

**Expression of *Mycobacterium avium* subsp.
paratuberculosis epitopic regions of MP16 and
MP25 as 22 kDa fusion protein for study its
serodiagnostic potential**



THESIS

*Submitted in partial fulfilment of the requirements for the degree
of*

Master of Veterinary Science

in

ANIMAL BIOTECHNOLOGY

By

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Roll No. 4876

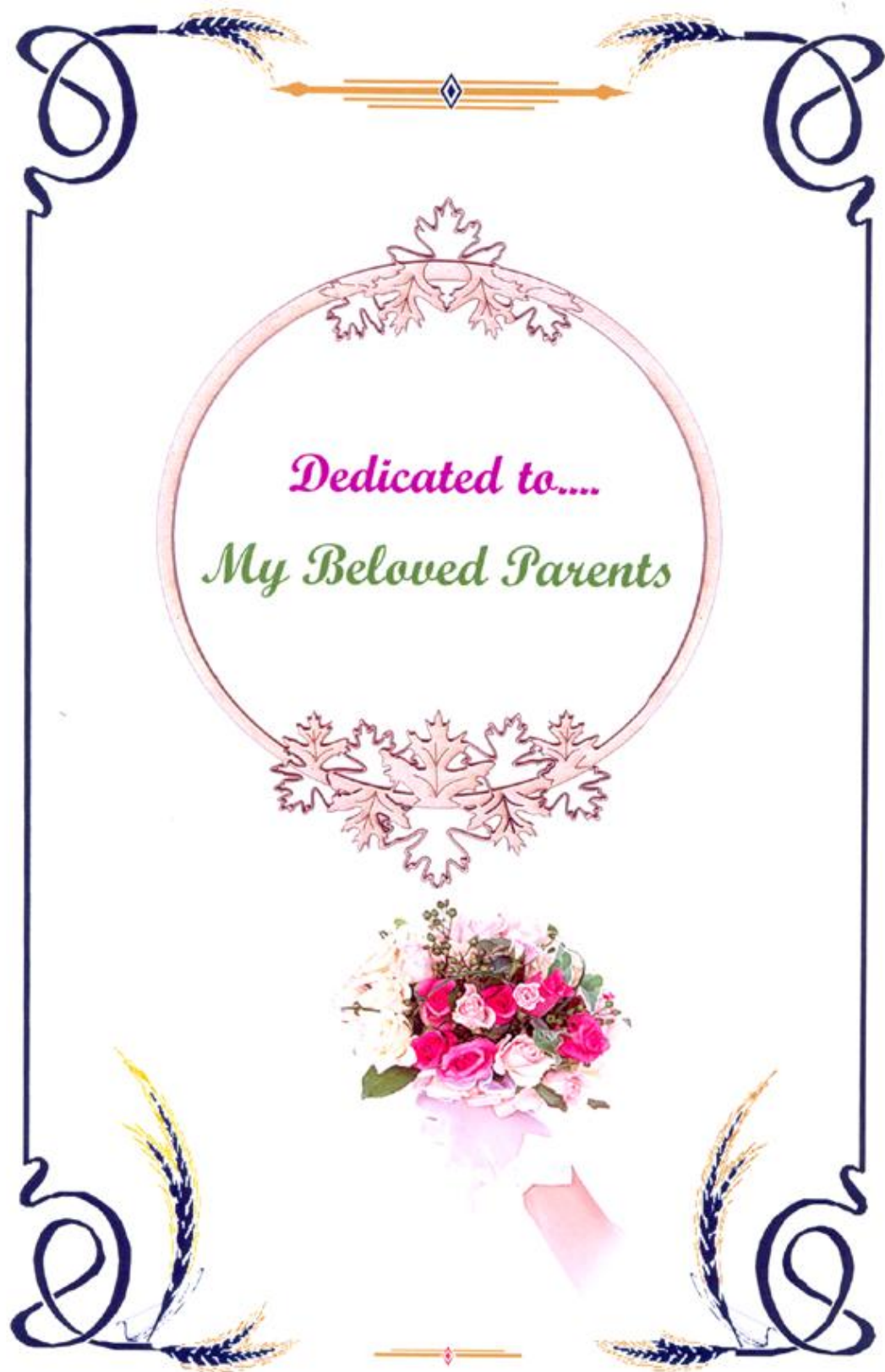
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भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Certificate

Certified that the research work embodied in this thesis entitled "Expression of Mycobacterium avium subsp. paratuberculosis epitopic regions of MP16 and MP25 as 22 kDa fusion protein for study its serodiagnostic potential" submitted by Dr. Sumanth B.E., Roll No. 4876, for the award of Master of Veterinary Science degree in Animal Biotechnology at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that Dr. Sumanth B.E., Roll No. 4676, has worked for more than 21 months in this Institute and has put in more than 150 days attendance under me from the date of registration for the degree of Master of Veterinary Science of the Deemed University, as required under the relevant ordinance.


(P.P. GOSWAMI)
Chairman
Advisory Committee

Certificate

Certified that the thesis entitled, "Expression of Mycobacterium avium subsp. paratuberculosis epitopic regions of MP16 and MP25 as 22 kDa fusion protein for study its serodiagnostic potential " submitted by Dr. Sumanth B.E., Roll No. 4876, in partial fulfilment of the requirement of Master of Veterinary Science degree in Animal Biotechnology, Deemed University, Indian Veterinary Research Institute, Izatnagar, embodies the original work done by the candidate. The candidate has carried out his work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of Master of Veterinary Science of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of Master of Veterinary Science of Indian Veterinary Research Institute.

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(Sumanth B E)

Abbreviation

%	:	Percentage
μl	:	Microlitre
mg	:	Milli gram(s)
ml	:	Milli litre
mM	:	Milli mole
°C	:	Degree celsius
A	:	Adenine
APS	:	Ammonium Persulfate
bp	:	Base pair
BSA	:	Bovine Serum Albumin
C	:	Cytosine
CMI	:	Cell Mediated Immunity
CTAB	:	Cetyl trimethyl ammonium bromide
DNA	:	Deoxyribo nucleic acid
dNTP	:	Deoxy nucleotide triphosphate
ds DNA	:	Double stranded DNA
DAB	:	Diamino benzidine
DNA	:	Deoxyribonucleic acid
DTH	:	Delayed type hypersensitivity
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
HRPO	:	Horse radish peroxidase
Hrs	:	Hours
His-22	:	Recombinant 22 kDa protein
IFA	:	Incomplete Freund's Adjuvant
IFN-γ	:	Interferon- gamma
iELISA	:	Indirect ELISA
IPTG	:	Isopropyl β-D-thogalactosidase
Kb	:	Kilobase pair
KDa	:	Kilodalton
KCl	:	Potassium Chloride

LB	:	Luria Bertani
MP	:	Mycobacterium paratuberculosis
MAP	:	Mycobacterium avium subsp. paratuberculosis
MgCl ₂	:	Magnesium Chloride
min	:	Minute
mA	:	Milli Ampere
Ni-NTA	:	Nickel- nitrilotriacetic acid
OADC	:	Oleic acid albumin dextrose catalase
ORF	:	Open Reading Frame
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
RE	:	Restriction Endonuclease
RNase	:	Ribonuclease
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBE	:	Tris borate EDTA
TGS	:	Tris glycine EDTA
TE	:	Tris EDTA
TEMED	:	NNN'N'-Tetra methylene diamine
Tris	:	Tris hydroxyl methyl amino methane
X-Gal	:	5 - B r o m o - 4 - c h l o r o - 3 - i n d o l y l - β - DGalactopyranoside
UV	:	Ultra violet

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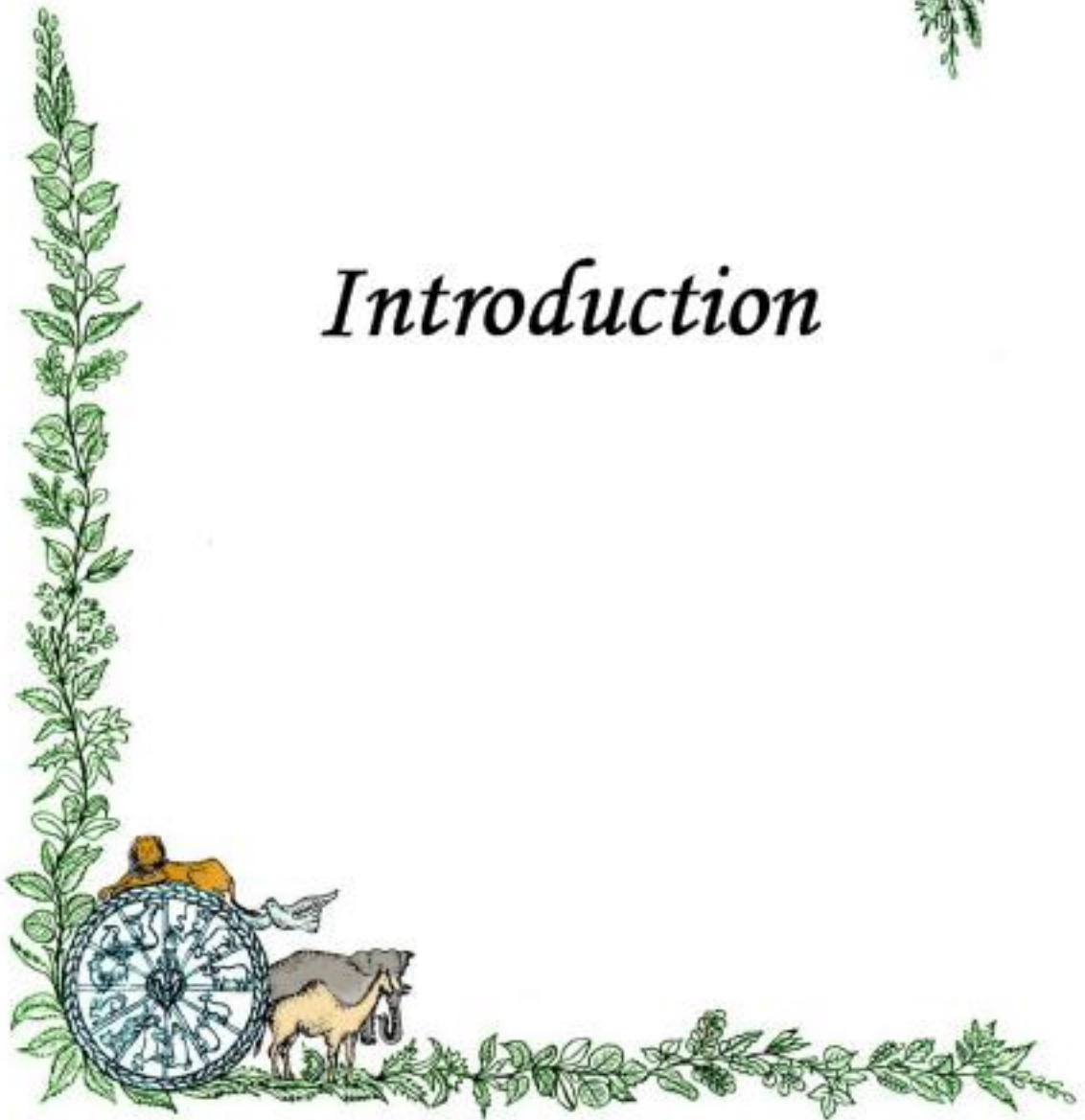
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Introduction





Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an intracellular pathogen that causes paratuberculosis (Johne's disease), a chronic granulomatous enteritis of domestic animals, wild ruminants and primates (Manning and Collins 2001).

Clinically infected animals develop chronic diarrhoea, progressive weight loss, debilitation, and eventually die. The organism is acid-fast, slow growing, fastidious bacterium requiring exogenous ferric mycobactin for its growth *in vitro* (Turenne *et al.*, 2007). After infection the animals remain in the subclinical stages of infection for two to five years and shed the organism in the faeces enabling the transmission to other animals (Johnsen *et al.*, 2001). This organism has also been implicated associated in Crohn's disease, a chronic multifactorial inflammatory bowel disease in humans (Uzagore *et.al*, 2007) Recently the MAP has also been characterized by hepatic granulomas in HIV positive and other immunocompromised patients (Toyoda *et al.*, 2009).

Johne's disease is widespread throughout the world and causes substantial economic losses to the farmers and the dairy industry. Johne's disease is considered as one of the most costly infectious disease of the Dairy herd (Hasonova *et al.*, 2006). The annual economic impact to the dairy industry is estimated at \$276 per cow per lactation for infected cows (Raizman *et al.*, 2009). The prevalence has increased more than three times in last one decade so that the expected loss

was much higher (USDA-APHIS, 2008; Kirkpatrick *et al.*, 2010). Paratuberculosis is enzootic disease declared by Office Internationale des Epizootics ('OIE') [List B disease as per the old classification].

At present no effective therapeutics or vaccine candidates are available that can completely eliminate viable MAP from the host (Hines *et al.*, 2007). Furthermore, vaccination induces antibody production and also a sensitization to delayed type hypersensitivity making difficult, thereby, the differential diagnosis of vaccinated animals from naturally infected animals.

In spite of several attempts, no effective diagnostics are available and early detection along with good management practices are the only way to control paratuberculosis (Harris and Barletta, 2001). One of the challenges to the effective management of affected herds is the identification of infected animals prior to the appearance of disease signs such as faecal shedding of MAP in particular, the sub-clinical infections are difficult to diagnose due to intermittent shedding of small numbers of bacteria (Bannantine *et al.*, 2004). The faecal culture test requires 12-16 weeks for cultivation and its sensitivity level is estimated to be approximately 38% (Whitlock *et al.*, 2000). Detection of MAP in faeces by PCR is sensitive and rapid but applicable only to late stage of the infection when animal starts shedding bacteria. Moreover, the tests (PCR and faecal culture) fail to differentiate between the pass through MAP and MAP colonization (Speer *et al.*, 2006).

Tests available for diagnosis of Johne's disease need to be improved due to deficiencies in their sensitivity or specificity. Problems of specificity are due to the high degree of similarity that exists between MAP and environmental mycobacteria, especially the closely related *M. avium* subsp. *avium* (MAA). It is not surprising therefore, that cell mediated immune assays such as the intradermal (Johnin) test, which

uses a complex, undefined MAP derived protein preparations to stimulate an immune reaction in the host. The test is less specific and prone to false-positive and associated with unspecific reactions due to cross-reactivity with similar proteins present in other mycobacteria (Huda *et al.*, 2004).

The identification of MAP antigens that could allow early, sensitive and specific detection of infection is critically needed to implement sound disease control measures. However, the current practice of using complex MAP protein mixtures as antigens, including Johnin purified protein derivative or whole cell extracts, compromises assay specificity due to the presence of conserved epitopes in those mixtures. Therefore, immunoassays are specific for the diagnosis of Johne's disease requires purified antigens unique to MAP (Bannantine *et al.*, 2010).

An *in vitro* assay used to measure gamma interferon (IFN- γ) levels in animals infected with MAP (Billman-Jacob *et al.*, 1992) was successful in identifying animals in the early stages of infection. However, IFN- γ assay have been reported to give higher degree of positive reactors suggesting a lack of specificity inherent in the assay (Huda *et al.*, 2003 and Storset *et al.*, 2005).

There are three widely used tests for the serodiagnosis of paratuberculosis: agar gel immuno-diffusion test (AGID), complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA). These antibody based assays for the diagnosis of paratuberculosis in small ruminants, have been reported to have similar discriminatory power for detecting clinical cases (Perez *et al.*, 1997). However, the AGID and ELISA seem to be more sensitive in identifying subclinically infected sheep than the CFT (Hilbink *et al.*, 1994).

The most frequently used test for paratuberculosis is based on serum antibody detection using an ELISA based techniques. While

being inexpensive, fast and able to handle numerous samples, it is of low diagnostic sensitivity during much of the course of the infection. Compromised specificity can also be a problem due to the extensive sharing of antigens or epitopes among MAP and other mycobacteria (Collins *et al.*, 1991).

It is largely accepted that major improvements are needed in currently available serodiagnostic tests to allow for an efficient control programme to be set up (Collins *et al.*, 2005; Sweeney *et al.*, 2006). Many laboratories have therefore chosen to improve the efficiency of serological assays by replacing the current common “extract antigen” by “purified MAP-specific subunit antigens” (Dupont *et al.*, 2005; Paustian *et al.*, 2004; Willemsen *et al.*, 2006). To date, a number of MAP specific immune targets have been identified and antigenicities of some proteins were evaluated. But the data about their reactivity in ELISA using large panels of sera, are still lacking (Bannantine *et al.*, 2004, 2008).

Different approaches have been used in the search for antigens unique to MAP eliciting immune response. Among immunoproteomic approaches, in which antigens are firstly selected on the basis of their antigenicity, and genomic approaches, in which antigens are selected in the finished genome of MAP on the basis of their sequence specificity (Li *et al.*, 2005). Recently, two large-scale immunoproteomic analyses have been reported very promising antigens, some of which can be used in detection of paratuberculosis infection by indirect ELISA (Cho *et al.*, 2006, 2007; Leroy *et al.*, 2009).

The identification of MAP antigen that could allow early, sensitive and specific detection of infection is critically needed to implement sound disease control practice. Bannantine *et al.* (2008), generated a partial protein array representing 92 MAP coding

sequences and showed that some MAP specific proteins were strongly detected with clinical sera at early post infection in experimental model.

The antibody response to MAP is complex with no evidence of a uniform response to a single antigen at any stage of infection. Therefore, there is a need to incorporate and express more than one antigen which may increase the chance of detecting antibodies to MAP with high sensitivity jeopardizing the specificity, which can be used for the development of an antibody based assay for paratuberculosis. Few studies have already been initiated in case of *M. tuberculosis* (Liu *et al.*, 2007; Rosenkrands *et al.*, 2008).

In our laboratory a number of MAP specific coding sequences have been identified and expressed in *E. coli*. Two MAP specific coding sequences from locus tag MAP 1087 and MAP 1204 encoding for 16 kDa (ABC peptide permease) and 25 kDa (putative invasion) proteins respectively were expressed individually in *E. coli* and their antigenicity have been evaluated (Goswami *et al.*, 2010). These antigens have also been reported to elicit antibody during early post infection in experimental MAP infected cattle (Bannantine *et al.*, 2008).

In the present study the tag MAP 1087 and tag MAP 1204 regions (MAP strain K10 complete genome accession no. AE016958) were selected which codes for 16 kDa (MP16) and 25 kDa (MP25) proteins respectively. The epitopic regions from these locus tags were used to generate 642 bp (627 bp + 15 bp linkers) fragments for expression of 22 kDa fusion protein. Further the fusion protein was used to study its sero diagnostic potential.

At present there is no published report available about the expression and characterization of the epitopic region of MAP specific coding sequence as fusion protein on these locus tag. Hence it was proposed to attempt this approach with the following objectives:

- To generate a construct containing B-cell epitopic region of MP-16 and s MP-25
- To clone and express the fragment in prokaryotic expression system as a fusion protein.
- To study the immunoreactivity and serodiagnostic potential of the expressed fusion protein.



*Review
of
Literature*





Review of Literature

GENERAL FEATURES

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an intracellular pathogen causes paratuberculosis (Johne's disease), a chronic granulomatous enteritis of domestic animals, wild ruminants and primates (Chiodini 1984, Manning and Collins 2001).

Paratuberculosis is a disease infecting domestic animals with chronic enteritis (diffuse thickening and corrugations of intestinal mucosa with presence of acid fast bacilli in the lesions) was first described by Johne and Frothingham in Germany in 1895 (Chiodini *et al.*, 1984). MAP has a broad host range starting from domestic ruminants (Cattle, sheep, goats), non ruminants (like pigs, dogs, horses and cats), wild ruminants, free ranging animals (blue bull, deer and rabbits) and primates (Chiodini *et al.*, 1984). The organism has been isolated from the systematically infected animals, particularly with the clinical disease (Antognoli *et al.*, 2008; Bower *et al.*, 2011). The reports of paratuberculosis in wild life including monogastric animals suggest additional reservoirs that may increase the risk of transfer of the disease (Beard *et al.*, 2001; Daniels *et al.*, 2003; Cleland *et al.*, 2010).

The causative agent, originally named *Mycobacterium enteritidis chronicae pseudotuberculosis bovis johne*, was then referred as *Mycobacterium paratuberculosis*. The classification of *M.paratuberculosis* has followed classical bacterial lines based on

the main criteria of extremely slow growth and the requirement of exogenous mycobactin. However, some strains of *M. avium* designated wood pigeon mycobacteria require exogenous mycobactin only on primary isolation but later become independent of it on subsequent passage.

MAP is a member of Mycobacterium avium complex (MAC). Other members of the MAC group include *M. avium* subsp. *avium* (MAA), *M. avium* subsp. *sylvaticum* (MAS), *M. avium* subsp. *hominis* (MAH) and a second species *M. intracellulare* (MI). Cattle can be infected systematically with MAA and MAH, but humans are infected preferentially with MAH. All members of the MAC are highly genetically similar, although the small distinctions between MAA and MI are now well established (Thorel *et al.*, 2001).

There is evidence of association of MAP with Crohn's disease, a chronic multifactorial inflammatory bowel disease in humans (Uzagore *et al.*, 2007; Behr, 2008). While some studies did not reveal MAP in CD patients. The meta-analysis demonstrated the clear association of MAP with CD (Abubakar *et al.*, 2008).

MAP is small, rod shaped (0.5 x 1.5 μ), Gram positive (weak) and acid fast organism. It requires mycobactin, an iron chelating compound, for its *in vitro* growth and produces smooth or rough, small (1-5mm) firm glistening colonies (Merkal and Curran 1974). MAP is an intracellular obligate pathogen with strong affinity for lamina propria of intestine remain in the soil and water less than one year without replication (Whitnigton *et al.*, 2011). It has a thick poorly permeable lipid rich cell wall containing mycolic acid which imparts taxonomically as well as diagnostically important features such as acid fastness, resistance to disinfectants, heat (pasteurization) and partially determines the narrow spectrum of antibiotics which may be used to treat the infection.

