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Molecular Cloning and Expression Of ESAT-6 Antigen of *Mycobacterium Bovis* For Differential Diagnosis of Bovine Tuberculosis

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ABSTRACT

In a search for developing new skin test reagents, the possibility of using ESAT-6 protein antigen as a candidate antigen for the diagnosis of bovine tuberculosis was investigated. Genomic DNA of *M. bovis* was extracted, purified and the *esat-6* gene was amplified by PCR. The gene was then ligated to an expression vector, PQE. After transformation of an *E. coli* TOPO host strain with the PQE plasmid, the expression was induced using 10 mM of IPTG. A band (18 KDa) was seen in the SDS-PAGE analysis. The His-tagged r ESAT-6 antigen was then purified by metal affinity chromatography using Ni-NTA agarose.

The serodiagnostic potential of the r ESAT-6 was evaluated in sera of experimentally infected guinea pigs with different strains of mycobacteria using ELISA in comparison with the PPD. The results indicated the efficiency of ESAT-6 but not PPD to discriminate between *M. bovis* infected guinea pigs and those sensitized with other mycobacterial strains at serum dilution of 1/50.

In a clinical trial to assess the diagnostic potential of r ESAT-6 antigen, serum samples from tuberculin positive (+ve) and tuberculin negative (-ve) cattle were tested by ELISA. All +ve animals were confirmed to be *M. bovis* infected by post mortem (PM) and bacteriological examination. It is concluded that r ESAT-6 could recognize the *M. bovis* infected, tuberculin +ve animals at serum dilution of 1/50.

INTRODUCTION

Tuberculosis (TB) is expected to be responsible for more than 2 million deaths and up to 8 million new infections during the coming years ***(1)*** in human and causes sever economical losses in animals. The identification of infected animals for culling is extremely difficult, as it is based on intradermal injection of tuberculin. Unfortunately, PPD contains many antigens widely shared among mycobacteria as it is a crude, poorly defined mixture of mycobacterial antigens containing both secreted and somatic proteins, and the specificity of this reagent is therefore low.

Several studies have demonstrated that PPD cannot reliably distinguish among previous *Mycobacterium bovis* infection, BCG vaccination and exposure to environmental mycobacteria, ***(2,3).*** Despite its low sensitivity and specificity, PPD has been used for many years as an in vivo skin test reagent in both humans and cattle. Alternatives to skin testing have been investigated, for example in 1990, an in vitro diagnostic test for *Mycobacterium bovis* infection in cattle was developed, based on the detection of gamma interferon (IFN-γ) liberated in whole blood cultures incubated in vitro with PPD ***(4).*** In 1994, an adaptation of this test was developed for the diagnosis of *M. tuberculosis* and *Mycobacterium avium* infection in humans, again using PPD-type antigens ***(5).***

Moreover in vitro methods for the specific diagnosis of *M. bovis* infection would avoid some of the practical difficulties associated with intradermal skin testing. In vitro methods based on antibody detection have had either limited sensitivity or limited specificity ***(6)*** when PPD antigen is used. Newer species specific antigen thus may eliminates this problem.

Thus there is an urgent need for reproducible and rigorous frameworks to assess new tests for the diagnosis of *Mycobacterium bovis* infection.

Recently, genes which are deleted from BCG have been identified ***(7),*** and some of

these also appear to be absent from most environmental mycobacteria. One such antigen, ESAT-6 (early secretory antigenic target -6), has shown promising results for use as an immunodiagnostic reagent ***(8).*** Recent studies with humans have found that a test based on the detection of ESAT-6-specific T cells via their production of gamma interferon (IFN-γ) ex vivo specifically discriminates between *M. tuberculosis* infection and exposure to other mycobacteria ***(9).***

Southern blotting of genomic DNA has shown the presence of the *esat-6* gene in *M. tuberculosis*, *M. africanum*, and virulent ***M. bovis***, whereas this gene could not be demonstrated in any BCG vaccine strains and in non tuberculous mycobacteria, with a few exceptions *(M. kansasii*, *M. szulgai*, and *M. marinum)* ***(10, 11).*** In agreement with this distribution, it was recently demonstrated that ESAT-6 was able to discriminate TB patients from both BCG-vaccinated individuals and ***M. avium*** patients ***(12).***

Thus this study aimed for cloning and expression of *east 6* gene from *M. bovis* and evaluation of its differential diagnostic potential of bovine tuberculosis.

MATERIAL AND METHODS

Mycobacterial strains

Standard strains of *Mycobacterium tuberculosis*. *M. bovis*, *M. bovis* BCG and *M. intracellulare* strain ATCC 13950 (VSVRI Abbassia Cairo) were used in this study. All strains were grown on modified Sauton or Middlebroke7H9 media for 4 weeks at 37°C, and the cells were harvested by centrifugation and stored at -20°C till used.

