# *A promising new ELISA diagnostic test for cattle babesiosis based on* Babesia bigemina *Apical Membrane Antigen-1*

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*Babesia bigemina*,
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**Summary**
Babesiosis due to *Babesia bigemina* is a relevant tick-borne disease, affecting cattle worldwide. Many surface proteins of the pathogen including the Apical Membrane Antigen 1 (AMA-1) - have been analysed for vaccine and diagnostic purposes. This study focused on *B. bigemina* AMA-1 and on its use for the assessment of diagnostic tests. After bioinformatic analyses, AMA-1 codifying region was amplified and cloned into an expression vector used to induce protein synthesis in *Escherichia coli* cells. AMA-1 was purified by affinity chromatography and used to set up the best condition for an ELISA protocol. Bovine field sera positive to *B. bigemina* were used to evaluate the presence of anti-AMA-1 antibodies. In order to verify the assay specificity, sera positive to *Babesia bovis* or to the piroplasm *Theileria annulata* were also included. Significant differences were obtained between sera negative to both *B. bigemina* and *B. bovis* and samples positive to *B. bigemina*, to *B. bovis* or to both pathogens. No significant reaction was observed with *T. annulata* positive sera. The results showed that AMA-1 protein is suitable to be used as antigen in diagnostic assays for babesiosis diagnosis in cattle, as it does not show any cross reaction with anti-*T. annulata* antibodies.

## *Un nuovo test ELISA per la diagnosi della babesiosi bovina basato sull'Apical Membrane Antigen-1 di* Babesia bigemina

**Parole chiave**
Anticorpi,
Apical Membrane Antigen-1,
*Babesia bigemina,*
Babesiosi,
ELISA,
Patogeni trasmessi da zecche,
Piroplasmosi,
Test diagnostici.

**Riassunto**
La Babesiosi causata da *Babesia bigemina* è una malattia trasmessa da zecche che colpisce gli allevamenti bovini in diverse aree del mondo. Molte proteine di superficie del patogeno - tra cui anche l'*Apical Membrane Antigen 1* (AMA-1) - sono state studiate al fine di realizzare nuovi vaccini e ottenere nuovi metodi diagnostici. Questo studio è stato condotto sulla proteina AMA-1 di *B. bigemina* e sul suo possibile utilizzo nella messa a punto di nuovi test diagnostici. In seguito a studi bioinformatici, la regione codificante la proteina AMA-1 è stata amplificata e clonata all'interno di un vettore di espressione. Il vettore ricombinante è stato utilizzato per indurre la sintesi proteica in cellule di *Escherichia coli*. La proteina AMA-1 è stata purificata mediante cromatografia d'affinità e utilizzata per ottimizzare le condizioni per il protocollo ELISA. Sieri di bovini di campo, risultati positivi a *B. bigemina*, sono stati utilizzati per rilevare la presenza di anticorpi anti-AMA-1. Al fine di verificare la specificità del saggio, sono stati inclusi nell'analisi sieri positivi a *Babesia bovis* o al piroplasma *Theileria annulata*. I sieri positivi a *B. bigemina*, *B. bovis* o ad entrambi i patogeni hanno mostrato risposte significativamente differenti rispetto ai sieri negativi sia a *B. bigemina* che a *B. bovis*. Non è stata osservata alcuna reazione significativa in presenza di sieri positivi a *T. annulata*. Lo studio ha mostrato che la proteina AMA-1 di *B. bigemina* può essere impiegata come antigene nei test per la diagnosi della babesiosi nei bovini, in quanto non mostra alcuna cross-reattività nei confronti degli anticorpi anti-*T. annulata*.

## Introduction

Cattle babesiosis is a tick-borne disease caused by apicomplexan protozoan such as *Babesia bovis* and *Babesia bigemina*. The pathology affects cattle in many areas of the world, strongly reducing meat and milk production (Bock *et al.* 2004, Torina and Caracappa 2007). Understanding the basic molecular mechanism of merozoite invasion of erythrocytes may improve the development of an effective control strategy. Host-cell invasion by apicomplexan parasites is a complex, multistep process (Black and Boothroyd 2000), as a number of proteins are implicated in it, although in most cases their precise functions remain unknown (Zhou *et al.* 2006).