The disease is ubiquitous and distributed throughout the world including India. Numerous reports of the infection are available in cattle, sheep and goats. Motiwala *et al.* (2004) have reported occurrence of the disease in wild ruminants and birds. Animals are apparently infected when young and shed the organism via faeces without showing the clinical symptoms for several years.

Johnes disease is considered as one of the most costly infectious disease of the Dairy herd (Hasonova *et al.*, 2006). The annual economic impact to the dairy industry is estimated at \$276 per cow per lactation for infected cows on a dairy (Raizman, 2009).

The organism may resist high heat and the presence of MAP in the milk has been reported for milk supplies including bulk milk at farm supplies and bulk milk at farm level (Stabel *et al.*, 2005) at processing level. Animals became infected as neonates primarily via the faeco-oral route but do not develop clinical sign of disease for years (Rossels *et al.*, 2006). The disease also transplacentally transmitted and remains in latency in tissues and depend on the macrophage for spread in the host (Whittington *et al.*, 2011). The infected animal shed the organism in the faeces while remain clinically in apparent (Begg *et al.*, 2008).

HOST IMMUNE RESPONSE

After oral uptake MAP is able to pass the intestinal wall with a topical preference for the distal part of the small intestine, the ileum. M cells, specialized in uptake of particles, preferentially bind bacteria and transport them to the basolateral side (Momotani *et al.*, 1988). Following release of MAP in the extracellular fluid of the lamina propria, they are taken up by macrophages, which normally process bacteria via enzymatic processes. MAP, however, is specialized in the blockage of phagosome-lysosome fusion, thereby promoting its survival

(Kuehnel *et al.*, 2001). Furthermore, they provide anti-apoptotic signals extending the life span of their host cell. MAP interacts with macrophages via different kinds of receptors, including Toll-like receptors (TLRs) (Ferwerda *et al.*, 2007; Koets *et al.*, 2010). MAP is able to upregulate interleukin (IL)-10, an immunomodulatory cytokine that suppresses killing of MAP by macrophages, as well as Th1-type immune responses, which are most desired to combat intracellular infections, by inhibition of IL-12 (Weiss *et al.*, 2005; Weiss *et al.*, 2002).

Macrophages are important cells of the innate immune system, but also provide signals to induce adaptive immune responses. MAP is able to interfere with antigen presentation, hence subsequent adaptive responses (Weiss and Souza, 2008). Our understanding of the adaptive immune response against mycobacteria in general, and against MAP more specifically, is far from complete. The chronic nature of the infection, its primary locus of infection (the intestinal wall), and the availability of tools to dissect the immune response provide interesting challenges. Much of our understanding is gained from longitudinal responses observed in peripheral blood from infected animals, or obtained by cross-sectional analysis at necropsy. For vaccine development it is critical to find correlates of protection, defined as specific immune responsiveness to a vaccine that is closely related to protection against infection or disease (Plotkin, 2008).

MAP-infected macrophages can activate T cells via antigens presented on major histocompatibility complex (MHC) class I and II molecules, or CD1 molecules. Activated T cells will clonally expand to generate sufficient numbers for an effective immune response. Subsequently, antigen-experienced T cells will differentiate into different memory subsets, called effector memory (Tem) and central memory (Tcm) T cells. Tem are able to directly exert effector functions

at the site of inflammation and are relatively short-lived. Tcm are long-lived immune cells, which can respond quickly upon secondary antigen encounter (Sallusto *et al.*, 2010).

Depending on the interaction between MAP and immune cells, T cell responses will be polarized and differentiated into defined subsets, like the classical Th1, Th2, and more recently identified Th17 cells. Mycobacterial infection primarily leads to an immune response characterized by a Th1 type reaction pattern, of which interferon-gamma (IFN-gamma) is the key effector cytokine (Flynn and Chan, 2001; Kaufmann, 2010). IFN-gamma plays an important role in the activation of T cells and macrophages. It is produced by CD4, CD8, and $\gamma\delta$ T cells, and natural killer (NK) cells (Bassey and Collins, 1997; Boysen and Storset, 2009; Rogers *et al.*, 2005). Experimental studies in cattle have shown that MAP-specific proliferative T cell responses and IFN-gamma can be detected as first marker of infection in the first year after challenge (Koo *et al.*, 2004; Stabel and Robbe-Austerman, 2011). At the primary locus of infection, the lamina propria where infected macrophages are situated, there is a constant interplay between MAP and the host immune system. In animals that progress to the clinical phase of paratuberculosis immune control is insufficient. It has been shown that the local immune response is altered by a progressive loss of a MAP-specific CD4 T cell population towards an increased proportion of potentially regulatory/anti-inflammatory $\gamma\delta$ T cells (Hoek *et al.*, 2009; Koets *et al.*, 2002). The chronic antigenic stimulation at the site of infection can lead to induction of regulatory T cells, which limit the pro-inflammatory response initiated by Th1 T cells (Coussens, 2004; de Almeida *et al.*, 2008).

The B cells respond to their antigen via triggering of the B cell receptor, often in orchestration with T cell help from CD4 T cells in

secondary lymphoid tissues (lymph node and spleen). Subsequent proliferation and differentiation gives rise to memory B cells and plasma cells, producing antibodies. Little is known about the development of the B cell response in the course of paratuberculosis. The consequence of the B cell response, indicated by serum antibodies against MAP, is used in diagnostic assays. They can be measured reliably from 2 years of age with the current diagnostic assays. Most of these diagnostic assays measure antibodies directed against crude antigens, like tuberculins. Tuberculins are complex mixtures of soluble protein antigens derived from mycobacteria grown in vitro. The lack of measurable antibody responses may be due to pathogenic mechanisms directing the host pathogen interaction away from antibody-mediated immune responses in the early phases of infection. An alternative explanation may be that antibodies are formed in the early phases of infection. However, the target antigens are not present or scarce in the tuberculin protein mixtures. A recent study by Bannantine *et al.* indicated that a transient antibody response can be detected at 70 days post-infection against some defined recombinant MAP antigens (notably MAP0862) (Bannantine *et al.*, 2008).

Our understanding of the immune response directed at MAP antigens present at different stages of infection, comparable to e.g. latency antigens in tuberculosis (Lin and Ottenhoff, 2008), will be boosted by increasing availability of recombinant antigens and multiplex antibody detection technologies.

VACCINE

Studies with whole-cell based vaccines, either live attenuated or inactivated, have collectively shown that occurrence of clinical symptoms and tissue colonization can be reduced, but infection is not prevented in cattle (Sweeney *et al.*, 2009; Uzonna *et al.*, 2003).

One of the major drawbacks of the use of whole-cell based vaccines is the interference with diagnosis of bovine tuberculosis and paratuberculosis (Kohler *et al.*, 2001; Muskens *et al.*, 2002).

One lead for development of new paratuberculosis vaccines is directed at the use of subunits, mostly proteins, potentiated with adjuvants. Several Th1 antigens have been identified by genomic and proteomic analysis: the antigen 85 complex proteins (A, B, and C) and superoxide dismutase (Shin *et al.*, 2005); Hsp65 and Hsp70 (Koets *et al.*, 1999); lipoprotein LprG ((Rigden *et al.*, 2006); PPE family proteins MAP1518 and MAP3184 (Nagata *et al.*, 2005). Only few of these have been tested in cattle, the target species, namely: the antigen 85 complex proteins together with superoxide dismutase, and heat shock protein 70 (Kathaperumal *et al.*, 2008; Koets *et al.*, 2006).

DNA vaccination was also shown to protect mice from MAP challenges (Huntley *et al.*, 2005; Park *et al.*, 2008 and Roupie *et al.*, 2008). Multi-antigen virally vectored vaccine found to be effective (Bull *et al.*, 2009).

DIAGNOSIS

Diagnosis of bovine paratuberculosis can be divided in assays that detect the infectious agent or detect a MAP-specific immune response in the host. At present, no single diagnostic test is available which can detect the disease at all stages. The detection in early stage is essential for instituting preventive measures and for removing carrier animals from herd.

Detection of organism

Acid-fast staining of faecal sample may reveal mycobacterial bacilli on microscopic examination, but the sensitivity of this method is low. It is often difficult to detect and distinguish MAP from other

non-pathogenic mycobacteria resulting a low specificity of the test (OIE Manual, 2004). Faecal culture is considered as the gold standard but the method requires several weeks before a test result is observed and the procedure is also costly.

The preferred and most reliable approach to date has been direct detection of *M.a.paratuberculosis* by Ziehl Neelsen's staining in faeces or biopsies of mesenteric lymph nodes, in which presence of more than one clump of 3-4 strongly acid-fast bacteria is considered positive for infection with paratuberculosis (OIE manual, 2004).

The faecal culture is not sufficient to detect the infection status of the animal; supportive histo-pathological lesions are considered as a standard method to diagnose Johne's disease (National Academy of Sciences Reports, 2003). Considerable research efforts aimed to develop better faecal processing and detection methods have failed to increase the sensitivity beyond 50% and to reduce the growth period which is currently 12 to 16 weeks or even more (Sackett *et al.*, 1992; Whitlock *et al.*, 2000).

A radiometric culture method, BACTEC, has been used to reduce the time for detection of MAP (Whittington *et al.*, 1998). The liquid medium contains radioisotope labeled nutrient source (CO₂ labeled palmitate) called BACTEC 460 which measures CO₂ released from palmitate in bacterial metabolism. However, the growth of other mycobacteria limits the possibility of detecting MAP.

Immunohistochemical methods such as direct fluorescent antibody Test (FAT) (Gilmour and Angus, 1976); indirect immunoperoxidase Test (Navarro *et al.*, 1991); avidin biotin complex techniques and colloidal immunogold technique have attempted for detection of MAP in tissues mainly as confirmation of pathologic lesions.

Detection of cellular immune response

The first detectable host response to mycobacterial response is the cell mediated immune response. This test evaluates the delayed type hypersensitivity (DTH) reaction after intradermal injections of *M. a. paratuberculosis* PPD (johnin), is an indicator of the CMI response of animals. However, cross reactivity with other mycobacteria preclude its use as a diagnostic tool for paratuberculosis (Manning and Collins, 2001). The intradermal johnin test, a delayed type of hypersensitivity reaction, is the only diagnostic test available at the field level, and it has been widely used for screening of paratuberculosis in small and large ruminants. It is mostly used as a single intradermal test. An increase in the skin thickness over 2 mm after 72 hr after inoculation is regarded as positive. Manning and Collins (2001) advocated an increase in skin thickness of 3 mm in 24-72 hr to be considered positive. Sigurdardottir (1999) also described an increase of skin thickness of >3.5 in 48 hr as positive Johnin test.

Lymphocyte stimulation test LST is a sensitive *in vitro* method for the detection of T-cell mediated immune response to different mycobacterial antigens. Hence, proliferative responses of lymphocytes either in peripheral blood or in the lymphocyte culture following stimulation with mycobacterial antigens have been utilized for the diagnosis of paratuberculosis (Tripathi *et al.*, 2002).

The IFN- γ assay has been reported in a number of studies for detection of sub-clinical infection in sheep (Perez *et al.*, 1999; Gwozdz *et al.*, 2000). Sensitivity of this test in clinical paratuberculosis in cattle ranged from 50-93%, when compared with repeated faecal culture results, and clinically affected cattle may also give negative results (Stable and Whitlock, 2001).

Perez *et al.* (1999) found that 55.4% sheep with paratuberculosis lesions and 6.1% sheep without lesions were

positive in IFN- γ assay. The IFN- γ assay also showed promise for detection of infected animals soon after exposure. In several studies, where lambs and kids were experimentally infected with large doses of MAP, IFN- γ assays yielded positive results after 9-18 weeks (Gwozdz *et al.*, 2000; Storset *et al.*, 2001).

A study by Storset (2005) revealed that the IFN- γ assay was more reliable test for the diagnosis of MAP infection in case of small ruminants than cattle. Interferon gamma (IFN- γ) assay has been shown in a number of studies in the detection of subclinical paratuberculosis in sheep (Perez *et al.*, 1999; Gwozdz *et al.*, 2000). To increase the sensitivity an ELISPOT assay, a highly sensitive technique for detection of cytokines has the potential to detect the Johne's disease both at early stages and later in advanced clinical cases of the disease. The assay detects the total number of IFN- γ producing cells than measuring the total amount of IFN- γ as is usual in traditional cytokine ELISA assay. The test is 10-200 times more sensitive to detect the cytokines than ELISA assays (Douglas *et al.*, 2009)

Detection of Humoral immune response

Humoral immune responses to an infection can be detected by measuring antibody production by the host, with the use of various serological methods. The serological tests most frequently employed are ELISA, AGID and CFT. It is widely believed that serological tests can be very sensitive, when antibody production is at its highest, as in clinical stage of disease, the appearance of clinical signs may be evident, before a positive test result is achieved.

AGID was amongst the first serological tests developed for the diagnosis of paratuberculosis and it was used as a supplementary method to faecal culture (Pavlik *et al.*, 2000). It has a high specificity (more than 90%) in cattle with clinical signs, stage III and stage IV, of

the disease while the sensitivity is estimated to be 30% in the early stage IV infections (Hermel, 1998). The AGID test is considered as a reliable test for confirmation of paratuberculosis in clinically suspected sheep (Shulaw *et al.*, 1993) and goats (Sherman *et al.*, 1984; Rajukumar *et al.*, 2001). The most widely used antigen in the AGID is the protoplasmic extract of *M. a. paratuberculosis*. Tripathi *et al.* (2006) recorded the sensitivity of AGID as 96.2% in multibacillary cases and 50% sensitivity in paucibacillary cases. In comparison to ELISA and CFT, AGID is considered less sensitive (Nielsen *et al.*, 2001)

The Complement Fixation Test (CFT) was considered valuable for identifying the paratuberculosis in early 1980s (Delisle *et al.*, 1980; Ratnamohan, 1986). However, it was unsatisfactory in subclinical infections with high proportions of false positives and false negatives (Abbas *et al.*, 1983).

ELISA is a more sensitive and specific test for serum antibodies, than any other serological tests and it has been widely used for screening herds (Clark *et al.*, 2008; Pinedo *et al.*, 2008). Detection of infection, by the ELISA technique, appears to be dependent upon the disease stage of the animal tested (Nielsen and Toft, 2008). Dargatz and co-workers (2001), who evaluated the sensitivity of ELISA using a commercial kit, found that the results ranged from 15.4% in cattle classified as light MAP shedders, to 80% for heavy shedders. Sergeant others (2003) also reported a variation in sensitivity of the ELISA in sheep with different histological lesion type, possibly indicating variation with the stage of disease. They found that sensitivity was higher in sheep with multibacillary lesions, than sheep with paucibacillary lesions.

Different ELISAs such as unabsorbed, absorbed, lipoarabinomannan (LAM) and affinity purified antigen (APA)-ELISA

have been developed (Manning and Collins, 2001). An absorbed ELISA, based on protoplasmic antigen, and a non absorbed use a lipoarabinomannan-based antigen was developed (Kenna et al., 2005) Later Speer *et al.*, (2006) reported an ELISAs using whole bacilli treated with formaldehyde (WELISA) as well as a surface antigen, obtained by treatment of MAP bacilli with formaldehyde and sonication (SELISA) appeared superior than commercial ELISA used for the diagnosis of JD.

Eda *et.al.*, (2006) demonstrated an ethanol vortex enzyme-linked immunosorbent assay (EVELISA) based on surface antigens of MAP which had diagnostic specificity and sensitivity rates of 96.9 and 100%, respectively. EVELISA identified 96.6% of the low fecal shedders and 100% of the midlevel and high-level shedders, and 96.2% if the high-level and mid-level shedders. The EVELISA may form the basis for a highly sensitive and subspecies-specific test for the diagnosis of JD.

Further a JTC ELISA based on using antigens secreted (early to mid-log phase) had a higher diagnostic sensitivity which can detect low level faecal shedders of Map (40%). The JTC ELISA worked effectively both on serum and milk samples for detection of cattle with subclinical MAP infections. (Shin *et al.*,2008)

A flow cytometric method (FCM) has also been used could be used to diagnose early as well as chronic Johne's disease by testing serum for antibodies against *M. a. paratuberculosis* (Eda *et. al.*, 2005). The FCM had diagnostic sensitivity and specificity levels greater than 95% and was capable of detecting Johne's disease 6 to 44 months earlier than the fecal test and 17 to 67 months earlier than commercial ELISA.

In contrast to the previous reports that the humoral immune responses prevail long after the cellular immune responses certain

recent studies have revealed that several MAP specific secretory proteins were expressed and released from the host cell during earlier stages of infection. These antigens make suitable targets for detection of antibodies during subclinical stages of infection due to their strong immunoreactivity *in vivo* (Koets *et al.*, 2001; Walters *et al.*, 2003)

Molecular approach

Advances in recombinant DNA technology are provides new approaches to research and are having considerable impact on progress in many areas including identification of genetic markers, strain differentiation, diagnosis and epidemiology. Further generation of species specific antigen will permit a more accurate analysis of the immune response to *M. a. paratuberculosis* in animals.

Molecular methods, such as the combination of DNA probes and PCR, are promising diagnostic tools for paratuberculosis (Soumya *et al.*, 2009), since they may be more sensitive than culture methods. Such methods also enable detection of MAP organisms, without having to grow the bacterium.

In most studies, the main DNA target has been the IS900 (Mobius *et al.*, 2008). Approximately 15 to 20 copies of this sequence are integrated into the genome of MAP (Green *et al.*, 1989). PCR methods targeting IS900 have been widely used for detection of the presence of MAP, in faecal samples (Irengue *et al.*, 2009), milk samples (Hertnek *et al.*, 2008), tissue samples (Pislak *et al.*, 2003) and buffy coat from the blood of cattle and sheep (Bhide *et al.*, 2006).

PCR, which targets MAP-specific sequences, is useful because of its rapid turn around time and high sensitivity (Brey *et al.*, 2006). However, reduced sensitivity of PCR has also been reported and it is

thought to be associated with several factors including the presence of PCR inhibitors, such as Ca^{2+} ions in the case of milk, and unsuccessful DNA isolation, due to inefficient extraction methods for mycobacterial DNA (Hertnek *et al.*, 2008). In addition, recent reports have challenged the sequences in mycobacteria unrelated to MAP (Tasara *et al.*, 2005).

In recent years, application of real-time (RT), nested and TaqMan PCR methods, along with immunomagnetic bead separation of the micro-organism and dot blot hybridization of PCR products to assays for the detection of MAP, have improved sensitivity and made it possible to perform semiquantitative analysis (Alinovi *et al.*, 2009). Ireng and co-workers (2009) carried out a triplex real time PCR (TRT-PCR), which targeted the multi-copy IS900, the single-copy MAP-restricted F57 and the unique-copy MAP-restricted ISMAP02 genetic targets, in order to improve PCR identification of MAP. Their study showed that inclusion of the F57 and/or the ISMAP02 targets are an important adjunct for rapidly confirming the presence of MAP in faecal samples. Application of RT-PCR also showed a higher sensitivity, compared to culture methods and this offered faster results for better on-farm decision making (Ireng *et al.*, 2009; Alinovi *et al.*, 2009).

MAP ANTIGENS

MAP has at least 44 antigen components, many of which are common with those of *M. bovis* and other *M. avium* strains (Gunnarsson and Fodstad, 1979). Glycolipid antigens such as glycopeptides-I and lipoarabinomannan are capable of evoking antibody responses (Camphousen *et al.*, 1988; Sugden *et al.*, 1989).

El Zaatari *et al.* (1995) screened expression library by monoclonal antibodies and identified 65 kDa genes having more than 90% homology to other mycobacterial species. Several antigens of *M.*

a. paratuberculosis were later on identified viz., 32 kDa (Anderson *et al.*, 1991); 35 kDa (El Zaatari *et al.*, 1997); Gro ES (Cobb *et al.*, 1999) 34 kDa (Coetsier *et al.*, 1998) and 34 kDa (Cameron *et al.*, 1994 and Sharp 1997). None of these proteins are unique to MAP thus limiting their utility as diagnostic reagents. Ideally immunogenic proteins unique to MAP would provide the desirable candidate for potential diagnostic reagent.

A 35 kDa protein of MAP reported by Banasure *et al.*, (2001) was expressed in *E. coli* (Basagoudanavar *et al.*, 2004). The expressed 35 kDa protein stimulated both T and B cell immune response (Basagoudanavar *et al.*, 2006). It was suggested that 35 kDa protein based ELISA could be useful for detecting antibodies in paratuberculosis infected animals (Shin *et al.*, 2005). Secreted MAP specific antigens of 9, 15 and 34 kDa (Map2609, Map2942c and Map0210c, respectively) were used for screening a genomic expression library with serum of a naturally infected clinical cow. The 9, 15 and 34 kDa antigens displayed strong homology to previously described *M. tuberculosis* antigens, TB8.4, MPT53 and Erp, respectively detect the clinical sera (Willman *et al.*, 2006). Most of the recombinant antigens discovered are homologous to the other mycobacterial species antigens resulting in cross reactivity that reduces the specificity in the detection of MAP.

The sequencing of the MAP genome ushered in a new era of research for finding diagnostic and prognostic indicators, construction of live-attenuated vaccines, and treatment targets for controlling JD (Li L *et al.* 2005). The pathogenic strain K-10 (Foley-Thomas EM *et al.* 1995) was used by Li *et al.*, as the best candidate of low *in vitro* passages to retain virulence factors and other genes of pathogenic importance. The MAP genome is 4.83 MB in size, encodes 4,344 open reading frames (ORFs), 45 tRNAs, one rRNA operon, and has a 69.3% GC

content. Of the ORFs, 60% have known functions, and 25% encode putative proteins of unknown functions. There is a high level of redundancy within the MAP genome due to gene duplication, approximately 50% of genes have duplications. Open reading frame sizes range from 114 bp (a ribosomal subunit encoding gene) to 19155 bp (a peptide synthetase). A total of 52.5% of genes are transcribed with the same polarity as the origin of replication. One hundred and fifty genes are regulatory in function. Insertion elements are abundant throughout the genome (20 different insertion elements with 58 copies) and one unique element (*IS900*) has been used in PCR diagnosis (Jayarao BM *et al.*, 2004).