For cloning and expression of the recombinant ESAT 6 protein, *E. coli* 10 TOPO (Invitrogen) and *E. coli* XL1 blue were used.

Serum samples

Serum samples were taken from tuberculin tested cattle (n=20) from different geographical locations in Egypt that were subjected to post mortem (PM) and bacteriological examinations (Table 1). For the experimental guinea pigs, the serum samples were collected 4 weeks post artificial infection.

Experimental sensitization

Thirty tuberculin negative guinea pigs were divided into 5 groups (5 animals each) each sensitized as described previously ***(13, 14)*** with one of the aforementioned mycobacterial strains and the last group remains unsensitized as a negative control.

Genomic DNA extraction

The genomic DNA of *M. bovis* AN5 was extracted by the Triazol® method according to the manufacture instruction and ***(15).*** Briefly, 5mg (wet weight) were extensively washed with TE buffer pH 8 and finally reconstituted in 1 ml TE buffer pH 8 and incubated at 37°C for 4 hours with 5µl lysozymes (final concentration 100µg/ml). Proteinase-K was added 50µl/1ml (final concentration 100µg/ml) and incubated for further 3 hours at 56°C with shaking then 1 ml of Triazol was added. After vortex for 30 sec, 0.5 ml of chloroform was added and centrifuged for 10 min at 14000Xg. The DNA in the interphase was precipitated with 0.5 ml of absolute ethanol, washed twice with 0.1M sod. citrate in absolute ethanol and finally redissolved in 50ul of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml HEPES (0.1 M). Two µl of RNAase were then added and incubated at 37°C for 1 hour. Purification of the genomic DNA was done using Wizard DNA clean up system (Promega).

Preparation and purification of recombinant *ESAT 6*

A. PCR amplification

PCR was performed in 50-µl reaction mixtures containing 50 mM KCl, 10mM Tris-HCl (pH8.8), 3 mM $MgCl_2$, 200 mM (each) deoxynucleoside triphosphate, 10µl Q solution (Qiagene) and 2.5 U of thermostable recombinant *pfu* DNA polymerase and 50 pMol of each oligonucleotide primer. The DNA sequence of the oligonucleotide primers used for amplifying of the *esat-6* gene were as follows: Forward primer (*5'- ATG ACA GAG*

CAG CAG TGG AAT-3'), corresponding to the position -8 to +10 and reverse primers (*5'-TGC GAA CAT CCC AGT GAA G- 3'*), corresponding to the position 652 to 673, and DNA samples (200 ng) were pipetted through the mixture. Thermal cycling was performed using T gradient (Biometra, Germany), the parameters for amplification were denaturated at 95 °C for 3 min for one initial cycle and then 40 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec. A final extension at 72°C for 10 min was also included. The size of the amplicon was determined by electrophoresis on 1% agarose and visualized under UV transillumination.

B. Cloning procedures

1. Construction of the cloning vector

The amplicon was ligated to the PcDNA3.1/V5-His TOPO TA cloning plasmid (Invitrogen Cat# K4800-01) at 25°C/5 min then placed on ice /10 min. The plasmid was then transformed to One Shot TOPO 10 *E. coli* (chemically competent cloning host) provided with the kit, and then spread over LB agar containing 200µg/ml ampicillin. The plates were incubated overnight at 37°C and the growing colonies were collected separately and overnight culture was made on LB broth.

2. Miniprep

Plasmid miniprep was prepared from the overnight culture using High pure plasmid isolation kit (Roche cat # 1 754 785) and a PCR was conducted to confirm the correct orientation of the amplicon using the forward primer provided with kit that anneal to the plasmid 5' upstream the amplicon and the reverse primer that anneal to the end of the amplicon. Only plasmid containing the amplicon in the correct orientation will give a 300 bp band when electrophoresed on 1% agarose.

3. Restriction digestion of the cloning vectore

The cloning vector was then digested with *Bam HI* and *SacII*, electrophoresed on 1% agarose and the 370 bp band (containing the *esat-6* gene) was sliced, purified with PCR purification wizard (Promega) and ligated to the PQE expression vector.

4.Construction the expression vector

To construct an expression vector, 3 arm ligation was done. First the PQE-50 plasmid (Qiagene) was digested with *Bgl II* and *Bam HI* and then electrophoresed, the 1038 bp fragment was cut from the low melting agarose and purified using wizard PCR purification kit (Promega) and eluted in TE buffer. For the PQE-16 plasmid, digestion was done with *Bgl II* and *SacII*, a 2084 bp fragment was isolated, purified as before. PCR product (insert) was treated as PQE-50. For 3 arm ligation to be done, PQE-50, PQE-16 and insert (amplicon) was mixed in a ratio 1:1:2 and incubated at 16°C/overnight with T4 DNA ligase. The electrophoresis pattern of the ligated mixture showed a single band that migrates at nearly 4 Kbp, which is corresponding to the theoretically calculated expression construct. The construct was then transformed into XL1 blue competent cells by heat shock method as described by the manufacture instruction.

