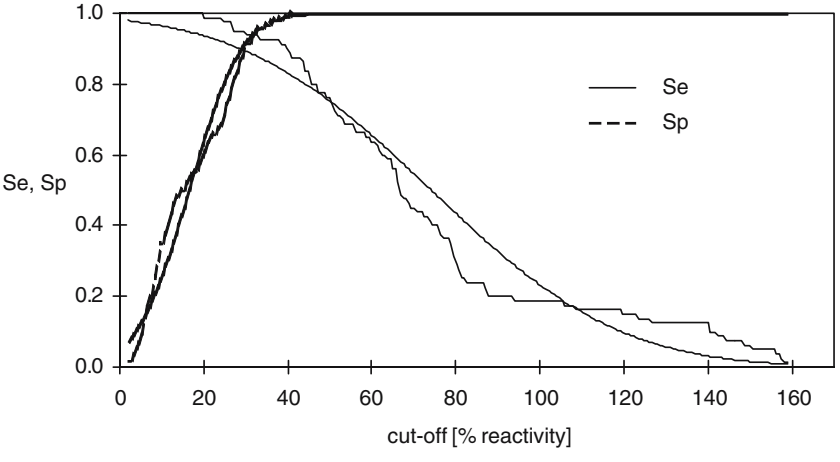

Fig. 6 The observed non-parametric values, Se and Sp, overlaid with the parametric estimates Se^{*} and Sp^{*}

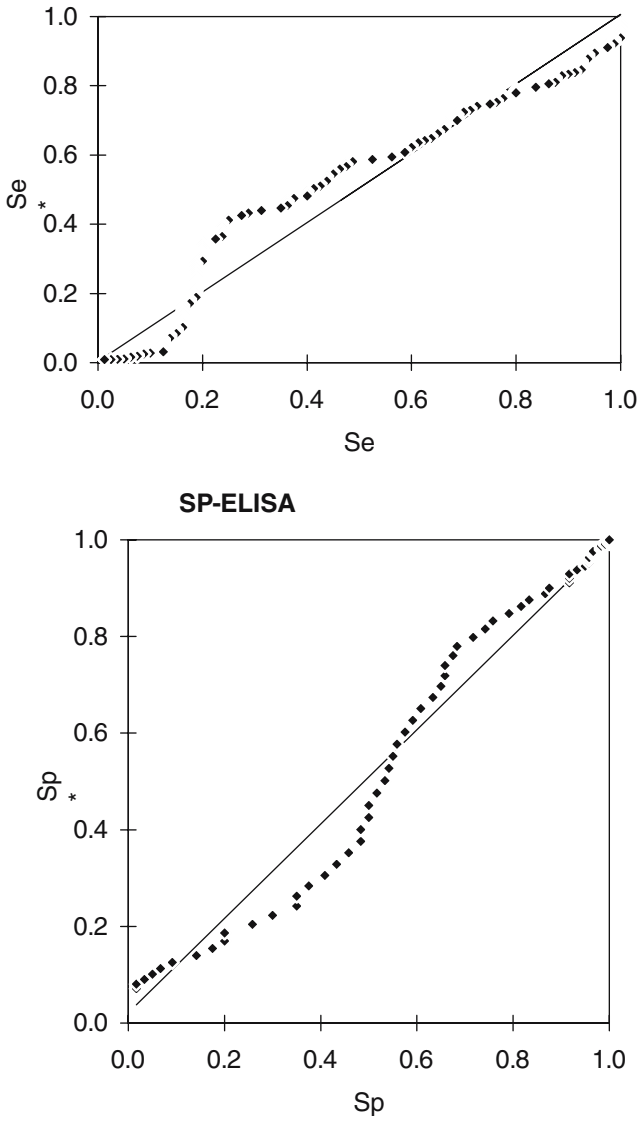

Fig. 7 Linear correlation analysis of Se against Se* (*upper*) and Sp against Sp* (*lower*)

tested for normality and showed significant skewness ($P < 0.05$). Thus, this method was not considered for further investigations. A new approach to define test cut-off values and performance has been proposed (Greiner 1995). The new approach utilizes the conventional ROC principle modified in such a way that the test sensitivity and specificity can be read directly from these plots. The modified ROC plot is known as a TG-ROC. By using this method the cut-off was set at 31.6 and 0.46 using the PP values and OD values, respectively, and both sensitivity and specificity were 93.5. On the basis of the OIE recommendation (Norval 1992), the cut-off obtained by this method using PP only was considered to be suitable for field investigations.

