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Molecular and Phylogenetic Analysis of the Partial *Tams1* Gene Sequence of a Vaccine Strain of *Theileria annulata*

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ABSTRACT

The polypeptide Tams1 is an immunodominant major merozoite piroplasm surface antigen of the protozoan parasite Theileria annulata *and is highly variable. In this study, the partial nucleotide (nt) sequence of the Tams1 (522 nt) gene of Iranian vaccine strain (Vaccine-ir68) recovered from an outbreak of disease in Iran was determined and compared with the corresponding sequences of eighteen previously published Tams1 genes. According to sequencing result, a novel amino acid substitution at the Tams1 region (K→Q) was found exclusively in isolate Vaccine-ir68. Genetic distance values, estimated from the sequence data, revealed striking sequence homology (approximately 99%) between Vaccine-ir68 isolate and Tunisian isolates, showing that they were same isolates of* T. annulata *which were spread in these areas. The phylogenetic tree constructed based on the sequence alignment of 19 Tams1 coding regions was distinctly divided into five lineages. There might be some unknown tick carrier birds immigrating to the different geographical regions. These birds have an effective role to distribute the* T. annulata *species in North Africa, Palestine and Iran.*

Key words: *Tams1*, *Theileria Annulata*, merozoite surface antigen, phylogenetic

INTRODUCTION

Tropical theileriosis is a debilitating disease of cattle caused by the protozoan parasite *Theileria annulata*. The parasite is transmitted by the ticks of the genus *Hyalomma* which exist in many countries across the northern Africa and southern Europe, extending through the Middle East, India, and southern Russia to China (Jensen et al., 2008; Kotti et al., 2001). The disease has a wide geographical distribution and is endemic in the Mediterranean basin. Theileriosis is the most economically important cattle disease in Iran, causing major losses in livestock production. It is a serious constraint to the cattle breeding programs designed to increase the milk and meat production in the Iran (Hashemi-Fesharki 1990). The mortality rate varies from 90% in introduced exotic breeds to 5% or less in indigenous breeds (Neitz 1957). It has been reported that the rate of mortality in Iranian pure and crossbred cattle reached 40 and 80%, respectively (Hashemi-Fesharki 1988).

Tams1, a well-characterised antigen of *T. annulata*, is a member of the major merozoite piroplasm surface antigen (mMPSA) family of polypeptides that have been identified on all the *Theileria* species studied to-date (Katzer et al., 2002). *Tams1* coding region encodes the *Tams1* polypeptide with a variable molecular mass of between 30 to 32 kDa (Katzer 1998). Generation and selection of divergent antigenic types has

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implications for the inclusion of the *Tams1* antigen in a subunit recombinant vaccine or use in the development of a diagnostic ELISA (Gubbels et al., 2000). d'Oliveira reported the use of PCR for sensitive and specific amplification of *T. annulata* DNA from blood samples obtained from carrier cattle in 1995 (Shiels et al., 1994; d'Oliveira et al., 1995). The advent of PCR technology followed by sequencing has greatly allowed the development of sensitive diagnostic assays and phylogenetic studies for *T. annulata* (Bishop 1992).

The present work was undertaken with an objective of studying the genetic relationship of Iranian vaccine strain (Vaccine-ir68) with other isolates from different parts of the world, based upon the incomplete sequence of *Tams1* gene. In addition, molecular analysis of partial *Tams1* region of Vaccine-ir68 isolate was also performed.

MATERIAL AND METHODS

Organism isolation and vaccine preparation

A virulent strain of *T. annulata* isolated locally from an outbreak in Iran-Tehran during 1968 was used to prepare an appropriate vaccine. The culture medium was made up of a mixture of Eagle's balanced salt solution and yeast extract lactalbuminhydrolysate (YLH; 20%). Schizont-infected lymphoid cells, obtained originally from the prescapular gland of an infected calf by the biopsy technique, were inoculated in the culture medium. These lymphoid cells were then successively subcultured at 4-day intervals in the form of suspension and were kept at 37°C in laboratory incubator. In the cultures, almost all of the lymphoid cells were infected with the schizonts. Large Roux flasks containing 80 to 100 ml of medium and 6 to 7 ml of suspension culture (4.2 to $4.9*10^6$ Schizont-infected lymphoid cells) were used in order to prepare the anti-theileriosis vaccine. After cells had completed the growth, the material was harvested and concentrated to 1.5-$2.0*10^6$ infected cells. The following step was performed by the addition of Glycerol (10%) and the final suspension (15 ml) was distributed into small 20-ml vials. The vials were transferred to a freezer maintained at -70 °C until required (Hashemi-Fesharki and Shad-Del, 1973). This strain has been used for the immunization of cattle against *T. annulata* in Iran, since 1973 (Hashemi-Fesharki 1998).

