DOI 10.1002/pmic.200600988 *Proteomics* 2007, *7,* 1164–1176

RESEARCH ARTICLE

Antigen discovery: A postgenomic approach to paratuberculosis diagnosis

Baptiste Leroy[1], Virginie Roupie[2], Isabelle Noël-Georis[1, 3], Valérie Rosseels[2], Karl Walravens[4], Marc Govaerts[4], Kris Huygen[2] and Ruddy Wattiez[1]

[1] Department of Proteomics and Protein Biochemistry, University of Mons-Hainaut, Mons, Belgium
[2] Laboratory of Mycobacterial Immunology, WIV-Pasteur Institute, Brussels, Belgium
[3] Institut de Recherches J.-M. Wiame (IRMW), Bruxelles, Belgium
[4] Department of Bacteriology and Immunology, Veterinary and Agrochemical Research Centre, Uccle, Belgium

Received: December 8, 2006
Accepted: December 30, 2006

Paratuberculosis is a chronic enteritis caused in domestic and wild ruminant species by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that is responsible for major economic losses to the agricultural industry. To date, no satisfactory therapeutic, vaccine, or diagnostic tools are available, globally impairing all control programs. In this study, we have undertaken a large-scale postgenomic analysis of MAP proteins, to identify specific antigens that could potentially improve the diagnosis of paratuberculosis. Two complementary approaches were implemented, the first one consisting in the systematic proteomic identification of proteins present in MAP culture filtrates (CFs), followed by the selection of MAP-specific proteins by BLAST query on available mycobacterial genomes. The resulting database represents the first established secretome of MAP and a useful source of potentially specific antigens. The second approach consisted in the immunoproteomic analysis of both MAP extracts and CFs, using sera from MAP-infected and uninfected cattle. Combining results obtained with both approaches resulted in the identification of 25 candidate diagnostic antigens. Five of these were tested in an ELISA assay for their diagnostic potential, on a limited panel of field sera, and the combination of three of them competed in performance with available commercial assays, reaching a test sensitivity of 94.74% and specificity of 97.92%.

Keywords:
Diagnostic antigens / ELISA / Immunoproteomics / *Mycobacterium avium* subsp. *paratuberculosis*

1 Introduction

Bovine paratuberculosis, also called Johne's disease, is a chronic granulomatous enteritis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Infected cattle develop diarrhea resulting in reduced milk production, severe emaciation, and substantial financial losses to the farming industry [1, 2]. Not only cattle are receptive, but also most of the other domestic and wild ruminant species [3], making Johne's disease a growing issue to be faced by the agricultural industry. To date, no effective therapeutic or vaccine agents are available, and early detection along with good management practices are the only ways to control paratuberculosis [1]. Unfortunately, control programs are hampered by the lack of simple and efficient diagnostic tests, especially for subclinically infected animals. Cultivation of bacteria excreted by animals at the clinical stage, for example, is highly specific, but only applicable to the latest stage of infection. Moreover, the slow growth of MAP translates into

Correspondence: Professor Ruddy Wattiez, Department of Proteomics and Protein Biochemistry. University of Mons-Hainaut, Av. du Champs de Mars, 6, B-7000 Mons, Belgium
E-mail: ruddy.wattiez@umh.ac.be
Fax: +32-65373320

Abbreviations: CE, cell extract; **CF,** culture filtrate; **IB,** immunoblot buffer; **MAP,** *Mycobacterium avium* subsp. *paratuberculosis*; **Se,** sensitivity; **Sp,** specificity

a 3-month waiting time to define the individual infection status. Detection of MAP in feces by PCR is not only rapid, but also applicable only to the late stage of infection, when animals start shedding bacteria. Serological and cell-mediated immunity (CMI)-based assays remain most promising, but so far specific immunodominant antigens are lacking [4]. Several antibody ELISA kits are commercially available, but most of them use crude cellular extracts and are based on preadsorption of the test sera on *Mycobacterium phlei*, to limit crossreactivity due to sensitization to the environmental mycobacteria. However, this preadsorption step is responsible for a considerable decrease in sensitivity (Se), particularly among low shedders [5, 6]. A recent longitudinal study [7] estimated the overall Se of current commercial diagnostic assays to be as low as 14%. Collins *et al.* [8] estimated that only one third of cattle infected with MAP and shedding bacteria in their feces could be detected by commercial antibody ELISAs. Moreover, it is not specified whether these tests are able to discriminate paratuberculosis from bovine tuberculosis caused by *M. bovis* [9].

Taken together, these shortcomings have led to an extensive effort by many laboratories to identify and characterize antigens able to increase the efficiency of Johne's disease immunodiagnosis [4, 9–19]. Several antigens with a diagnostic potential in antibody or IFN-γ ELISA assays have been characterized, but have not been included yet in an efficient diagnostic test [4].

Genomic screening of the fully sequenced MAP genome further allowed identification of prospectively MAP-specific immune targets [4, 10, 20]. Antigenicity of these proteins was evaluated by Western blot using sera from a small number of cattle naturally infected with MAP. Till date, a number of antigens have been identified using this genomic approach but data about their reactivity in ELISA using larger panels of sera are still lacking. Bannantine and coworkers [21] have further obtained interesting results with MAP surface antigens. Using a surface antigen-based ELISA (EVELISA), they were able to identify 98.4% of the fecal culture-positive animals with 100% specificity (Sp). However, the capacity of this ELISA to discriminate MAP- from *M. bovis*-infected animals was investigated on a very limited scale since the study only integrated three sera from animals experimentally infected by *M. bovis*.

