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Cristian Piras*, Alessio Soggiu, Viviana Greco, Isabella Alloggio, Luigi Bonizzi, Paola Roncada

Peptidomics in veterinary science: focus on bovine *paratuberculosis*

Abstract: Bacterial infections represent a serious burden both for animal production and human health (zoonosis). Faster and more reliable diagnosis are mandatory in order to avoid economic losses and antibiotics misuse. The development of new potential diagnostic strategies for the immunodetection of pathogens is closely linked to the discovery of small polypeptides with immunogenic or immunoreactive activity. The candidate peptides used for this purpose must have several properties principally represented by their specificity and their location in the bacterial cell. Both proteomics, peptidomics and bioinformatics represent powerful complementary tools to discover specific immunoreactive peptides useful for diagnosis or vaccine. Peptidomics of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) represents a good example of the potential of this discovery-phase. This review reports a comprehensive update of the current scientific knowledge about proteins and peptides of MAP with already documented humoral response. These findings, together with bioinformatics tools available, could be extremely useful to design a better strategy for subclinical bovine *paratuberculosis* diagnosis. The knowledge provided also represents a reliable example on the workflow to be followed in the direction of the diagnosis of other diseases through a peptidomic approach.

Keywords: immunoreactive peptides, bioinformatics, bovine *paratuberculosis*, diagnostic peptides

***Corresponding author: Cristian Piras:** Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli studi di Milano, Milano, Italy, E-mail: cristian.piras@unimi.it
Alessio Soggiu, Isabella Alloggio, Luigi Bonizzi, Paola Roncada: Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli studi di Milano, Milano, Italy
Viviana Greco: Fondazione Santa Lucia – IRCCS, Rome, Italy
Viviana Greco: Dipartimento di medicina sperimentale e chirurgia, Università degli studi di Roma TorVergata, Roma, Italy
Paola Roncada: Istituto Sperimentale Italiano L. Spallanzani, Milano, Italy

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1 Introduction

The development of new potential diagnostic strategies for the immunodetection of pathogens is closely linked to the discovery of small polypeptides with immunogenic or immunoreactive activity. For this purpose, it would be better if these peptides are specific and located in the bacterial cell surface. Specificity is negatively related to the conservation of the sequence of that epitope among bacterial species that could be in contact with the same host: If the immunoreactive epitope has a very well conserved sequence, the probability that the host is immunized against that epitope is high and could bring to a high rate of cross-reaction. Moreover, the location in the bacterial cell wall also represents a critical element. In fact, the immunization against a bacterial protein is easier if the protein is located in the surface because it has higher probability to be in contact with host immune system [1].

The increasingly large availability of fully sequenced pathogens genomes has increased the possibility to study in silico the presence of epitopes useful for the diagnosis. Therefore synthetic peptides could be created for the preparation of antigenic cocktails useful as high-throughput platform for immunological testing. However, it is not feasible to perform the complete in silico bioinformatic analysis totally avoiding a bench approach. The bench approach is necessary in order to obtain targeted preliminary data and to proceed with the in silico analysis (Fig.1).

Vaccination also represents a key point in public health advances with important goals such as worldwide eradication of diseases as smallpox and poliomyelitis. Vaccines are usually made with the pathological agent attenuated or killed and the immunization is made by injection. This practice has, as positive effect, the immunization of the patient but also presents some negative effects. For example, these negative effects

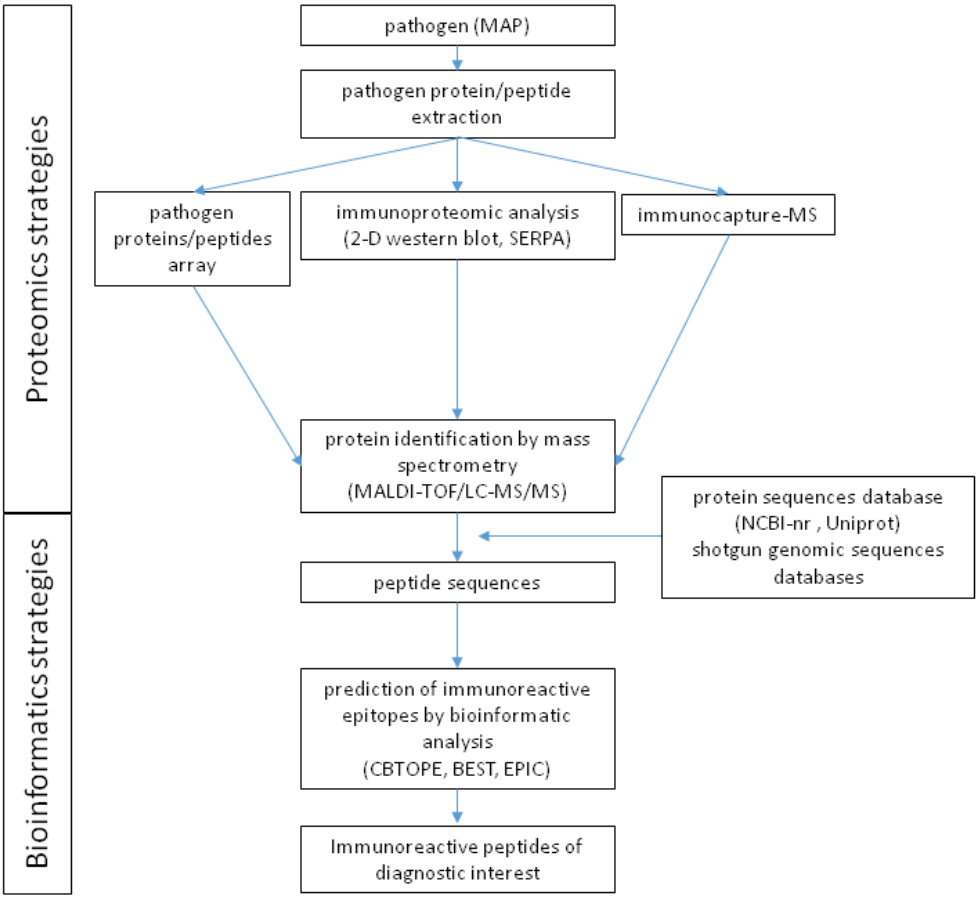

Figure 1. Peptidomics workflow for detection of antigenic peptides.

could be due to the stimulation of a high and unwanted host immune response against self-structures linked to molecular mimicry mechanisms. Mainly for these reasons, the vaccination through specific synthetic peptides could represent the solution to overcoming these problems. One of the most significant contributions of this approach resulted in the production of antigens used for vaccination against cancer [2].

There are two different types of epitopes that could be recognized by immune cells or antibodies, the continuous and the discontinuous ones. The continuous epitopes consist in short linear fragments of an antigen; the discontinuous ones are formed by aminoacid residues brought together by the secondary structure of a protein ad are dependent from the protein folding. According to a peptidomic point of view, among these two classes of antigens, the most important are the continuous ones because of the simpler procedure of synthesis that is independent from protein folding [3].