C. Expression of the recombinant protein

The constructed plasmid was transformed in *E. coli* XL1 blue competent cells by heat shock method ***(15)*** and +ve colonies that express the recombinant protein was identified by colony hybridization method using mouse anti-His primary antibodies (fig 2). Positive colonies were then grown on LB medium containing 10mM IPTG (iso-propyle-thioglycoside) for induction of the expression of r-ESAT 6 protein. Cells were taken at 1hr intervals and lysed in lyses buffer (6mM guanidine hydrochloride) and subjected to SDS-PAGE (fig2).

D. Purification and refolding of the protein

After induction with 10 mM IPTG for 5 hrs the recombinant cells were harvested by centrifugation and lysed. The supernatant was passed through Ni-NTA agarose column .After washing with (8 mM urea pH 6.5) the recombinant protein was eluted in (8 mM urea pH 4.5) and refolded in (30% polyethylene glycol), and electrophoresed on 12% SDS-PAGE.

E. Western blotting analysis

The electrophoretic transfer of polyacrylamide gel resolved proteins to the nitrocellulose membrane was carried out by electroblotting as previously described ***(16)*** using BioRad Electro Transfere unit. The unoccupied sites on the nitrocellulose membrane were blocked with blocking buffer (Tris buffered saline TBS, pH 7.2 containing 0.1% Tween-20, 1% (w/v) western blot grade gelatin and 0.05% Triton X100). The nitrocellulose membrane was then incubated with RGS-penta His antibody solution (1:100 in blocking buffer) at 37°C for 1 hour followed by washing three times with TBS-tween 20. The membrane was then incubated at 37°C for 1 hour in anti-mouse peroxidase labeled dilution of 1:5000 in TBS- tween 20. The membrane was then washed as above and incubated in freshly prepared substrate solution (10 mg aminoethyle carbazone in 50 ml PBS containing 50 µl of 30 % H_2O_2) for 3-4 min for colour development and visible bands were developed, then the reaction was stopped by washing the membrane with running distilled water.

ESAT-6-specific Ig^G ELISA

ELISA plates (His-Sorb microtiter plates, Qiagene) were coated with recombinant ESAT-6 (50 ng /well) overnight at 4°C. Free binding sites were blocked by 3% bovine serum albumin–PBS. Individual serum samples from the experimentally infected guinea pigs or cattle were analyzed in two fold dilutions starting with 1/50. Protein G HRP labeled secondary antibodies was diluted 1/15000. OPD was used as a substrate and the O.D was read at 450 °A. the cut off value was then calculated according to ***(17)***. A serum dilution was considered positive if it yielded a mean OD of each group equal to/or greater than the cut off value.

Lymphocyte proliferation assay

Lymphocyte proliferation assay was carried out using Cell proliferation ELISA BrdU (Colorimetric) kit Roche applied Science (Cat # 1 647 229) according to the manufacture instruction. Briefly, blood lymphocytes (PMNC) were purified on a density gradient (using Ficole hypobaque). Cells were cultured in microtiter wells (96-well plates; Nunc, Roskilde, Denmark) containing 3 $X10^5$ cells in a volume of 100 ml of RPMI 1640 supplemented with 50 µM 2-mercaptoethanol, 100 µg/ml penicillin-streptomycin, 1 mM glutamine, and 10% (vol/vol) fetal calf serum. Each sample was run in 6 well to the 1st two wells phytoheamagglutinin (PHA) was added in a final concentration of 0.5µg/well, and to the 2nd two wells r EAST-6 was added in a final concentration of 5µg/well and the last 2 wells remain unstimulated. The plates were then incubated for 5 days in a humid chamber at 37°C and 5% CO_2. The plates were then pulsed with 10µl /well of BrdU labeling solution for 120min, centrifuged at 1500rpm/10min at RT, placed in hot air oven at 60°C/90 min then fixed with 100µl / well of the fixative solution (provided with the kit) for 30 min. the plates were then decanted and washed twice with the washing solution and the 100µl of anti-BrdU monoclonal antibodies was added and incubated for 120min/37°C, washed as before and 100µl/well of the substrate solution was added and incubated in dark place /5 min and then the O.D. value was measured at wave length of 450°A. The results were then undergo statistically analyzed.

RESULTS

The *esat 6* gene was amplified from the genomic DNA of *M. bovis* by PCR using cloned *Pfu* polymerase which have high validity, when DNA concentration was 200µg/reaction a clear visible band migrate at 300 pb was visualized under the UV illumination (Fig 1 lane 2).