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from the schizont-infected lymphoid cells by phenol-chloroform method as described previously (Sambrook et al., 1989). PCR was carried out using the specific primers to amplify a 522-bp fragment of the *Tams1* gene. DNA prepared as described above was amplified in 50 µl of reaction mixture containing 20 µl of 10x reaction buffer, 5 µl of mixed dNTPs (2.5 mM each), 0.5 unit of Taq DNA polymerase enzyme (Roche Diagnostic, Germany), 1 µl of each primer (10 pmol each) designed with DNASIS software: forward (5'-CAT AGA ATT CTT TGA GAT GTT GTC C-3') and reverse (5'-GAT ATC TAG ATA CGA ACA TGG GTT-3'), 4 µl of DNA template (100ng), 3 µl 25mM $MgCl_2$ (1.5mM final concentration), and up to 50 µl with ddH_2O. The fragments were amplified using a PCR program of: 94ºC for 3 min, 30 cycles of (94ºC for 30 s, 52ºC for 30 s, 72ºC for 40 s), 72ºC for 5 min.

Cloning of PCR product, DNA sequencing and data analysis

The PCR amplicons were analyzed on 1% agarose gel to check for the correct fragment size. In this regards, amplicons were visualised with ethidium bromide and subsequently extracted and purified from the agarose gel by using the gel extraction kit (Roche). The DNA fragments amplified by PCR were cloned into pTZ57R/T vector (Fermentas, Germany) using standard protocols. DNA sequencing was carried out using T7 promoter primer in both directions in MWG Co, Germany. Percent identity was made by using the MegAlign project of DNAStar software package (version 5.1). Multiple sequence alignments were analyzed using the BioEdit, version 7 (Hall 1999). Table 1 shows the description of *T. annulata* isolates used in the study. Phylogenetic tree (with bootstrap values) was created using the CLUSTAL X (2.0) and was shown using the NJPLOT program.

Table 1 - Description of *T. annulata* field isolates used to determine the sequence variability in the *Tams1* coding region.

Serial No.	Isolate	Country	Accession No.
1	Vaccine-ir68	Iran	AY672541
2	Vaccine	Iran	EF092914
3	Boein-zahra/1	Iran	EF092918
4	Boein-zahra/2	Iran	EF092919
5	Karaj/78	Iran	EF092915
6	Ankara	Turkey	XM948626
7	Ankara	Turkey	TAU22887
8	na31	Turkey	AF214908
9	tp12	Portugal	AF214828
10	tb39	Bahrain	AF214802
11	tH5	India	AF214844
12	492e	Tunisia	AF214903
13	234	Tunisia	AF214895
14	233	Tunisia	AF214894
15	231	Tunisia	AF214893
16	08#06	Iraq	GU130192
17	08#03	Iraq	GU130189
18	08#02	Iraq	FJ159695
19	tI11	Italy	AF214863