Bannantine *et al.* (2002) analysed 21 predicted coding sequences *in silico* which were found to be present in all MAP isolates and absent from all other mycobacterial species when examined by PCR amplification. Gene sequencing followed by BLAST analysis also revealed the presence of these genes only in the MAP. The DNA sequences from these 21 unique predicted coding regions were amplified and cloned into two different *E. coli* expression vectors encoding polyhistidine or maltose binding protein (MBP) affinity purification tags. All 21 of the expressed proteins were successfully purified under denaturing conditions and were evaluated in immunoblotting studies with sera from rabbits and mice immunized with MAP. These studies showed that 5 of the 21 gene products are produced by MAP are antigenic when tested with a panel of sera from 9 healthy cattle and 10 cattle with clinical disease. These findings may have a major impact on improved diagnostics for Johne's disease (Bannantine *et al.*, 2004).

Computational and PCR-based analysis of the complete genome sequence of MAP (Paustian *et al.*, 2004) identified 13 open reading

frames with no identifiable homologs. One of these sequences is a putative insertion element present in six copies on the MAP genome.

These MAP genes were cloned into *E. coli* expression vectors, and nine were successfully expressed as recombinant fusion proteins. Five of these proteins were purified in sufficient amounts to allow immunoblot analyses of their reactivity with sera from naturally infected cattle. Two proteins were recognized by nearly all of the sera tested, including those from cattle in the clinical stages of disease. Collectively, the results of this study demonstrate the utility of genomic data to identify potential diagnostic sequences.

POSTGENOMICS AND PROTEOMICS

Cho *et al.* (2006) identified MAP proteins of potential interest which were first separated by 2-DE. Roughly 240 CF protein spots were detected on CBB-stained gels using Phoretix 2-D software. Of these, 83% reacted with serum from MAP infected cattle in immunoblots. When bovine serum was absorbed with *M. phlei* antigens, however, only 37 of these antigenic protein spots were reactive. Twenty-four of these spots were selected for identification based on their immunoblot staining intensity and differences in isoelectric pH and mass. A total of 14 proteins were ultimately identified by MS and BLAST searches as ModD, PepA, ArgJ, CobT, Antigen 85C, and nine hypothetical proteins. N-terminal peptide analysis of PepA, Antigen 85C, ModD, MAP1693c, MAP2168c, and MAP1022c showed that each protein has 27–39 amino acids that may function as a signal sequence suggesting that they are secreted through a Sec-dependent pathway. These 14 proteins from MAP CF are strong candidates for use as antigens for improved serodiagnostic tests for bovine paratuberculosis.

Cho *et al.*, (2007) further expressed these 14 ORFs in *E. coli* pET-22b (+) vector. Immunoblot analysis using ant CF Map JTC303

strain serum revealed that five of the expressed proteins were derived from CF components: ModD, Antigen 85C, PepA, MAP1693c, and MAP2168c. ELISA using ModD as solid-phase antigen on a set of paratuberculosis infected and noninfected bovine sera revealed that there was strong serum antibody reactivity to rModD.

Leroy *et al.* (2007) identified specific antigens by systematic proteomic identification of proteins present in MAP culture filtrates (CFS), followed by the selection of MAP-specific proteins by BLAST query on available mycobacterial genome. It resulted in the generation of database for secretome of MAP specific antigens. The second approach consisted in the immunoproteomic analysis of both MAP extracts and CFs, using sera from MAP-infected and uninfected cattle. Combining results obtained with both approaches resulted in the identification of 25 candidate diagnostic antigens. Among them three proteins identified, when used in an ELISA test that was equally sensitive (94.74%) and more specific (97.92%) than the existing commercial tests, and the combination of three of them competed in performance with available commercial assays, reaching a test sensitivity of 97.4% and specificity of 97.92%.

Bannantine *et al.* (2008) developed a partial protein array using 43 recombinant MAP specific proteins on dot blot format. Dot blot arrays were used to profile antibody responses in a rabbit and mouse exposed to MAP as well as in cattle showing clinical signs of Johne's disease. The MAP heat shock protein DnaK, encoded by ORF MAP 3840 and a membrane protein (MAP 2121c) were identified as the most strongly immunoreactive both in the mouse and rabbit hosts, respectively. MAP3155c, which encodes a hypothetical protein, was most strongly immunoreactive to sera from Johne's disease cattle. This study has enabled direct comparisons of antibody reactivity for an entire panel of over 40 proteins and has laid the foundation for

future high throughput production and arraying of MAP surface proteins for immune profiling experiments in cattle. Bannantine *et al.* (2008) again developed a partial protein array using 92 Map specific recombinant proteins using the sera from experimentally infected cattle and evaluated the humoral immune response on 70,194 and 321th day post infection. The studies revealed that the proteins encoded by MAP 0862, MAP 1087 and MAP 1204 elicited a strong humoral immune response as early on 70th day post infection. However the antibody titre against the protein encoded by MAP 0862 reduced with progression of time. Moreover antibodies against these three proteins were detected in the sera from naturally infected animals.

Leroy *et al.* (2009) screened the complete genome of MAP for identifying specific coding sequences that could be valuable in the design of a sensitive and specific serological assay. Among eighty seven MAP-specific sequences three candidate antigens were selected by *in silico* analysis based on their putative immunogenecity and have been analysed for their serodiagnostic potential. These antigens were cloned in *E. coli*, expressed, purified and tested in ELISA using a well characterized panel of 18 and 48 sera from MAP infected and uninfected cattle, respectively. Two of these antigens, antigen 6 and MAP1637c, yielded a sensitivity of 72% and 82%, respectively, for a specificity of 98%. It was particularly noticeable that, when probed, with the most widely used European paratuberculosis commercial seroassay (Pourquier test) the same serum panel yielded a sensitivity of 72% for a specificity of only 92%.

Santema *et al.* (2009) analyzed the qualitative protein composition of PPD of the major mycobacterial pathogens. Using onedimensional gel electrophoresis followed by tandem mass spectrometry of PPD from MAP, MAA and MB identified 156, 95 and 132 proteins, respectively. Comparative sequence analysis led to the

selection of one MAP specific protein (MAP1718c). Expression in *E. coli* of this and other diagnostic candidate proteins (MAP3515c and MAP1138c (LprG) enabled evaluation of their immunogenicity. The serum antibody levels for MAP1138c in paratuberculosis infected cows were higher than in control animals, despite the conserved nature of this protein.

Bannantine *et al.* (2010) using DNA micro array, studied the host responses to the infection with MAP yielding insight into the genetic regulation and biology of Johne's disease. Zhiguo *et al.* (2010), using SDS PAGE followed by LC-MS/MS, identified and characterized the cell wall associated antigens of MAP K10. They identified 309 different proteins including 101 numbers of previously identified hypothetical proteins creating a profile of subproteome. The combination of proteomics and genomics, starting from complex protein mixtures, present in PPD revealed some novel proteins for use in immunodiagnostics.



*Materials
and
Methods*





Materials and Methods

MATERIALS

Chemicals

All the chemicals and biologicals used in the present study were of molecular biology grade as mentioned in Table no. 1.

Glassware and plasticware

All glass and plastic wares used in the study were procured from Borosil India. Microfuge tubes and micropipette tips were supplied by Axygen, USA. ELISA plates from Nunc, Germany.

Buffers and other reagents

The compositions of various buffers/reagents used in this study are mentioned in the appendix.

Oligonucleotide primer

The sets of specific oligonucleotide primers (MP 1, 2, 3 and 4) were designed based on sequence information of MAP strain K10 complete genome accession no. AE016958 tag MAP 1087 and MAP 1204 which codes for 16 kDa (MP16) and 25 kDa (MP 25) proteins. The epitopic regions from these locus tags MAP 1087 and MAP 1204 were used to generate 642 bp fragment encoding 22 kDa fusion protein.

The primers were synthesized by integrated DNA technologies, inc. Coralville, IA USA. The nucleotide sequence of these primers were as follows:

Table 1 : List of chemicals and biologicals used in present study

Chemicals/kits	Source
- Restriction endonucleases - T₄ DNA ligase, Insta PCR Cloning Kit - DNA molecular weight marker, dNTPs solution, protein molecular weight marker - Agarose, Ammonium persulphate, Acrylamide, Bovine serum albumin, Bis-acrylamide, β-mercaptoethanol, Bromophenol blue, Calcium chloride, Coomassie brilliant blue 250, Magnesium chloride, Sodium chloride, Potassium Acetate, Tween-20, Isopropyl thiogalactose pyranoside (IPTG), X-Gal, lysozyme, Sodium dodecyl sulphate, Diamino-benzidine, TEMED, Glycine, Ethidium Bromide, Proteinase-K, RNase A, Triton X-100, PMSF, Trypan blue, Fetal calf serum, GMEM, Goat anti-rabbit IgG HRP conjugate, Rabbit anti-goat IgG HRP conjugate, Dialysis tube and antibiotics (ampicillin and kanamycin) - Potassium chloride, Disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, Hydrogen peroxidase, Certrimide, Agar, CTAB and Glucose - Glycerol, Sodium nitrite and Orthophosphoric acid - Sodium carbonate, Sodium bicarbonate, Isopropanol, Phenol, Chloroform, Ethylene diamine tetra-acetic acid, Boric acid, Glacial acetic acid, isoamyl alcohol, Sodium hydroxide, Sodium acetate, Sodium dihydrogen phosphate, Magnesium sulphate - QIAEX II Gel Extraction Kit - Plasmid isolation kit - Luria-Bertani Media, Middlebrook 7H10 agar and OADC salt - Mycobactin J	New England Biolabs, UK MBI Fermentas, Germany MBI Fermentas, Germany Sigma Chemicals, USA., Life Technology, USA Qualigens, India Merck, India SRL, India QIAGEN, Germany Au-Prep, USa Difco, USA Allied Monitor, USA

Primer Code Sense/ Antisense Sequence

MP 1(1087 F) Forward F 5'	GGA TCC ATG GAC ATC GTC GCG CAG GTC AAG 3' 27 mer
MP 2(1087 R) Reverse 5'	CCA CCG CCC CCG AGC CCA CCG CCC CCG AGG TCG AAA CGT GGG GAC CGC GCC 3' 51mer
MP 3(1204 F) Forward F 5'	GGT GGC GGC GGC TCG GGT GGC GGG GGC TCC ATG CGC CGC AAC CGG TTT CGT 3' 51 mer
MP 4(1204 R) Reverse R 5'	AAG CTT TCA CGG CGT GGC CGC GGC CGC 3' 27 mer

MAP strain

Bacterial strain of *M. a. paratuberculosis* 316F were obtained from *Central Diengenees Kunding Tieh Instituut, Lelystad, The Netherlands* and maintained at Gene Expression lab, Division of Veterinary Biotechnology, IVRI, Izatnagar. The Culture Filtrate (CF) antigen was prepared from the grown culture of MAP 316F and was already been available in the laboratory.

Plasmid and host strains

Prokaryotic expression vector pQE-30 UA and *E.coli* strain (M15, pREP4) purchased from QIAGEN, Germany was available in the laboratory. The plasmid contains a T5 promoter and a 6xHis-tag coding sequence at 5' to the multiple cloning region. The plasmid also contains an ampicillin resistance marker.

Laboratory animals

New Zealand white rabbits were obtained from Laboratory Animal Resource Section, IVRI, Izatnagar. Standard prescribed guidelines for care and use of laboratory animals were followed during the experimentation with these animals.

METHODS

Isolation of genomic DNA from *M. a. paratuberculosis*

The genomic DNA from *M. a. paratuberculosis* was isolated by the method of Portillo *et al.* (1991) with a few modifications. The bacterial colonies were scrapped from two months old Middle brook 7H10 agar slants in 1.5 ml microfuge tube, washed thrice with 1X TE and resuspended in 500 µl of 1X TE. Lysozyme was added to the final concentration of 5 mg/ml and incubated at 37°C for two hrs. SDS and proteinase K were added to a final concentration of 1% and 250 µg/ml respectively and incubated further at 65°C for 30 min. To this, 80 µl of 5M NaCl was added and vortexed. This was followed by addition of 64 µl of CTAB/NaCl solution and vortexed. The suspension was again incubated at 65°C for 30 min. DNA was extracted once with phenol, once with phenol: chloroform (1:1) and finally with chloroform: isoamyl alcohol (24:1). The aqueous phase containing DNA was pelleted by centrifugation and washed with 80% ethanol, dried and redissolved in 200 µl of 1X TE. RNA was removed from DNA by incubating with 100 µg/ml RNase treatment was given for one hour at 37°C, followed by phenol: chloroform extraction and ethanol precipitation.

The DNA was quantitatively estimated using the following formula, by recording the absorbance at 260nm wavelength:

$$\text{Concentration of dsDNA } (\mu\text{g/ml}) = A_{260} \times 50 \times \text{dilution factor}$$

Agarose gel electrophoresis

Agarose gel (1.2%) was prepared by boiling the agarose (low EEO, Sigma, USA) in an appropriate volume of 0.5X TBE. It was allowed to cool to about 50°C and then ethidium bromide (10 µg /ml) was added to a final concentration of 0.5 µg/ml. About 30 ml of agarose gel was poured into the casting tray, it was allowed to solidify

at room temperature for 30-45 min after insertion of the comb. Prior to electrophoresis, 2 µl of 6X loading buffer (MBI Fermentas, Germany) was added to 10 µl of PCR product. 12 µl of the samples were loaded into the well of the gel along with a 100 bp DNA ladder as molecular weight marker (MBI Fermentas). Electrophoresis was performed @ 6 volts/cm. The mobility was monitored by the migration of the loading dye. The gels were visualized under UV transillumination and documented by photography in Uvitech Gel Documentation System (Austria).

Polymerase chain reaction

Specific amplification of sequences containing probable B-cell epitopic regions MP 16 and MP 25 from genomic DNA of MAP was carried out in two tubes separately using the above mentioned primers (MP1 2, and 3, 4 respectively). In the second PCR reaction the amplified products each from two reaction were used as template to generate a 642 fragment with primers (MP1 and MP4). The PCR was carried out in 25 µl reaction volume containing 1 µl of genomic DNA (10 ng) as template, 2.5 µl of PCR buffer, 1 µl of $MgCl_2$ (1.5 mM), 1 µl (25 µM) of each primers, 1 µl of dNTP mix (200 µM of each dNTP) and 1 µl of Taq DNA polymerase. The volume was made up to 25 µl by adding DNase free water. The thermal cycling steps were carried out in PTC-200 thermocycler (MJ Research Inc, USA) with initial denaturation at 94°C for 5 min followed by 30 cycles with denaturation at 94°C for 1 min, annealing at 58°C for 1min, extension at 72 °C for 40 Seconds and final extension at 72°C for 10 min. Size of the amplified product was determined on a 1.2% agarose gel, visualized under UV transillumination and documented by photography in Uvitech Gel documentation System (Austria).

Fusion of gene sequences containing B-cell epitopic regions MP16 and MP25 was carried out by overlap extension PCR technology (Liu *et al.*, 2007).

Recovery of PCR product from agarose gel

The DNA fragment of interest was eluted from agarose gel using QIAEXII gel extraction kit (Qiagen, Germany). While running the gel two samples of the 642 bp PCR amplified fragment was loaded on the gel having at least leaving the gap of two wells. The fragment from first sample was exposed to UV light. The fragment from second sample was excised out of the gel using a sharp clean scalpel without exposing the fragment to UV light. The gel fragment was weighed, sliced and placed in a microfuge tube. QXI buffer three times to the weight of the gel piece was added and incubated at 50°C for 10-15 min with intermittent vortexing every 2 min to solubilize the gel. To it 10 µl of QIAEXII solution was added, the tube was gently vortexed, kept at room temperature for 20 min, followed by centrifugation at 10,000 rpm for 1 min. The pellet obtained was washed twice with 300 µl of buffer PE, air-dried and resuspended in 20 µl 1X TE by vortexing and kept at room temperature for 10 min. The suspended silica was pelleted at 12,000 rpm for 2 min and supernatant containing the eluted DNA was collected in a fresh sterile tube. Eluted Product was confirmed by agarose gel electrophoresis and visualized under UV transillumination.

Cloning of 642 bp fragment in prokaryotic (*E.coli*) expression system

Ligation

1 µl (50ng) of pQE 30 UA, 1 µl (50 ng) of eluted PCR product 3 µl nuclease free water and 5 µl of 2X ligation master mix were mixed in order in a sterile microcentrifuge tube. The 10 µl ligation mixture was kept at 16°C for 6 hours and stored at -20°C

Transformation

The transformation was carried out using kit from MBI Fermentas following the protocol as described by suppliers. In brief

the culture of DH5 α cells was inoculated into prewarmed C-medium without antibiotic for overnight growth at 37°C in shaker. When the OD600 of the culture reached about 0.5, 200 μ l of the culture was transferred to afresh C-medium and again incubated for one hr at 37°C in shaker. The T solution was prepared after thawing and mixing 250 μ l each of T-solution (A) and T-solution (B). The cells were pelleted at 6,000 rpm for 1 min at 4°C. The pellet was gently resuspended in 300 μ l of T-solution incubated on ice for 5 minutes. The cells were again pelleted at 6,000 rpm for 1 min at 4°C and resuspended with 120 μ l of chilled T-solution and again incubated on ice for 2 minutes to make the cells competent. 5 μ l of the ligation mixture was then added to the competent cells, mixed well by pipetting and incubated on ice for 5 minutes. The transformed cells were spread on LB agar plate containing ampicillin (100 μ g/ml) and kanamycin (25 μ g/ml). Appropriate positive and negative controls were processed simultaneously. Plates were incubated at 37°C overnight and later stored at 4°C.

Screening for recombinant pQE 642 clones

About twenty five randomly selected colonies were picked up and grown on LB broth containing ampicillin and kanamycin and incubated at 37°C overnight in a shaker incubator at 180 rpm. Plasmid DNA was extracted by miniprep plasmid isolation method (Sambrook *et al.*, 2001). Identification of positive clones was carried out by colony PCR and subsequently confirmed by resolving the expressed fusion protein on SDS PAGE. Positive clones were designated as pQE 642.

Expression of the recombinant 22 kDa protein

Two positive colonies were chosen from the master plate for induction. The colonies were picked up with a sterile toothpick and inoculated in 4 ml of LB broth ampicillin (100 μ g/ml) and kanamycin

(25 µg/ml) and incubated overnight at 37°C with constant shaking at 180 rpm for 12- 16 h. Ten milliliters of fresh LB broth containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) was then inoculated with 100 µl of the overnight culture and further incubated at 37°C with constant shaking until mid-log phase to reach an OD₆₀₀ of 0.6. One milliliter of the culture was collected from each tube and kept as an uninduced control. To the rest of the culture, IPTG was added at a final concentration of 1mM and kept at 37°C, with constant shaking at 180 rpm. All the cultures collected were pelleted by centrifugation at 6,000 rpm for 5 minutes and kept at -20°C till further use. Expression of the His-tagged 22 kDa (His 22) protein was analysed by SDS-PAGE analysis.

Purification of the recombinant 22 kDa protein

For purification of the His 22 protein, 100 ml of LB broth containing ampicillin and kanamycin antibiotics was inoculated with one microlitre of overnight grown *E. coli* culture from pQE 642 and incubated for 4 hrs in shaker till the growth reached an OD₆₀₀ of 0.6. The IPTG was added to the culture with the final concentration of 1 mM. After further incubation of 4 hrs at shaker, the cells were centrifuged and the pellet was weighed and lysed with 5 volumes of lysis buffer containing 8M urea (Buffer B, see appendix). The cell suspension was kept at room temperature for 1-2 h with intermittent vortexing. Subsequently, the debris was removed by centrifugation at 12,000 rpm for 30 min and the clear supernatant was transferred to a clean tube. Then 1/4th of Ni-NTA agarose slurry containing 2.5 mM imidazole (37.5 µl) and 20 mM β -mercaptoethanol (5 µl) was added and mixed thoroughly and kept on a tube rotatory shaker for 1 h with thorough mixing. The lysate-resin mixture was loaded on to 5 ml polypropylene column (Qiagen) equilibrated 39 with 1X Trisphosphate buffer (pH 8.0). The column was washed with 500 µl of

buffer-C (pH 6.3) thrice to remove proteins and other cellular debris leaving His tagged 22 kDa protein conjugated to Ni-NTA agarose. The column was further equilibrated with 500 µl buffer D (pH 5.9), followed by 500 µl buffer E (pH 4.5). The flow through passed through the column was collected in different tubes. The protein is quantitated by Bradford assay (Bradford, 1976) and analysed on SDS-PAGE to determine the purity.