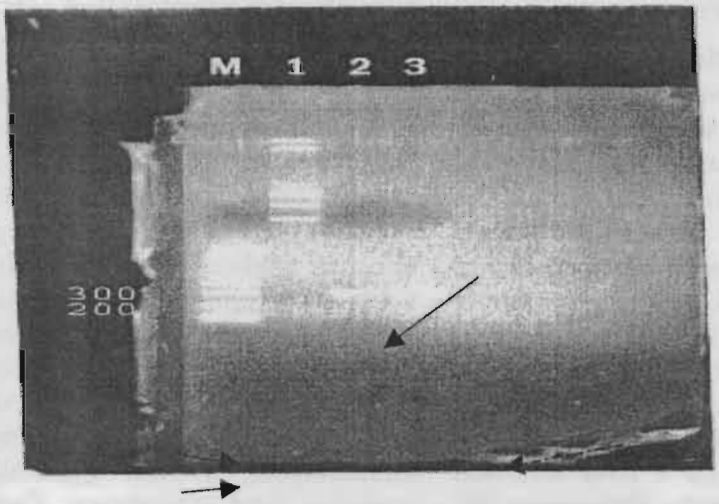

Figure 1. Agarose gel electrophoresis of *Bam HI* digested PQE 50 plasmid (lane 1) amplified *esat 6* from genomic DNA of *M. bovis* (lane 2) or plasmid miniprep (lane 3), 100pb DNA ladder (Finzyme) (lane M). note the presence of 2084 pb band corresponding to the digested PQE 16 plasmid with the 2 enzymes (solid arrow) and the 300pb band represents the amplified *esat 6* gene (dashed arrow)

After cloning of the *esat 6* gene in the pcDNA3.1cloning vector, the plasmids with correct orientation was checked by PCR using forward primer provided with kit that anneal to the plasmid 5' upstream the amplicon and the reverse primer that anneal to the end of the amplicon. A band with 300 bp was seen only with plasmids carrying the amplicon in the correct orientation (Fig 1 lane 3).

After subcloning of the *esat 6* gene in PQE expression vector, *E. coli* XL1 blue competent cells were heat shock transformed with the constructed plasmid, over than 20 cells /plate were grown by overnight incubation at 37°C. Colony hybridization (fig 2) showed that over 80% of the colonies were positive (expressing the r ESAT 6 antigen).

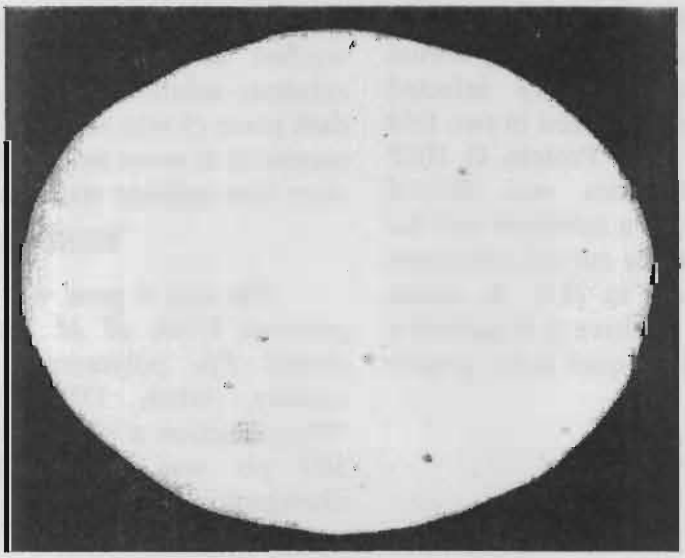

Fig. 2. Colony hybridization of the transformed *E. coli* cells.

For induction of expression overnight culture of *E. coli* XL1 blue cells were used to inoculate a 10ml of LB-medium containing antibiotics the $OD_{600} \approx 0.6$ after 0.5-2 hours. Then IPTG was added. Fig. 3 show the SDS-pattern of induced and non-induced cells harvested at the time intervals indicated. A clear visible band that migrate at 18 KDa, the intensity of the bands increased by time and reached the maximum after 4 hours.

The expressed protein was then identified by western blotting analysis using anti-HIS monoclonal antibody that reacts with the 6 histadine tag of the expressed ESAT 6 protein antigen.(fig 4).