RESULTS

Partial nucleotide sequence coding for *Tams1* polyprotein was determined from the cloned fragments of DNA by PCR amplification and sequencing. As shown in Figure 1, the nucleotide and predicted amino acid sequence of Vaccine-ir68 (currently used as vaccine strain) was aligned with the corresponding sequences of eighteen previously published *Tams1* genes. The number of *Tams1* sequences analyzed (19 isolates) is listed in Table 1. The nucleotide sequence analysis demonstrated significant similarity (>99% nucleotide identity) between the Vaccine-ir68 and Tunisian isolates (Fig. 2). The comparison analysis of *Tams1* sequences retrieved from the GenBank revealed a nucleotide exchange at position 376 of the Vaccine-ir68 sequence. Figure 1a showed that the nucleotide 376 was changed from A to C, resulting in a change in amino acid 126 from Lysine to Glutamine (see Fig. 1b). In contrast to Vaccine-ir68, the remaining eighteen isolates showed no corresponding changes in the same position. Figure 3 shows a phylogenetic tree constructed based on the sequence alignment of 19 gene sequences. The phylogenetic clustering in the *Tams1* coding region of all the nineteen field isolates included in the analysis was distinctly divided into five lineages (A-E). The isolate Vaccine-ir68 was clustered with two Iranian and four Tunisian isolates into a separate branch from other *T. annulata* isolates (lineage C). Two Turkish isolates and lone isolate tp12 were grouped closely together in lineage D. The resulting phylogenetic tree indicated that four isolates (tb39 and tH5) and (tI11 and na31) differed from the Vaccine-ir68 and could be placed in the lineages E and A, respectively. They showed genetic relationship with 91.6-98.5% nucleotide identity among themselves and 92.9-94.3% identity to isolate Vaccine-ir68. Phylogenetic analysis showed that two other Iranian isolates were clustered in the lineage B and are linked to three other isolates from Iraq, 08#02, 08#03, and 08#06, suggesting that they had a close genetic relationship. These isolates might be actually genetically the same isolates of *T. annulata*. The overall topology of the phylogenetic tree presented in Figure 3 suggested that, among all these sequences the Vaccine-ir68 shared the highest homology with the Vaccine isolate and Tunisian isolates (99% similarity), and the lowest homology with Iraqi and Iranian isolates presented in lineage B (89% similarity).

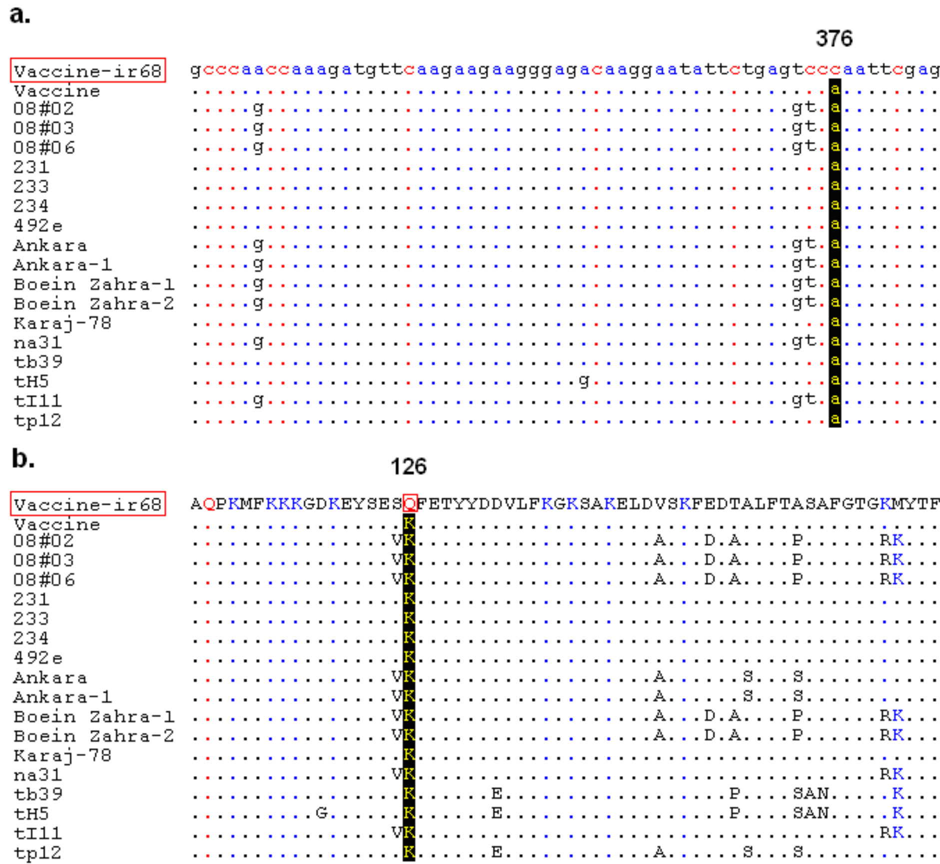

Figure 1 - (a) Alignment of partial nucleotide sequences of *Tams1* region. Mutation relevant in this study is highlighted. Dot (.) indicates sequence identity in relation to the Iranian vaccine strain (Vaccine-ir68). (b) The partial amino acid sequence of *Tams1* polypeptide of the Iranian vaccine strain (Vaccine-ir68) is shown in the top lane in the one letter code. Aligned to it are sequences of other *T. annnulata* isolates. Specific residue is marked with a red box. The numbers (126 and 376) above the sequences indicate the positions of substitution from the start of the *Tams1* coding region.