One of the molecules potentially involved in the erythrocyte invasion by *B. bigemina* is the Apical Membrane Antigen-1 (AMA-1), an apically located protein that is shared by many Apicomplexa and has a role in the host red blood cells invasion process. While the biological function of AMA-1 is not completely understood, its cellular localization, a phase-specific expression, and the secretion during the host cell invasion suggest that this protein could have a key role in the red blood cell penetration by the parasite.

The most characterized AMA-1 protein is the one codified by *Plasmodium falciparum* (PfAMA) (Peterson *et al.* 1989). This protein has been extensively studied as it is one of the most promising erythrocyte stage candidates among the few anti-malarial vaccine candidates under development (Latitha *et al.* 2008). *Babesia bovis* AMA-1 protein (BbAMA-1) was identified and characterized in 2004 (Gaffar *et al.* 2004) and it showed structural features similar to those of other AMA-1 proteins (Yokoyama *et al.* 2006). It is supposed that also *Babesia bigemina* invades erythrocytes using an Apical Membrane Antigen-1. *Babesia bigemina* AMA-1 is a 65.9KDa protein showing several common features with other AMA-1 family members. It also exhibits a high degree of similarity with the orthologue gene of *B. bovis*. Structural features of *B. bigemina* AMA-1 protein were recently described (Torina *et al.* 2010).

This study was devoted to the synthesis of *B. bigemina* AMA-1 recombinant protein into a prokaryotic expression system and its use to asses a new ELISA test useful for diagnosis of cattle babesiosis.

## Material and methods

### *B. bigemina* AMA-1 production in *E. coli*

Bioinformatic analyses were performed using ClustalW2.0.10 (Larkin *et al.* 2007) software for sequence alignment with the purpose to select a *B. bigemina* surface antigen conserved enough among different strains of the pathogen. DAMBE (Xia and Xie 2001) and MEGA (Kumar *et al.* 2008, Tamura *et al.* 2007) software were used to obtain similarity percentage among the analysed sequences.

Apical Membrane Antigen-1 codifying region was amplified from DNA extracted from blood of a bovine naturally infected by *B. bigemina* and the amplification product was cloned into the expression vector pET160/GW/D-TOPO (Life Technologies Corporation™, Carlsbad, CA, United States), following the manufacturer's instruction. The construct was initially used to transform One Shot® TOP10 Chemically Competent *Escherichia coli* cells (Life Technologies Corporation™, Carlsbad, CA, United States). The plasmid was thus extracted, purified, and sent to Macrogen Europe (Amsterdam, The Netherlands) for sequencing using T7F and T7R universal primers in order to test if the PCR product was cloned in the correct direction. The recombinant plasmid was then used to transform *E. coli* BL21 Star™ (DE3) cells (Life Technologies Corporation™, Carlsbad, CA, United States). Bacterial cells were cultured in LB-Broth at 37°C. Bacterial cell growth was monitored by reading the Optical Density (O.D.) at 600 nm up to the value of approximately 0.6 and then the protein production was induced by 0.75 mM IPTG (isopropil-tio-β-Dgalattoside). After 3 hours of induction, bacteria were collected and treated as previously described (Gallo *et al.* 2012). Briefly, pellets were cleaned 3 times with a washing solution (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 4 mg/ml leucopeptin, 0.7 mg/ml pepstatin, 5 mg/ml benzamidin). Following re-suspension in washing solution containing 0.3% SDS, bacterial cells were disrupted by sonication on ice (output control 4, 4 x 15 s, Vibra Cell, Sonics & Materials, Inc., Newtown, CT, USA). Samples were then boiled (5 minutes) and rapidly cooled down on ice (15 minutes). DNase (100 µg/ml) and RNase (50 µg/ml) were added in ice for 20 minutes. Cell debris and non-broken cells were separated by centrifugation at 15,000 x g, for 15 minutes, at 4°C. Protein solutions were precipitated by using 3 volumes of acetone, at -20°C, overnight, and re-suspended in mQ $H_2O$.