Renaturation of the protein

The fractions containing *His* 22 protein were extensively dialyzed at 4°C against PBS, pH 7.4, to renature the protein. The dialysis tube (8 kDa cut-off) was prepared by the standard protocol (Sambrook *et al.*, 2001). Dialysis of the eluted denatured recombinant protein (I in 8M urea) was carried out against decreasing molar concentration of urea as 6M, 4M, 2M and PBS buffer (pH 7.4) at 4°C. The protein was analyzed on SDS-PAGE gel and quantitated by Bradford assay and was found to be 15 mg/L. The protein solution was sterilized by filtration and aliquots were stored at -20°C, until used.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE)

The induced recombinant pQE 642 pellets collected and the controls were resuspended in 50 µl of SDS-PAGE sample buffer (2X). The volume was made to 100 µl by addition of distilled water to the samples. The pellets were boiled for 10 min in a water bath in order to lyse and denature the bacterial proteins. Samples were then spun at 12,000rpm to pellet the cellular debris, following which 20 µl of the supernatant was loaded into wells, SDS-PAGE was carried out on a vertical slab mini apparatus (Atto, Japan) as described by Laemmli (1970). 12% separating and 5% stacking polyacrylamide gel containing 0.1% SDS was used. Electrophoresis was carried out in 1X Trisglycine electrode buffer (pH 8.3) at 100V for 2-3 hr till the bromophenol blue

migrated to the bottom of the gel. The gels was stained for 2h r with Coomassie brilliant blue G 250 (Sigma, USA) and destained in solution containing methanol and acetic acid as per procedure described by Towbin *et al.* (1979). The fractions containing the uninduced, induced and purified his 22 protein was resolved. The size of the protein was determined against the protein molecular weight marker.

Antibody production

Two New Zealand White 8-10 weeks old rabbits weighing approximately 1.5 kg were immunized to raise antibody against the His 22 protein .Rabbits were inoculated intradermally with 250 µg of the immunogen each in 0.5 ml PBS (pH 7.4) along with equal volumes of incomplete Freund's adjuvant (IFA) making an oil in water emulsion. After three weeks, first booster with 150 µg (in 0.5 ml) his 22 making an emulsion with 0.5ml IFA was given subcutaneously. Second booster was given intramuscularly with 300 µg His 22 in PBS 14 days after first booster. The rabbits were bled 10 days after second booster. The sera were separated and stored in aliquots at -20°C for further use. All the sera used in this study were preadsorbed with *E. coli* antigens following the procedure of Harlow and Lane (1988). Goat serum was obtained from a goat with clinical paratuberculosis confirmed by Ziehl-Nielsen staining of faecal smears and also by PCR amplification of the *M .a. parartuberculosis* specific IS 900 sequence (Collins *et al.*, 1989) from the faecal sample.

Western Blot analysis

For western blotting, the proteins from the gels were transferred to nitrocellulose membranes (0.45 iM) using semidry electroblotting (Atto, Tokyo, Japan) at 0.8 mA/cm², following the method of Bjerrum and Schaffer-Nielsen (1986). The blots were blocked with 2% skimmed milk powder in PBS-T buffer (PBS containing 0.1% Tween-20) for 2

hr at room temperature. After washing with PBS-T buffer three times, the membranes were incubated for 2 hr at 37°C with rabbit anti- His 22 protein antibodies (1:2000 in PBS). Following further washings, the blots were incubated with a 1:500 dilution of HRP-labelled goat anti-rabbit IgG (Genei®, India) for 1 hr. Similarly the reactivity of the MAP culture filtrate (CF) was also carried out using rabbit anti-His 22 antisera. The sero-reactivity of His 22 protein with the clinically positive goat serum (1:50 dilution) was recognized using a 1:500 dilution of a rabbit anti-goat HRP conjugate (Genei®, India). Antigens were visualized on the blots by incubation with 0.02% di amino benzidine suspended in PBS containing 0.03% hydrogen peroxide. The sera from a healthy goat was taken as -ve control.

Determination of antibody titre in rabbit sera against His 22 protein and detection of anti His 22 specific antibodies in paratuberculosis infected goats sera by ELISA

The optimum concentration of antigen (His 22 protein) and conjugate were determined using block titration as described by Engwall and Pearlman (1971). The wells of ELISA plates (Grenier, Germany) were coated with 25-100 ng/well of antigen diluted in carbonate bicarbonate buffer and the plates were incubated at 4°C overnight. The plates were washed thrice with PBS-Tween 20 (PBS-T) and blocked with 5% skim milk powder in PBS-T for 2hr at 37°C. Then, 1:200 dilution of serum in 100 µl volume of PBS-T were added in duplicate and incubated at 37°C for one hour. The plates were washed thrice with PBS-T for 3min at each wash. Conjugate anti-mouse IgG HRPO at dilution of 1: 2,000 in 100 µl volume was added to each well and the plates were incubated for 1hr at 37°C. The plates were then washed three times with PBS-T and colour was developed with 100 µl of 10 mg/ml OPD with 10 µl of 30% H₂O₂ in substrate buffer. After sufficient colour development the reaction was stopped

by the addition of 50 μ l 1N H₂SO₄ and the plates were read at 490nm in an ELISA reader (Tecan, Austria).

Similarly the MAP culture filtrate antigen and the His 22 protein was also used for detecting antibodies in paratuberculosis infected goats sera.

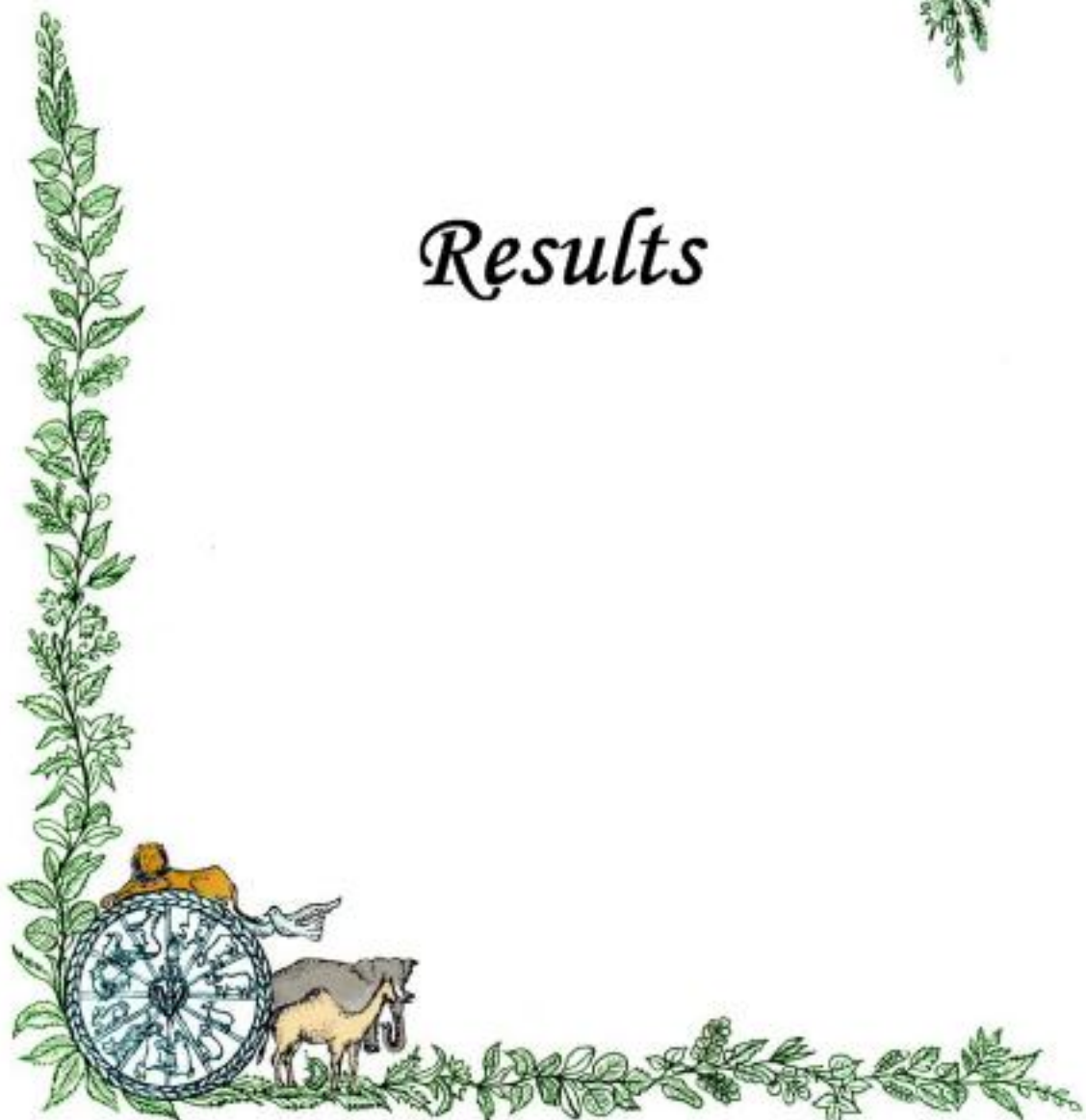
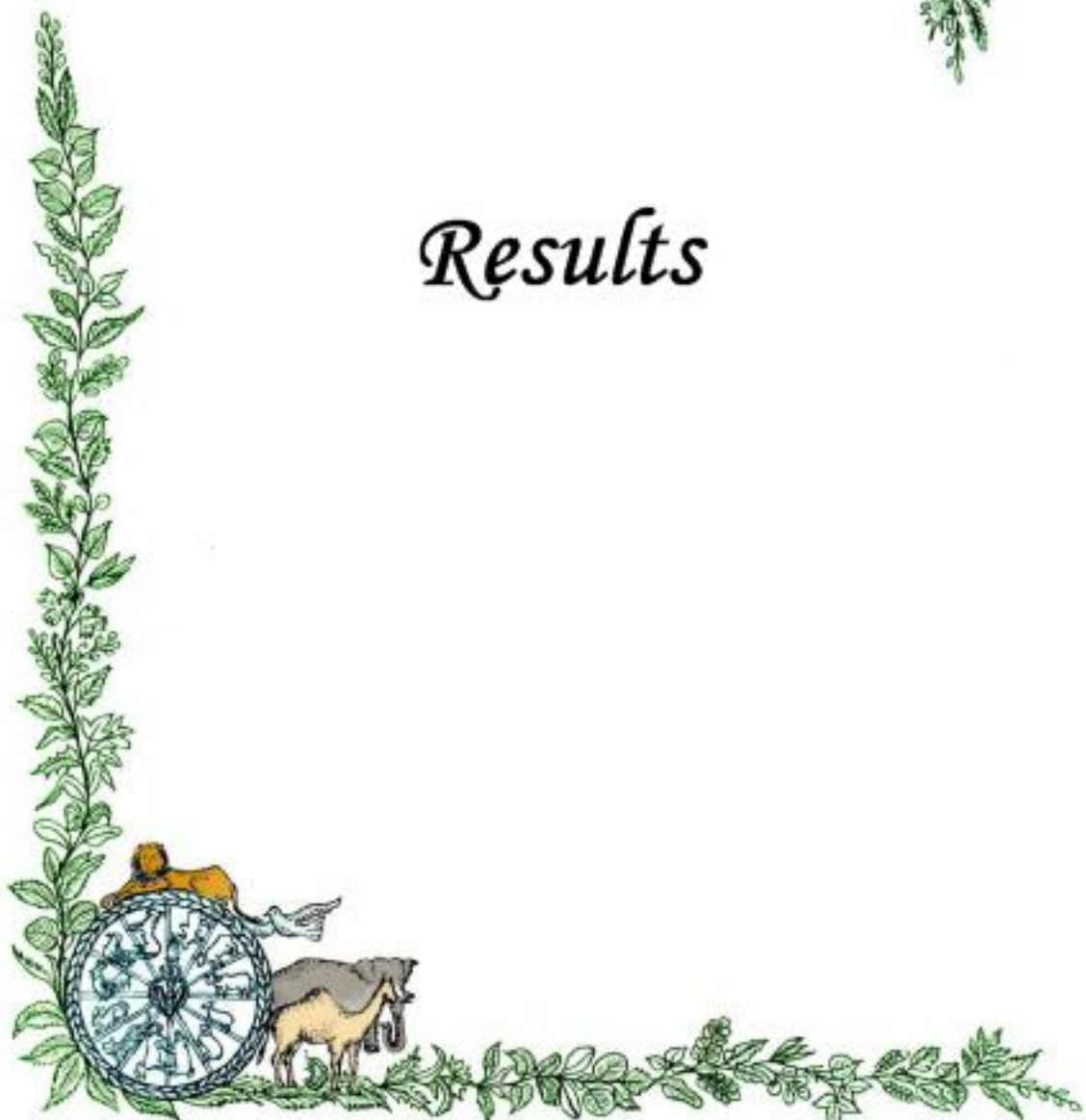
DNA Sequence and deduced amino acid analysis

DNA sequencing of the plasmid pQE 642 was carried out commercially. The deduced amino acid sequence of cloned DNA fragment encoding 22 kDa fusion protein was analyzed for hydrophobic domains according to Kyte and Doolittle algorithm (Kyte and Doolittle, 1982) using Lasergene software (DNASTAR, Madison, USA).





Results



GENOMIC DNA ISOLATION

The *M. a. paratuberculosis* culture 316F, grown in Middlebrook 7H10 agar as well as in Middlebrook 7H9 liquid media supplemented with OADC and mycobactin J, produced visible growth after eight weeks. The yield of the purified DNA was about 10-15 μ g per slant and 60-75 μ g per 100mg wet cells as analyzed by agarose gel electrophoresis.

PCR amplification of 642bp fragment

Amplification of DNA fragment each MAP locus 1087 and 1204 respectively with MAP genomic DNA using the specific set of primers in 30 cycles produced the product of 300bp each and overhangs (Figure 1, Lane 1 and 2). In a next PCR reaction a 642bp fragment was obtained using two PCR amplified products 300bp each and overhangs as template in 30 cycles reaction. The product was analysed on agarose gel electrophoresis showed the single band (Figure 2, Lane 3).

Elution of PCR product

The recovery of the eluted PCR amplified fragment of 642bp from agarose gel was approximately 70-80% of the initial PCR product used. The DNA was found to be single band without smear. (Figure 3, Lane 2).

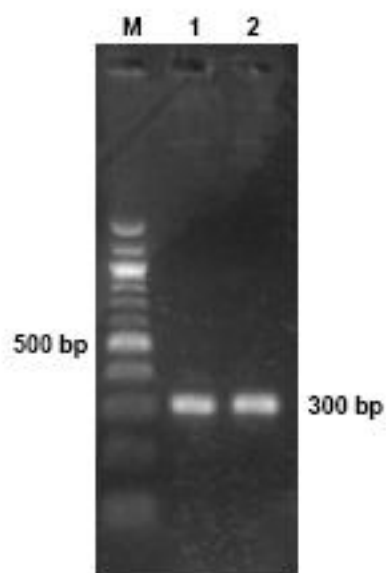


Fig. 1 : Agarose (1.2%) gel electrophoresis showing PCR amplified 300 bp (MAP1087) and 300 bp (MAP1204) fragments from genomic DNA

Lane M : DNA molecular weight marker 100bp ladder
 Lane 1 : PCR amplified 300 bp (MAP 1087) DNA fragment
 Lane 2 : PCR amplified 300 bp (MAP 1204) DNA fragment

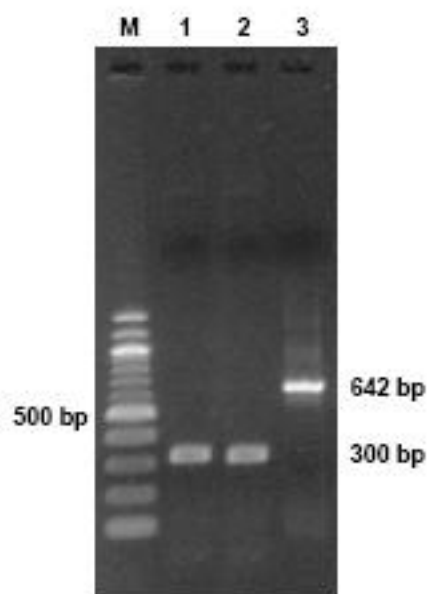


Fig. 2 : Agarose (1.2%) gel electrophoresis showing PCR amplified 300 bp (MAP1087), 300 bp (MAP1204) and 642 bp fragments

Lane M : DNA molecular weight marker 100bp ladder
 Lane 1 : PCR amplified 300 bp (MAP 1087) DNA fragment
 Lane 2 : PCR amplified 300 bp (MAP 1204) DNA fragment
 Lane 3 : PCR amplified 642bp fusion gene fragment

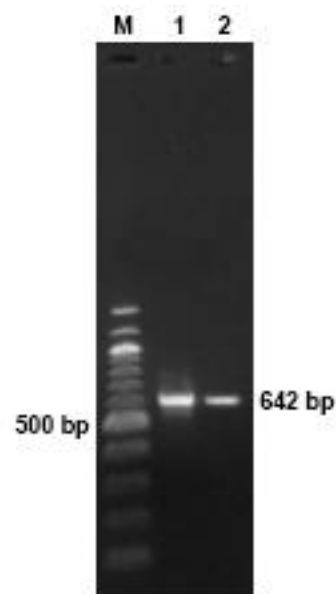


Fig. 3 : Agarose (1.2%) gel electrophoresis showing eluted 642 bp fragment
Lane M : DNA molecular weight marker 100 bp ladder
Lane 1 : PCR amplified 642 bp fragment
Lane 2 : Eluted 642 bp fragment

Construction of plasmids pQE 642 and screening of recombinant clones

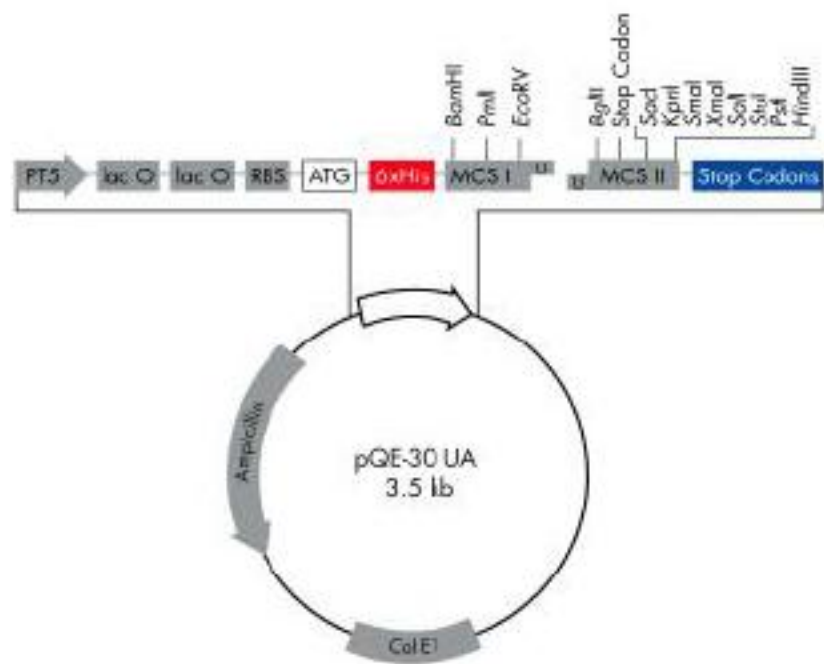
PCR amplified 642 bp product encoding 22 kDa fusion protein was ligated to the expression vector pQE30UA in the insert: vector ratio of 6:1. The construct was designated as pQE 642 (Plate 1). On transformation of construct pQE 642 into *E.coli* M15 cells produced approximately 1×10^2 colonies on LB agar plate supplemented with ampicilline and kanamycin.. Twenty five colonies were selected randomly and two positive colonies were identified by the amplification of the desired size fragment by colony PCR (Figure 4, Lane 3 and 4). Release of the identical size fragment upon digestion of the DNA from plasmid pQE 642 with Bam HI and Hind III also confirmed the positive clones (Figure 5, Lane 2).

Expression of the 22 kDa recombinant protein in *E .coli*

IPTG induced *E. coli* cells harbouring the recombinant plasmid pQE 642 generated a prominent band of molecular weight 24.2 k Da (22 kDa fusion protein and 2.2 kDa His tag and vector sequences) on SDS-PAGE (Figure 6, Lane 3 and 4). The recombinant fusion protein was designated as His 22. No such band was observed in IPTG induced *E. coli* M15 cells and uninduced *E. coli* M15 cells harbouring recombinant plasmid pQE 642 (Figure 6, Lane 1 and 2).