As stated before, the availability of a high number of bacterial sequences enhances the possibility of in silico analysis. However, a bench approach is necessary in order to obtain experimental data useful to support bioinformatics results. The methods able to provide important information are mainly related to proteomics approaches. One of the most powerful techniques is represented by 2D electrophoresis followed by immunoblotting (2D immunoblotting) and mass spectrometry analysis for the identification of immunoreactive protein spots [4] (fig. 1). This approach provides reliable data on the presence of immunogenic proteins and on their sequence. Moreover if the experimental design also includes a comparison between infected and control samples, it is possible to evaluate if these proteins are specifically recognized by infected host [5]. Another promising approach is based on the enzymatic shaving of bacterial surfaces [6]. This procedure consists in two steps: treatment of the intact bacterial cells with trypsin and mass spectrometry analysis of shaved peptides. This provides, with good confidence, the knowledge about proteins that are partly exposed out of the bacterial cell wall.

The biggest challenge in veterinary medicine is represented by the constant request of detection methods for an early diagnosis of pathologies in their subclinical stage. Several pathologies are indeed diagnosed once

the advanced clinical status is coming leading to an increase of expenses in the management [7]. In this field, one of the worst pathologies is represented by bovine *paratuberculosis*. A huge work has been done for the detection of novel epitopes useful for its diagnoses. Nevertheless, to date there is no well applicable documented progress and the subclinical form is still very difficult to diagnose.

The aim of this review is to summarise all the advances that have been made to date in the field of the diagnosis and vaccine through the discovery of immunogenic peptides. In recent years many protein and peptides have been widely described, but it is still missing a complete list of all obtained data useful to provide exhaustive and comprehensive information for future studies on putative diagnoses and vaccines.

In this context it will be underlined how the complementary use of both bench and in silico approaches could lead to reliable results useful to the synthesis of immunoreactive peptides to be used as vaccines or diagnostic tools.

2 Bovine *paratuberculosis* (Johne's disease/JD) and its impact on veterinary industry and on human health

The widespread use of antibiotics and their relation with the increase of antibiotic resistance is a great concern worldwide. In this field, a correct and rapid diagnosis plays a pivotal role. The later the diagnoses is performed, the higher is the possibility of infection spread and the amount of antibiotics needed in order to counteract the infection [8]. This topic is relevant in the light of the evidences of the increasing problems of drug and multidrug resistance [9]. For these reasons, bovine *paratuberculosis* has been chosen as the keynote study.

Bovine *paratuberculosis* is caused by MAP and is characterized by a systemic infection and chronic inflammation of the intestine in animals [10]. It is spread worldwide and is the cause of huge economic loss for animal production [11]. The economic loss is due to lower conversion index of food, to higher veterinary costs, lower milk and meat production, lower value at slaughterhouse and of the whole breeding. Considering that cattle business represents the most important part of animal products for human consumption and that bovine *paratuberculosis* is spread worldwide, the financial loss is currently still underestimated [12].

Moreover, there is considerable evidence that documents how this disease could also be a zoonosis [13]. Crohn's disease (CD) is a chronic inflammation of the intestine in humans very similar to Johne's disease which was spread in Europe and North America. There are many pieces of experimental evidence that document how MAP could be the etiologic agent. One of the most interesting type of studies in this field is related to the evaluation of CD incidence in individuals with environmental or occupational exposure [10]. This study has been performed in US and demonstrated how this cluster of individuals was associated with a significantly reduced death rate due to inflammatory bowel diseases (IBD) [14]. This evidence demonstrates both that a link between these two pathologies could exist, both that exposure to antigens, even in humans, could generate an immunization process against this pathogen. Another really interesting result comes from the study of population movement and this pathogen [10]. Particularly interesting is the study that describes what happened in Iceland: after introduction of MAP via Karakul sheep imported from Germany, the increasing incidence of JD rate in sheep, cattle, and, afterwards, incidence of CD in humans increased up to 18 folds [15, 16].

There are many pieces of experimental evidences that document the presence of MAP in blood and mucosal biopsies [17-20].

Several cases document how MAP infection could be involved in the onset of autoimmune diseases in humans. Many MAP proteins, such as hsp65, have been previously identified as immunodominant antigens that stimulate humoral and T-cell mediated response during mycobacterial infection [21]. hsp65 has, in its sequence, characteristic epitopes that resemble humans structures and, the immunization against this protein could generate the production of antibodies against self-structures. This similarity is the base of the proposed etiopathological mechanism (molecular mimicry) that stimulates the production of autoantibodies associated with autoimmune diseases as type 1 diabetes, Hashimoto's thyroiditis, and multiple sclerosis [22].

These evidences contribute to underline the hazards that this pathogen represents for public health [23].

3 Bovine *paratuberculosis* diagnosis and vaccines

The biggest actual challenges of veterinary industry against Johne's disease are still the diagnosis and vaccination. The disease spread could be avoidable

only through the diagnosis of the subclinical infection. What is actually happening is that, considering a herd, for every cow that has been successfully diagnosed, there is an average number of roughly 15-20 cows that are infected but cannot be diagnosed [24]. Among diagnostic methodologies there are the serological tests, such as ELISA, that are mostly used to confirm the presence of this pathology in cattle with evident clinical signs. Because of its relatively short turnaround time and low cost it is the most frequently used. However commercial ELISA tests are unable to detect early infections and the use of this technique for the JD diagnosis is still questionable. ELISA is able to detect about 40% of diseased cattle that could be detected by faecal culture methods [25]. Considering that faecal culture has a sensitivity of about 50%, the final rate of sensitivity of ELISA test is very low.

Methods for the investigation of cell-mediated immunity, as hypersensitivity methods (skin test), could also be used for diagnosis. However, these methods fail to be useful for a reliable diagnosis because of the lack of specificity and high rates of false positive and false negatives [26].

As a reliable test is needed for subclinical *paratuberculosis* diagnosis, effective vaccine is also required. To date, the *paratuberculosis* vaccines are made with live attenuated or killed bacteria. The vaccination is necessary for the prevention of clinical cases but it interferes with the interpretation of skin tests for bovine tuberculosis. This represents the main reason why vaccination is prohibited in many countries.

4 Literature on diagnoses and putative peptides to be used

As stated before, to date, for bovine *paratuberculosis* diagnosis, several serological tests have been developed. However, lack of sensitivity and specificity [27] still represents a problem and the selection of proteins belonging form MAP surface could represent a possible solution [28].

Many studies have been conducted in order to enhance knowledge and methods for bovine *paratuberculosis* diagnosis. The recent advantages in the field of proteomics and peptidomics have contributed to an increase in the number of alternative approaches for *paratuberculosis* diagnosis. The high resolution of 2D gel-electrophoresis coupled to the powerful tool of mass spectrometry lead to the identification of several hundred interesting MAP proteins.

Mikkelsen and colleagues [29] provided a table containing all information separated according to the type of antigen. Antigens list was divided in two groups, antigens responsible for cell-mediated immunity and antigens responsible for humoral immunity that is the most promising application in the diagnostic field.

In this review table 1 summarises all the details about the antigens responsible for the humoral immunity, modified from Mikkelsen and colleagues [29], and updated with all the currently available data. In the previous study, all the immunoreactive epitopes both for humoral and cell-mediated immunological response have been listed. In this context they have been revised all the epitopes closely linked to humoral response whose protein sequence was well annotated in order to provide the basis for further in silico analysis of the immunoreactive epitopes. The overall representation of these data has been filtered considering protein information available. According to experimental evidences and functions, these proteins play a key role in the pathogenicity of MAP and in its ability to stimulate the host humoral response. In table 1 it has been described, where possible, the main cellular function in which every protein could be involved and the most relevant GO terms. The cellular compartment has been also provided where possible according to GO annotations. For each protein it has been provided the Uniprot accession number in order to facilitate the availability of the protein and gene sequence that could be useful for further studies.