Recombinant AMA-1 protein was thus purified using HisGraviTrapTM kit (GE HealthCare, Little Chalfont, niUted Kingdom) following manufacturer's instruction, and then digested with AcTEV Protease (Life Technologies Corporation™, Carlsbad, CA, United States) for 3 hours at 30°C to remove the His Tag from the fusion protein. After the digestion, proteins were newly purified with HisGraviTrapTM kit (GE HealthCare, Little Chalfont, Buckinghamshire, United Kingdom) to eliminate the protease, the undigested recombinant proteins and the cleaved tags. Protein extracts were analysed

by SDS-gel electrophoresis after staining with Lumio™ Green Detection Kit (Life Technologies Corporation™, Carlsbad, CA, United States). The Lumio™ Green Detection Reagent is non-fluorescent and becomes fluorescent upon binding to the tetracysteine Lumio™ tag codified by the expression vector, allowing the specific visualization of the recombinant protein at UV light. As an alternative to the Lumio™ Green Detection, the protein was stained with 0.005% Blue Coomassie (Coomassie Brillant Blue R-250) (w/v). The protein was quantified by Bradford protein assay.

## *B. bigemina* AMA-1 based ELISA optimization

The obtained protein was used to set up an ELISA protocol. ELISA tests were performed in 96 well plates (MaxiSorp, NUNC, Roskilde, Denmark). Apical Membrane Antigen-1 protein in carbonate/bicarbonate buffer (0.1 M $NaHCO_3$ in distilled water pH 8.2 Sigma-Aldreich, St. Louis, Missouri, United States) was used to coat plate wells at 4°C over nigth. Then, 5% BSA solution to saturate free sites of the wells, serum samples, and Anti-bovine IgG secondary antibody conjugated with Peroxidase (Sigma- Aldreich, St. Louis, Missouri, United States) were added in this order. After the addition of the specific substrate (SigmaFastTM OPD, Sigma-Aldreich, St. Louis, Missouri, United States), O.D. values were determined at 405 nm at spectrophotometer (Mod 680, Biorad, Hercules, CA, United States) or, if the plate could not be read immediately, 3M $H_2SO_4$ were added and the stopped reactions were read at 490 nm.

Different concentrations and incubation times for all of these reagents were tested to find the optimal conditions for the assay. To validate our results, serum samples were tested in duplicate and in all plates, foetal bovine serum (FBS) was used as negative control, while blank samples were included replacing sera with the same volume of Phosphate-buffered saline (PBS). Field serum samples were previously selected and tested by serological and molecular techniques, as described in the section 'Field samples tested in the study', in order to verify their positivity/negativity to *B. bigemina* and their closely related organisms *B. bovis* and *Theileria annulata*.

One sample, which resulted positive at both serological and molecular tests, was then used as positive control. Another sample, which was negative at serological and molecular tests, was subsequently used as negative control. These 2 samples were included in all the plates to verify result reproducibility. Antibody titers were considered positive when they yielded an O.D. 405 nm value at least twice as high as the negative field sera.

## Field samples tested in the study

In order to investigate the specificity of the new *B. bigemina* AMA-1 based ELISA, the assay was used to analyse field serum samples that had been previously tested by serological and molecular techniques and to verify their positivity/negativity to *B. bigemina* and its closely related organisms *B. bovis* and *T. annulata*. In particular, bovine serum samples were analysed using a commercial ELISA kit for the presence of antibodies against *B. bigemina* (Svanova Biotech AB, Uppsala, Sweden) and an IFA kit for the detection of antibodies against *B. bovis* (Fuller Laboratories, Fullerton, CA, United States) following the manufacturer's instructions. The presence of antibodies against *T. annulata* was assessed by immunofluorescence using a protocol modified from Burridge and Kimber (Burridge and Kimber 1972) so to find out the serum antibody titres to *Theileria* infection. For molecular analysis, DNA was extracted from EDTA-treated blood samples using the PureLink Genomic Mini kit (Life Technologies Corporation™, Carlsbad, CA, United States) following the manufacturer's instructions. DNA samples were analysed by polymerase chain reaction (PCR) to detect the presence of DNA from *Babesia bovis* (Figueroa *et al.* 1993), *B. bigemina* (Figueroa *et al.* 1993), and *T. annulata* (D'Oliveira *et al.* 1995). PCRs were performed in a reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, forward and reverse primers at a concentration of 0.4 mM, and 0.025 U/µl of Taq DNA polymerase (5 U/µl) (Promega, Madison, WI, United States). For each reaction, a positive control consisting of pathogen DNA and a negative control in which DNA was replaced by water were used. Polymerase chain reaction products were visualized after agarose gel electrophoresis containing 10 µg/ml ethidium bromide.