Percent Identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		99.8	89.3	89.3	90.4	99.6	99.6	99.6	99.6	95.4	95.4	90.8	90.8	98.4	94.3	93.7	92.9	94.1	97.1	**1**	Vaccine-ir68
2	0.2		89.9	89.9	90.8	99.8	99.8	99.8	99.8	95.5	95.5	91.2	91.2	98.6	94.3	93.5	92.6	94.1	97.1	**2**	Vaccine
3	10.9	10.5		100.0	100.0	89.9	89.9	89.9	89.9	92.7	92.7	100.0	100.0	89.3	94.5	90.2	91.7	94.2	91.1	**3**	08#02
4	10.9	10.5	0.0		100.0	89.9	89.9	89.9	89.9	92.7	92.7	100.0	100.0	89.3	94.5	90.2	91.7	94.2	91.1	**4**	08#03
5	9.9	9.6	0.0	0.0		90.8	90.8	90.8	90.6	92.5	92.5	100.0	100.0	89.9	95.9	89.5	92.9	94.6	91.9	**5**	08#06
6	0.4	0.2	10.5	10.5	9.6		100.0	100.0	99.6	96.0	96.0	91.2	91.2	98.8	94.6	94.1	93.3	94.6	97.1	**6**	231
7	0.4	0.2	10.5	10.5	9.6	0.0		100.0	99.6	96.0	96.0	91.2	91.2	98.8	94.6	94.1	93.3	94.6	97.1	**7**	233
8	0.4	0.2	10.5	10.5	9.6	0.0	0.0		99.6	96.0	96.0	91.2	91.2	98.8	94.6	94.1	93.3	94.6	97.1	**8**	234
9	0.4	0.2	10.5	10.5	9.9	0.4	0.4	0.4		96.0	96.0	91.0	91.0	98.8	94.4	94.1	92.9	94.3	97.1	**9**	492e
10	4.6	4.7	7.1	7.1	7.4	4.2	4.2	4.2	4.2		100.0	92.9	92.8	96.5	92.9	93.1	91.9	92.7	97.3	**10**	Ankara-1
11	4.5	4.7	7.1	7.1	7.4	4.1	4.1	4.1	4.1	0.0		92.9	92.8	96.5	92.9	93.1	92.0	92.7	97.3	**11**	Ankara
12	9.3	9.2	0.0	0.0	0.0	9.1	9.1	9.1	9.3	7.1	7.1		100.0	90.4	96.1	90.0	93.3	94.9	92.3	**12**	Boein-zahra-1
13	9.4	9.2	0.0	0.0	0.0	9.2	9.2	9.2	9.4	7.1	7.1	0.0		90.4	96.1	90.0	93.3	94.9	92.2	**13**	Boein-zahra-2
14	1.6	1.4	10.9	10.9	10.3	1.2	1.2	1.2	1.2	3.6	3.6	9.8	9.9		93.5	94.1	92.5	93.3	95.7	**14**	Karaj-78
15	5.6	5.7	5.7	5.7	4.2	5.4	5.4	5.4	5.6	7.1	7.0	4.0	4.0	6.4		91.8	94.8	98.5	93.1	**15**	na31
16	6.0	6.2	9.8	9.8	10.3	5.6	5.6	5.6	5.6	6.8	6.8	9.8	9.8	5.5	7.9		96.0	91.6	92.7	**16**	tb39
17	6.8	7.1	8.1	8.1	7.0	6.4	6.4	6.4	6.8	7.9	7.9	6.6	6.6	7.1	5.0	3.7		94.4	92.3	**17**	tH5
18	5.8	6.0	6.1	6.1	5.6	5.4	5.4	5.4	5.8	7.3	7.3	5.3	5.3	6.6	1.5	8.1	5.4		92.5	**18**	tl11
19	2.9	2.9	9.1	9.1	8.4	2.9	2.9	2.9	2.9	2.7	2.7	8.0	8.0	4.4	7.0	7.0	7.5	7.7		**19**	tp12
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**		

Figure 2 - Percent identity and divergence between Vaccine-ir68 isolate and eighteen published sequences of *Tams1* gene.