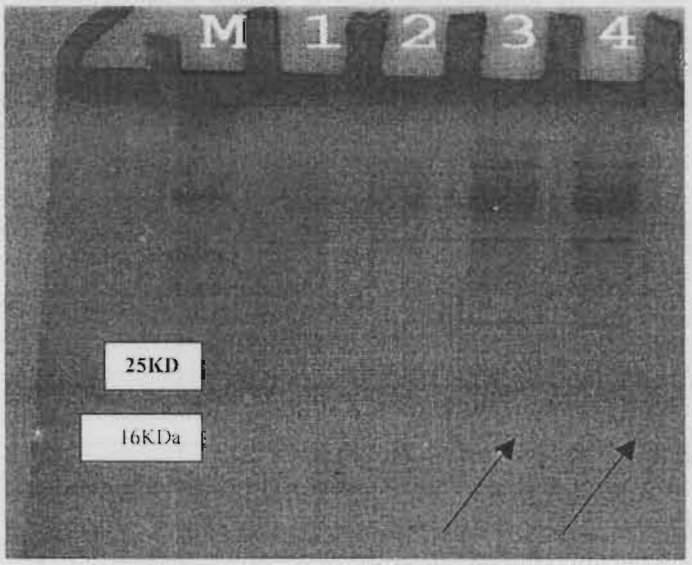

Fig. 3. SDS-PAGE of non-induced XL1 blue *E. coli* cells (lane 1). after 2,3,4 hours of induction (lane 2,3,4 respectively). M (marker).notice the presence of a band at 18 that increase in the intensity by the time of incubation.

Fig 4. The western blotting analysis of r ESAT 6 protein antigen shown a clear distinct band at 18 KDa (arrowed)

The recombinant ESAT 6 protein antigen was evaluated for its potential differential diagnosis of bovine tuberculosis in the experimentally infected guinea pigs with different mycobacterial species, serologically using ELISA or using the cellular proliferation assay

Table (1) and fig (5) shows the ELISA results, and table (2) and figure (6) the cellular proliferation assay of sera and PMNC obtained from the experimentally infected guinea pigs respectively.

Table 1. ELISA mean results of guinea pig sera 4 weeks post experimental infection using recombinant ESAT 6 as an antigen.

Group#	strain	Mean O.D. value of ELISA results of guinea pig sera 4 weeks post experimental infection				
		Serum dilution				
		1/50	1/100	1/200	1/400	1/800
١	*M. tuberculosis*	0.450	0.343	0.184	0.043	0.025
٢	*M. bovis*	0.829	0.773	0.720	0.625	0.465
٣	*M. Bovis BCG*	0.063	0.068	0.055	0.069	0.083
٤	*M. intracellulare*	0.061	0.083	0.058	0.067	0.064
٥	*Negative control*	0.067	0.080	0.060	0.074	0.080

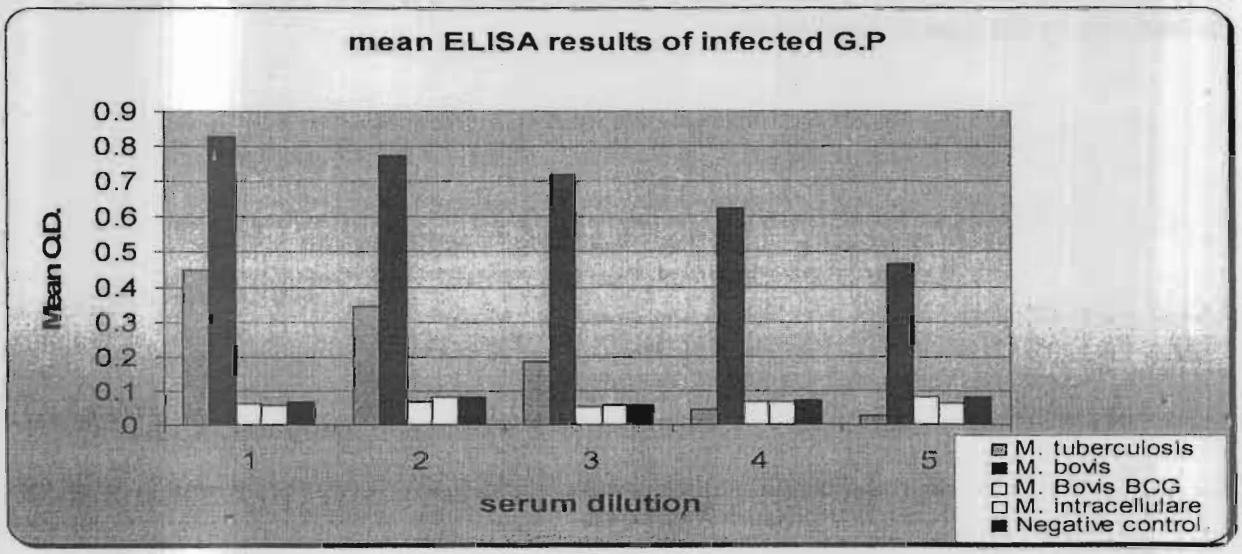

Fig 5. ELISA mean results of guinea pig sera 4 weeks post experimental infection using recombinant ESAT 6 as an antigen.

Table 2. Mean O.D. value of the stimulated and non stimulated PMNC cells of the guinea pigs with either r ESAT 6 antigen or PHA.