Following serological and molecular analyses, 136 sera were divided into 6 groups according to their positivity/negativity for *B. bigemina*, *B. bovis*, and *T. annulata* pathogens. In detail, Group 1 included samples positive for *B. bigemina, B. bovis* and *T. annulata* (26 samples). Group 3 comprised 16 samples positive for *B. bovis* and *B. bigemina* but negative for *T. annulata*. Group 2 comprehended samples that were positive only for *B. bovis* (26 samples), Group 4 comprehended samples positive only for *B. bigemina* (28 samples). Samples that were positive (22 samples) only for *T. annulata* were enclosed in Group 5 while Group 6 included 18 samples negative to all three pathogens.

Statistical analysis of the results was performed by ANOVA test. Threshold O.D. values were determined to discriminate *B. bigemina*/*B. bovis* positive from negative sera. The threshold O.D. values were obtained by analysing the distribution frequencies of the O.D. values normalized according to Gauss' curves.

## Results

### Recombinant *B. bigemina* AMA-1 in *E. coli* production

The region codifying AMA-1 protein was amplified by PCR and the amplification product was inserted into the expression vector. The correct orientation of the cloned fragment was confirmed by sequence analysis. Obtained recombinant plasmid was used to transform BL21 Star™ (DE3) *E. coli* cells (Life Technologies Corporation™, Carlsbad, CA, United States). Protein expression was induced by IPTG and, after 3 hours, pellets were collected. Obtained cell suspensions were lysed and treated to extract the total protein pattern that was separated by SDS-PAGE and visualized by staining with Lumio Green™ (Life Technologies Corporation™, Carlsbad, CA, United States) as shown in Figure 1A. Protein purification by affinity chromatography was verified by SDS-PAGE (Figure 1B).

### *B. bigemina* AMA-1 ELISA results

Recombinant protein was used for the assessment of an ELISA test. The optimum conditions of the test included the coating of individual wells of a microtiter plate (Nunc) with 0.1 µg/well of AMA-1 antigen overnight at 4°C in an antigen coating buffer (bicarbonate/carbonate at pH 8.2, Sigma-Aldreich, St. Louis, Missouri, United States). After 5 washes with PBS Tween, plated were saturated with 5% BSA in PBS (100 µl/well) for 2 hours and 30 minutes at 37°C. Plates were washed 5 times with PBS Tween, sera diluted (1:100) in PBS Tween were added and incubated for 1 hour at 37°C (100 µl/well). After 5 washes with PBS Tween, secondary antibody (Anti-bovine IgG-Peroxidase antibody, Sigma-Aldreich, St. Louis, Missouri, United States) diluted 1:10,000 in PBS-Tween was added and incubated for 1 hour at 37°C (100 µl/well). Plates were washed with PBS Tween 5 times and, then, fast OPD substrate (100 µl/well) was added (Sigma-Aldreich, St. Louis, Missouri, United States) and plates were read at a spectrophotometer at 405 nm after 20 minutes of incubation at room temperature. All the plates showed O.D. values of 0.1 for FBS and between 0.2 and 0.1 for blank samples.