These revised information about all describes epitopes could provide an important starting point for the in silico analysis of immunoreactive specific epitopes. According to our knowledge, the proteins and relative peptides in the following table are able to stimulate antibodies production in animals.

Table 1. Table integrating all protein epitopes up to date and according to Mikkelsen [29]. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

5 Laboratory methods and bioinformatics tools for immunoreactive epitope prediction

Antibodies reflect health: they are a feature in diseases such as autoimmune diseases, cancer or infections. One important feature of antibodies is their relative easy availability. Because they are carried in the bloodstream, their screening is simply a matter of blood sample

Table 1. Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

	Antigen	Locus (ENA)	Size	Uniprot		GO Biological process	GO Cellular compartment	GO Molecular function	Reference
Energy Metabolism	Uncharacterized protein	MAP0593c	14,819	Q743J2	Metabolism	metabolic process		catalytic activity	Gumber et al. (2009) (Kawaji, Gumber et al. 2012)
	Uncharacterized protein	MAP4308c	33,645	Q73RX0	Metabolism	glycolytic process,		fructose-bisphosphate aldolase activity	Leroy et al. (2007)
	Uncharacterized protein	MAP1637c	52,073	Q73ZG6	Metabolism	ubiquinone biosynthetic process	plasma membrane	FMN binding, carboxy-lyase activity, oxidoreductase activity	Leroy et al. (2009)
	MoaA3	MAP3932c	41,553	Q73SZ0	Metabolism	Mo-molybdopterin cofactor biosynthetic process			Hughes et al. (2008)
	Uncharacterized protein	MAP0334	34,517	Q744K7	Metabolism	cellular metabolic process		catalytic activity, coenzyme binding	Hughes et al. (2008)
	aceAb	MAP1643	85,213	Q73ZG0	Metabolism	carboxylic acid metabolic process		isocitrate lyase activity	Bannantine et al. (2007)
	Uncharacterized protein	MAP 2020	26,873	Q73YD6	Metabolism			hydrolase activity	Mon et al. (2012)
	Enoyl-CoA hydratase	MAP_1197	28,761	Q740Z5	Metabolism			catalytic activity	(Nagata, Kawaji et al. 2013)
	DesA2	MAP2698c	31,469	Q73WG2	Fatty acid metabolism	fatty acid metabolic process		acyl-[acyl-carrier-protein] desaturase activity	Gurung et al. (2014, 2012)
	ATP synthase epsilon chain	MAP2450c	13,124	Q73X60	Metabolism	plasma membrane ATP synthesis coupled proton transport	plasma membrane	ATP binding, proton-transporting ATP synthase activity, rotational mechanism	Gurung, 2013
	EchA20	MAP0516c	26,847	Q743S5	Metabolism			catalytic activity	Gurung, 2013
	EchA8_1	MAP1017c	27,884	Q741S0	Metabolism			catalytic activity	Gurung, 2013
	FadE3_2	MAP3651c	44,051	Q73TR7	Metabolism			acyl-CoA dehydrogenase activity, flavin adenine dinucleotide binding	Gurung, 2013
	Uncharacterized protein	MAP2487c	17,900	Q73X23	Metabolism			carbonate dehydratase activity, zinc ion binding	Gurung, 2013
	pstA	MAP1242	430,487	Q740V0				catalytic activity, phosphopantetheine binding	Wu et al. (2009)
	Malate dehydrogenase	MAP_2541c	34,631	P61976		cellular carbohydrate metabolic process, tricarboxylic acid cycle		L-malate dehydrogenase activity	Piras et al. (2014)
	FixA	MAP_3061c	27,847	Q73VF3				electron carrier activity	Piras et al. (2014)

continued **Table 1.** Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

	Antigen	Locus (ENA)	Size	Uniprot	GO Biological process	GO Cellular compartment	GO Molecular function	Reference
Oxidative stress response and redox pathways	Mpt53	MAP2942c	18,294	Q73VR9	Oxidative stress response	cell redox homeostasis	oxidoreductase activity	Willemsen et al. (2006)
	Uncharacterized protein	MAP2411	15,538	Q73X98	Oxidative stress response	oxidation-reduction process	FMN binding	Gumber et al. (2009) (Kawaji, Gumber et al. 2012)
	Superoxide dismutase [Mn]	MAP0187c	23,030	P53647	Oxidative stress response	superoxide metabolic process	extracellular region	metal ion binding	Liu et al. (2001); Shin et al. (2004);
	10 kDa chaperonin	MAP4264	10,748	P60533	Stress response	Stress response	Cytoplasm	Chaperone	Cobb and Frothingham (1999)
	Protein GrpE	MAP3841	23,709	Q73T78	Stress response	protein folding, response to stress	cytoplasm	adenyl-nucleotide exchange factor activity	Hughes et al. (2008)
	Hsp65	MAP_3936	56,643	P42384	Stress response	protein refolding		Chaperone	Piras et al. (2014)
	Chaperone protein DnaK	MAP3840	66,518	Q00488	Stress response	protein folding, response to stress		ATP binding	Bannantine et al. (2007,2008)
	Uncharacterized protein	MAP4147	42,224	Q73SC7	Stress response	cell redox homeostasis		flavin adenine dinucleotide binding, oxidoreductase activity	Hughes et al. (2008)
	Uncharacterized protein	MAP2182c	15,789	I3NID4	Stress response			oxidoreductase activity	Bannantine et al. (2008)
	Uncharacterized protein	MAP3567	30,184	Q73U01	Stress response			oxidoreductase activity	Gurung et al. (2014, 2012)
	Uncharacterized protein	MAP 2513c	36,563	Q73WZ7	Stress response			oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	Mon et al. (2012)
	Uncharacterized protein	MAP_0388	43,851	Q744F3	Stress response			heme binding, peroxidase activity	Roupie et al. 2012
	Uncharacterized protein	MAP_3743	36,544	Q73TH5	Stress response			oxidoreductase activity	Roupie et al. 2012
	Hsp65K	MAP_3936	56,643	P42384	Stress response	protein refolding	cytoplasm	ATP binding	ElZaatari et al. (1995)
	Hsp70	MAP_3840	66,518	Q00488		protein folding, response to stress		ATP binding	Langelaar et al. (2002)
	Uncharacterized protein	MAP1339	15,437	Q740L1	Stress response	response to stress			Gurung, 2013
	Alkyl hydroperoxide reductase AhpD	MAP1588c	18,842	Q73ZL4	Stress response	response to oxidative stress		hydroperoxide reductase activity, peroxidase activity	Gurung, 2013
	AhpC	MAP1589c	21,641	Q73ZL3	Stress response	antioxidant activity, oxidoreductase activity			Gurung, 2013

