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Short communication

Evaluation of indirect TaSP enzyme-linked immunosorbent assay for diagnosis of tropical theileriosis in cattle (*Bos indicus*) and water buffaloes (*Bubalus bubalis*) in Egypt

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ABSTRACT

The aim of the present study was to evaluate the validity of *Theileria annulata* surface protein (TaSP)-ELISA, in comparison with traditional microscopic test, for the diagnosis of *T. annulata* infection among Egyptian baladi cattle (*Bos taurus*) and water buffaloes (*Bubalus bubalis*). Molecular confirmation of infection using *T. annulata* merozoite surface (Tams-1) target amplification by PCR was used as a gold standard. A total of 76 clinically suspected animals including 64 baladi cattle and 12 water buffaloes were investigated in the current study by the three methods. Based on the PCR-confirmed results, the evaluation study revealed higher sensitivity of TaSP-ELISA (72.9% and 75%) as compared to microscopic examination (58.3% and 50%) among cattle and buffaloes, respectively. On the other hand, the specificity of TaSP-ELISA in diagnosis of *T. annulata* infection was higher (87.5%) in baladi cattle as compared to water buffaloes (37.5%). In conclusion, TaSP-ELISA was shown to be suitable for the diagnosis of *T. annulata* infection in cattle under field conditions.

Tropical theileriosis (*Theileria annulata* infection, also known as Mediterranean fever) is among the tick-borne diseases that cause losses in the productivity as well as fatalities in bovine breeds (Dolan, 1989). In Egypt, the disease is one of the most destructive obstacles to live stock production, infecting both cattle and water buffaloes (Nagaty, 1947; Ezzat, 1960; EL-Deeb and Younis, 2009). While, Egyptian cattle act as reservoir hosts for *T. annulata*, water buffaloes (*Bubalus bubalis*) are reported as relatively more resistant to *Theileria* species infection as compared to cattle (Ezzat, 1960; Osman and Al-Gaabary, 2007). Previous studies on cattle have shown that long-lasting carrier status is a common finding in the field among recovered animals. These carrier animals have an important role in the transmission of the infection by *Hyaloma* ticks (D'Oliveira et al., 1995).

Acute *T. annulata* infection is traditionally diagnosed by the demonstration of schizont-infected cells in superficial lymph nodes draining the site of tick bite, also by observation of typical piroplasms in peripheral red blood cells (Anon., 1997). In recovered carrier animals, the piroplasm is the only stage that can be demonstrated, often with great difficulty (Kirvar et al., 2000).

Serological assays are best suitable for the diagnosis in later phases of the disease and in carrier animals in which the antibody titers are usually higher and the piroplasm parasitemia drops to microscopically undetectable levels (Bakheit et al., 2004). Recently, *T. annulata* surface protein (TaSP) has been characterized and its application in

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indirect ELISA has been documented and validated in cattle (Schnittger et al., 2002; Bakheit et al., 2004; Salih et al., 2007). TaSP is present as a single copy within the parasite genome and transcribed in the sporozoite and schizont stage. Sequence analysis of TaSP showed high identity with the corresponding *T. annulata* surface protein from different geographical locations (Bakheit et al., 2004).

The application of PCR for molecular diagnosis of bovine theileriosis has shown recent advances (D'Oliveira et al., 1995). Tams-1 (a 30 kDa protein) is the most abundant and immunodominant antigen on the surface of merozoites and piroplasms of *T. annulata* (Shiels et al., 2000). Tams-1 PCR assay has been documented by several studies as the most preferred method for detection of *Theileria* species in epidemiological studies. It shows superior sensitivity and specificity in diagnosing *T. annulata* infection under field conditions over both microscopic and serological methods. As compared to serological assays, PCR-based molecular assays can differentiate between true positive cases and those cases with false positive results due to cross-reaction or old infection (Martin-Sanchez et al., 1999; Aktas et al., 2002; Vantansever and Nalbantoglu, 2002).