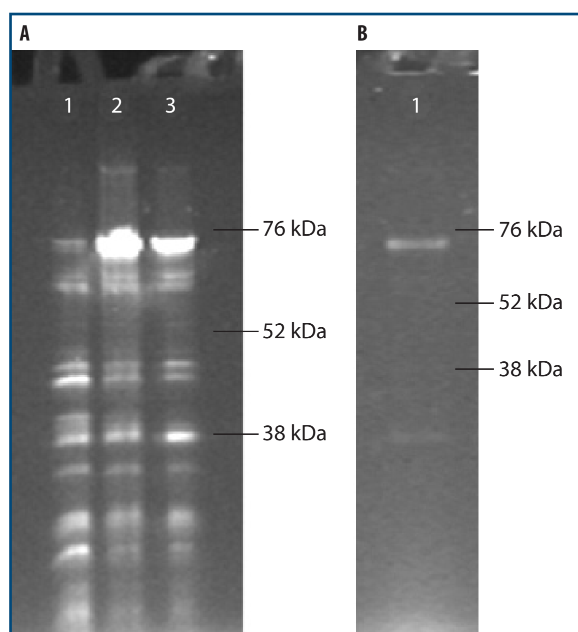


**Figure 1. *A.*** *SDS-PAGE of total protein extracts from* E.coli *cells allowed to grow for 3 hours in absence (lane 1) or in presence (lane 2 and 3) of IPTG, stained with Lumio™ Green Detection Kit (Life Technologies Corporation™, Carlsbad, CA, United States) and observed at UV light.* ***B.*** *SDS-PAGE of the recombinant protein after purification by affinity chromatography, stained with Lumio™ Green Detection Kit (Life Technologies Corporation™, Carlsbad, CA, United States) and observed at UV light.*

The average values of each individual group are shown in Table I.

The statistical analysis performed by ANOVA test among the different groups confirmed a significant difference between samples that were positive for *B. bigemina* and/or *B. bovis* and samples that were negative for these 2 pathogens. No significant reaction was observed in presence of *T. annulata* positive sera.

Moreover, we did not find significant differences between the samples of Groups 1 and 3 positive for both *B. bovis* and *B. bigemina*, which were then grouped in Group A, so it was the case for Group 2 and Group 4 (*B. bovis* or *B. bigemina* positive, respectively), grouped in Group B, and samples of Group 5 and Group 6 (negative for both *B. bovis* and *B. bigemina*, positive or negative for *T. annulata*) unified in group C. It was not possible to unify the samples of Groups A and B into a single group because the values of the samples of Group A showed O.D. values significantly higher (1.15 ± 0.12) than those of Group B (0.94 ± 0.15).

Values obtained by the ELISA test for groups A, B, and C with their means, standard deviations, and number of samples are summarized in Table II.

Cut-off value to discriminate *B. bigemina*/*B. bovis* positive sera from the negative ones was recognised at 0.55 O.D., in such a way as to favour the sensitivity with respect to the specificity of the assay. In particular, it can be established that, following the specific optimised protocol, samples with O.D. lower than 0.55 can be considered reasonably negative. In contrast, values of O.D. greater than 0.69 indicate positivity to at least 1 of the 2 considered *Babesia*. The intermediate value of O.D. (between 0.55 and 0.69) could be considered doubtful, so for these



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**Table I.** *Results of* B. bigemina *Apical Membrane Antigen-1 ELISA assay performed on serum samples divided into 6 groups according to their positivity/negativity to* B. bigemina, B. bovis *and* T. annulata *pathogens*. The field serum samples included in the study were taken from a panel of sera and DNA samples collected by the Istituto Zooprofilattico Sperimentale della Sicilia from 2011 to 2012 for diagnostic purposes. The samples had been previously tested by serological and molecular techniques.

| Group number | *B. bigemina* | *B. bovis* | *T. annulata* | N° of samples | Samples tested by serological analyses | Samples tested by molecular analyses | Mean O.D. value | Standard Deviation |
|---|---|---|---|---|---|---|---|---|
| 1 | POS | POS | POS | 26 | 26 | 1 | 1.15 | 0.11 |
| 2 | NEG | POS | NEG | 16 | 9 | 7 | 0.99 | 0.18 |
| 3 | POS | POS | NEG | 26 | 10 | 16 | 1.15 | 0.14 |
| 4 | POS | NEG | NEG | 28 | 21 | 7 | 0.9 | 0.13 |
| 5 | NEG | NEG | POS | 22 | 10 | 12 | 0.4 | 0.13 |
| 6 | NEG | NEG | NEG | 18 | 12 | 6 | 0.42 | 0.11 |
| | | | **Total** | **136** | **87** | **49** | | |

samples it may be appropriate to repeat the test on a withdrawal made after 2 weeks.