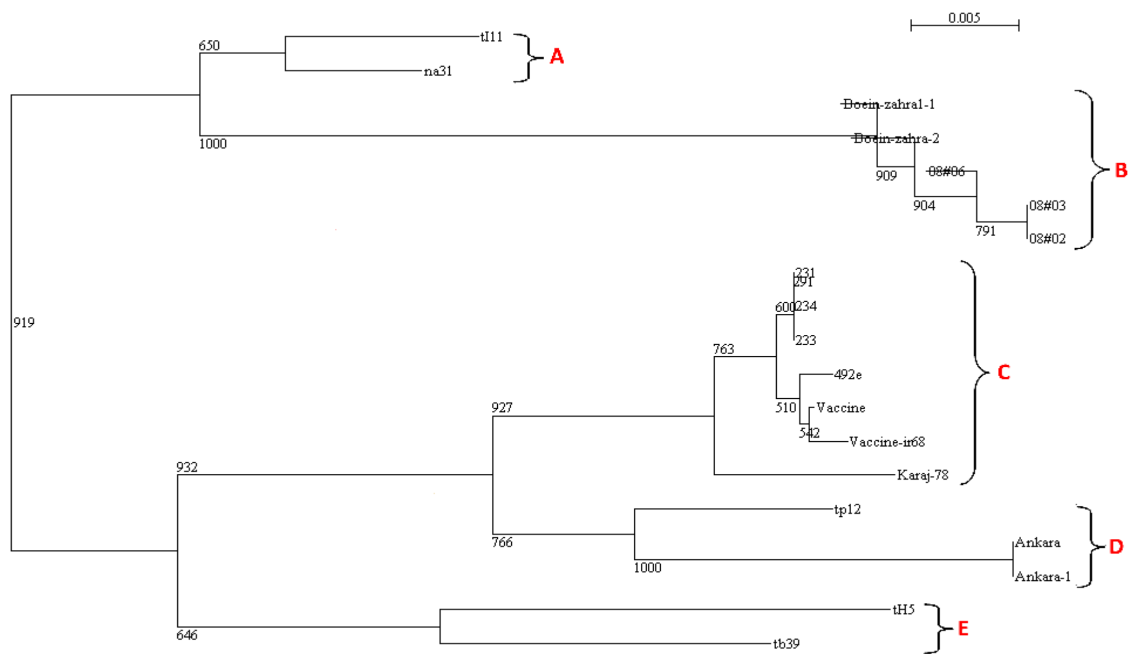

Figure 3 -Phylogenetic tree based on a comparison of Vaccine-ir68 isolate to other *T. annulata* field isolates, established with *Tams1* coding sequences (522 bp). The lineages and bootstrap values are shown on the tree.

DISCUSSION

Of the few polypeptides that have been located to the merozoite/piroplasm stages of *T. annulata*, the major merozoite piroplasm surface antigen (mMPSA) *Tams1* has been characterized in great detail. The function of *Tams1* and other mMPSA polypeptides have not been defined conclusively. *Tams1* appears to be coded by a single copy gene in the haploid genome of the parasite (Gubbels et al., 2000). The partial nucleotide sequence of the *Tams1* (522 nt) gene of Iranian vaccine strain (Vaccine-ir68) enabled us to perform a phylogenetic analysis using *Tams1* sequences, originated from various geographical locations, available in the Genbank database. Figure 2 indicates a high level of sequence variability among the isolates. The high level of genetic diversity within the *Tams1* polyprotein has been confirmed by the phylogenetic analysis. Gubbels reported that the analysis of the sequences provided evidence for the generation of sequence diversity through the intragenic recombination. It seemed that *Tams1* polymorphism evolved by the selection of amino acid substitutions in the particular regions and exchange of these regions between the alleles during the sexual recombination. Advent of diversity through the positive selection of amino acid substitutions and intragenic recombination have been reported for a number of viral, bacterial, rickettsial and protozoan antigen genes (Gubbels et al., 2000; Katzer et al., 1998). *Tams1* sequences and mMPSA sequences from other classical *Theileria* species reveal the regions where amino acids appear to be conserved. It is believed that the conserved regions play a functional/structural role, while the variable regions are exposed to the immune response (Gubbels et al., 2000). *Tams1* gene encodes a protein of 281 amino acids (aa) containing a putative hydrophobic N-terminal signal peptide, which is highly variable (d'Oliveira et al., 1996; Gubbels et al., 2000).