Group #	strain	Mean O.D. value of the PMNC cells of the guinea pigs		
		r- ESAT 6 stimulated cells	PHA stimulated cells	Non stimulated cells
١	*M. tuberculosis*	0.683	0.615	0.189
٢	*M. bovis*	1.528	0.635	0.199
٣	*M. Bovis BCG*	0.175	0.554	0.198
٤	*M. intracellulare*	0.186	0.599	0.185
٥	Negative control	0.173	0.567	0.168

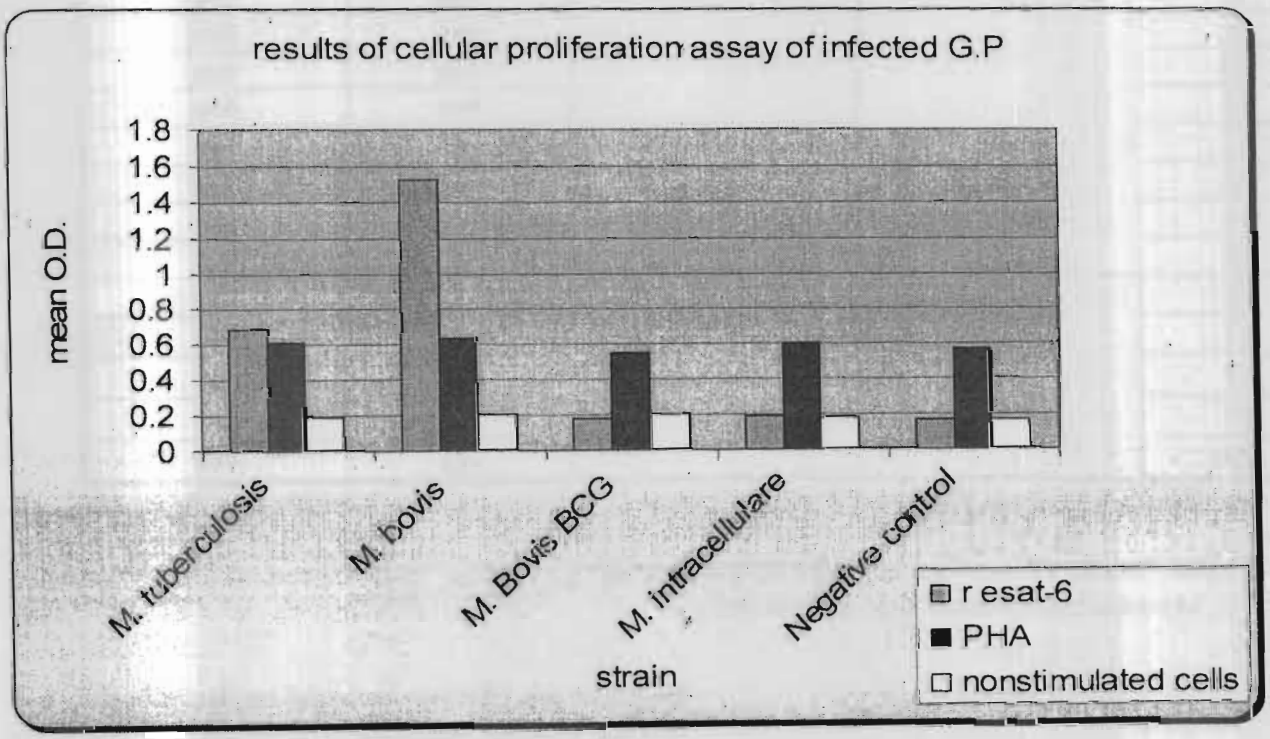

Fig 6. Mean O.D. value of the stimulated and non stimulated PMNC cells of the guinea pigs with either r ESAT 6 antigen or PHA.

For field evaluation of the recombinant ESAT 6 antigen as a differential diagnostic reagent, 20 serum samples from cattle were tested by ELISA using r ESAT 6 as an antigen, in comparison to the traditional bacteriological and tuberculin test (Table 3).

Table 3. ELISA results of the cattle sera as tested by r ESAT 6 antigen.

Animal #	Tuberculin test	Bacteriological examination	ELISA results	
			r ESAT-6	Bovine PPD
1	+ve	*M. bovis*	*+ve	+ve
2			+ve	+ve
3			+ve	+ve
4			+ve	+ve
5			+ve	+ve
6			+ve	+ve
7			+ve	+ve
8			+ve	+ve
9			+ve	+ve
10			+ve	+ve
11			+ve	+ve
12		**MOTT	-ve	+ve
13			-ve	+ve
14			-ve	-ve
15			-ve	-ve
16	-ve	-ve	-ve	-ve
17			-ve	-ve
18			-ve	-ve
19			-ve	-ve
20			-ve	+ve

*A serum dilution considered positive if it yielded a mean O.D. value equal to or greater than the cut off value

Cut off value = mean O.D. of the negative sera plus 2 SD

** MOTT. Mycobacterium other than tuberculosis

DISCUSSION

The ideal diagnostic tool for bovine tuberculosis would be one that accurately and uniquely detects infection of individuals with *M. bovis*. The current diagnostic agent, PPD, although sensitive and inexpensive, has specificity problems, yielding positive results in animals sensitized by environmental, nonpathogenic mycobacteria. Furthermore, the use of PPD as a skin test reagent is not simple and requires two field visits, first to administer PPD intradermally and 3 days later to read the reaction. It would therefore also be desirable if future tests were simpler to perform. It has been recognized that improvements in diagnosis are likely to be based on the use of a species specific mycobacterial antigen. Since mycobacterial infections generally induce a strong Th1 response, most work has focused on defining T-cell responses to candidate proteins.

