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
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 and 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

![Peptidomics workflow for detection of antigenic peptides.](image)
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
| 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) (Kawai, 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 | FMN binding, carboxylase activity, oxidoreductase activity | Leroy et al. (2009) |
| MoaA3            | MAP3932c    | 41,553  | Q73ZS0    | Metabolism            | Mo-molybdoeprotein 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            | Metabolism              | hydrolytic activity     | Mon et al. (2012)                |
| Enoyl-CoA hydratase | MAP_1197   | 28,761  | Q740Z5    | Metabolism            | Metabolism              | catalytic activity      | (Nagata, Kawai 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 | MAP2650c | 13,124  | Q73X60    | Metabolism            | plasma membrane ATP synthesis coupled proton transport | ATP binding, proton-transporting ATP synthase activity, rotational mechanism | Gurung, 2013 |
| EchA20           | MAP0516c    | 26,847  | Q740S5    | Metabolism            | Metabolism              | catalytic activity      | Gurung, 2013 |
| EchA8-1          | MAP1017c    | 27,884  | Q74150    | Metabolism            | Metabolism              | catalytic activity      | Gurung, 2013 |
| FadE3-2          | MAP3651c    | 44,051  | Q73TR7    | Metabolism            | Metabolism              | acyl-CoA dehydrogenase activity, flavin adenine dinucleotide binding | Gurung, 2013 |
| Uncharacterized protein | MAP2687c   | 17,900  | Q73X23    | Metabolism            | Metabolism              | carbonate dehydratase activity, zinc ion binding, catalytic activity, phosphopantetheine binding | Gurung, 2013 |
| pstA             | MAP1242     | 430,487 | Q740V0    | Metabolism            | Metabolism              | L-malate dehydrogenase activity | Wu et al. (2009) |
| Malate dehydrogenase | MAP_2541c  | 34,631  | P61976    | Metabolism            | Metabolism              | cellular carbohydrate metabolic process, tricarboxylic acid cycle | Piras et al. (2014) |
| FixA             | MAP_3061c   | 27,847  | Q73VF3    | Metabolism            | Metabolism              | electron carrier activity | Piras et al. (2014) |

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].
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| 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 stresscell redox homeostasis response | Oxidoreductase activity | Willemsen et al. (2006) |
| Uncharacterized protein | MAP2411 15,538 | Q73X98  | Oxidative stress oxidation-reduction response process | FMN binding | Gumber et al. (2009) (Kawai, Gumber et al. 2012) |
| Peroxide dismutase [Mn] | MAP0187c 23,030 | P53647  | Oxidative stress superoxide metabolic response 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 | Hughes et al. (2008) activity |
| Hsp65 | MAP_3936 56,643 | P42384  | Stress response protein refolding | Chaperone | Piras et al. (2014) |
| Chaperone 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  | Stress response protein folding, response to stress | ATP binding | Langelaar et al. (2002) |
| Uncharacterized protein | MAP1339 15,437 | Q740L1  | Stress response | oxidoreductase activity | Gurung, 2013 |
| Alkyl hydroperoxide reductase AhpD | MAP1588c 18,842 | Q73ZL4  | Stress response | oxidoreductase activity | Gurung, 2013 |
| AhpC | MAP1589c 21,641 | Q73ZL3  | Stress response | Antioxidant activity, oxidoreductase activity | Gurung, 2013 |
| Hsp65K | MAP_3936 56,643 | P42384  | Stress response | cytoplasm ATP binding | ElZaatari et al. (1995) |
| Hsp70 | MAP_3840 66,518 | Q00488  | Stress response | ATP binding | Langelaar et al. (2002) |
| Uncharacterized protein | MAP1339 15,437 | Q740L1  | Stress response | oxidoreductase activity | Gurung, 2013 |
| Alkyl hydroperoxide reductase AhpD | MAP1588c 18,842 | Q73ZL4  | Stress response | oxidoreductase activity | Gurung, 2013 |
| AhpC | MAP1589c 21,641 | Q73ZL3  | Stress response | Antioxidant activity, oxidoreductase activity | Gurung, 2013 |
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       | 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 phosphoribosyltransferase | MAP3857       | 18,871  | Q73T62          | DNA metabolism                                           | ‘de novo’ UMP biosynthetic process | Hughes et al. (2008) |
| Transcription elongation factor GreA | MAP1027c      | 17,924  | Q741R0          | DNA metabolism                                           | Transcription, DNA binding F | Gumber et al. (2009) |
| Uncharacterized protein         | MAP2963c        | 97,054  | Q73VQ0          | DNA metabolism                                           | Transcription, DNA binding F | 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 | Gurung, 2013       |
| Single-stranded DNA-binding protein | MAP0068       | 17,590  | Q744V5          | DNA metabolism                                           | DNA replication               | Gurung, 2013       |
| N5-carboxyaminoimidazole ribonucleotide mutase | MAP3393c      | 17,608  | Q73UH5          | DNA metabolism                                           | ‘de novo’ IMP biosynthetic process | Gurung, 2013       |