The validity of the TaSP-ELISA, as compared to microscopic examination method, for the diagnosis of tropical theileriosis among Egyptian baladi cattle and water buffaloes under field conditions was evaluated. Tams-1 PCR assay was used for molecular confirmation of *T. annulata* among suspected investigated animals as the gold standard test. The validation parameters included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and combined predictive value (CPV) that were calculated using previously established and described formulas (Thrusfield, 2005).

A total of 76 animals, suspected of being infected with *T. annulata* were selected from different localities in EL-Fayoum, EL-Minia, Assuit, Sohage and EL-Wady EL-Gaded governorates in Upper Egypt during the period of May to August 2008. Investigated animals were selected after clinical examination and included 64 baladi cattle (*Bos taurus*) and 12 water buffaloes (*B. bubalis*). The inclusion criteria included those animals with different degrees of tick infestation with or without suspected signs of acute or chronic forms of tropical theileriosis. Direct blood sample was collected from the ear vein of investigated animals for preparation of blood films. Two whole blood samples were collected from the jugular vein of each investigated animals with and without sodium EDTA anticoagulant for molecular and serological assays, respectively. In addition, lymph node aspiration was also collected from suspected animals and was used for preparation of lymph node impression smears for microscopical diagnosis, and other part was kept for molecular diagnostic assay. Collected blood, lymph and serum samples were stored at −20 °C till time of use. Thin blood films and lymph node impression smears were prepared immediately after sampling and were stained with Giemsa stain as previously described (Charles, 2002). Four thin blood films and 4 lymph node impression smears were examined for each animal for detection of suspected piroplasms and/or trophozoite stages and macroschizonts and/or microschizonts stages of *Theileria* species, respectively.

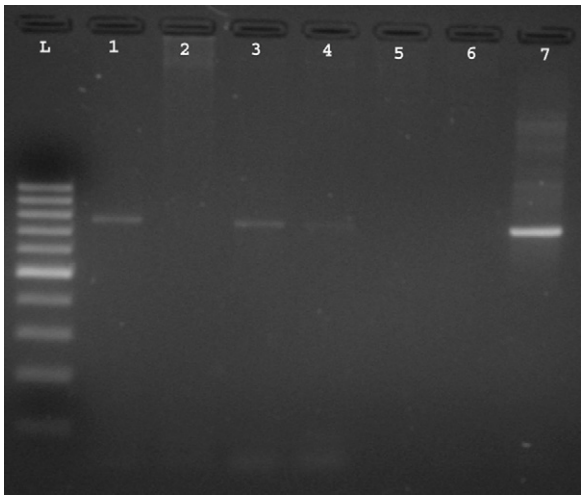

Fig. 1. Representative gel showing amplification of Tams-1 target from suspected *T. annulata* infection isolates. Lane L: 100 bp. DNA ladder: lanes 1, 3, 4 and 7: positive isolates yielded 785 bp. product; lanes 2 and 5: negative isolates; lane 6: negative control.

Serological diagnosis was carried out using TaSP-based indirect ELISA kit (Research Center Department, Borstel, Germany) as recommended by the manufacturer and as previously described (Bakheit et al., 2004). The samples were set in duplicate, with the mean value being used for recording and calculations. The absorbance at 450 nm was measured using an ELISA reader Stat fax® 2100 reader (Awareness, Technology Inc., Palm City, FL). Results were expressed as percent positivity (PP), which was calculated as the mean OD values of duplicate tested samples multiplied by 100 and divided by the mean OD values of the four replicates of the positive control. The cut off was set at 44 PP with specificity and sensitivity of 93.5% as recommended by manufacture using the TWO-ROC software (Salih et al., 2005).

Tams-1-based PCR assay was carried out for the molecular detection of *T. annulata* from blood samples lymph node specimens of all suspected animals using primer set Tams1F (ATGCTGCAAATGAGGAT) and Tspm1R (GGACTGATGAGAAGACGA TGAG) to amplify a 785 base-pair fragment of the Tams-1 gene as previously described (Kirvar et al., 2000).