## Discussion and conclusions

This work describes the optimisation of a prokaryotic system for AMA-1 recombinant protein production and the use of the produced antigen for a new diagnostic ELISA test. Obtained results showed a significant signal difference among the analysed serum samples, confirming the presence of *B. bigemina* antibodies directed against AMA-1 protein in infected bovine sera and suggesting that *B. bigemina* AMA-1 protein is sufficiently immunogenic and conserved to be recognised by antibodies against *B. bovis*, as it can be observed also in the amino acidic alignment showed in Figure 2. The higher O.D. values of the samples of Group A are probably due to presence in these sera of antibodies to both *B. bigemina* and *B. bovis* able to determine a stronger response to the antigen.

The use of the negative controls (SBF) and blank samples showed that the values obtained were consistent and very low in all the plates, suggesting the absence of non-specific signals in the plate wells.

In this paper a new ELISA test reliable, reproducible, and specific for *B. bovis* and *B. bigemina* is described based on *B. bigemina* AMA-1 protein, which is an apically located protein shared by many Apicomplexa. These proteins show common features and are enclosed in the AMA-1 protein family whose members are characterised by the presence of a Signal Peptide for membrane targeting, a Trans-membrane helix (TM), and disulphure bonds dividing the protein in functional domains. Apical Membrane Antigen-1 members have a key role in the host red cell invasion process and are extensively studied for their possible inclusion in a subunit vaccine. Sequence analyses showed the presence of regions highly conserved among the different examined organisms (Torina *et al.* 2010), so the choice to include samples positive for *B. bovis* and *T. annulata* in the analysis is justified.

**Table II.** *Results of* B. bigemina *Apical Membrane Antigen-1 ELISA assay performed on serum samples divided into 3 groups obtained by the association of the samples of Group 1 and Group 3 (Group A), Group 2 and Group 4 (Group B), Group 5 and Group 6 (Group C), after ANOVA analysis.* The field serum samples included in the study were taken from a panel of sera and DNA samples collected by the Istituto Zooprofilattico Sperimentale della Sicilia from 2011 to 2012 for diagnostic purposes. The samples had been previously tested by serological and molecular techniques.

| Group | *B. bigemina* | *B. bovis* | *T. annulata* | N° of samples | Mean O.D. value | Standard Deviation |
|---|---|---|---|---|---|---|
| A | **POS** | **POS** | POS | 52 | 1.15 | 0.12 |
| | **POS** | **POS** | NEG | | | |
| B | NEG | **POS** | NEG | 44 | 0.94 | 0.15 |
| | **POS** | NEG | NEG | | | |
| C | NEG | NEG | **POS** | 40 | 0.41 | 0.12 |
| | NEG | NEG | **NEG** | | | |

Obtained results suggest the use of this assay for screening purposes in order to enable a rapid diagnosis and lower costs of the reaction. In case of a negative reaction, in fact, it should not be necessary to carry out species-specific serological tests that would be performed only on those serum samples positive at the first screening.

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CLUSTAL 2.1 multiple sequence alignment


BbigeminaAMA1      ------MQCILNRIT------------LLATPVIFFLWLSTEISPTGCAFVTFQNEPTST 42
BbovisAMA1         MQLHYKMQSTSPKYNYKRMLC------MVFVPVILSSFFAEDALASNSTLFAFHREPTNR 54
                         **.  : .              :: .***:  ::: :  .:..::.:*:.***.