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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        | P60805  | DNA metabolism        | cytoplasm               | ATP binding, ATP phosphoribosyltransferase activity, magnesium ion binding               | Gurung, 2013                                   |
| Uncharacterized protein       | MAP 0834c       | Q742K1  | Transcription process | DNA binding, DNA-templated | DNA binding, phosphorelay response regulator activity                                  | Gurung, 2013                                   |
| 50S ribosomal protein L10     | MAP4125         | Q735E9  | Translation, ribosome biogenesis | ribosome                | LSU rRNA binding, structural cons-Gurung, 2013                                        |                                               |
| Proteins with structural function |                 |         |                       |                         |                                                                                       |                                               |
| modD                          | MAP1569         | I3NIE1  | Structural            | extracellular region    | extracellular matrix binding                                                           | Cho et al. (2006, 2007); Facciuolo et al. (2013) (Souza, Rodrigues et al. 2011) |
| Csp                           | MAP 0209c       | Q745J8  | Structural            | N-acetylmuramoyl-L-alanine amidase activity, zinc ion binding, heparin binding |                                                 | Mon et al. (2012)                             |
| Uncharacterized protein        | MAP3968         | Q735V5  | Structural/Pathogenesis | cell surface            |                                                                                       | Gurung, 2013 Bannantine et al. (2008)         |
| Protein metabolism and folding |                 |         |                       |                         |                                                                                       |                                               |
| PepA                          | MAP3527         | I3NID9  | Protein metabolism   | cytoplasm               | translation release factor activity, codon specific                                     | Hughes et al. (2008)                          |
| ATP-dependent Clp protease    | MAP2281c        | Q73XM8  | Protein metabolism   | cytoplasm               | serine-type endopeptidase activity                                                     | Cho et al. (2006, 2007); Gumber et al. (2009) |
| Peptidyl-prolyl cis-trans isomerase A | MAP1693c   | Q73ZB0  | Protein folding       | proteolysis              | serine-type endopeptidase activity                                                     |                                               |
| Phosphoribosyl isomerase A    | MAP1297         | P60583  | Protein metabolism   | cytoplasm               | peptidyl-prolyl cis-trans isomerase activity                                            | Leroy et al. (2007)                           |
| 4-hydroxy-tetrahydrodipicolinate reductase | MAP2878c | Q73Y3    | Protein metabolism   | cytoplasm               | 1-(5-phosphoribosyl)-5-[(5-phosphoribosylaminomethylidene neamine-4-carboxamide isomerase activity, phosphoribosylanthranilate isomerase activity, NAD binding, 4-hydroxy-tetrahydrodipicolinate reductase, oxidoreductase activity, acting on CH or CH2 groups, NAD or NADP as acceptor | Hughes et al. (2008)                           |
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                                                                                                                                                                                                       | cytoplasm                | amino acid binding                                                                         | Hughes et al. (2008)      |
| MetC                          | MAP3657     | 47,748 | Q73UB1  | Protein metabolism                                                                                                                                                                                                       | cytoplasm                | 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                                                                                                                                                                                                       | cytoplasm                | histidine biosynthetic process, protein folding                                             | Hughes et al. (2008)      |
| Peptidyl-prolyl cis-trans isomerase | MAP 1693    | 18,330 | Q73ZB0  | Protein metabolism                                                                                                                                                                                                       | cytoplasm                | threonine-type endopeptidase activity                                                       | Gurung, 2013              |
| Proteasome subunit             | MAP1834c    | 28,078 | Q73YW9  | Protein metabolism                                                                                                                                                                                                       | cytoplasm                | serine-type endopeptidase activity                                                         | Gurung, 2013              |
| ATP-dependent Clp protease     | MAP2280c    | 23,292 | Q73XM9  | Protein metabolism                                                                                                                                                                                                       | cytoplasm                | protein dephosphorylation                                                                  | Gurung, 2013              |
| 4-hydroxy-tetrahydrodipicolinate synthase | MAP2864c   | 30,973 | Q73VZ7  | Protein metabolism                                                                                                                                                                                                       | cytoplasm                | 4-hydroxy-tetrahydrodipicolinate synthase, amine-lyase activity                           | Gurung, 2013              |
| Phosphate metabolism           | Inorganic pyrophosphatase-MAP0435c | 18,615 | Q744A6  | Phosphate metabolism                                                                                                                                                                                                     | 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 | phosphatidylycholine metabolism | 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) | Gurung, 2013 |
| Wag31                          | MAP1889c    | 28,050 | Q73YR6  | Cell cycle | cell cycle, cell division | cytoplasm | |