Diagnostic performance was evaluated by assessing the area under the ROC (AUC) and VRP, which were found to be 0.98 and 0.973, respectively. The closer to 1 the indices are, the better the test performance is (Mboloi et al. 1999). In this study, the VRP was 0.973 (Table 3), indicating a high performance of this ELISA in identifying the animals according to their true health state. Moreover, the AUC is a quantitative, descriptive expression of how close the ROC curve is to the perfect one (AUC = 1.0) (Greiner et al. 1995). The *Ta*SP ELISA showed a high performance because the index AUC was 0.98 (Fig. 4). The IR, which defines results that are neither positive nor negative, was zero using 90% accuracy level (AL) and four using 95% AL. In an ideal test, the AUC and VRP would be 1.0 and the IR would be zero (Greiner et al. 2000). In this study, these parameters were close to those of an ideal test. However, the IR used to describe non-positive and non-negative test results was 4, in cases where the AL is 95%, because the lower limit of the IR is greater than the upper limit. In this study, the IR using 90% accuracy level was zero; hence, over 90% of cattle were correctly diagnosed. The interpretation of intermediate test results depends on the specific diagnostic purpose of the test (Mboloi et al. 1999). Because of the ambiguity of borderline results, it is appropriate to consider only one cut-off value and indicate the test parameters (Se, Sp and LR) for a given cut-off value selected for an epidemiological situation (Greiner et al. 2000). In clinical diagnosis, the values that fall between the IR limit would require testing by a confirmatory assay or re-testing for the detection of seroconversion (Kiltz et al. 1986; Simel et al. 1987).

Previously, the cut-off value using TG-ROC for Tams1 ELISA was set at 10.6 PP leading to a sensitivity and specificity of 87% with an IR of between 10.2 and 11.8 PP using a 95% accuracy level (Gubbels et al. 2000). This cut-off was determined for sera collected from experimentally infected calves with IFA test positive sera before 3 months of post-inoculation. In the second group (IFA test positive sera after 3 months of post-inoculation) the cut-off was set at 12.7 PP leading to 100% sensitivity and specificity (Gubbels et al. 2000). There is a large disagreement between these results and the results obtained in the present study. This disagreement may be mainly due to the sera used in each study, whereas in the previous study they used experimental sera, which sometimes produce overoptimistic estimate of accuracy (Mboloi et al. 1999). In the present study sera collected from the field were used. In addition, the antigens tested in the two studies were recombinant proteins, although they had originated from different stages of parasite life cycle. Gubbels et al. (2000) tested Tams1 antigen, which was derived from the merozoite, whereas in the present study we tested *Ta*SP antigen, which was derived from the surface protein of macroschizont stage (Schnittger et al. 2002).

Further validation of the test precision needs to be done according to the ISO 5725-1986 international procedure and authenticated by interlaboratory comparisons of the ELISA results. In this study, we have attempted to calculate cut-off values for *Ta*SP ELISA by using sera from cattle exposed and unexposed to *T. annulata* under field conditions using IFA test as a ref-

erence. Moreover, a study on the determination of the cut-off for *Ta*SP ELISA in the absence of gold standard is going on in order to further validate the *Ta*SP ELISA. It will be of great interest to repeat this study using known positive and negative experimental sera to check whether the cut-off value obtained from experimental sera is applicable to the situation in the field.

Acknowledgements This research was partially supported by the International Atomic Energy Agency (IAEA) through the Project No. SUD/05/027 entitled "Control of Ticks and Tick-borne Diseases". Additional support was provided by the European Union (INCO-Dev programme, contract number ICA4-CT-2000-300028). An additional support was provided by the ICTTD Concerted Action Project on Integrated Control of Ticks and Tick-borne Diseases, supported by the INCO-DC Program of the European Union under contract no. IC18-CT95-0009. We thank Dr. R. Hashemi-Fesharki, Head Department of Protozoology and Protozoa Vaccine, Razzi Institute, Iran for the kind provision of the positive control serum and Dr. E. D. M. ELAmin, CVRL, Sudan for his critical review of the manuscript. Our thanks are also extended to Dr. M. Greiner for his valuable suggestions and comments. This work is published by kind permission of the Director-General Animal Resources Research Corporation.

References

Bakheit MA, Schnittger L, Salih DA, Boguslawski K, Beyer D, Fadl M, Ahmed JS (2004) Application of the recombinant *Theileria annulata* surface protein in an indirect ELISA for the diagnosis of tropical theileriosis. Parasitol Res 92:299–302

Barajas-Rojas JA, Riemann HP, Franti CE (1993) Notes about determining the cut-off value in enzyme linked immunosorbent assay (ELISA). Prev Vet Med 15:231–233

Boulter NR, Brown CGD, Kirvar E, Glass E, Campbell J, Morzaria S, Nene V, Musoke A, d'Oliveira C, Gubbels MJ, Jongejan F, Hall R (1998) Different vaccine strategies used to protect against *Theileria annulata*. Ann NY Acad Sci 849:234–246

Brown CGD (1990) Control of tropical theileriosis (*Theileria annulata* infection of cattle). Parasitologia 32:23–31

Dolan TT (1989) Theileriosis: a comprehensive review. Rev Sci Tech Off Int Epiz 8:11–36

d'Oliveira C, van der Weide M, Habela MA, Jacquiet P, Jongejan F (1995) Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. J Clin Microbiol 13:2665–2669

de Kok JB, d'Oliveria C, Jongejan F (1993) Detection of the protozoan parasite *Theileria annulata* in *Hyalomma* ticks by the polymerase chain reaction. Exp Appl Acarol 17:839–846