continued **Table 1.** Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

	Antigen	Locus (ENA)	Size	Uniprot	GO Biological process	GO Cellular compartment	GO Molecular function	Reference
	Probable thiol peroxidase	MAP1653	16,685	I3NID7	Stress response cell redox homeostasis		thioredoxin peroxidase activity	Gurung, 2013
	Thioredoxin	MAP4340	12,451	I3NIE3	Stress response cell redox homeostasis, glycerol ether metabolic process		protein disulfide oxidoreductase activity	Gurung, 2013
	Uncharacterized protein	MAP0508	27,551	Q743T3	Stress response		oxidoreductase activity	Gurung, 2013
	FabG5_2	MAP2872c	26,793	Q73VY9	Stress response		oxidoreductase activity	Gurung, 2013
	FadB4	MAP3190	33,405	Q73V26	Stress response		oxidoreductase activity, zinc ion binding	Gurung, 2013
	FabG3_2	MAP_3577	25,922	Q73TZ1	Stress response		oxidoreductase activity	Gurung, 2013
	Uncharacterized oxidoreductase	MAP3007	30,013	Q73VK6	Stress response		oxidoreductase activity	Gurung, 2013
	Uncharacterized protein	MAP3538	16,000	Q73U30	Stress response		oxidoreductase activity	Gurung, 2013
DNA metabolism and gene expression transcription and regulation	Orotate hosphoribosyltransferase	MAP3857	18,871	Q73T62	DNA metabolism	'de novo' UMP biosynthetic process	magnesium ion binding	Hughes et al. (2008)
	Transcription elongation factor GreA	MAP1027c	17,924	Q741R0	DNA metabolism	Transcription, DNA binding F	DNA binding	Gumber et al. (2009)
	Uncharacterized protein	MAP2963c	97,054	Q73VQ0	Gene expression regulation		sequence-specific DNA binding	Paustian et al. (2004)
	Pseudouridine synthase	MAP3422c	31,807	Q73UE6		pseudouridine synthesis	RNA binding, pseudouridine synthase activity	Bannantine et al. (2011)
	CspB	MAP_0810	15,231	Q742M5	DNA metabolism	regulation of transcription, cytoplasm DNA-templated	DNA binding	Gurung, 2013
	Single-stranded DNA-binding protein	MAP0068	17,590	Q744V5	DNA metabolism	DNA replication	single-stranded DNA binding	Gurung, 2013
	N5-carboxyaminoimidazole ribonucleotide mutase	MAP3393c	17,608	Q73UH5	DNA metabolism	'de novo' IMP biosynthetic process	5-(carboxyamino)imidazole ribonucleotide mutase activity	Gurung, 2013

continued **Table 1.** Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

	Antigen	Locus (ENA)	Size	Uniprot		GO Biological process	GO Cellular compartment	GO Molecular function	Reference
	ATP phosphoribosyltransferase	MAP1846c	30,542	P60805	DNA metabolism	histidine biosynthetic process	cytoplasm	ATP binding, ATP phosphoribosyltransferase activity, magnesium ion binding	Gurung, 2013
	Uncharacterized protein	MAP 0834c	25,025	Q742K1	Transcription process	transcription, DNA-templated		DNA binding, phosphorelay response regulator activity	Gurung, 2013
	50S ribosomal protein L10	MAP4125	20,168	Q73SE9	Transcription process	Translation, ribosome biogenesis	ribosome	LSU rRNA binding, structural constituent of ribosome	Gurung, 2013
Proteins with structural function	modD	MAP1569	36,116	I3NIE1	Structural		extracellular region	extracellular matrix binding	Cho et al. (2006, 2007); Facciuolo et al. (2013) (Souza, Rodrigues et al. 2011)
	Csp	MAP 0209c	56,522	Q745J8	Structural	peptidoglycan catabolic process		N-acetylmuramoyl-L-alanine amidase activity, zinc ion binding	Mon et al. (2012)
	Uncharacterized protein	MAP3968	21,291	Q73SV5	Structural/ Pathogenesis	cell adhesion, pathogenesis	cell surface	heparin binding	Gurung, 2013 Bannantine et al. (2008)
Protein metabolism and folding	Peptide chain release factor 2	MAP3175c	41,520	Q73V41	Protein metabolism	Protein biosynthesis	cytoplasm	translation release factor activity, codon specific	Hughes et al. (2008)
	PepA	MAP3527	35,709	I3NID9	Protein metabolism	proteolysis		serine-type endopeptidase activity	Cho et al. (2006, 2007)
	ATP-dependent Clp protease proteolytic subunit 2	MAP2281c	21,665	Q73XM8	Protein metabolism	proteolysis	cytoplasm	serine-type endopeptidase activity	Gumber et al. (2009) (Kawaji, Gumber et al. 2012
	Peptidyl-prolyl cis-trans isomerase	MAP1693c	18,330	Q73ZB0	Protein folding	protein folding		peptidyl-prolyl cis-trans isomerase activity	Leroy et al. (2007)
	Phosphoribosyl isomerase A	MAP1297	25,402	P60583	Protein metabolism	histidine biosynthetic process, tryptophan biosynthetic process	cytoplasm	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase activity, phosphoribosylanthranilate isomerase activity	Hughes et al. (2008)
	4-hydroxy-tetrahydrodipicolinate reductase	MAP2878c	25,561	Q73VY3	Protein metabolism	diaminopimelate biosynthetic process, lysine biosynthetic process via diaminopimelate	cytoplasm	NAD binding, 4-hydroxy-tetrahydrodipicolinate reductase, oxidoreductase activity, acting on CH or CH2 groups, NAD or NADP as acceptor	Hughes et al. (2008)

continued **Table 1.** Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

	Antigen	Locus (ENA)	Size	Uniprot		GO Biological process	GO Cellular compartment	GO Molecular function	Reference
	Ornithine carbamoyltransferase	MAP1365	33,595	Q740I5	Protein metabolism	arginine biosynthetic process	cytoplasm	amino acid binding	Hughes et al. (2008)
	MetC	MAP3457	47,748	Q73UB1	Protein metabolism	cellular amino acid metabolic process		pyridoxal phosphate binding, transferase activity, transferring alkyl or aryl (other than methyl) groups	Hughes et al. (2008)
	Histidinol dehydrogenase	MAP1293	49,373	P60860	Protein metabolism	histidine biosynthetic process		NAD binding, histidinol dehydrogenase activity, zinc ion binding	Hughes et al. (2008)
	Peptidyl-prolyl cis-trans isomerase	MAP 1693	18,330	Q73ZB0		protein folding		peptidyl-prolyl cis-trans isomerase activity	Mon et al. (2012)
	Proteasome subunit alpha	MAP1834c	28,078	Q73YW9	Protein metabolism	proteolysis involved in cellular protein catabolic process	cytoplasm	threonine-type endopeptidase activity	Gurung, 2013
	ATP-dependent Clp protease proteolytic subunit 1	MAP2280c	23,292	Q73XM9	Protein metabolism		cytoplasm	serine-type endopeptidase activity	Gurung, 2013
	4-hydroxy-tetrahydrodipicolinate synthase	MAP2864c	30,973	Q73VZ7	Protein metabolism	diaminopimelate biosynthetic process, lysine biosynthetic process via diaminopimelate	cytoplasm	4-hydroxy-tetrahydrodipicolinate synthase, amine-lyase activity	Gurung, 2013
Phosphate metabolism	Inorganic pyrophosphatase	MAP0435c	18,615	Q744A6	Phosphate metabolism	phosphate-containing compound metabolic process	cytoplasm	inorganic diphosphatase activity	Gumber et al. (2009)
Proteins involved in cell signalling, pathways regulation and cell cycle	Uncharacterized protein	MAP0865	45,726	Q742H0	Cell cycle	cell cycle	integral component of membrane	ATP binding, DNA binding	Bannantine et al. (2008)
	Uncharacterized protein	MAP1012c	37,505	Q741S5	Cell signalling	phosphatidylcholine metabolic process		lysophospholipase activity	Hughes et al. (2008)
	ptpA	MAP1985	18,065	Q73YH1	Cell signalling	protein dephosphorylation		protein tyrosine phosphatase activity	Gurung et al. (2014), Begg et al (2014)
	Wag31	MAP1889c	28,050	Q73YR6	Cell cycle	cell cycle, cell division	cytoplasm		Gurung, 2013