Purification of His 22 protein

The histidine tagged 22 kDa (His 22) fusion protein was accumulated in the cytoplasm of the *E. coli* as inclusion bodies. The grown culture, was pelleted and proteins lysed under denaturing conditions using the lysis buffer. The His 22 protein was fractionated from the cell lysate using affinity chromatographic separation for purification using the Ni-NTA agarose column The purified His 22 protein was eluted in buffer E with a monomeric band of 24.2 kDa on



**Prelinearized
pQE-30 UA vector**

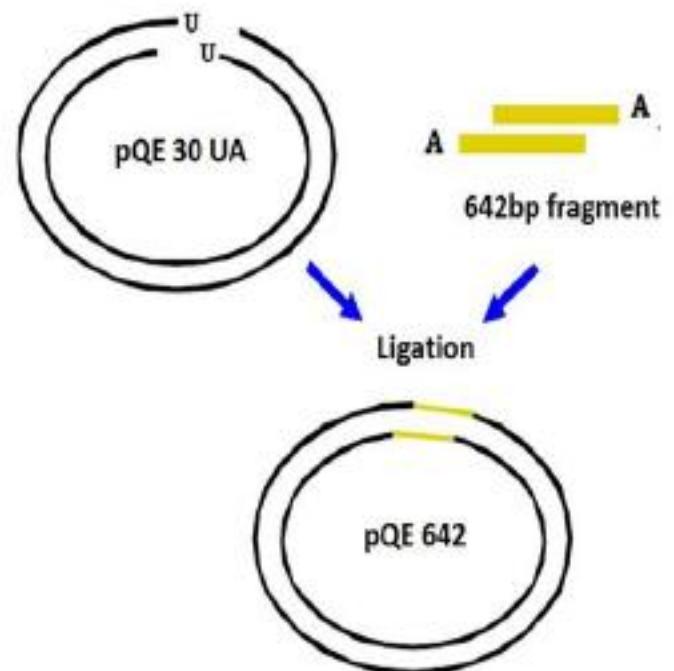


Plate 1 : Cloning strategy of PCR amplified 642bp fragment in prokaryotic expression vector pQE 30UA

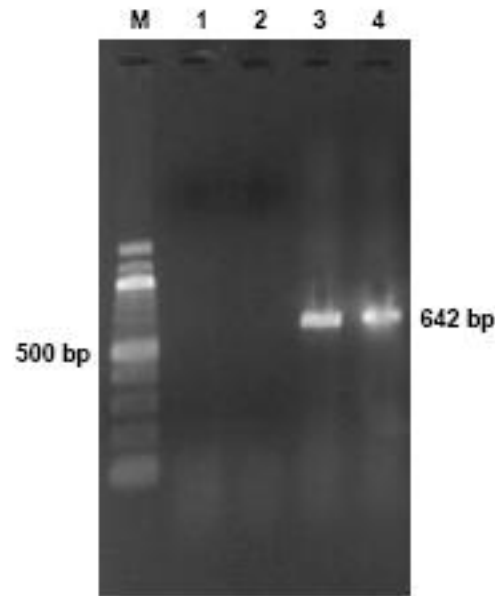


Fig. 4 : Agarose (1.2%) gel electrophoresis showing PCR amplified 642bp fragment by cPCR of pQE 642

Lane M : DNA molecular weight marker 100 bp ladder
 Lane 1, 2 : No amplification
 Lane 3, 4 : cPCR amplified 642 bp fusion gene fragment

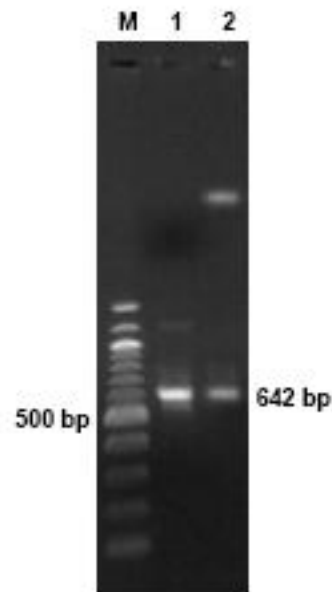


Fig. 5 : Agarose (1.2%) gel electrophoresis showing 642 bp fragment in pQE 30 UA expression vector

Lane M : DNA molecular weight marker 100 bp ladder
 Lane 1 : PCR amplified 642 bp fragment
 Lane 2 : Release of 642 bp gene fragment after digestion with *Bam*HI and *Hind*III from pQE 642

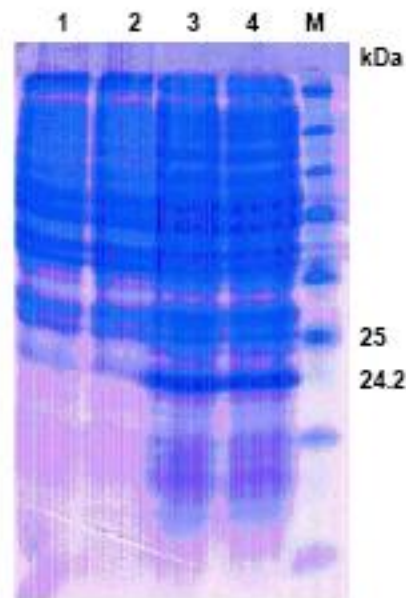


Fig. 6 : SDS PAGE showing expression of 642 bp fragment encoding His 22 fusion protein

- Lane M : Protein molecular weight marker
 Lane 1,2 : Whole cell extract of transformed *E. coli* M15 harboring construct pQE 642 (IPTG Uninduced)
 Lane 3,4 : Whole cell extract of transformed *E. coli* M15 harboring construct pQE 642 (IPTG induced)

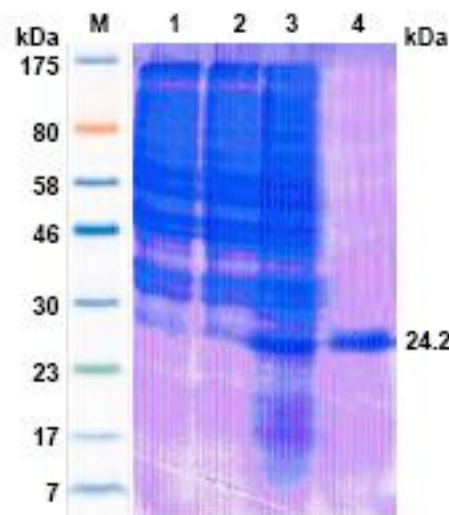


Fig. 7 : SDS PAGE showing purified His 22 fusion protein

- Lane M : Protein molecular weight marker
 Lane 1 : Whole cell extract of *E. coli* M15
 Lane 2 : Whole cell extract of *E. coli* M15 harboring construct pQE 642 (IPTG Uninduced)
 Lane 3 : Whole cell extract of *E. coli* M15 harboring construct pQE 642 (IPTG induced)
 Lane 4 : Purified His 22 protein

analysis by 12% SDS PAGE (Figure 7, Lane 4). The yield of the purified protein was about 10 mg/l at shake flask culture. Dialysis was carried out which removed urea and renatured the protein to get native conformation which can be efficiently used for immunization of rabbit.

Immunoreactivity of the recombinant protein

The pooled polyclonal sera raised against purified His 22 protein in rabbits could bind to 4 hour IPTG induced *E. coli* (M15) cells harbouring pQE 642 on western blot and produced a brown colour band (Figure 8, Lane 3). No reaction was observed in induced *E. coli* (M15) cells and uninduced *E. coli* (M15) cells harbouring recombinant plasmid pQE 642 (Figure 8, Lane 1 and 2).

Furthermore the anti-His 22 sera also reacted with MAP culture filtrate and showed two brown colour bands migrated at size 16 kDa and 25 kDa (Figure 8, Lane 5) on western blot. The purified His 22 protein as well as IPTG induced *E. coli* cells harbouring plasmid pQE 642 was used as positive controls (Figure 8, Lane 3 and 4). Specific binding of the recombinant His 22 protein was also observed with a band of 24.2 kDa when probed with anti MAP culture filtrate sera on immunoblot. (Figure 9, Lane 2).

Reactivity of His 22 protein with clinical sera

The purified His 22 protein was also recognized by the serum from a goat naturally infected with paratuberculosis on western blot assay. However no such band was appeared with the healthy goat sera (Figure 10).

ELISA

High titre of antibody was obtained in antisera raised in rabbits against His 22 protein (Figure 11), whereas no significant anti His 22.8 kDa antibody titre was observed with healthy sera. Further

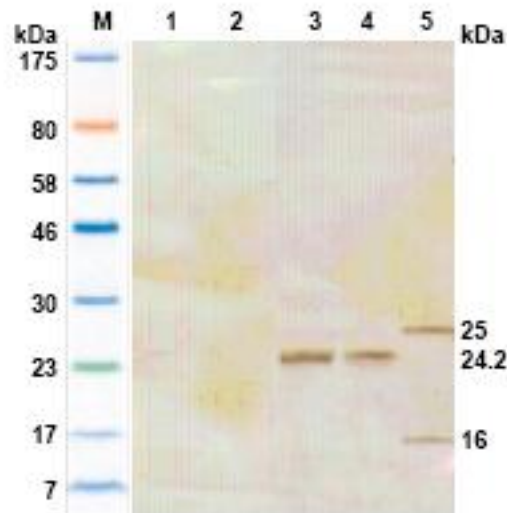


Fig. 8 : Western blot of the His 22 protein expressed in *E. coli*, purified His 22 protein and MAP culture filtrate probed with anti His 22 sera

- Lane M : Protein molecular weight marker
- Lane 1 : Whole cell extract of *E. coli* M15
- Lane 2 : Whole cell extract of *E. coli* M15 harboring construct pQE 642 (IPTG Uninduced)
- Lane 3 : Whole cell extract of *E. coli* M15 harboring construct pQE 642 (IPTG induced)
- Lane 4 : Purified His 22 protein
- Lane 5 : MAP culture filtrate antigen showing 16 kDa and 25 kDa proteins

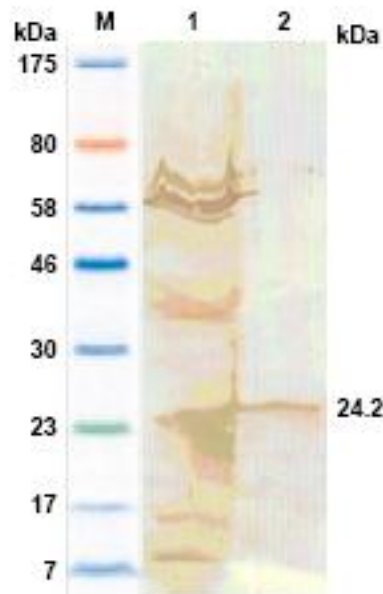


Fig. 9 : Western blot of MAP culture filtrate and purified His 22 protein probed with anti culture filtrate sera

- Lane M : Protein molecular weight marker
- Lane 1 : MAP culture filtrate antigen
- Lane 2 : Purified His 22 protein



Fig. 10 : Western blot showing seroreactivity of purified His 22 protein

- 1 : His 22 protein probed with clinical goat sera
- 2 : His 22 protein probed with healthy goat sera

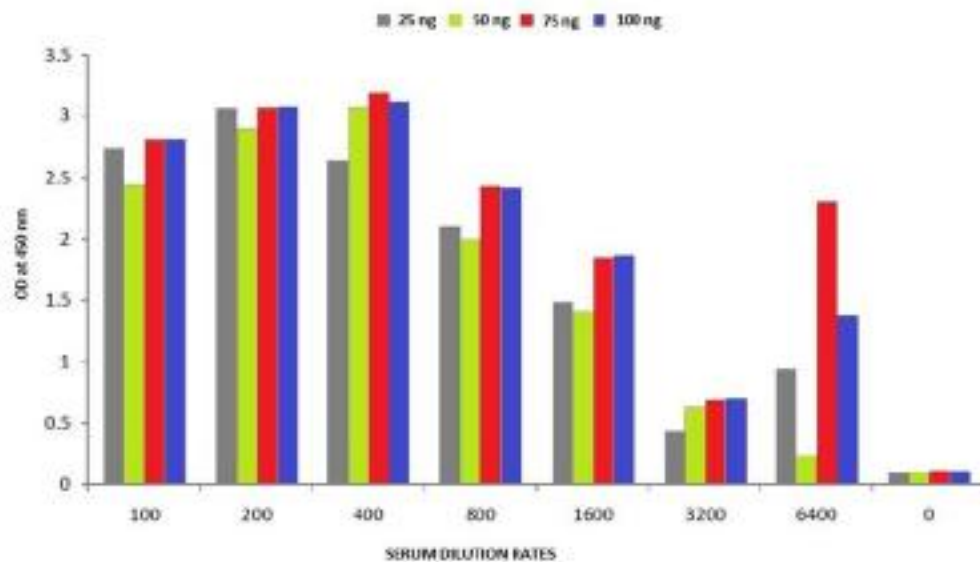


Fig. 11 : ELISA showing antibody titre of anti His 22 sera (Checker board)

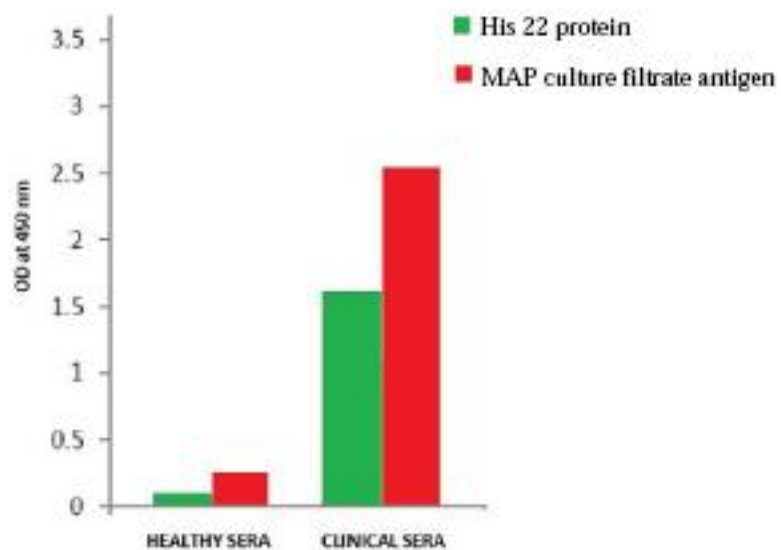


Fig. 12 : ELISA result for 20 healthy and 10 MAP infected sera

optimization of antigen in ELISA revealed that 75ng of His 22 protein detected the antibody consistently and resolved between positivity and negativity. The clinical sera from goats infected with paratuberculosis gave distinguishable absorbance than the healthy sera using His 22 and MAP culture filtrate as coating antigen. However higher absorbance was obtained with MAP culture filtrate antigen (Figure 12).

DNA sequence analysis and deduced amino acid analysis

The nucleotide sequence used from locus tag MAP 1087 and MAP 1204 of the plasmid pQE 642 revealed 100% homology 642 bp DNA sequences. The predicted primary protein composed of 211 amino acid followed by stop codon

Analysis of the deduced amino acid sequence (211 amino acids) of the recombinant 22 kDa protein, according to the Kyte and Doolittle algorithm for hydropathy index, indicated that the protein contained five major hydrophobic regions (Plate 2).



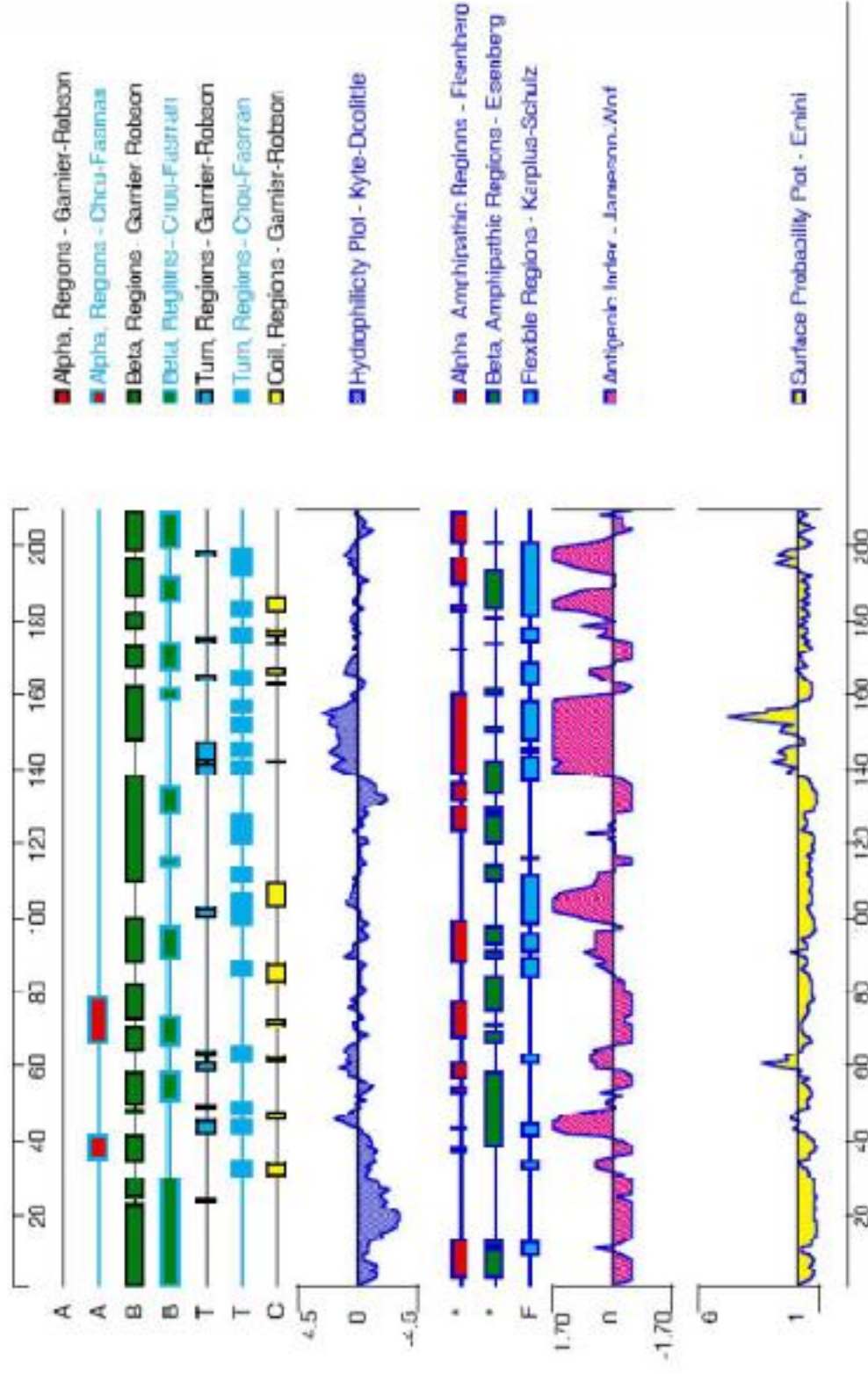


Plate 2 : Structure prediction of 22 kDa fusion protein using DNA STAR laser gene protean software



Discussion



Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a chronic granulomatous enteritis resulting in wasting of domestic animals and also wild ruminants. It is also associated in Crohn's disease and other immunosuppressive diseases in man. Successful preventive or diagnostic methods are not available and the curative measures are not rewarding. This has resulted in the spread of the disease by expanding its host range and geography (Whittington *et al.*, 2011). Good management, periodic screening and eliminating the infected animals help in reducing the menace.

The faecal culture, the current gold standard test, detects MAP during subclinical and clinical stages of infection, but it is time consuming requiring more than 12 weeks of incubation and also labour intensive. PCR is sensitive and rapid, but applicable in bacterial shedders. Further both faecal culture and PCR could not differentiate between the pass through MAP and MAP colonization. Due to the intracellular nature of the organism the assay of cell mediated immunity like interferon gamma assay, lymphocyte proliferation assay and DTH response through skin test are used. Strong homology between various mycobacterial species is the reason for cross-reactivity and low sensitivity. Serological tests used in diagnosis of paratuberculosis such as agar gel precipitation, ELISA and CFT are relatively easy to perform but suffer from lack of specificity (Clarke *et al.*, 1996; Colgrove *et al.*, 1989). Currently, the antigen based

diagnostic tests for paratuberculosis use complex mixtures of antigens, many of which are highly conserved among mycobacterial species.

Genomic screening of the fully sequenced genome of MAP allowed identification of some prospective MAP specific immune targets and the antigenicity of some of them have been evaluated. Efforts have been made to identify MAP specific immunodominant antigens from culture filtrate and cellular extracts either by using two dimensional electrophoresis (2 DE) followed by mass sepectroscopy (MS) and/or immunoproteomic analysis (Leroy *et al.*, 2007; Bannantine *et al.*, 2008). Similar strategy had yielded several antigens from *Mycobacterium tuberculosis* (MTB). In case of paratuberculosis although some of these antigens appear to show promising results, none has been used for routine and specific diagnosis.

Antigenic screening of MAP culture filtrate through MS and later BLAST search identified 14 specific proteins with secretory signal at their N terminals. Each of these specific proteins may function as candidate antigen for use in serodiagnosis of paratuberculosis. Data based on secretome of MAP specific antigens was established by systemic proteome analysis of MAP culture filtrate and followed by BLAST search identified 25 candidate antigens (Leroy *et al.*, 2007). Further evaluation of humoral immune response with sera from experimentally infected animals by development of partial protein array using the MAP specific recombinant proteins expressed in *E. coli* revealed that the detection of antibody against certain MAP specific secretory protein fraction was possible as early as 70 days post infection (Bannantine *et al.*, 2008).

Therefore, the present study was envisaged to address the critical importance of selecting B cell epitopic regions from two MAP specific antigens secreted at early stages of infection through cloning and

heterologous expression to generate fusion protein containing two B-cell epitopic regions. Expression of a 22 kDa fusion protein containing the B-cell epitopic regions from MAP specific antigen MP-16 and MP-25 encoded by tag MAP 1087 and MAP 1204 in *E. coli* from the MAP genomic DNA was chosen. The above proposition was undertaken based on generation of a fusion protein from *M. tuberculosis* (MTB) proteins MTB.11, MTB 8, MTB.48 and 38 kDa protein found efficient in the detection of tuberculosis by I-ELISA (Houghton *et al.*, 2002). With a similar strategy, Liu *et al.* (2007) had also produced another fusion protein for detection of bovine tuberculosis.

Hence specific oligo nucleotide primers were designed based on the sequence information of most probable B-cell epitopic regions MP 16 and MP 25 of tag MAP 1087 and MAP1204. The 642 bp product produced by PCR amplification was ligated into prokaryotic expression vector pQE 30UA for direct cloning of the PCR product with the 3' dA overhangs that is generated during the PCR using Taq polymerase. For efficient ligation the reaction was carried out at 16 °C for 4 hrs followed by overnight at 4 °C. On transformation into *E. coli* cells produced numbers of positive colonies on ampicillin and kanamycin LB agar plates. The positive clones were identified by cPCR and resolving of the expressed proteins by SDS PAGE. A similar strategy of cloning in pQE series expression vector system was applied to generate high levels of *Clostridium perfringens* epsilon toxin (Goswami *et al.*, 1996) 35 kDa protein and 34.9 kDa PPE proteins of MAP Basgoudanavar *et al.*, 2004; Deb and Goswami 2010).

Expression of recombinant (His-22) protein is induced by, IPTG which binds to the *lac* repressor protein, inactivating it leading to transcription of sequences downstream of the promoter. High level expression of the recombinant His 22 protein of molecular weight 24.2 k Da (22 kDa fusion protein and 2.2 kDa His tag and vector

sequences) were observed in pQE 642 four hours post-induction with 1mM IPTG on SDS-PAGE. Using pQE-30 vector, Goswami *et al.*, (1996) and Basgoudanavar *et al.* (2004) got maximum expression of the recombinant protein 4 to 6 hr post induction. Therefore, a period of 4 hr of post induction was adopted to isolate protein from *E. coli* culture.