El Ghali AA, ElHussein AM (1995) Diseases of livestock in EdDamer Province, El Nile State, Sudan: a Two-year retrospective study. Sud J Vet Anim Husb 34:37–45

Elhaj MT, Hamid ME (2003) Haematological and serum chemistry studies on crossbred calves infected with *Theileria* species. Sud J Vet Sci Anim Husb 42:128–140

FAO (1984) Tick and tick-borne diseases control. A practical field manual, vol II. Food and Agriculture Organization of the United Nations, Rome

Galen RS (1986) Use of predictive value theory in clinical immunology. In: Rose NR, Friedman H, Fahey JL (eds) Manual of clinical laboratory immunology, 3rd edn. American Society for Microbiology, Washington, DC, pp 966–970

Greiner M (1995) Two-graph receiver-operating characteristic (TG-ROC): a Microsoft-EXCEL template for the selection of cut-off values in diagnostic tests. J Immunol Methods 185:145–146

Greiner M, Franke CR, Bohning D, Schlattman P (1994) Construction of an intrinsic cut-off value for the sero-epidemiological study of *Trypanosoma evansi* infections in a canine population in Brazil: a new approach towards an unbiased estimation of prevalence. Acta Trop 56:97–109

Greiner M, Sohr D, Gobel P (1995) A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. J Immunol Methods 185:123–132

Greiner M, Pfeiffer D, Smith RD (2000) Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. Prev Vet Med 45:23–41

Gubbels JM, d'Oliveira C, Jongejan F (2000) Development of an indirect Tams1 enzyme-linked immunosorbent assay for diagnosis of *T. annulata* infection in cattle. Clin Diag Lab Immunol 3:404–411

Ilhan T, Williamson S, Kirvar E, Shiels B, Brown CGD (1998) *Theileria annulata:* carrier and immunity. Ann NY Acad Sci 849:109–125

Jacobson R (1996) Principles of validation of diagnostic assays for infectious diseases. In: Office international des epizooties standards commission (ed) Manual of standards for diagnostic tests and vaccines, 3rd edn. Office international des epizooties paris, France, pp 8–15

Kiltz HH, Uilenberg G, Franssen FFJ, Perié NM (1986) *Theileria orientalis* occurs in Central Africa. Res Vet Sci 40:197–200

Manuja A, Nichani AK, Kumar R, Rakha NK, Kumar B, Sharma KD (2000) Comparison of cellular schizont, soluble schizont and soluble piroplasm antigens in ELISA for detecting antibodies against *Theileria annulata*. Vet Parasitol 87:93–101

Martin SW (1984) Estimating disease prevalence and the interpretation of screening test results. Prev Vet Med 2:463–472

Mboloi MM, Cornelis PJ, Kruitwagen C, Greiner M, Jongejan F (1999) Validation of the indirect MAP1-B enzyme-linked immunosorbent assay for diagnosis of experimental *Cowdria ruminantium* infection in small ruminants. Clin Diag Lab Immun 6:66–72

Metz CE (1998) ROCKIT 0.9B. Beta Version. www-radiology.uchicago. edu/krl/toppage11.htm

Norval RA (1992) In: Perry BD, Young AS (eds) The epidemiology of Theileriosis in Africa. Academic Press, NY, 481pp

Pipano E, Cahana M (1969) Fluorescent antibody test for the serodiagnosis of *Theileria annulata*. J Parasitol 55:765

Schnittger L, Katzer F, Biermann R, Shayan P, Boguslawski K, Mckellar S, Beyer D, Shiels BR, Ahmed JS (2002) Characterization of a polymorphic *Theileria annulata* surface protein (*Ta*SP) closely related to PIM of *Theileria parva*: implications for use in diagnostic tests and subunit vaccines. Mol Biochem Parasitol 120:247–256

Simel DL, Feussner JR, DeLong ER, Matchar DB (1987) Intermediate, indeterminate, and uninterpretable diagnostic test results. Med Decis Making 7:107–114

Smith RD (1991) Evaluation of diagnostic test. In: Smith RD (ed) Veterinary clinical epidemiology. Butterworth-Heinemann, Stoneham MA, pp 29–43

Voller A, Bidwell DE, Bartlett A, Edwards R (1977) A comparison of isotopic and enzyme-immunoassays for tropical parasitic disease. Trans R Soc Trop Med Hyg 71:431–437

Williamson S, Tait A, Brown D, Walker A, Beck P, Shiels B, Fletcher J, Hall R (1989) *Theileria annulata* sporozoite surface antigen expressed in *Escherichia coli* elicits neutralizing antibody. Proc Natl Acad Sci USA 86:4639–4643

Wright PF, Nilsson E, Van Rooij EMA, Lelenta M, Jeggo MH (1993) Standardization and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev Sci Tech Off Int Epiz 12:435–450

Youden D (1950) Index for rating diagnostic tests. Cancer 3:32–35

Zweig MH, Campbell G (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 39:561–577