Current results revealed that Tams-1 PCR assay was able to detect specific DNA target of *T. annulata* (Fig. 1) and to confirm the infection in 48 (75%) and 4 (33.3%) cases out of the 64 and 12 clinically suspected baladi cattle and water buffaloes, respectively. Out of these, 28 and 35 baladi cattle, and 2 and 3 water buffaloes were diagnosed positive by microscopic examination and TaSP-ELISA, respectively. Only 15 baladi cattle and one water buffalo were found positive by both microscopic examination and TaSP-ELISA. On the other hand, out of the PCR-confirmed negative cases, 2 baladi cattle and 5 water buffaloes were found positive for *T. annulata* infection by TaSP-ELISA (Table 1).

Based on the PCR results, microscopical examination of Giemsa stained blood and lymph node impression smears was found to have high specificity (100%) for the detection of *T. annulata* infection among both baladi cattle and

Table 1
Results obtained using all three diagnostic techniques: microscopic examination, TaSP-ELISA and Tams-1 PCR.

	Tams-1 PCR$^+$		Tams-1 PCR$^-$		Total	
	Cattle	Buffaloes	Cattle	Buffaloes	Cattle	Buffaloes
Mic.$^+$ TaSP$^+$	15	1	0	0	15	1
Mic.$^+$ TaSP$^-$	13	1	0	0	13	1
Mic.$^-$ TaSP$^+$	20	2	2	5	22	7
Mic.$^-$ TaSP$^-$	0	0	14	3	14	3
Total	48	4	16	8	64	12

water buffaloes. On the other hand, low sensitivity (58.3% and 50%) was recorded among baladi cattle and water buffaloes, respectively (Table 2). This could be attributed to the low parasitemia or chronic cases of the disease. Using conventional microscopic method for diagnosis of tropical theileriosis, although cheap and simple, is limited to the detection of acute cases especially with high level of parasitemia. The test was found less sensitive during the chronic form of the disease or in case of carrier animals (Martin-Sanchez et al., 1999; Tabidi et al., 2006). Therefore, microscopic examination is recommend during acute stages when the level of the parasitemia is high enough where trophozoites can be detected in Giemsa stained thin blood films and/or schizonts can be detected in Giemsa stained lymph smears (Jabbar et al., 1997; Tabidi et al., 2006).

The evaluation study of the TaSP-ELISA revealed that ELISA, as compared to microscopic examination, has higher sensitivity (72.9% and 75%) for diagnosis of tropical theiriasis in both baladi cattle and water buffaloes, respectively (Table 2). The currently recorded sensitivity of TaSP-ELISA, although higher than microscopic examination method, it is considered relatively low when compared to its reported sensitivity (99.1%) during previous evaluation study in Sudan (Bakheit et al., 2004). Given the superior sensitivity of Tams-1 PCR assay over IFAT (Martin-Sanchez et al., 1999), the different evaluation values could be attributed to the different comparison standards used in the current study as compared to the previous one, where PCR and IFAT, respectively, were used as reference tests. In general the relatively low sensitivity could be attributed to the inability of the test to detect the acute infection in cattle due to low titer of specific-antibodies (Salih et al., 2007).