Although a wide variety heterologous expression systems have been developed to produce recombinant proteins, the purification of proteins is still problematic (Makrides, 1996; Goswami *et al.*, 1996). The presence of 6x histidine residues at N-terminal of the recombinant protein enabled us to purify this protein by single step affinity purification using Ni-NTA resin. The 6x his tag is poorly immunogenic, it neither interferes with the immunogenicity nor with the structure and function of recombinant proteins (Mukhiza *et al.*, 1995; Pillai *et al.*, 1996). So the tag was not removed by protease cleavage (Sisk *et al.*, 1994).

The protein was purified the cell lysate under denaturation condition using mild denaturant 8M urea in a column containing the NINTA resin which led to high affinity to the 6x histidine. Dialysis of the fusion protein in PBS with descending concentration of urea to renature and also to bring back the native conformation the His 22 protein.

The pooled polyclonal sera raised in rabbits against the purified His 22 protein reacted strongly to the *E. coli* expressed as well as the purified His 22 protein.

The strong binding of anti His 22 sera with MAP culture filtrate antigen on western blot with MP16 and MP25 proteins not only confirmed the heterologous expression of 22 kDa recombinant protein, but also indicated that these proteins are secreted by MAP. Moreover, the recombinant His 22 protein retained its antigenicity even after fusion of the epitopic regions and expression in the prokaryotic system. The

immunoblot assay clearly identified the expression of the recombinant protein also suggested that the His 22 protein has retained its antigenicity. The binding of His 22 protein with anti MAP culture filtrate sera indicated that the protein retained its native conformation.

Furthermore, the reactivity of His 22 protein with the clinical serum not only indicated the presence of anti MP16 and anti MP25 kDa antibodies in it but also that it could be a useful antigen for detection of antibodies in MAP infection in animals.

The use of single recombinant antigen in serodiagnosis of MAP infection produced result with high specificity but low sensitivity. The use of antigens from two proteins or even multi-antigenic proteins that have been used in detection of tuberculosis in cattle by indirect ELISA, which increased the sensitivity jeopardizing the specificity (Liu *et al.* 2007).

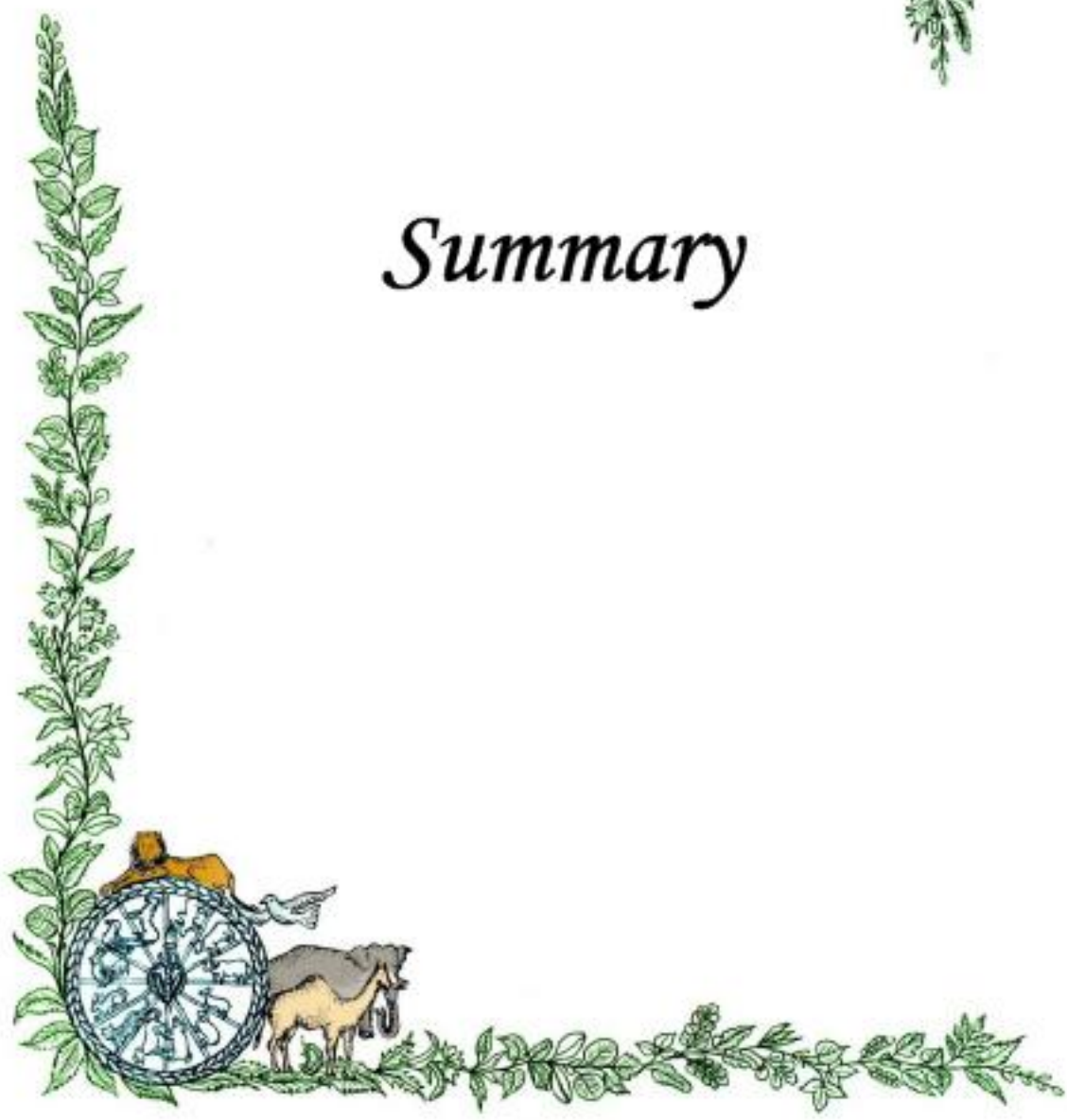
To determine the immunoreactivity of His 22 protein, standardisation ELISA was performed with different concentrations of antigens and anti- His 22 polyclonal antibodies in serum sample. A high antibody titre (1:6400) against the His 22 protein was obtained. After standardisation of ELISA His 22 protein was compared with MAP culture filtrate antigen. Though high absorbance was obtained with MAP culture filtrate antigen, it was reported to be cross reactive with other mycobacteria as well. But His 22 protein is specific to MAP and suitable for detection of MAP specific antibody.

Initially promising though, large number of clinical serum samples need to be tested to establish the antigen as a potential diagnostic agent for detection of MAP infection in animals in the ELISA based assay.





Summary



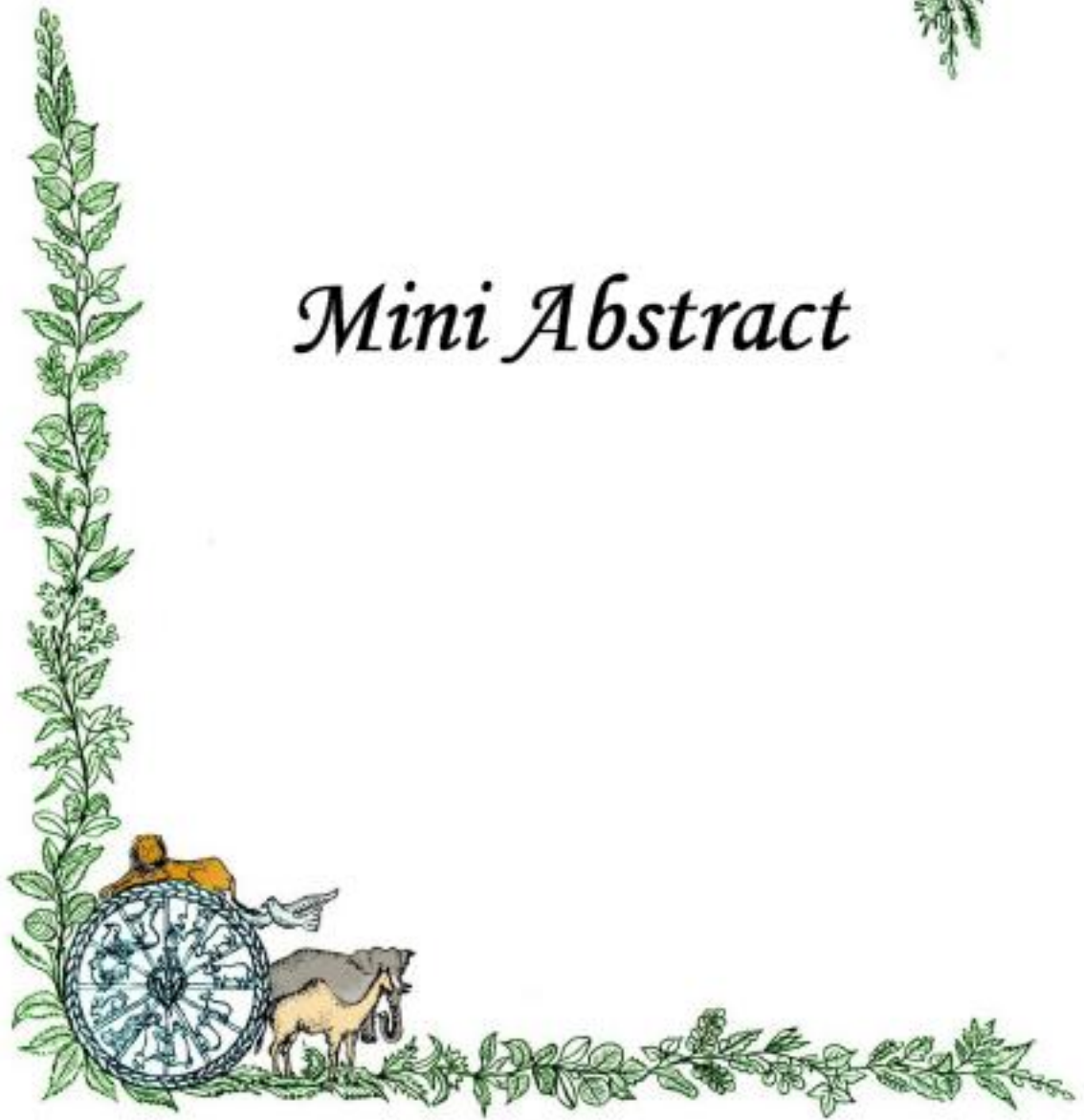
1. Specific amplification of sequences containing probable B-cell epitopic regions MP 16 and MP 25 from MAP locus 1087 and 1204 respectively of genomic DNA was carried out in two tubes separately using the specific set of primers (MP1 2, and 3, 4 respectively) to generate product of 300bp each and overhangs. In the second PCR reaction the PCR amplified products 300bp each and overhangs each from two reaction were used as template to generate a 642 fragment with primers (MP1 and MP4).
2. Cloning of the 642 bp fragment encoding 22 kDa into *E. coli* expression vector pQE30 UA in frame resulted a construct designated as pQE 642. Transformation into *E. coli* M15 cells and plating on LB agar containing ampicillin and kanamycin generated recombinant colonies.
3. The amplified product of 642 bp was obtained from positive recombinant clones by cPCR. DNA from positive clones were confirmed by release of identical size fragment with restriction enzymes and also by sequencing.
4. Expression of the *E. coli* cells harboring the clones pQE 642 achieved by induction with IPTG produced expressed 24.2 kDa (22 kDa fusion protein and 2.2 kDa His tag and vector sequences) as analysed on SDS-PAGE.
5. Purification of the His 22 protein with Ni-NTA under denaturing condition followed by dialysis yielded the purified protein of about 10 mg/l.

6. Polyclonal antisera raised in rabbits against the purified His 22 protein reacted with induced *E. coli* whole cell lysate expressing 22 kDa protein, purified His 22 protein and MAP culture filtrate on western blot.
7. The purified His 22 protein also reacted with clinical sera from goats infected with paratuberculosis on western blot. The binding of His 22 protein with anti MAP culture filtrate sera indicated that the protein retained its native conformation.
8. High titre of antibodies was produced in polyclonal antisera raised in rabbits against the purified His 22 protein as determined using indirect ELISA. The His 22 protein also showed higher reactivity with serum of goats infected with paratuberculosis.
9. After standardisation of ELISA His 22 protein was compared with MAP culture filtrate antigen. Though high absorbance was obtained with MAP culture filtrate antigen, it was reported to be cross reactive with other mycobacteria as well. But His-22 protein is specific to MAP.





Mini Abstract



Mini Abstract

The present study was undertaken to generate a construct having the 642 bp fragment encoding 22 kDa fusion protein having B-cell epitopic regions of MP16 and MP25 from locus tag MAP 1087 and MAP 1204 of *M. a. paratuberculosis* (MAP). PCR amplification of DNA fragment from locus tag MAP 1087 and MAP 1204 respectively, with Map genomic DNA using specific primers produced the product of 300bp each with overhangs. In the next step the 642bp fragment was further generated using the PCR product of 300bp each with overhangs as template with specific primers. The amplified product of 642 bp was cloned into prokaryotic expression vector pQE 30 UA and the construct was designated as pQE 642. On transformation into *E. coli* (M15) cells and plating on LB agar containing ampicillin and kanamycin produced recombinant colonies. The positive clones were confirmed by cPCR, and release of the desired fragments by RE analysis with Bam HI and Hind III and also by DNA sequencing. The DNA sequence analysis of plasmid pQE 642 revealed homology with the B-cell epitopic regions of locus tag MAP 1087 and MAP 1204 of *M. a. paratuberculosis* (MAP) selected. The recombinant clones thus selected on induction with IPTG generated the His 22. The purified protein, showed a monomeric band of 24.2 kDa (22 kDa fusion protein + 2.2 kDa His tag and vector sequences) size on SDS-PAGE. The yield of the purified His 22 protein was about 10 mg/l of culture. Expression of the recombinant protein was confirmed by immunoblotting. Polyclonal antisera raised in rabbits against the purified His 22 protein reacted with induced recombinant clones and purified His 22 protein. The anti His 22 sera could also bind at 16kDa and 25kDa on western blot. Moreover, the anti MAP culture filtrate sera revealed a band at 24.2kDa indicating that the His 22 protein retained its antigenicity. The protein was also recognised by serum from goat with clinical paratuberculosis. High titre of antibodies were observed in antisera from His 22 protein as determined by ELISA. After standardising the checker board His 22 protein and the MAP culture filtrate antigen was used as coating antigen for detecting antibodies in healthy and MAP infected goats. The study carried out indicated that the His 22 fusion protein may be used as future diagnostic reagent for specific and sensitive detection of MAP at early post infection.

लघु सारांश

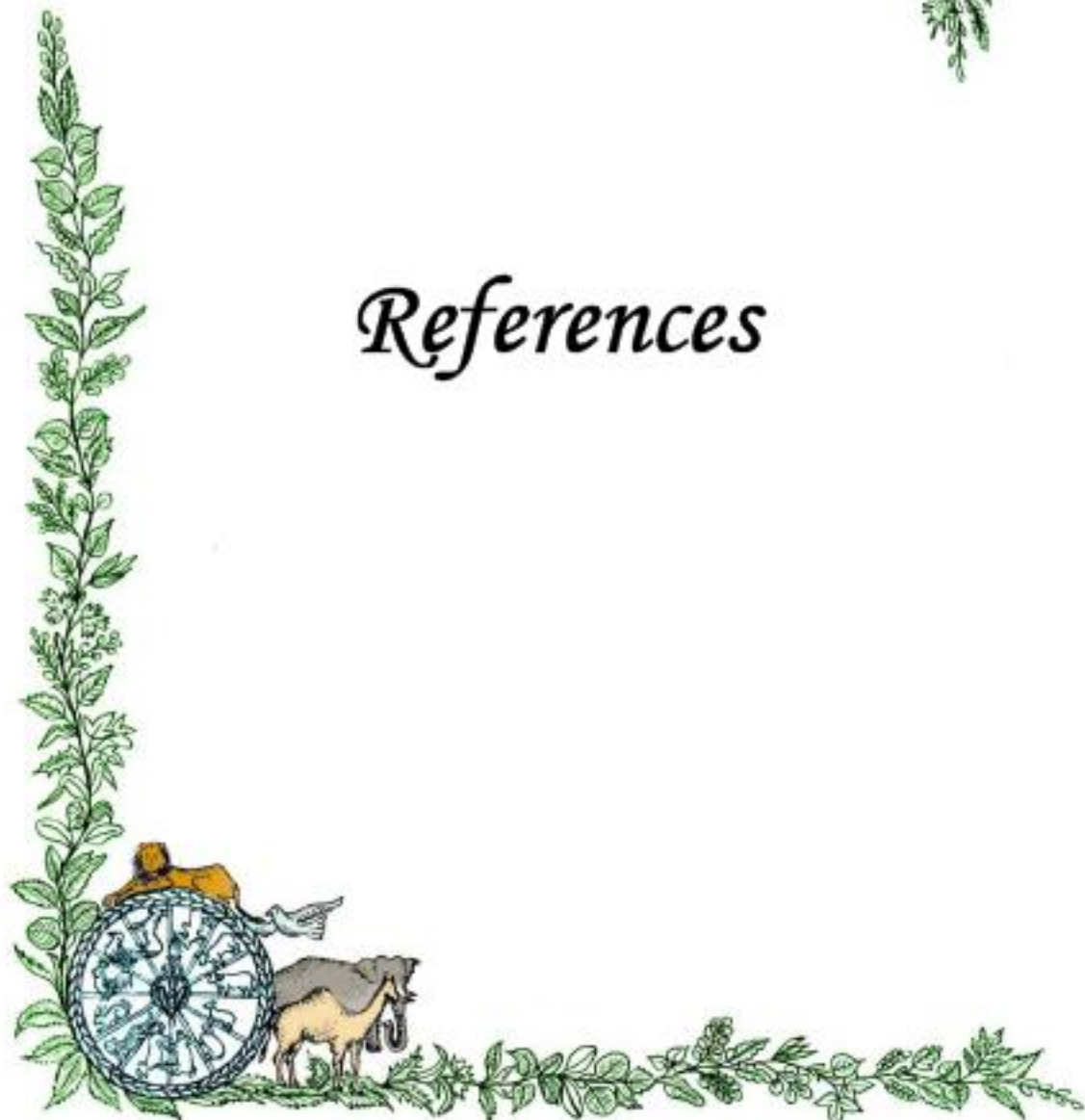


लघु सारांश

प्रस्तुत अध्ययन में 22 के.डी.ए. मिश्रित प्रोटीन से सांकेतिक 642 बेस पेयर का प्रतिरूप उत्पन्न किया गया, जिसमें एम.ए. पैराट्यूबरकुलोसिस (मैप) के लोकस टैग एम.ए.पी. 1087 और एम.ए.पी. 1204 के एम.पी.16 और एम.पी. 25 के एपिटोपिक क्षेत्र की बी-सेल है। लोकस टैग एम.ए.पी.1087 और एम.ए.पी. 1204 का एम.ए.पी. जिनोमिक डी.एन.ए. से विशिष्ट प्राइमर का उपयोग करके पी.सी.आर. विस्तारीकरण किया गया। जिसमें 300 बेस पेयर का उत्पाद मिला। पुनः 300 बेस पेयर के दो उत्पाद को लेकर पी.सी.आर. विस्तारीकरण द्वारा 642 बेस पेयर का उत्पाद उत्पन्न किया गया। विस्तारित उत्पाद को एक्सप्रेस वेक्टर पी.क्यू.ई.30 यू.ए. में क्लोन किया गया। जिसका पी.क्यू.ई.642 नाम रखा गया। ट्रांसफार्मेशन के फलस्वरूप रिकाम्बिनेन्ट कालोनी प्राप्त हुई। सकारात्मक क्लोन का पी.सी.आर, आर.ई. एन्जाइम विश्लेषण द्वारा यह पता चला कि पी.क्यू.ई.642, पैराट्यूबरकुलोसिस की लोकस टैग एम.ए.पी.1087 और एम.ए.पी.1204 की एपिटोपिक क्षेत्र की बी. सेल के साथ समानता है। रिकाम्बिनेन्ट क्लोन का आई. पी.टी.जी. से उत्प्रेरित करके हिंस 22 (22 के.डी.ए. + 2.2 के.डी.ए. हिंस-टैग) के साथ चयनित किया गया। शोधित प्रोटीन का एस.डी.एस. पेज पर 24.2 के. डी.ए. प्रोटीन प्राप्त हुई। शोधित हिंस. 22 के.डी.ए. प्रोटीन 10 मिग्रा/लीटर के हिसाब से प्राप्त हुई। इम्यूनोब्लॉटिंग द्वारा प्रोटीन एक्सप्रेसन की पुष्टि की गयी। हिंस-22 प्रोटीन के विरुद्ध खरगोश में पालीक्लोन सीरा उत्पन्न किया गया। एम.ए.पी. कल्चर फिल्ट्रेस सीरा से 24.2 के.डी.ए. बैंड प्राप्त हुआ। जिससे यह संकेत मिलता है कि हिंस-22 प्रोटीन में एंटीजेनेसिटी उपस्थित है। बकरी के मैप संक्रमित सीरा से भी प्रोटीन की पहचान की गयी। एलाइजा करने पर हिंस 22 सीरा में अधिक मात्रा में प्रतिरोधक का पता चला। चेकर-बोर्ड के मानकीकरण द्वारा एम.ए.पी. कल्चर फिल्ट्रेट का कोटिंग एंटीजन के रूप में स्वस्थ और एम.ए.पी. संक्रमित बकरी में प्रतिरोधक की पहचान के लिये प्रयोग किया गया। इस अध्ययन से यह पता चलता है कि हिंस 22 प्रोटीन को भविष्य में डायग्नोस्टिक अभिक्रमक के रूप शुरू संक्रमण का पता करने के लिये प्रयोग किया जा सकता है।



References



- Abubakar, I., Myhill, D., Aliyu, S. H. and Hunter, P. R. (2008). Meta analysis demonstrates a clear association of MAP with CD. Detection of MAP from patient with crohn's Disease using Nucleic acid –based technique: a systemic review and meta analysis. *Inflammatory Bowel Disease*. 14: 401-410.
- Alinovi, C.A., Ward, M.P., (2009). Real time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium paratuberculosis*. *Veterinary Microbiology*, 136, 177-179.
- Andersen, P., Doherty, T.M. (1991). TB subunit vaccines – putting the pieces together. *Microbes Infect.*, **7**: 911-921.
- Bannantine, J. P., Baechlor, E., Zhang, Q., Li, L. L., Kapur, V. (2002). Genome scale comparison of *Mycobacterium avium* subsp.*Paratuberculosis* with *Mycobacterium avium* subsp.*avium* reveals potential diagnostic sequences. *J. Clin. Microbiol.* 40(4): 1303-1310.
- Bannantine, J. P., Hansen, J. K., Paustian, M. L., Amonsin, A., Li, L. L., Stabel, J. R. and Kapur, V. (2004). Expression and immunogenicity of proteins encoded by sequences specific to *Mycobacterium avium* subsp. *Paratuberculosis*. *J. Clin. Microbiol.* 42: 106-114.
- Bannantine, J. P., Paustian, M. L., Waters, W. R., Stabel, J. R., Palmer, M. V., Li, L., Kapur, V. (2008). Prolonged bovine antibody responses to *Mycobacterium avium* subsp. *paratuberculosis* infection by using protein arrays. *Infect. Immun.* 76: 739–749.
- Bannantine, J. P., Stabel, J. R., Bayles, D. O., Geisbrecht, B. V. (2010). Characteristics of an extensive *Mycobacterium avium* subspecies *paratuberculosis* recombinant protein set. *Protein Express. Purif.* 72: 223–233.