continued **Table 1.** Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

	Antigen	Locus (ENA)	Size	Uniprot		GO Biological process	GO Cellular compartment	GO Molecular function	Reference
	Uncharacterized protein	MAP3200	14,789	Q73V16	Cell signalling			phosphorelay response regulator activity	(Kawaji, Gumber et al. 2012)
	CysQ_2	MAP2058c	25,484	Q73Y98	Cell signalling	phosphatidylinositol phosphorylation			Gurung, 2013
Proteins with function still not described	Uncharacterized protein	MAP2609	11,397	Q73WQ1					Willemsen et al. (2006)
	Uncharacterized protein	MAP2168c	18,279	Q73XZ0					Cho et al. (2006, 2007)
	Uncharacterized protein	MAP3199	19,697	Q73V17					Leroy et al. (2007)
	Uncharacterized protein	MAP1272c	33,436	Q740S0					Li et al. (2007); Mon et al. (2012)
	PirG	MAP0210c	30,705	Q745J7					Willemsen et al. (2006)
	FbpC2	MAP3531c	37,769	Q73U37					Cho et al. (2006, 2007); Shin et al. (2004); Dheenadhayalan et al. (2002)
	FbpA	MAP0216	36,086	I3NIE0					Shin et al. (2004); Dheenadhayalan et al. (2002)
	FbpB	MAP1609c	34,707	Q73ZJ4					Shin et al. (2004); Dheenadhayalan et al. (2002)
	Uncharacterized protein	MAP0586c	33,137	Q743J9					Leroy et al. (2007)
	Uncharacterized protein	MAP2677c	14,559	Q73WI3					Leroy et al. (2007)
	Uncharacterized protein	MAP1087	15,436	Q741K3					Bannantine et al. (2008)
	Uncharacterized protein	MAP2121c	33,671	I3NID5					Bannantine et al. (2008)
	Uncharacterized protein	MAP1204	25,415	Q740Y8					Bannantine et al. (2008)
	Uncharacterized protein	MAP1506	39,695	Q73ZU5					Newton et al. (2009)
	Uncharacterized protein	MAP3817c	33,478	Q73TA1					Bannantine et al. (2008)

continued **Table 1.** Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

Antigen	Locus (ENA)	Size	Uniprot	GO Biological process	GO Cellular compartment	GO Molecular function	Reference
Uncharacterized protein	MAP3420c	38,738	Q73UE8				Newton et al. (2009)
Hypothetical protein	MAP3155c						Bannantine et al. (2008)
Uncharacterized protein	MAP0864	14,304	Q742H1				Leroy et al. (2009)
Uncharacterized protein	MAP2685	21,337	Q73WH5				Hughes et al. (2008)
Uncharacterized protein	MAP1564c	23,146	Q73ZN7				Hughes et al. (2008)
Uncharacterized protein	MAP3627	23,016	Q73TU1			O-methyltransferase activity	Hughes et al. (2008)
Uncharacterized protein	MAP0268c	23,953	R4N2J7			S-adenosylmethionine-dependent methyltransferase activity	Hughes et al. (2008)
Uncharacterized protein	MAP3491	28,293	Q73U77			hydrolase activity	Hughes et al. (2008)
Uncharacterized protein	MAP0860c	32,285	Q742H5				Bannantine et al. (2008); Paustian et al. (2004)
Uncharacterized protein	MAP0862	39,695	Q742H3				Paustian et al. (2004); Bannantine et al. (2008)
Uncharacterized protein	MAP1730c	35,859	Q73Z73				Bannantine et al. (2008)
Uncharacterized protein	MAP2154c	20,747	Q73Y04				Paustian et al. (2004)
Uncharacterized protein	MAP3732c	24,683	Q73TI6				Paustian et al. (2004)
Uncharacterized protein	MAP0471	28,432	Q743X0				Facciuolo et al. (2013)
Uncharacterized protein	MAP1981c	27,294	Q73YH5				Facciuolo et al. (2013)
Uncharacterized protein	MAP0196c	46,636	Q745I9				Facciuolo et al. (2013)
Uncharacterized protein	MAP 0038	48,749	Q744U0				Mon et al. (2012)
PirG	MAP 0210c	30,705	Q745J7				Mon et al. (2012)
Lpp34	MAP1473c	19,861	Q73ZX8				Gioffre et al. (2006)

continued **Table 1.** Table resuming all protein epitopes up to the date both according with Mikkelsen and colleagues [29] and from other more recent literature. All references are on the right column, only first author has been reported, whole reference is present in the references section [4, 30-57].

Antigen	Locus (ENA)	Size	Uniprot	GO Biological process	GO Cellular compartment	GO Molecular function	Reference
Pra Para-LP-01	MAP1025	25,259	Q741R2				Bannantine et al. (2011) (Thirunavukkarasu, Plain et al. 2013)
Uncharacterized protein	MAP_3733c	22,653	Q73TI5				(Cossu, Rosu et al. 2011)
Uncharacterized protein	MAP_3738c	26,908	Q73TI0				(Cossu, Rosu et al. 2011)
Uncharacterized protein	MAP3555	18,898	Q73U13				(Kawaji, Gumber et al. 2012)
35 kDa		33,671	Q9RAJ4				Shin et al. (2004)
Hsp18_3	MAP3268	16,433	Q73UU8				Gurung, 2013
Hsp	MAP_3701c	16,303	Q73TL7				Gurung, 2013
Uncharacterized protein	MAP_0184c	24,703	Q745H9				Gurung, 2013
Uncharacterized protein	MAP3864	16,413	Q73T55				Gurung, 2013
Uncharacterized protein	MAP1586	17,010	Q73ZL6				Gurung, 2013
Uncharacterized protein	MAP0540	17,618	Q743Q1				Gurung, 2013
Uncharacterized protein	MAP1560	15,298	Q73ZP1				Gurung, 2013
Uncharacterized protein	MAP1885c	18,441	Q73YS0				Gurung, 2013
Uncharacterized protein	MAP_1386c	27,654	Q740G5				Piras et al. (2014)
Uncharacterized protein	MAP2705c	13,983	Q73WF5				Gurung, 2013

withdrawal. However, it is not always easy to find the right epitopes to get reliable antibody response that well reflects the disease status.

In this field a great progress has been made through immunoproteomics. One of the most powerful approaches is characterized by 2D electrophoresis approach followed by immunoblotting and MS (serological proteome analysis, SERPA). This approach allows the detection and the identification of specific immunoreactive epitopes and proteins and represents one of the most used [58].

Among gel-free approaches, there are many methods to discover and identify immunogenic proteins. Protein array is probably the most common approach and is characterized by the fractionation and immobilization of antigens according to their features such as hydrophobicity or pI [59-61]. The advantage of this technique is that, in most cases, the protein structure remains intact and antibodies can even bind nonlinear epitopes. On the contrary, the immunocapture-MS approach requires the immobilization of antibodies that will bind antigens that afterwards can be detected through mass spectrometry technique [58, 62-64].

Once immunoreactive protein has been detected, it is important to go on with epitope mapping through the synthesis of libraries of immunoreactive peptides. To avoid the synthesis of peptides that have no possibility to be immunoreactive, several bioinformatics tools have been developed.