Great variation in the cellular response, both between individuals and over time within an individual, to mycobacterial antigens purified from *M. bovis* culture filtrate by gel electrophoresis has been previously described in cattle infected with *M. bovis* ***(18).*** Such variation has important ramifications for the evaluation of potential diagnostic reagents ***(19,20).*** Some researcher set out to delineate the kinetics of T-cell (proliferation, IFN-γ, and

IL-2) responses to a panel of defined mycobacterial antigens in cattle experimentally infected intratracheally with an isolate of *M. bovis* from Great Britain. The antigen chosen for this study was selected on the basis of their ability to induce cellular immune responses in bovine or human tuberculosis ***(21- 23)***.

ESAT-6 and CFP-10 have previously been identified as antigens for a T-cell based diagnostic assay for bovine tuberculosis ***(22, 25, 24).***

The present study investigates the possibility of the recombinant ESAT 6 protein antigen of *M. bovis* as a differential diagnostic reagent for bovine tuberculosis.

In order to amplify *esat 6* gene from the genomic DNA of *M. bovis* to increase the copy number, a PCR technique was applied using a designated PCR primers that recognize the specific sequence in the *esat 6* gene. The nucleotide sequence of *M. bovis esat 6* (http://www.sanger.ac.uk/Projects/M_bovis) was studied and a specific primer was designated to span the whole open reading fram *(ORF)* of the gene with the deletion of the stopping condone of the gene allowing the co-expression of 6 histidin tag in the expression PQE plasmid.

The amplified *esat 6* gene (amplicon) was first cloned in pcDNA3.1 vector using the advantage of the TA technology that allows the cloning of amplicon generated by *taq* polymerase that inserts a 5' protruded A base and the correct orientation was examined by PCR that uses the forward primer that anneal to the 5" upstream of the cloning vector and the reverse primer that anneal to the 3" end of the gene. From about 50 colonies only 3 colonies yielded a plasmid with the correct orientation, these plasmids were digested and the insert was subcloned in the PQE expression vector.

For cloning and expression of ESAT 6 protein antigen, an expression vector was constructed and used for the transformation of the expression *E. coli* host strains XL1 blue. Recombinant protein expressing transformants were selected by 2 means, 1st by the antibiotic resistance of the +ve transformants and 2nd by the colony hybridization. Constructed expression vector will transfer ampicillin resistance to the transformed *E. coli* through part of *bla* gene sequence from PQE 16 and the other completing sequence from 50 plasmids to form complete transcriptable sequence, thus only transformants that receive the constructed plasmid will survive in the ampicillin-containing medium.Colony hybridization was performed using penta-His MAb that recognize at least 5 successive molecules of histidin regardless of the surrounding amino acid context ***(26,27)*** and the constructed expression vector will add 6 histidin molecules at the C-terminal of the recombinant ESAT 6 (His-tag) so that only fully expressed protein will be detected in the hybridization method. It was noticed that about 40% of the *E. coli* transformants gave +ve signals in the colony hybridization and it is worth mentioned that some of these signals was highly intense and these transformants were traced back and selected for the further steps of induction and purification. Expression was induced by the use of 10mM of IPTG (fig. 3), lower molar concentrations as 1mM, yield no or very little concentration of the r ESAT 6 protein, the explanation was that the mycobacterial genes are difficult to be induced so that needs high inducer concentration (Qiagen tech information, personal communication).

SDS-PAGE analysis revealed a distinct band at ~ 18 KDa that vary in concentration during time coarse evaluation analysis (fig. 3). This may be due to trimmers from the glycosylated or non glycosylated form ESAT 6 because cloning was done with the signal sequence of the gene so the recombinant protein was directed to the periplasmic space where partial glycosylation occur. In the glycosylated form the carbohydrates are covalently linked to the protein and do not dissociate even in the presence of SDS and dissociating agents such as guanidine and urea, also it is possible that the linkage of the sugar to the protein is not conventional glycoprotein linkage as found in the eukaryotes and the

carbohydrate moiety could be a part of heteropolysaccharide such as liposaccharide linked on the protein by acylation on cysteine residues ***(28).***