- Basagoudanavar, S. H. Goswami P. P. Tewari, V. (2006) Cellular immune response to 35 kDa recombinant antigen of *Mycobacterium avium* subsp. *paratuberculosis*. *Vet. Res. Commun.* **30**; 357-367.
- Basagoudanavar, S. H., Goswami, P. P., Tiwari, V., Pandey, A. K. and Singh, N. (2004). Heterologous expression of a gene encoding 35 kDa protein of *Mycobacterium avium subsp. paratuberculosis* in *Escherichia coli*. *Veterinary Research Communication*, **28**: 209-224.
- Bassey, E.O., Collins, M.T., 1997, Study of T-lymphocyte subsets of healthy and *Mycobacterium avium subsp. paratuberculosis*-infected cattle. *Infect Immun* 65, 4869-4872.
- Beard PM (2001). Paratuberculosis Infection of Nonruminant Wildlife in Scotland. *Journal of Clinical Microbiology*;39:1517-21.
- Begg, D. J., Whittington, R. J., (2008).Experimental animal infection models for Johne's disease, an infectious enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis*. *Vet.J.*, 176: 129-145.
- Bhide, M., (2006). IS900-PCR-based detection and characterisation of *Mycobacterium paratuberculosis* from buffy coat of cattle and sheep. *Veterinary Microbiology*, 112, 33-41.
- Billman-Jacobe, H., Cockrum, F., Carner, L. A. and Gill, I. J. (1992).Acomparision of IFN-gamma assay with adsorbed ELISA for diagnosisof Johne's disease in cattle.*Australian Vet. J.* 69: 25-28.
- Bjerrum, O.J. and Schafer-Nielson, C. (1986). Buffer systems and transfer parameters for semidry electroblotting with a horizontal apparatus. *In:Dunn (Ed.) Electrophoresis* 86. VCH, Weinheim, Germany, 315-327.
- Boysen, P., Storset, A.K., 2009, Bovine natural killer cells. *Vet Immunol Immunopathol* 130, 163-177.
- Bull, T. J., Linedale, R., Hinds, J., Hermon-Taylor, J. (2009). A rhodanine agent active against non-replicating intracellular *Mycobacterium avium* subspecies *paratuberculosis*. *Gut Pathog.* 1: 25.

- Camerson, R. M., Stevenson, K., English, N. F., Kalusen, J. and Sharp, J. M. (1994). Identification and characterization of putative serine protease expressed in vivo by *M. a. paratuberculosis*. *Microbiology*. **140**: 1977-1982.
- Camphousen, R. T., Jones, R. L. and Brenna, P. J. (1988). Antigenic relationship between *M. a. paratuberculosis* and *M. avium*. *Am. J. Vet. Res.* **49**: 1307-1310.
- Chiodini, A. and Kruiningen, H. J. (1984). Ruminant Paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Veterinarian*, 74: 218-262.
- Cho, D., Shin, S. J., Talaat, A. M., Collins, M. T. (2007). Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. *Prot. Expr. Purif.*, 53: 411-420.
- Cho, D., Sung, N., Collins, M. T. (2006). Identification of proteins of potential diagnostic value for bovine paratuberculosis. *Proteomics*. 6: 5785-5794.
- Clarke, C. J. and Little, D. (1996). The pathology of ovine paratuberculosis : Gross and histopathological changes in the intestine and other tissues. *J. Comp. Patho.* 114: 410-437.
- Cleland, P. C., Lehmann, D .R., Phillips, P. H., Cousins, D. V., Reddacliff, L. A., Whittington, R. J.(2010).A survey to detect the presence of *Mycobacterium avium* subspecies *paratuberculosis* in Kangaroo Island macropods. *Vet .Microbiol.* 145: 339-346.
- Coetsier, C., Havanx, X., Mattlelard, F., Sadatte, S., Cormont, F., Buergelt, K., Limbourg, B., Latinine, D., Bazin, H., Denef, J. F. and Cocito C. (1998). Detection of *M. a. paratuberculosis* in infected tissues by new species specific immunohistological procedures. *Clin. Diagn. Lab. Immunol.* **5**: 456-451.
- Colgrove, C.S., Thoen, C.O., Blackburn, B.O. and Murphy, C.D. (1989). Paratuberculosis in cattle a comparison of three serological tests with results of 18 faecal culture. *Vet. Micrbiol.* **19**:183-187.

- Collins, M.T., Sockett, D.C., Ridge, S., Cox, J.C. (1991). Evaluation of a commercial enzyme-linked immunosorbent assay for Johne's disease. *J. Clin. Microbiol.* 29: 272-276.
- Collins, M.T., Welis, S.J., Petrini, K.R., Collins, J.E. (2005). *J. Clin. Diagn. Lab Immunol.*, 12: 685-692.
- Coussens, P.M., 2004, Model for immune responses to *Mycobacterium avium* subspecies paratuberculosis in cattle. *Infect Immun* 72, 3089-3096.
- Daniels, M. J., Henderson, D., Greig, A., Stevenson, K., Sharp, J. M., Hutchings, M. R. (2003). The potential role of wild rabbits *Oryctolagus cuniculus* in the epidemiology of paratuberculosis in domestic ruminants. *Epidemiol. Infect.* 130: 553-559.
- de Almeida DE, Colvin CJ, Coussens PM. Antigen-specific regulatory T cells in bovine paratuberculosis. *Vet Immunol Immunopathol.* 2008;125:234-45.
- Deb, R, Goswami, P.P, Saxena, V.K, Prasad, N.S, Molecular cloning of a DNA fragment encoding 34.9 kDa carboxy terminal PPE protein of MAP in *E.coli*. *Indian J. Anim. Res.*. 44(2) 131-134, 2010
- DeLisle, G.W., Seguin, P., Samagh, B.S., Corner, A.H. and Duncan, J.R. (1980). Bovine paratuberculosis. : A herd study using complement fixation test and Intradermal tests. *Can. J. Comp. Med.*, **44**: 177-182.
- Dupont, C., Thompson, K., Heuer, C., Gicquel, B., Murray, A. (2005). Identification and characterization of an immunogenic 22 kDa exported protein of *Mycobacterium avium* subspecies paratuberculosis. *J. Med. Microbiol.*, 54: 1083-1092.
- Eda, S., Bannantine, J. P., Waters, W. R., Mori, Y., Whitlock, R. H., Scott, M. C., Speer, C. A. (2006). A highly sensitive and subspecies specific surface antigen enzyme-linked immunosorbent assay for diagnosis of Johne's disease. *Clin. Vaccine Immunol.*, **13**: 837-844.
- Eda, S., Elliott, B., Scott, M. C., Waters, W. R., Bannantine, J. P., Whitlock, R. H. and Speer, C. A. (2005). New method of serological testing for *Mycobacterium avium* subsp paratuberculosis (Johne's disease) by flow cytometry. *Food borne Pathog. Dis.*, **2**: 250-262.

- El Zaatari, F. A., Naser, S. A., Graham, D. Y. (1997). *J. Clin. Microbiol*, **35**: 1794-1799.
- El-Zaatari, F. A., Naser, S. A., Engstrand, L., Burch, P. E., Hachem, C. Y., Whipple, D. L. and Graham, D. Y. (1995). Nucleotide sequence analysis and seroreactivities of the 65K heat shock protein from *Mycobacterium paratuberculosis*. *Clin. Diagn. Lab. Immunol*, **2**: 657-664.
- Ferwerda, G., Kullberg, B.J., de Jong, D.J., Girardin, S.E., Langenberg, D.M., van Crevel, R., Ottenhoff, T.H., Van der Meer, J.W., Netea, M.G., 2007, *Mycobacterium paratuberculosis* is recognized by Toll-like receptors and NOD2. *J Leukoc Biol* 82, 1011-1018.
- Flynn, J.L., Chan, J., 2001, Immunology of tuberculosis. *Annu Rev Immunol* 19, 93-129.
- Gilmour, N. J. S. and Angus, K. W. (1976). The specificity and sensitivity of fluorescent antibody test for *Mycobacterium johnei* infection in abattoir and farm cattle. *Res. Vet. Sci.*, 20: 10.
- Green, E. P., Tizard, M. L. V., Moss M. T., et al (1989). Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. *Nucleic Acids Research*, 17, 9063-9073.
- Gunnarsson, E. and Fodstad, F. (1979). Analysis of antigens in *Mycobacterium paratuberculosis*. *Acta Vet. Scand*, **20**: 200-215.
- Gwozdz, J. M., Reichel, M. P., Murray, A., Manktelow, W., West, D. M. and Thompson, K. G. (1997). Detection of *Mycobacterium avium* subsp. *paratuberculosis* in ovine tissues and blood by the polymerase chain reaction. *Veterinary Microbiology*, 51: 233-244.
- Harris, N.B. and Barletta, R.G. (2001). *Mycobacterium avium* subspecies *paratuberculosis* in Veterinary Medicine. *Clin. Micro. Rev.*, 14(3): 489-512.
- Hasonova, L., Pavlik, I. (2006) Economic importance of paratuberculosis in dairy cattle herds: a review. *Veterinarni medicina*, 51: 193-211.
- Hendrick SH., (2005). Effect of paratuberculosis on culling, milk production, and milk quality in dairy herds. *J Am Vet Med Assoc*. 2005;227:1302-8.

- Hermel, S. R (1998). Testing for Johne's. *Angus Journal*, 3, 194-202
- Herthnek, D., Nielsen, S. S., (2008). A robust method for bacterial lysis and DNA purification to be used with real time PCR for detection of *Mycobacterium paratuberculosis* in milk. *Journal of Microbiological methods*, 75, 335-340.
- Hilbink, F.; West D.M; de Lisle, G.W., et al.,(1994), comparison of a complement fixation test, a gel diffusion test and two absorbed and unabsorbed ELISAs for the diagnosis of paratuberculosis in sheep. *Vet. Microbiol.*, 41; 107-116
- Hines, M.E., 2nd, Stabel, J.R., Sweeney, R.W., Griffin, F., Talaat, A.M., Bakker, D., Benedictus, G., avis, W.C., de Lisle, G.W., Gardner, I.A., Juste, R.A., Kapur, V., Koets, A., McNair, J., Pruitt, G., Whitlock, R.H., 2007, Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Vet Microbiol* 122, 197-222.
- Hoek, A., Rutten, V.P., Kool, J., Arkesteijn, G.J., Bouwstra, R.J., Van Rhijn, I., Koets, A.P., 2009, Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo. *Vet Res* 40, 6.
- Huda A et al. Diagnosis of bovine paratuberculosis by interferon-gamma (IFNgamma) test. *Acta Vet Scand.* 2003;44:281.
- Huda, A., Lind, P., Christoffersen, A. B., Jungersen, G. (2003). Analysis of repeated test for interferon-gamma (IFN-gamma) response and faecal excretion for diagnosis of subclinical paratuberculosis in Danish cattle. *Vet. Immunol and .Immunopathol.*94 (1-2): 43-53
- Huntley, J. F., Stable,J. R., Bannantine, J. P.(2005). Immunoreactivity of the *Mycobacterium avium* subsp. *Paratuberculosis* 19 kDa lipoprotein. *BMC Microbiol* 5(5), 3:10. 1186/1471-2180-5-3
- Ireng, L. M., Walravens, k., (2009). Development and validation of a triplex real time PCR for rapid detection and specific identification of *Mycobacterium paratuberculosis* in faecal samples. *Veterinary Microbiology*, 136, 166-172.

- Jayarao BM et al. Evaluation of IS900-PCR assay for detection of *Mycobacterium avium* subspecies *Paratuberculosis* infection in cattle using quarter milk and bulk tank milk samples. *Foodborne.Pathog.Dis* 2004;1:17-26.
- Kathaperumal, K., Park, S.U., McDonough, S., Stehman, S., Akey, B., Huntley, J., Wong, S., Chang, C.F., Chang, Y.F., 2008, Vaccination with recombinant *Mycobacterium avium* subsp. *paratuberculosis* proteins induces differential immune responses and protects calves against infection by oral challenge. *Vaccine* 26, 1652-1663.
- Kaufmann, S.H., 2010, Future vaccination strategies against tuberculosis: thinking outside the box. *Immunity* 33, 567-577.
- Kirkpatrick, B.W., Shi, X., Shook, G. E., Collins, M. T. (2010). Whole-Genome association analysis of susceptibility to paratuberculosis in holstein cattle. *Anim. Genet.*, pii: AGE2097, doi:10.1111/j.1365-2052.2010.02097.
- Koets A et al. Progressive bovine paratuberculosis is associated with local loss of CD4(+) T cells, increased frequency of gamma delta T cells, and related changes in T-cell function. *Infect Immun* 2002;70:3856-64.
- Koets, A., Hoek, A., Langelaar, M., Overdijk, M., Santema, W., Franken, P., van Eden, W., Rutten, V., 2006. Mycobacterial 70 kD heat-shock protein is an effective subunit vaccine against bovine paratuberculosis. *Vaccine* 24, 2550-2559.
- Koets, A., Santema, W., Mertens, H., Oostenrijk, D., Keestra, M., Overdijk, M., Labouriau, R., Franken, P., Frijters, A., Nielen, M., Rutten, V., 2010, Susceptibility to paratuberculosis infection in cattle is associated with single nucleotide polymorphisms in Toll-like receptor 2 which modulate immune responses against *Mycobacterium avium* subspecies *paratuberculosis*. *Prev Vet Med* 93, 305-315.
- Koets, A.P., Rutten, V.P., de Boer, M., Bakker, D., Valentin-Weigand, P., van Eden, W., 2001, Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine *Mycobacterium avium* subsp. *paratuberculosis* infection. *Infect Immun* 69, 1492-1498.

- Koets, A.P., Rutten, V.P., Hoek, A., Bakker, D., van Zijderveld, F., Muller, K.E., van Eden, W., 1999, Heat-shock protein-specific T-cell responses in various stages of bovine paratuberculosis. *Vet Immunol Immunopathol* 70, 105-115.
- Kohler, H., Gyra, H., Zimmer, K., Drager, K.G., Burkert, B., Lemser, B., Hausleithner, D., Cubler, K., Klawonn, W., Hess, R.G., 2001, Immune reactions in cattle after immunization with a *Mycobacterium paratuberculosis* vaccine and implications for the diagnosis of *M. paratuberculosis* and *M. bovis* infections. *J Vet Med B Infect Dis Vet Public Health* 48, 185-195.
- Koo, H.C., Park, Y.H., Hamilton, M.J., Barrington, G.M., Davies, C.J., Kim, J.B., Dahl, J.L., Waters, W.R., Davis, W.C., 2004, Analysis of the immune response to *Mycobacterium avium subsp. paratuberculosis* in experimentally infected calves. *Infect Immun* 72, 6870-6883.
- Kuehnle, M.P., Goethe, R., Habermann, A., Mueller, E., Rohde, M., Griffiths, G., Valentin-Weigand, P., 2001, Characterization of the intracellular survival of *Mycobacterium avium ssp. paratuberculosis*: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cell Microbiol* 3, 551-566.
- Leroy, B., (2009). Li, L., Bannantine, J. P., Zhang, Q., Amonsin, A., May, B. J., Alt, D., Banerji, N., Kanjilal, S., Kapur, V. (2005). The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proc. Natl. Acad. Sci., USA* 102: 12344-12349.
- Leroy, B., Roupie, V., Noel-Georis, I., Rosseels, V., Wairavens, K., Govaerts, M., Huygen, K. and Wattiez, R. (2007). Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. *Proteomics*. 7: 1164-1176.
- Leroy, B., Viart, S., Trincherro, N., Roupie, V., Govaerts, M., Letesson, J.J., Huygen, K., Wattiez, R., 2009. Use of *Mycobacterium avium subsp. paratuberculosis* specific coding sequences for serodiagnosis of bovine paratuberculosis. *Vet. Microbiol.* 135, 313-319.

- Li, L., Bannantine, J. P., Zhang, Q., Amonsin, A., May, B. J., Alt, D., Banerji, N., Kanjilal, S., Kapur, V. (2005). The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 102: 12344-12349.
- Lin, M.Y., Ottenhoff, T.H., 2008, Not to wake a sleeping giant: new insights into host-pathogen interactions identify new targets for vaccination against latent *Mycobacterium tuberculosis* infection. *Biol Chem* 389, 497-511.
- Macrides, S. C. (1996). Strategies for achieving high level expression of genes in *E. coli*. *Microbiol. Rev.*, **60**: 512-538.
- Manning, E. J. and Collins, M. T. (2001). *Mycobacterium avium* subsp. *Paratuberculosis* : pathogen, pathogenesis and diagnosis. *Reviue Scientifique Techique*, 20: 133-150.
- McKenna SLB et al. Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. *Veterinary Microbiology* 2005;110:105-11.
- Merkal, R. S. and Curran, B. J. (1974). Growth and metabolic characteristic of *Mycobacterium paratuberculosis*. *Appli. Microbiol.* 28: 276-279.
- Momotani, E., Whipple, D.L., Thiermann, A.B., Cheville, N.F., 1988, Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Peyer's patches in calves. *Vet Pathol* 25, 131-137.
- Motiwala, A. S., Janagama, H. K., Paustian, M. L., Zhu, X., Bannantine, J. P.,Kapur, V., Sreevatsan, S.(2006). Comparative transcriptional analysis of human macrophages exposed to animal and human isolates of *Mycobacterium avium* subspecies *paratuberculosis* with diverse genotypes. *Infect. Immun.* 74: 6046-6056
- Mukhija, R., Rupa, P., Pillai, D. and Garg, L.C. (1995).High-level production and one-step purification of biologically active human growth hormone in *Escherichia coli*.*Gene*. **165**(2):303-6.
- Muskens, J., van Zijderveld, F., Eger, A., Bakker, D., 2002, Evaluation of the long-term immune response in cattle after vaccination against paratuberculosis in two Dutch dairy herds. *Vet Microbiol* 86, 269-278.

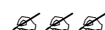
- Nagata R et al. Expression cloning of gamma interferon-inducing antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Infect Immun* 2005;73:3778-82.
- National Academy of Science Report (2003). Diagnosis and control of Johne's disease <http://books.nap.edu/openbook/0309086116/html/index.html>.
- Navarro, J. A., Bernabe, A., Gometz, M. A., Sanchez, J. And Gomes, S. (1991). Mycobacterial antigen detection by immunohistochemistry in goat paratuberculosis. *J. Vet. Med.*, Sec. B, 38: 231-237.
- Nielsen, S.S., Toft, N., 2008, Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. *Vet Microbiol* 129, 217-235.
- Park, H. D., Guinn, K. M., Harrell, M. I., Liao, R., Voskuil, M. I., Tompa, M., Schoolnik, G. K., Sherman, D. R., (2008). Rv 3133c /dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. *Mol. Microbiol.* 48: 833-843.
- Paustian, M. L., Amonsin, A., Kapur, V., Bannantine, J. P. (2004). Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis* : implications for diagnosis of Johne's disease. *J. Clin. Microbiol.* 42: 2675-2681.
- Pavlik, I., BArtl, J., Dvorska, L., (2000). Epidemiology of paratuberculosis in wild ruminants studied by RFLP in the Czech Republic during the period 1995-1998. *Veterinary Microbiology*, 77, 231-251.
- Perez, V., Tellechea, J., Corpa, J. M. J., Gutierrez, M., Garcia, Marin, J. F. (1999). Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis. *Am. J. Vet .Re.* 58: 799-803.
- Pillai, D. Dixit A., and Garg, L.C. (1996). *FEBS Lett.*, **387**: 23-26
- Plotkin, S.A., 2008, Vaccines: correlates of vaccine-induced immunity. *Clin Infect Dis* 47, 401-409.
- Portillo,P.D., Murillo,L.A. and Patarroyo,M.E. (1991). Am Mark, T. D. (1998). Genetically engineered vaccines: An overview (review). *Plasmid*, **39**: 100-113.