As previously stated, two types of epitopes exist, the continuous and the discontinuous. The continuous ones are constituted by peptides in their linear sequence and are easier to prepare because the epitope formation is not dependent on the protein folding. However, there are many regions of a protein that have no chances to become a B-cell epitope (i.e. the trans-membrane regions). For this reason, in order to avoid the random synthesis and screening of peptides to be tested, some key bioinformatics tools have been proposed to suggest the best sequences for the synthesis [65].

Several studies have already successfully detected immunoreactive B-cell epitopes using computational approaches and some of them are described here [66-68]. CBTOPE (http://www.imtech.res.in/raghava/cbtope/) is an algorithm from Ansari and colleagues for the prediction of B-cell immunoreactive epitopes starting from a protein FASTA sequence [1]. There are many algorithms that can be used for this purpose as Lbtope (http://crdd.osdd.net/raghava/lbtope/) [69], BEST [70] and many others [71-75].

EpiC (http://bioware.ucd.ie/epic/) [76] and IEDB Analysis Resource (http://tools.immuneepitope.org/bcell/) [77] also represent complete tools for epitope analysis and require as input a fasta sequence or uniprot accession number.

Another problem to overcome before accurately choosing the peptide to synthesize is related to the conserved sequences of epitopes. It is always better not to choose conserved sequences in order to avoid cross-reactivity problems. In order to overcome these problems it is really important to use the tool made by Marchler-Bauer and colleagues (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) that is useful for the search of conserved domains [78].

6 Future perspectives

Increasing resistance to antibiotics has led to a necessary improvement of side strategies to overcome infections such as early diagnosis or vaccination. Genomics, proteomics, immunoproteomics and peptidomics are able to provide necessary information in order to improve research and outcome on this field. For what it concern bovine *paratuberculosis*, several progresses have been made both from bench and in silico approach. In this review it has been provided the latest summary of experimental evidences of the proteins involved in the stimulation of humoral immunity against MAP (Table 1). All listed information, together with bioinformatics approaches described in paragraph 6, are able to provide the bases for future studies starting from the synthesis of immunoreactive antigens (peptides) to be screened as putative vaccines or as diagnostic tools. This approach could be applied in the research field of many infectious diseases.

Despite the several advances in the study and in the prediction of linear epitopes for diagnosis and vaccines, a lot of work still has to be done, in particular about the topic of post-translational modifications. Indeed, the antibody-antigen interaction could be due not only to a linear motif, but to the glycans attached to amminoacid residues.

In the light of future perspective, a promised application in the discovery of vaccine candidates, is represented by the study of post translational modification as reported by Facciuolo and Mutharia. In this work, they focus attention on glycosylation as targeted candidate for both diagnosis and vaccine [79].

Glycans often represent the first host-pathogen contact and in particular O-antigen heterogeneity is often used for diagnostic purposes in other bacterial species and serotypes [80].

About this topic it would be relevant to test the described antigens both before and after protein

deglycosylation in order to evaluate the putative role of glycans in a specific protein immunoreaction. However, there are several novel tools that are able to predict where a protein glycosylation may occur and could be successfully used in the epitopes prediction process. One of the future perspective is represented by the development of novel methods for the characterization and the study of bacterial glycosylation process and glycan structures. Some databases able to help for this task are already present. However, even if the structure has been characterized, their putative synthesis remains a difficult task to be feasible in order to cast diagnostic tools. For this reasons, to date, it remains a better choice to focus efforts on the prediction and on the synthesis of peptides which are easier to sequence and synthesise.

References

[1] Ansari, H. R., Raghava, G. P., Identification of conformational B-cell Epitopes in an antigen from its primary sequence. *Immunome research* 2010, *6*, 6.

[2] Purcell, A. W., McCluskey, J., Rossjohn, J., More than one reason to rethink the use of peptides in vaccine design. *Nature Reviews Drug Discovery* 2007, *6*, 404-414.

[3] Carter, J. M., Loomis-Price, L., B cell epitope mapping using synthetic peptides. *Current Protocols in Immunology* 2004, 9.4. 1-9.4. 23.

[4] Piras, C., Soggiu, A., Bonizzi, L., Greco, V., *et al.*, Identification of immunoreactive proteins of *Mycobacterium avium* subsp. *paratuberculosis*. *PROTEOMICS* 2014, n/a-n/a.

[5] Sousa, R., Osório, H., Duque, L., Ribeiro, H., *et al.*, Identification of Plantago lanceolata Pollen Allergens Using an Immunoproteomic Approach. *J Investig Allergol Clin Immunol* 2014, *24*, 177-183.

[6] Castro-Borges, W., Dowle, A., Curwen, R. S., Thomas-Oates, J., Wilson, R. A., Enzymatic shaving of the tegument surface of live schistosomes for proteomic analysis: a rational approach to select vaccine candidates. *PLoS neglected tropical diseases* 2011, *5*, e993.

[7] Turk, N., Milas, Z., Mojcec, V., Ruzic-Sabljic, E., *et al.*, Molecular analysis of Leptospira spp. isolated from humans by restriction fragment length polymorphism, real-time PCR and pulsed-field gel electrophoresis. *FEMS microbiology letters* 2009, *300*, 174-179.

[8] Gilchrist, M. J., Greko, C., Wallinga, D. B., Beran, G. W., *et al.*, The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environmental health perspectives* 2007, *115*, 313.

[9] Turk, R., Piras, C., Kovačić, M., Samardžija, M., *et al.*, Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis. *Journal of proteomics* 2012, *75*, 4412-4428.

[10] Hermon-Taylor, J., *Mycobacterium avium* subspecies *paratuberculosis*, Crohn's disease and the Doomsday scenario. *Gut pathogens* 2009, *1*, 1-6.

[11] Manning, E., Collins, M., *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. *Revue scientifique et technique (International Office of Epizootics)* 2001, *20*, 133.

[12] Behr, M. A., Collins, D. M., *Paratuberculosis: organism, disease, control*, CABI 2010.

[13] Piras, C., Soggiu, A., Greco, V., Cassinotti, A., *et al.*, Serum protein profiling of early and advanced stage Crohn's disease. *EuPA Open Proteomics* 2014, *3*, 48-59.

[14] Cucino, C., Sonnenberg, A., Occupational mortality from inflammatory bowel disease in the United States 1991–1996. *The American journal of gastroenterology* 2001, *96*, 1101-1105.

[15] Hermon-Taylor, J., El-Zaatari, F., The *Mycobacterium avium* subspecies *paratuberculosis* (MAP) problem, and its relation to the causation of Crohn's disease. *Pathogenic Mycobacteria in Water* 2004.

[16] Fridriksdottir, V., Gunnarsson, E., Sigurdarson, S., Gudmundsdottir, K., *Paratuberculosis* in Iceland: epidemiology and control measures, past and present. *Veterinary microbiology* 2000, *77*, 263-267.

[17] Naser, S. A., Ghobrial, G., Romero, C., Valentine, J. F., Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. The Lancet

[18] Bentley, R. W., Keenan, J. I., Gearry, R. B., Kennedy, M. A., *et al.*, Incidence of *Mycobacterium avium* subspecies *paratuberculosis* in a population-based cohort of patients with Crohn's disease and control subjects. *The American journal of gastroenterology* 2008, *103*, 1168-1172.