Partial sequences of the *Tams1* gene were compared with the Iranian strain (Vaccine-ir68) to estimate the extent of *Tams1* diversity throughout the regions included in this analysis. The deduced amino acid sequences investigated here were variable when compared with each other. The number of sequence differences exhibited by each of the isolates revealed that the Vaccine-ir68 contains amino acid substitution at position 126 of

the amino acid sequence. This isolate was characterized by a mutation at nucleotide position 376, resulting in a Lys-to-Gln substitution (Fig. 1a). An interesting observation was that in relation to the Vaccine-ir68 isolate the entire isolates revealed no such change. Polymorphism in *Tams1* may be associated with the evasion of the bovine immune system and possibly balancing selection (Weir et al., 2007). Another possible explanation for the high levels of genetic diversity found among the isolates of *T. annulata* could be random mutation of nucleotides during asexual reproduction (Gubbels et al., 2000).
In this work, the similarities of Tunisian and Iranian isolates of *T. annulata* were investigated. The resulting homology showed that they were analogous and closely resembled each other. Information on the prevalence of tick-borne pathogens in the potential vector ticks of the region is essential for the epidemiology of tick-borne diseases (Ica et al., 2007). According to the sequence similarity observed among the isolates of Iran, Tunisia and Turkey, and also the location of Palestine as a land bridge between the Africa and Asia, it could be inferred that some unknown immigrant birds might have an effective role to distribute the ticks of the *T. annulata* species from the north Africa to Palestine and then to Turkey, Russia and Iran. In support of this hypothesis, there have been many studies on the wild birds indicating that the wild birds could carry different ectoparasites such as species of *Hyalomma* ticks (Calvete et al., 2003; Grigor'ev et al., 2001; Kotti et al., 2001; Millan et al., 2004; Mumcuoglu et al., 2005). The sequence analysis based on the *Tams1* gene is in complete agreement with a previous report documenting that there is divergence between *T. annulata* vaccine strains and other available isolates in Iran (Habibi et al., 2007). The mentioned isolates of *T. annulata* sharing 99% similarity are economically valuable for the vaccine manufacturing industry in order to make the vaccines more affordable for use in developing countries such as Iran, Tunisia and Algeria. The extensive diversity of *Tams1* sequences may make this antigen a difficult candidate for the vaccine production. The attenuated strain of *T. annnulata* in Iran, which is now applied routinely in the vaccine development against the bovine tropical theileriosis is local one and has been originally obtained from an infected calf from Vasfenard village, located in south of Tehran, Iran. In order to consider the Iranian vaccine strain as an appropriate vaccine, all necessary quality and quantity control methods have been implemented. As a result of this project, the vaccine has proved to be effective in prevention of infection in cattle without any clinically significant abnormality and adverse side effects. The duration of vaccine-induced immunity lasted more than one year. Furthermore, different strains with diversity of virulence and clinical symptoms they induced in infected animals (parasitical and thermal reactions) were isolated in Iran from infected cattle. Studies done on inoculating the Algerian *T. annulata* vaccine (Schizont infected blood named Cuba vaccine) into Iranian cattle indicated that Algerian vaccine induced protective immunity against the Iranian strain of *T .annnulata*. The protective immunity induced by the Algerian vaccine was perhaps the decisive reason showing the cross immunity between the strains of Iran and Algeria (Hashemi-Fesharki et al., 1988). The existence of functional motifs conserved across *Tams1* polypeptides must be taken into consideration in the design and manufacture of vaccine, if they are found to be associated with the immune protection. Therefore, all different immunogenic forms of *Tams1* antigen should be noted in the vaccine production trends in respect of their biological characteristics.

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