BbigeminaAMA1      RGTRRSSRSSRHQQATSSTSEAGAGEATERAGGRTAGSKLIPQTPWTRYMIKYDIARCHG 102
BbovisAMA1         RLTRRASR-G--QLLNSRRGSDDTSESSDRYSGRSGGSKNSGQSPWIKYMQKFDIPRNHG 111
                   * ***:** .  *  .*  .. .:.*:::* .**:.***   *:** :** *:**.* **

BbigeminaAMA1      SGIFVDIGGYEAVGNKYYRMPTGKCPVMGKVISLASGADLLEPISADNPRYRGLAFPETV 162
BbovisAMA1         SGIYVDLGGYESVGSKSYRMPVGKCPVVGKIIDLGNGADFLDPISSEDPSYRGLAFPETA 171
                   ***:**:****:**.* ****.*****:**:*.*..***:*:***::* *********.

BbigeminaAMA1      IKHTGASAGALTNAGNI-HGNLSPVSAADLRKWGYKGNAVTNCAEYASNIVPGSDQRTKY 221
BbovisAMA1         VDSNIPTQPKTRGSSSVTAAKLSPVSAKDLRRWGYEGNDVANCSEYASNLIPASDKTTKY 231
                   :. . .:     .:..:  .:****** ***:***:** *:**:*****::*.**: ***

BbigeminaAMA1      RYPFVYDGKEEMCYILYSPMQYNQGTRYCDEDGSAKEGPSSLLCMKPYKSEADAHLYYGS 281
BbovisAMA1         RYPFVFDSDNQMCYILYSAIQYNQGNRYCDNDGSSEEGTSSLLCMKPYKSAEDAHLYYGS 291
                   *****:*..::*******.:*****.****:***::**.***********  ********

BbigeminaAMA1      ARIDPKWDQNCPMKPIKDAIFGTWVSGACVALESAFEEYVDSAEECAAILFENSAADVDI 341
BbovisAMA1         AKVDPDWEENCPMHPVRDAIFGKWSGGSCVAIAPAFQEYANSTEDCAAILFDNSATDLDI 351
                   *::**.*::****:*::*****.* .*:***: .**:**.:*:*:******:***:*:**

BbigeminaAMA1      DIDSERYNEISELYNGLKNLQLQQIAFSLFAPMAKSAASATLSKGVGMNWANYESETGVC 401
BbovisAMA1         EVVNEEFNELKELTSGLKRLNLSKVANAIFSPLSNVAGTSRISRGVGMNWATYDKDSGMC 411
                   :: .*.:**:.** .***.*:*.::* ::*:*::: *.:: :*:*******.*:.::*:*

BbigeminaAMA1      RILNATPTCLIINAGSLAMTALGSPLESDAINYPCHIDTLGYVEPRKRNSREDGDRNSGI 461
BbovisAMA1         ALINETPNCLILNAGSIALTAIGSPLEYDAVNYPCHIDTNGYVEPRAKTTNKYLDVPFEV 471
                    ::* **.***:****:*:**:***** **:******** ****** :.:.:  *    :

BbigeminaAMA1      TTALNMKTLKCTKYVHSKYSESCGTYYYCSEEKSGYLSRLYQFMCSHNVKKAAV-ISTAL 520
BbovisAMA1         TTALSTKTLKCNAYVHTKYSDSCGTYFLCSDVKPNWFIRFLHMIGLYNTKRIVIFVCCTT 531
                   ****. *****. ***:***:*****: **: *..:: *: :::  :*.*: .: :. :

BbigeminaAMA1      VLLCLAIYWIYQRLWSTKKGRQHDDYDRLMSKYEYD-DVSHDNIEPEHQLRTDAYIWGEA 579
BbovisAMA1         TAIVLTI-WIWKRFIKAKKEPAPPSFDKYLSNYDYDTTLDADN-ETEQRLDSSAYSWGEA 589
                   . : *:* **::*: .:**     .:*: :*:*:**  :. ** *.*::* :.** ****

BbigeminaAMA1      AARPSDITPVHMAKLN 595
BbovisAMA1         VQRPSDVTPVKLSKIN 605
                   . ****:***:::*:*
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**Figure 2.** *Amino acidic alignment of the* B. bigemina *and* B. bovis *Apical Membrane Antigen 1 (AMA-1).*

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