Cellular and humeral evaluation of the potential differential diagnoses of bovine tuberculosis was done under experimental condition. First sera from expermintally infected guinea pigs were tested with ELISA that utilizes r ESAT 6 protein as antigen. As shown in table 1 the cut off value is 0.09 so for *M. tuberculosis* the ELISA gave +ve reaction till serum dilution of 1/200 were as for *M. bovis* serum dilution of 1/800 still gave +ve reaction. For other mycobacterial strains ELISA results gave –ve reaction even at serum dilution of 1/50. These results can be explained on the basis of species distribution of the *esat 6* gene in different mycobacterial strains and the homology in the DNA amino acid sequence in these strains. In a previous trial conducted before ***(29)*** the species distribution of ESAT-6 in different MOTT were examined. Molecular analyses of cultured isolates from bovine lymph node specimens of 48 cattle identified a wide variety of mycobacterial species including *M.nonchromogenicum*, *M. malmoense*, *M. bohemicum*, *M. paratuberculosis*, *M. avium*, *M. kansasii*, *M. holsaticum*, *M. palustre*, M. sp. IWGMT 90210, M. sp. LIV-2129, a potentially novel mycobacterial species (EMBL/GenBank/DDBJ Accession Number AJ617495) and *Rhodococcus equi* revealed the absence of the ESAT-6 protein antigen from all tested strains except *M. kansassii.*

Second, the differential diagnostic potential of the ESAT-6 protein was assessed with cellular proliferation assay using cell proliferation ELISA BrdU kit which is a colorimetric immunoassay for the quantification of cell proliferation based on the measurement of bromo-uridine incorporation during DNA synthesis (a non radioactive alternative to the [^{3}H]– thymidine incorporation assay). Table (2) and figure (5) shows the results of cellular proliferation assay of PMNC cells of the experimentally infected guinea pigs. As with the ELISA, the PMNC isolated from guinea pigs infected with MOTT was not reactive to the ESAT 6 due to the lack of this antigen in such stains where only *M. bovis* and *M. tuberculosis* infected guinea pigs reacted to this antigen but the intensity of the reaction to *M. bovis* was greater than that to *the M tuberculosis*. It may be explained on the basis of the heterology of ESAT 6 *M. bovis* than that of M *tuberculosis* on the level of amino acid sequence.

In a field trial, 15 tuberculin +ve and 5 tuberculin –ve cattle were examined by ELISA that utilize either recombinant ESAT 6 or bovine PPD as antigen, as shown in table 3 r ESAT-6 but not PPD could differentiate between *M. bovis* infected and mycobacterium other than tuberculosis (MOTT) sensitized cattle and that is due to the presence of high level of shared antigen between MOTT strains and PPD antigen where the ESAT 6 antigen is completely absent from all MOTT ***(29).***

From this study it could be recommended that recombinant ESAT 6 antigen could replace the traditional tuberculin test for the differential diagnosis of bovine tuberculosis.

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الملخص العربي

كلونة و تعبير الأنتيجين esat-6 من ميكروب السل البقري للتشخيص التفاضلي للسل البقري

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المعمل المركزي للرقابة علي المستحضرات الحيوية البيطرية

في بحثٍ لتَطوير كواشفِ إختبار الجلدِ الجديدةِ، تم إختبار الأنتيجين البروتيني ESAT 6 لتشخيص مرض السل في الأبقار. فتم عزل و تنقية الحامض النووي لميكروب سل الأبقار و بواسطة إختبار تفاعل إنزيم البلمرة المتسلسل تم تحضير الجين الخاص بالبروتين ESAT 6 . ثم إلحاق هذا الجين بالبلازميد PQE و نقله لخلايا ***E. coli*** ثم تحفيز تعبير الجين داخل هذا الخلايا بإستخدام 10 mM IPTG و تحديد طوله كحزمة عند الطول ١٨ كيلودالتون عند اجراء اختبار التحليل الكهربى . وقد تمَ تنقية البروتين المستنسخ بواسطة الكروماتوجرفي .

الإمكانيةُ التشخيصيةُ للأنتيجين المستنسخ ESAT 6 تم إختباره في خنازير غينيا - المعداه صناعيا بالعترات المختلفة لميكروب السل – بواسطة إختبار الأليزا و قد أكدت الأختبارات تفوق هذا الأنتيجين علي PPD في تحديد الحيوانات المصابة بميكرب السل البقري عند تخفيف ١:٥٠ للسيرم.

في تجربة حقلية لتَقييم الإمكانيةِ التشخيصيةِ للأنتيجين المستنسخ ESAT 6 تم بواسطة الأليزا أختبار السيرم المأخوذ من ماشية إجابية و سلبية رد الفعل لأختبار التيوبركلين , حيث تم التأكيد من أصابة الماشية -إجابية رد الفعل لأختبار التيوبركلين – بميكروب السل البقري بإجراء الصفة التشريحية وفحص بكتريولوجي. و يتضح من النتائج أن إستخدام الأنتيجين المستنسخ ESAT 6 كانت له القدرة علي تحديد الإصابة بميكروب السل البقري عند تخفيف ١:٥٠ للسيرم الأبقار.