[19] Kirkwood, C. D., Wagner, J., Boniface, K., Vaughan, J., *et al.*, *Mycobacterium avium* subspecies *paratuberculosis* in children with early-onset Crohn's disease. *Inflammatory bowel diseases* 2009, *15*, 1643-1655.

[20] Feller, M., Huwiler, K., Stephan, R., Altpeter, E., *et al.*, *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. *The Lancet infectious diseases* 2007, *7*, 607-613.

[21] Nagabhushanam, V., Praszkier, J., Cheers, C., Molecular and immunological characterization of *Mycobacterium avium* 65 kDa heat shock protein (Hsp65). *Immunology and cell biology* 2001, *79*, 454-461.

[22] Dow, C. T., M. *paratuberculosis* Heat Shock Protein 65 and Human Diseases: Bridging Infection and Autoimmunity. *Autoimmune Diseases* 2012, *2012*.

[23] Agrawal, G., Borody, T. J., Chamberlin, W., 'Global warming'to *Mycobacterium avium* subspecies *paratuberculosis*. *Future microbiology* 2014, *9*, 829-832.

[24] Collins, M. T., Diagnosis of *paratuberculosis*. *The Veterinary clinics of North America. Food animal practice* 1996, *12*, 357-371.

[25] Whitlock, R., Wells, S., Sweeney, R. W., Van Tiem, J., ELISA and fecal culture for *paratuberculosis* (Johne's disease): sensitivity and specificity of each method. *Veterinary microbiology* 2000, *77*, 387-398.

[26] Kalis, C., Collins, M., Hesselink, J., Barkema, H., Specificity of two tests for the early diagnosis of bovine *paratuberculosis* based on cell-mediated immunity: the Johnin skin test and the gamma interferon assay. *Veterinary microbiology* 2003, *97*, 73-86.

[27] Lavers, C. J., Barkema, H. W., Dohoo, I. R., McKenna, S. L. B., Keefe, G. P., Evaluation of milk ELISA fordetection of

Mycobacterium avium subspecies *paratuberculosis* indairy herds and association with within-herd prevalence. *Journal of Dairy Science* 2014, *97*, 299-309.

[28] He, Z., De Buck, J., Localization of proteins in the cell wall of *Mycobacterium avium* subsp. *paratuberculosis* K10 by proteomic analysis. *Proteome Sci* 2010, *8*, 21.

[29] Mikkelsen, H., Aagaard, C., Nielsen, S. S., Jungersen, G., Review of *Mycobacterium avium* subsp. *paratuberculosis* antigen candidates with diagnostic potential. *Veterinary microbiology* 2011, *152*, 1-20.

[30] Willemsen, P. T., Westerveen, J., Dinkla, A., Bakker, D., *et al.*, Secreted antigens of *Mycobacterium avium* subspecies *paratuberculosis* as prominent immune targets. *Veterinary microbiology* 2006, *114*, 337-344.

[31] Gumber, S., Taylor, D. L., Whittington, R. J., Evaluation of the immunogenicity of recombinant stress-associated proteins during *Mycobacterium avium* subsp. *paratuberculosis* infection: Implications for pathogenesis and diagnosis. *Veterinary microbiology* 2009, *137*, 290-296.

[32] Paustian, M. L., Amonsin, A., Kapur, V., Bannantine, J. P., Characterization of novel coding sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*: implications for diagnosis of Johne's disease. *Journal of clinical microbiology* 2004, *42*, 2675-2681.

[33] Cho, D., Sung, N., Collins, M. T., Identification of proteins of potential diagnostic value for bovine *paratuberculosis*. *Proteomics* 2006, *6*, 5785-5794.

[34] Cho, D., Collins, M. T., Comparison of the proteosomes and antigenicities of secreted and cellular proteins produced by Mycobacterium *paratuberculosis*. *Clinical and vaccine immunology* 2006, *13*, 1155-1161.

[35] Cho, D., Shin, S.-J., Talaat, A. M., Collins, M. T., Cloning, expression, purification and serodiagnostic evaluation of fourteen *Mycobacterium paratuberculosis* proteins. *Protein expression and purification* 2007, *53*, 411-420.

[36] Liu, X., Feng, Z., Harris, N. B., Cirillo, J. D., *et al.*, Identification of a secreted superoxide dismutase in *Mycobacterium avium* ssp. *paratuberculosis*. *FEMS microbiology letters* 2001, *202*, 233-238.

[37] Li, L., Munir, S., Bannantine, J. P., Sreevatsan, S., *et al.*, Rapid expression of *Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins for antigen discovery. *Clinical and Vaccine Immunology* 2007, *14*, 102-105.

[38] Dheenadhayalan, V., Shin, K.-S., Chang, C.-F., Chang, C.-D., *et al.*, Cloning and characterization of the genes coding for antigen 85A, 85B and 85C of *Mycobacterium avium* subsp. *paratuberculosis*. *Mitochondrial DNA* 2002, *13*, 287-294.

[39] Shin, S. J., McDonough, S. P., Original Articles: Comparative antibody response of five recombinant antigens in related to bacterial shedding levels and development of serological diagnosis based on 35 kDa antigen for *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of veterinary science* 2004, *5*, 111-117.

[40] Bannantine, J. P., Bayles, D. O., Waters, W. R., Palmer, M. V., *et al.*, Early antibody response against *Mycobacterium avium* subspecies *paratuberculosis* antigens in subclinical cattle. *Proteome Sci* 2008, *6*.

[41] Bannantine, J. P., Paustian, M. L., Waters, W. R., Stabel, J. R., *et al.*, Profiling bovine antibody responses to *Mycobacterium avium* subsp. *paratuberculosis* infection by using protein arrays. *Infection and immunity* 2008, *76*, 739-749.

[42] Bannantine, J. P., Rosu, V., Zanetti, S., Rocca, S., *et al.*, Antigenic profiles of recombinant proteins from *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne's disease. *Veterinary immunology and immunopathology* 2008, *122*, 116-125.

[43] Bannantine, J. P., Waters, W., Stabel, J. R., Palmer, M. V., *et al.*, Development and use of a partial *Mycobacterium avium* subspecies *paratuberculosis* protein array. *Proteomics* 2008, *8*, 463-474.

[44] Newton, V., McKenna, S. L., De Buck, J., Presence of PPE proteins in *Mycobacterium avium* subsp. *paratuberculosis* isolates and their immunogenicity in cattle. *Veterinary microbiology* 2009, *135*, 394-400.

[45] Cobb, A. J., Frothingham, R., The GroES antigens of *Mycobacterium avium* and *Mycobacterium paratuberculosis*. *Veterinary microbiology* 1999, *67*, 31-35.

[46] Hughes, V., Bannantine, J. P., Denham, S., Smith, S., *et al.*, Immunogenicity of proteome-determined *Mycobacterium avium* subsp. *paratuberculosis*-specific proteins in sheep with *paratuberculosis*. *Clinical and vaccine immunology* 2008, *15*, 1824-1833.

[47] Bannantine, J. P., Radosevich, T. J., Stabel, J. R., Sreevatsan, S., *et al.*, Development and characterization of monoclonal antibodies and aptamers against major antigens of *Mycobacterium avium* subsp. *paratuberculosis*. *Clinical and Vaccine Immunology* 2007, *14*, 518-526.

[48] Leroy, B., Viart, S., Trinchero, N., Roupie, V., *et al.*, Use of *Mycobacterium avium* subsp. *paratuberculosis* specific coding sequences for serodiagnosis of bovine *paratuberculosis*. *Veterinary microbiology* 2009, *135*, 313-319.