Note: The table continues with more entries not shown here for brevity.
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                    |                         | phosporelay 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  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP3199     | 19,697  | Q73V17  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP1272c    | 33,436  | Q740S0  |                                 |                         |                                                           |                                 |
| PiiG                  | MAP0210c    | 30,705  | Q745J7  |                                 |                         |                                                           |                                 |
| FbpC2                 | MAP3531c    | 37,769  | Q73U37  |                                 |                         |                                                           |                                 |
| FbpA                  | MAP0216     | 36,086  | I3NIE0  |                                 |                         |                                                           |                                 |
| FbpB                  | MAP1609c    | 34,707  | Q73ZJ4  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP0586c   | 33,137  | Q743J9  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP2677c   | 14,559  | Q73W13  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP1087    | 15,436  | Q741K3  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP2121c   | 33,671  | I3NID5  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP1204    | 25,415  | Q740Y8  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP1506    | 39,695  | Q73ZU5  |                                 |                         |                                                           |                                 |
| Uncharacterized protein | MAP3817c   | 33,478  | Q73TA1  |                                 |                         |                                                           |                                 |
Tab. 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  | 0-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  | 0-methyltransferase 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 | Q73T16  |                        |                         | Paustian et al. (2004)            |
| Uncharacterized protein | MAP0471      | 28,432 | Q743X0  |                        |                         | Facciulo et al. (2013)           |
| Uncharacterized protein | MAP1981c     | 27,294 | Q73YH5  |                        |                         | Facciulo et al. (2013)           |
| Uncharacterized protein | MAP0196c     | 46,636 | Q74519  |                        |                         | Facciulo et al. (2013)           |
| Uncharacterized protein | MAP 0038     | 48,749 | Q744U0  |                        |                         | Mon et al. (2012)                |
| PirG         | MAP 0210c    | 30,705 | Q74517  |                        |                         | Mon et al. (2012)                |
| Lpp34        | MAP1473c     | 19,861 | Q73ZX8  |                        |                         | Gioffre et al. (2006)            |
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         | MAP1025 25,259   | Q741R2  |                       |                         |                       | Bannantine et al. (2011)                          |
| Para-LP-01  |                  |         |                       |                         |                       | (Thirunavukkarasu, Plain et al. 2013)            |
| Uncharacterized protein | MAP_3733c 22,653 | Q73T15  |                       |                         |                       | (Cossu, Rosu et al. 2011)                        |
| Uncharacterized protein | MAP_3738c 26,908 | Q73T10  |                       |                         |                       | (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   | Q73U8   |                       |                         |                       | 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 pl [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 really is 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 aminoacid 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 perspectives 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.

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