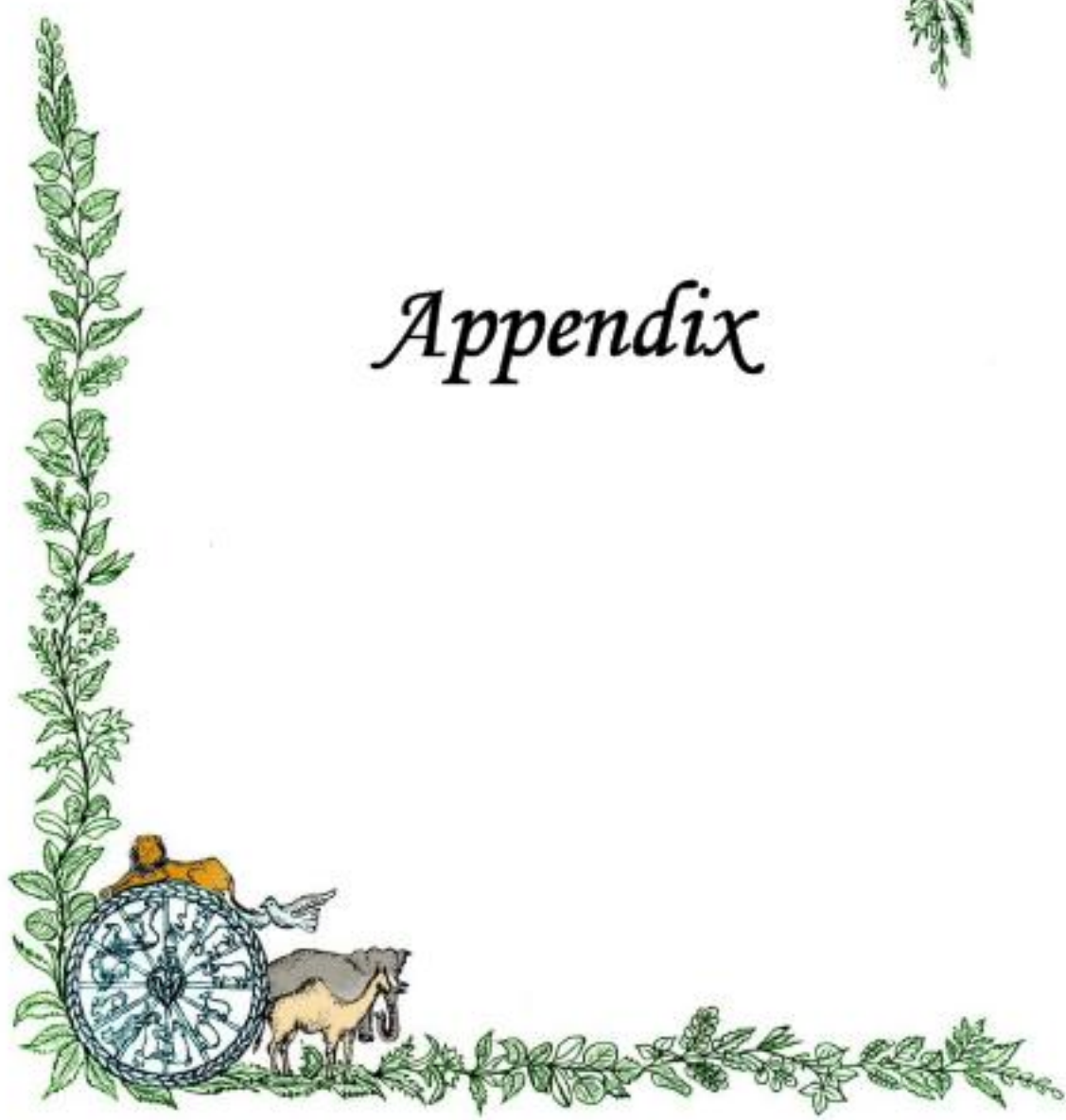
- Raizman EA, Fetrow JP, Wells SJ. Loss of income from cows shedding *Mycobacterium avium subspecies paratuberculosis* prior to calving compared with cows not shedding the organism on two Minnesota dairy farms. *J Dairy Sci* 2009;92:4929-36.
- Rajukumar, K., Tripathi, B. N., Kurade, N. P. And Parihar, N. S. (2001). An enzyme-linked immunosorbent assay using immuoaffinity purified antigen in the diagnosis of caprine paratuberculosis and its comparison with conventional ELISAs. *Vet. Res. Comm.* **25**: 539-553.
- Rigden, R.C., Jandhyala, D.M., Dupont, C., Crosbie-Caird, D., Lopez-Villalobos, N., Maeda, N., Gicquel, B., Murray, A., 2006, Humoral and cellular immune responses in sheep immunized with a 22 kilodalton exported protein of *Mycobacterium avium subspecies paratuberculosis*. *J Med Microbiol* 55, 1735-1740.
- Rogers, A.N., Vanburen, D.G., Hedblom, E.E., Tilahun, M.E., Telfer, J.C., Baldwin, C.L., 2005, Gammadelta T cell function varies with the expressed WC1 coreceptor. *J Immunol* 174, 3386-3393.
- Rosenkrands, I., Aagaard, C., Weldingh, K., Brock, I., Dziegiel, M.H., Singh, M., Hoff, S., Ravn, P., Anderson. P. (2008) identification of RV 0222 from RD4 as a novel serodiagnostic target for tuberculosis. *Tuberculosis*. 88: 335-343.
- Rosseels, V., March, S., Roupie, V., Govaerts, M., Godfroid, J., Walravens, K., Huygen.K. (2006). Members of the 30-to 32-kilodalton mycolyl transferase family (Ag85) from culture filtrate of *Mycobacterium avium* subsp. *Paratuberculosis* are immunodominant Th1-type antigens recognized early upon infection in mice and cattle. *Infect. Immun.*74 (1): 202-212.
- Sallusto, F., Lanzavecchia, A., Araki, K., Ahmed, R., 2010, From vaccines to memory and back. *Immunity* 33, 451-463.
- Sambrook and Russell. *Molecular Cloning - A Laboratory Manual*, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York . 2001. Ref Type: Generic

- Santema, W., Overdijk, M., Barends, J., Krijgsveld, J., Rutten, V., Koets, A., 2009. Searching for proteins of *Mycobacterium avium* subspecies *paratuberculosis* with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculins. *Vet. Microbiol.* 138, 191–196.
- Shin, J. S., Cho, D. and Collin, T.M. (2008) Diagnosis of Bovine Paratuberculosis by a novel Enzyme-linked Immunosorbent Assay Based on Early secreted Antigen of *Mycobacterium avium subsp. paratuberculosis* *Clinic. Vac. Immunol*, **15**;1277-1281
- Shin, S.J., Chang, C.F., Chang, C.D., McDonough, S.P., Thompson, B., Yoo, H.S., Chang, Y.F., 2005, In vitro cellular immune responses to recombinant antigens of *Mycobacterium avium subsp. paratuberculosis*. *Infect Immun* 73, 5074-5085
- Shulaw, W. P., Bech-Nielsen, S., Rings, D. M., Getzy, D. M. and Woodruff, T. S. (1993). Serodiagnosis of paratuberculosis in sheep by use of agar gel immunodiffusion. *Am. J. Vet. Res.* **54**: 184-189.
- Sockett DC et al. Evaluation of four serological tests for bovine paratuberculosis. *J Clin Microbiol* 1992;30:1134-9.
- Speer, C.A., Scott, M.C., Bannantine, J. P., Waters, W. R., Mori, Y., Whitlock, R. H., Eda, S. (2006) A novel- enzyme linked immune sorbent assay for diagnosis of *Mycobacterium avium subsp. paratuberculosis*(johnes's disease) in cattle. *Clini. Vacc. Immun.* 13: 535-540.
- Stabel, J. R. and Whitlock, R. H. (2001). An evaluation of a modified interferon-gamma assay for the detection of paratuberculosis in dairy herds. *Vet. Immunol. Immunopathol.* 79: 69-81.
- Stabel, J.R., Robbe-Austerman, S., 2011, Early immune markers associated with *Mycobacterium avium subsp. paratuberculosis* (MAP) infection in a neonatal calf model. *Clin Vaccine Immunol.*, Jan 12 (epub)
- Stable, S. B., Verma I, Khuller. G. K. (2005). Multicomponent antituberculous subunit vaccine based on immunodominant antigens of *Mycobacterium tuberculosis*. *Vaccine.* 23 (32) : 4175–84.

- Storset, A. K., Hasvold, H. J., Valheim, M., Brem-Hansen, H., Bernsten, G., Whist, S. K., Djonne, B., Press, C. McL., Holstad, G. and Larsen, H. J. S. (2001). Subclinical paratuberculosis in goats following experimental infection. An Immunological and Microbiological study. *Vet. Immunol. Immunopathol.* 80: 271-287.
- Storset, A. K., Hasvold, H. J., Valheim, M., Brem-Hansen, H., Bernsten, G., Whist, S. K., Djonne, B., Press, C. McL., Holstad, G. and Larsen, H. J. S. (2001). Subclinical paratuberculosis in goats following experimental infection. An Immunological and Microbiological study. *Vet. Immunol. Immunopathol.*, **80**: 271-287.
- Sugden, E., Corner, A., Samagh, B., Turcotte, C., Nielsen, K., Stewart, R. And Duncan, J. (1989). Serodiagnosis of Ovine paratuberculosis, using lipoarabinomannan in Enzyme Linked Immunosorbent Assay. *Am. J. Vet. Res.* **50**: 850-854.
- Sweeney, R. W., Whitlock, R. H., McAdams, S. and Fyock, T. (2006). Longitudinal study of ELISA seroreactivity to *Mycobacterium avium* subsp. *Paratuberculosis* in infected cattle and culture negative herd mates. *J. Vet. Diagn. Investig.* 18: 2-6.
- Sweeney, R.W., Whitlock, R.H., Bowersock, T.L., Cleary, D.L., Meinert, T.R., Habecker, P.L., Pruitt, G.W., 2009, Effect of subcutaneous administration of a killed *Mycobacterium avium* subsp *paratuberculosis* vaccine on colonization of tissues following oral exposure to the organism in calves. *Am J Vet Res* 70, 493-497.
- Thorel, M. F., Krichevsky, M. and Levy-Frebault, V. V. (1990). Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. and *Mycobacterium avium* subsp. *silvaticum*. *International J. Syst. Bacteriol.* 40: 254-260.
- Towbin, Staehelin, T. and Gordon, J. (1997). Electrophoretic transfer of protein from polyacrilamide gels to nitrocellulose sheet: Procedure and some applications. *Proct. Natl. Acad. Sci. USA* **76**:4350-4354.
- Tripathi, B. N., Munjal, S. K. and Paliwal, O. P. (2002). An overview of partuberculosis (Johne's disease) in animals. *Indian J. Vet. Pathol.*, 26(1&2): 1-10.

- Tripathi, B. N., Sivakumar, P., Paliwal, O. P., Singh, N. (2006). Comparison of tissue PCR, bacterial culture, johnin and serological test in the diagnosis of naturally occurring paratuberculosis in goats. *Veterinary Microbiology*, 116: 129-137.
- Uzonna, J.E., Chilton, P., Whitlock, R. H., Habecker, P. L., Scott, P., Sweeney, R. W. (2003). Efficacy of commercial and field-strain *Mycobacterium paratuberculosis* vaccinations with recombinant IL-12 in a bovine experimental infection model. *Vaccine*. 21(23):3101-9.
- Weiss, D.J., Evanson, O.A., de Souza, C., Abrahamsen, M.S., 2005, A critical role of interleukin-10 in the response of bovine macrophages to infection by *Mycobacterium avium subsp paratuberculosis*. *Am J Vet Res* 66, 721-726.
- Weiss, D.J., Evanson, O.A., Moritz, A., Deng, M.Q., Abrahamsen, M.S., 2002, Differential responses of bovine macrophages to *Mycobacterium avium subsp. paratuberculosis* and *Mycobacterium avium subsp. avium*. *Infect Immun* 70, 5556-5561.
- Weiss, D.J., Souza, C.D., 2008, Review paper: modulation of mononuclear phagocyte function by *Mycobacterium avium subsp. paratuberculosis*. *Vet Pathol* 45, 829-841.
- Whitlock, R.H., Wells, S.J., Sweeney, R.W. and Van Tiem, J. (2000). ELISA and fecal culture for paratuberculosis (Johne's disease): sensitivity and specificity of each method. *Vet. Microbiol.*, 77: 387-398.
- Whittington RJ et al. Rapid Detection of *Mycobacterium paratuberculosis* in Clinical Samples from Ruminants and in Spiked Environmental Samples by Modified BACTEC 12B Radiometric Culture and Direct Confirmation by IS900 PCR. *Journal of Clinical Microbiology* 1998;36:701-7.
- Whittington, R. J., Begg, D. J., Silva, K. D., Plain, K. M., Purdie, A. C. (2011). Comparative immunological and microbiological aspects of paratuberculosis as a model mycobacterial infection. *Vet. Immunology and immunopathology*. (In press VETIMM-8482; No. of Pages19).





Appendix

Appendix

MEDIUM

Luria Bertani Agar

Tryptone	10 gm
Sodium chloride	10 gm
Yeast extract	5 gm
Distilled water	800 ml

Adjust the pH to 7.2 to 7.5 by 10 N NaOH. Make the volume to 1 Lit. Sterilized by autoclaving. LB agar was prepared by adding 1.5 % agar to LB medium prior to autoclaving.

Middlebrook 7H10 Agar

Dissolve 3.8 gm of Middlebrook agar in 180 ml of distilled water containing 1 ml of bactoglycerol. Boil to dissolve completely. Autoclave at 121 C at 15 lb/sq. inch for 10 min. Aseptically add 20 ml OADC at 50 – 55 C. Mycobactin J (2mg/l) is included for the growth of *M.a.paratuberculosis*. Prepare slants, keep in incubator for 24 hr and then store at 4 C.

Middlebrook 7H9 broth

Suspend 4.7 gm of the powder in 900 ml of purified water (containing 2 ml glycerol or 0.5 gm polyabsorbate 80 if desired). Autoclaved at 121 C for 10 min. Aseptically add 100 ml OADC at 45 C. Mycobactin J (2 mg/l) is included for the growth of *M.a.paratuberculosis*. Prepare slants, keep in incubator or 24 hr and then store at 4 C.

GENERAL BUFFERS AND REAGENTS**TSS**

LB medium
 10% polyethylene glycol
 MgSO₄
 DMSO

TCM (1 X)

1 M Tris.HCL, pH 8	11 ml
1 M CaCl ₂	11 ml
1 M MgCl ₂	11 ml
Sterile DNase free water	967 ml

SOB Medium

Tryptone	2 gm
Yeast Extract	0.5 gm
NaCl	0.05 gm
Distilled Water	90 ml
Add KCl	2.5 mM

Adjust pH to 7.0 with NaOH, make volume to 100 ml and sterilized by autoclaving. Add sterile MgCl₂ to 10mM.

SOC medium

SOB	98 ml
Glucose (1 M)	2 ml

Phosphate Buffer Saline

Sodium Chloride	8.0 gm
Disodium Hydrogen Phosphate	1.16 gm
Potassium Chloride	0.2 gm
Potassium Dihydrogen Phosphate	0.2 gm

Add distilled water to make 1000 ml. Sterilized by autoclaving for 20 min at 15-lb/sq inch. Store at room temperature.

Sodium Dodecyl Sulphate (SDS) - 10 %

SDS	1 gm
Distilled water	10 ml

Ethylene Diamine Tetraacetate (EDTA) - 0.5 M pH- 8

EDTA	18.612
Distilled Water	80 ml

Stir vigorously on a magnetic stirrer to mix. Adjust the pH to 8.0 and make the volume to 100 ml. Autoclave and store at 4 C.

Sodium Hydroxide (NaOH) - 10 N

NaOH	4 gm
Distilled Water	10 ml

Sodium Acetate (3 M)

Sodium Acetate	
Distilled Water	100 ml

Proteinase K (10 mg/ml)

Proteinase K	10 mg
Distilled Water	1 ml

Store at -20°C.

RNase (10 mg/ml)

RNase	10 mg
Distilled Water	1 ml

Store at -20°C.

Tris - EDTA (TE) Buffer 1 X - pH 8.0

Tris-HCl	10 mM
EDTA	1 mM

Ampicillin Stock Solution (25 mg/ml)

Ampicillin Sodium salt	250 mg
Distilled Water	10 ml

Sterilize by Filtration.

Kanamycin Stock Solution (25 mg/ml)

Kanamycin	250 mg
Distilled Water	10 ml

Sterilize by Filtration.

Isopropyl Thiogalactosidase (IPTG) - 1 M

IPTG	238 gm
Distilled Water	1 ml

Sterilize by filtration. Store at – 20°C

X- Gal

X-Gal	25 mg
Diethyl formamide	1 ml

Sterilize by filtration. Store at – 20 C.

REAGENTS FOR PLASMID DNA ISOLATION**Solution P1**

Tris- HCl
EDTA
RNase

Store at 4°C.

Solution P2

NaOH	0.2 N
SDS	1 %

Store at room temperature.

Solution P3

5 M Potassium acetate
Glacial Acetic acid

Distilled Water.

REAGENTS FOR GENOMIC DNA ISOLATION**Tris Glucose EDTA**

Tris (1 M) pH 8.0	5 ml
EDTA (0.5 M) pH 8.0	2.5 ml
Glucose (1 M)	33 ml

Distilled Water made upto 100 ml.

Lysozyme Solution (20 mg/ml)

Lysozyme	100 mg
Tris Glucose EDTA	5 ml

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS**Tris Borate EDTA (TBE) Buffer (5 X Stock Solution)**

Tris base	54 gm
Boric Acid	27.5 gm
EDTA (0.5 M) pH 8.0	20 ml

Distilled Water to make 1000 ml.

6 X loading Dye

Intercalating Dye	
Ethidium Bromide	10 mg
Distilled Water	1 ml

REAGENTS FOR PURIFICATION OF RECOMBINANT PROTEINS UNDER DENATURING CONDITION**Lysis buffer**

Urea	8 M
Sodium dihydrogen phosphate (NaH_2PO_4)	0.1 M
Tris Cl	0.01 M

Adjust pH to 8.0.

Wash buffer

Urea	8 M
Sodium dihydrogen phosphate (NaH_2PO_4)	0.1 M

Tris Cl	0.01 M
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Adjust pH to 6.3.

Elution Buffer I

Urea	8 M
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Sodium dihydrogen phoshate (NaH_2PO_4)	0.1 M
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Tris Cl	0.01 M
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Adjust pH to 5.9.

Elution Buffer II

Urea	8 M
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Sodium dihydrogen phoshate (NaH_2PO_4)	0.1 M
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Tris Cl	0.01 M
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Adjust pH to 4.5.

REAGENTS FOR SDS PAGE AND WESTERN BLOTTING

Acrylamide-Bisacrylamide Solution (30%)

Acrylamide	30 gm
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Bisacrylamide	0.8 gm
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Distilled Water	100 ml
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Filter and store at 4°C.

4 X Separating Gel Buffer 1.5 M (pH 8.8)

Tris base	18.17 gm
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SDS	0.4 gm
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Distilled Water	100 ml
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4 X Stacking Gel Buffer 1.0 M (pH 6.8)

Tris base	6.05 gm
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Distilled Water	100 ml
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2 X loading Buffer (15 ml)

Stacking Gel Buffer	1.7 ml
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10 % SDS	9.5 ml
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β -mercaptoethanol	1.0 ml
Glycerol	1.0 ml
Bromophenol Blue	2 mg
Distilled Water	1.8 ml

1 X Electrode Buffer for SDS PAGE

Tris base	3 gm
Glycine	14.4 gm
SDS	1 gm
Distilled Water	1000 ml

Western Blot Transfer Buffer

Tris base	2.5 gm
Glycine	11 gm
Add distilled water to	800 ml

Adjust pH to 8.0. Add 200 ml methanol.

Comassie Brilliant Blue (CBB) Staining Solution (0.2 %)

Dissolve 0.2 % of CBB in methanol and mix the water, methanol and acetic acid in the ratio of 60: 30: 10.

Destaining Solution

Mix water, methanol and acetic acid in the ratio of 60: 30: 10.

Resolving Gel (12 %)

Distilled Water	3.3 ml
30 % Acrylamide bisacrylamide Solution	4.0 ml
4 X Resolving Gel Buffer	2.5 ml
10 % SDS	100 μ l
1 % Ammonium persulfate	100 μ l
TEMED	4 μ l

Stacking Gel (5 %)

Distilled Water	3.4 m
30 % Acrylamide bisacrylamide Solution	830 μ l
4 X Stacking Gel Buffer	630 μ l

10 % SDS	50 µl
1 % Ammonium persulfate	50 µl
TEMED	5 µl

REAGENTS FOR IMMUNOBLOT DETECTION

Blocking Solution

Skimmed milk powder	200 mg
PBST Solution	10 ml

PBS-T Solution

PBS, pH 7.4	100 ml
Tween 20	100 µl

PBS-T- BSA Solution

PBS, pH 7.4	20 ml
Tween – 20	10 µl
BSA	200 mg

Substrate Solution

Diaminobenzidine	5 gm
PBS, pH 7.4	10 ml
30 % H ₂ O	10 µl

REAGENTS FOR ELISA

Coating buffer (0.05 M pH 9.6)

Sodium carbonate	1.59 gm
Sodium bicarbonate	2.93 gm
Distilled Water	1000 ml

Store at 4°C.

Substrate/Citrate Buffer (0.1 M pH 4.6)

Solution A

Citric Acid	2.1 gm
Distilled Water	100 ml

Solution B

Sodium Citrate	2.94 gm
Distilled Water	100 ml

25.4 ml Solution A mixed with 24.5 ml Solution B stored at 4°C.

Substrate solution

Citrate buffer	100 ml
H ₂ O ₂ (30 % v/v)	12 µl
OPD.HCl	40 mg



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