On the other hand, lower specificity (87.3% and 37.5%) was shown by the TaSP-ELISA among baladi cattle and water buffaloes, respectively, as compared to microscopic examination (Table 2). This could be attributed to the cross-reaction possibility with other closely related blood parasites as described with other serological assays (Bakheit et al., 2004; Salih et al., 2007). The apparent variation in the specificity of TaSP-ELISA for detection of tropical theileriosis among buffaloes (37.5%) and cattle (87.5%) could be attributed to the low number of buffaloes involved in the current study as compared to that of cattle. However, another possible explanation could be the fact that the protozoan parasite has low affinity to peripheral blood mononuclear cells of water buffaloes, which were reported as relatively more resistant to *Theileria* species infection as compared to cattle (Ezzat, 1960; Osman and Al-Gaabary, 2007). The low capacity of *Theileria* spp. to establish infections in water buffalo compared with cattle could be explained in the light of previous studies on African buffaloes, which attributed this to the lower frequency of buffalo PBM cells, which undergo transformation into continuously replicating cell lines after infection (Stagg et al., 1983; Baldwin et al., 1986). Interestingly, higher proportion of piroplasms-infected peripheral RBC versus schizont-infected lymph node cells was noticed in cattle as compared to water buffaloes in the current study. This would support the up mentioned attribution. However, this assumption could not be confirmed due to the low numbers of currently investigated water buffaloes. Another possible explanation could be that water buffaloes can resist the infection through innate factors and cell mediated immune response (Ahmed et al., 2008). Therefore, water buffaloes, the more resistant host, may have low or no parasitemia with subsequent negative PCR, however, the high titer of persistent antibodies or the presence of other cross-reactive antibodies results in positive ELISA. This could explain the relatively higher sensitivity and lower specificity of the TaSP-ELISA in buffaloes.

Table 2
Evaluation of TaSP-ELISA in comparison with microscopic examination for diagnosis of tropical theileriosis in cattle and buffaloes based on the PCR-confirmed results.

Suspected animals	Diagnostic methods	Test results				Validity parameters (%)				
		TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV	CPV
Cattle (64)	Microscopic examination	28	16	0	20	58.3	100	100	44.4	68.8
	TaSP-ELISA	35	14	2	13	72.9	87.5	94.6	51.9	76.6
Buffaloes (12)	Microscopic examination	2	8	0	2	50	100	100	80	83.3
	TaSP-ELISA	3	3	5	1	75	37.5	37.5	75	50

TP and FP, true and false positive; TN and FN, true and false negative; PPV, NPV and CPV, positive, negative and combined predictive value.

The predictive values usually give an indication about the disease's probability in population during further investigation. In the current study, TaSP-ELISA revealed a relatively high PPV (94.6%) in baladi cattle that is comparable to that (100%) of microscopic examination. However, a considerably low PPV (37.5%) was revealed by TaSP-ELISA in water buffaloes (Table 2). These findings indicate that during further investigations of the tropical theileriosis, the ability of TaSP-ELISA to correctly identify true positive cases as being positive is high in baladi cattle and low in water buffaloes. On the other hand, the NPVs of TaSP-ELISA as revealed in the current study showed lower NPV (51.9%) in baladi cattle as compared to water buffaloes (75%), which were comparable to values of microscopic examination (44% and 80%, respectively) (Table 2). These results indicate lower ability of TaSP-ELISA to detect negative cases as being negative in baladi cattle as compared to water buffaloes during further investigation of the disease. The combined predictive values as recorded in the current study revealed relatively higher CPV (76.6%) of TaSP-ELISA as compared to microscopic examination (68.8%) for diagnosis of tropical theileriosis in cattle. Moreover, the CPV of TaSP-ELISA in baladi cattle was considerably higher than the CPV of the same test in water buffaloes (50%). However, higher CPV (83.3%) was recorded for microscopic examination in water buffaloes as compared to baladi cattle.

In conclusion, the results of the current study would recommend the use of TaSP-ELISA for epidemiological survey of tropical theileriosis in baladi cattle under field conditions. However, day to day examination for detection of new cases, where the titer of the specific antibodies is still low, is not recommended to avoid possible false negative results. Alternatively, microscopic examination of blood and/or lymph node smears is recommended here. On the other hand, although the current results indicated the unreliability of TaSP-ELISA for epidemiological survey of *T. annulata* in water buffaloes, the low number of water buffaloes used in the current study do not allow for proper validation of TaSP-ELISA in water buffaloes. Therefore, further investigation using larger numbers of suspected cases of water buffaloes need to be considered in future work.

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