[49] Facciuolo, A., Kelton, D. F., Mutharia, L. M., Novel secreted antigens of Mycobacterium *paratuberculosis* as serodiagnostic biomarkers for Johne's disease in cattle. *Clinical and Vaccine Immunology* 2013, *20*, 1783-1791.

[50] Gurung, R., Purdie, A., Whittington, R., Begg, D., Cellular and humoral immune responses in sheep vaccinated with candidate antigens MAP2698c and MAP3567 from *Mycobacterium avium* subspecies *paratuberculosis*. *Name: Frontiers in Cellular and Infection Microbiology* 2014, *4*, 93.

[51] Mon, M., #237, Laura, a., Viale, M., *et al.*, Search for *Mycobacterium avium* Subspecies *paratuberculosis* Antigens for the Diagnosis of *Paratuberculosis*. *Veterinary Medicine International* 2012, *2012*, 9.

[52] Gioffré, A., Caimi, K., Zumárraga, M. J., Meikle, V., *et al.*, Lpp34, a novel putative lipoprotein from *Mycobacterium avium* subsp. *paratuberculosis*. *Journal of Veterinary Medicine, Series B* 2006, *53*, 34-41.

[53] Wu, C.-w., Schmoller, S. K., Bannantine, J. P., Eckstein, T. M., *et al.*, A novel cell wall lipopeptide is important for biofilm formation and pathogenicity of *Mycobacterium avium* subspecies *paratuberculosis*. *Microbial pathogenesis* 2009, *46*, 222-230.

[54] Bannantine, J. P., Stabel, J. R., Lamont, E. A., Briggs, R. E., Sreevatsan, S., Monoclonal antibodies bind a SNP-sensitive epitope that is present uniquely in *Mycobacterium avium* subspecies *paratuberculosis*. *Frontiers in microbiology* 2011, *2*.

[55] Gurung, R. B., Begg, D. J., Purdie, A. C., Bach, H., Whittington, R. J., Immunoreactivity of protein tyrosine phosphatase A

(PtpA) in sera from sheep infected with *Mycobacterium avium* subspecies *paratuberculosis*. *Veterinary immunology and immunopathology* 2014.

[56] Roupie, V., Viart, S., Leroy, B., Romano, M., *et al.*, Immunogenicity of eight *Mycobacterium avium* subsp. *paratuberculosis* specific antigens in DNA vaccinated and Map infected mice. *Veterinary Immunology and Immunopathology* 2012, *145*, 74-85.

[57] Gurung, R. B., Begg, D. J., Purdie, A. C., Bannantine, J. P., Whittington, R. J., Antigenicity of Recombinant Maltose Binding Protein-*Mycobacterium avium* subsp. *paratuberculosis* Fusion Proteins with and without Factor Xa Cleaving. *Clinical and Vaccine Immunology* 2013, *20*, 1817-1826.

[58] Tjalsma, H., Schaeps, R. M., Swinkels, D. W., Immunoproteomics: From biomarker discovery to diagnostic applications. *PROTEOMICS-Clinical Applications* 2008, *2*, 167-180.

[59] Robinson, W. H., Antigen arrays for antibody profiling. *Current opinion in chemical biology* 2006, *10*, 67-72.

[60] Hueber, W., Robinson, W. H., Proteomic biomarkers for autoimmune disease. *Proteomics* 2006, *6*, 4100-4105.

[61] Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B., *et al.*, Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nature medicine* 2002, *8*, 295-301.

[62] Pan, C., Xu, S., Zhou, H., Fu, Y., *et al.*, Recent developments in methods and technology for analysis of biological samples by MALDI-TOF-MS. *Analytical and bioanalytical chemistry* 2007, *387*, 193-204.

[63] Villar-Garea, A., Griese, M., Imhof, A., Biomarker discovery from body fluids using mass spectrometry. *Journal of Chromatography B* 2007, *849*, 105-114.

[64] Philip, R., Murthy, S., Krakover, J., Sinnathamby, G., *et al.*, Shared immunoproteome for ovarian cancer diagnostics and immunotherapy: potential theranostic approach to cancer. *Journal of proteome research* 2007, *6*, 2509-2517.

[65] Carmona, S. J., Sartor, P. A., Leguizamón, M. S., Campetella, O. E., Agüero, F., Diagnostic peptide discovery: prioritization of pathogen diagnostic markers using multiple features. *PloS one* 2012, *7*, e50748.

[66] Goto, Y., Carter, D., Reed, S. G., Immunological dominance of Trypanosoma cruzi tandem repeat proteins. *Infection and immunity* 2008, *76*, 3967-3974.

[67] Cooley, G., Etheridge, R. D., Boehlke, C., Bundy, B., *et al.*, High throughput selection of effective serodiagnostics for Trypanosoma cruzi infection. *PLoS neglected tropical diseases* 2008, *2*, e316.

[68] List, C., Qi, W., Maag, E., Gottstein, B., *et al.*, Serodiagnosis of Echinococcus spp. infection: explorative selection of diagnostic antigens by peptide microarray. *PLoS neglected tropical diseases* 2010, *4*, e771.

[69] Singh, H., Ansari, H. R., Raghava, G. P., Improved method for linear B-cell epitope prediction using Antigen's primary sequence. *PloS one* 2013, *8*, e62216.

[70] Gao, J., Faraggi, E., Zhou, Y., Ruan, J., Kurgan, L., BEST: improved prediction of B-cell epitopes from antigen sequences. *PloS one* 2012, *7*, e40104.

[71] Odorico, M., Pellequer, J. L., BEPITOPE: predicting the location of continuous epitopes and patterns in proteins. *Journal of Molecular Recognition* 2003, *16*, 20-22.

[72] Rubinstein, N. D., Mayrose, I., Martz, E., Pupko, T., Epitopia: a web-server for predicting B-cell epitopes. *BMC bioinformatics* 2009, *10*, 287.

[73] Saha, S., Bhasin, M., Raghava, G. P., Bcipep: a database of B-cell epitopes. *BMC genomics* 2005, *6*, 79.

[74] Sweredoski, M. J., Baldi, P., PEPITO: improved discontinuous B-cell epitope prediction using multiple distance thresholds and half sphere exposure. *Bioinformatics* 2008, *24*, 1459-1460.

[75] Liang, S., Zheng, D., Standley, D. M., Yao, B., *et al.*, EPSVR and EPMeta: prediction of antigenic epitopes using support vector regression and multiple server results. *BMC bioinformatics* 2010, *11*, 381.

[76] Haslam, N. J., Gibson, T. J., EpiC: an open resource for exploring epitopes to aid antibody-based experiments. *Journal of proteome research* 2010, *9*, 3759-3763.

[77] Vita, R., Zarebski, L., Greenbaum, J. A., Emami, H., *et al.*, The immune epitope database 2.0. *Nucleic acids research* 2009, gkp1004.

[78] Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., *et al.*, CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic acids research* 2011, *39*, D225-D229.

[79] Facciuolo, A., Mutharia, L. M., Mycobacterial glycoproteins: a novel subset of vaccine candidates. *Frontiers in cellular and infection microbiology* 2014, *4*.

[80] Comstock, L. E., Kasper, D. L., Bacterial glycans: key mediators of diverse host immune responses. *Cell* 2006, *126*, 847-850.