The aim of the present study was to screen cell extract (CE) and culture filtrate (CF) of MAP in search for specific immunodominant antigenic proteins. In this context, we established the first secretome of MAP, allowing us to build a database of potential candidate antigens, *i.e.* proteins expressed in CFs and specific of MAP. In parallel, sera of paratuberculosis-infected cattle were used to screen the MAP CE and CF for immunodominant targets. The Sp of the antigens identified was evaluated using sera from *M. bovis*-infected cattle, and by sequence comparison with available mycobacterial genomes. Using these two approaches, we were able to establish a database of proteins with a potential for Johne's disease diagnosis. Five-specific MAP proteins were subsequently cloned in *Escherichia coli*, expressed and purified. The combination of three of these proteins resulted in a very promising, highly specific, and sensitive ELISA-based diagnostic test.

2 Materials and methods

2.1 Sample preparation

2.1.1 Cell extract

The MAP type strain ATCC19698 was grown as a surface pellicle at 39°C in mycobactin J-supplemented synthetic Sauton medium to stationary phase, as described previously [22]. Cells harvested by centrifugation were washed three times with 10 mM PBS, pH 7.2 and suspended 1:1 (v/v) in 40 mM Tris–HCl buffer, pH 7.2, 8 M urea, 4% w/v CHAPS. An equivalent volume of 106 μm glass beads was then added and the sample homogenized for 2 min in a Mini-beadbeater (Biospec Product, Bartlesville, USA). After one freeze/thaw cycle and a 2 min sonication, centrifugation at 18 000 × *g* was performed to recover the supernatant. The same procedure was repeated twice to yield a quantitative extraction.

2.1.2 Culture filtrates

MAP strain ATCC19698 was grown as a surface pellicle on mycobactin J-supplemented Sauton medium for 4 wk at 39°C. Culture filtrates were separated from bacteria by filtration and proteins were recovered by ammonium sulfate precipitation. Precipitate was extensively dialyzed against 10 mM PBS, pH 7.2.

Protein concentration in CFs and extracts was determined using the BioRad protein Assay kit (BioRad, USA).

2.2 MAP secretome analysis

The MAP CF secretome was analyzed by SDS-PAGE and by 2-DE, followed by the systematic identification of all CBB-stained protein bands/spots by MS.

For SDS-PAGE, CF samples were diluted in Laemmli sample buffer and 50 μg of proteins were electrophoretically separated on a 12% vertical acrylamide gel (Hoefer, Amersham Bioscience) at 250 V, 40 mA.

For 2-DE, MAP CF proteins were reprecipitated using TCA, and pellets were solubilized in a minimal volume of sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, and 50 mM DTT), and cleared by centrifugating at 18 000 × *g*. For the first dimension, 500 μg of proteins were subjected to IEF on IPG strips (pH 3–10; NL; 11 cm; Amersham Pharmacia Biotech, Sweden). The first-dimensional IEF was carried out, as previously described [23]. The second dimension vertical slab SDS-PAGE was run for 4 h at 30 mA/gel using the Criterion apparatus (BioRad) and precast gradient gels (10–20%).

The SDS-PAGE and 2-DE gels were stained with CBB R-250 (Amresco, Solon, OH, USA).

2.3 Protein identification by MS

Protein bands/spots were excised and submitted to trypsinolysis, as described previously [24]. Briefly, gel pieces were washed twice in 25 mM NH_3HCO_3 for 15 min under gentle agitation at room temperature, followed by two 15-min washes in 25 mM NH_3HCO_3, 50% v/v ACN. After speed vacuum dehydration, 10 μL of a 0.02 μg/μL trypsin solution in 25 mM NH_3HCO_3 (Promega Madison), was added and samples were incubated overnight at 37°C. Tryptic digestion was stopped by the addition of 1 μL of 5% v/v formic acid. For MALDI-TOF analysis, 1 μL of each sample was mixed with 1 μL of matrix (5 mg/mL CHCA and 0.5 pmol/μL rennin as an internal standard, in 25% v/v ethanol, 25% v/v ACN, 0.05% v/v TFA), then spotted onto a MALDI sample plate and allowed air drying. MALDI-TOF was performed using a M@ldi™ mass spectrometer (Micromass, Manchester, UK) equipped with a 337 nm nitrogen laser. The instrument was operated in the positive reflectron mode with 15 kV of source voltage, 2.5 kV of pulse voltage, and 2 kV of reflecting voltage. The resulting peptide masses were automatically searched for in a copy of the Release 7.0 of the UniProt Knowledgebase (February 06) composed of the UniProtKB/Swiss-Prot Protein Knowledgebase release 49.0 and the UniProtKB/TrEMBL Protein Database release 32.0 containing 2 812 716 sequence entries (UniProtKB/Swiss-Prot section: 207 132; UniProtKB/TrEMBL section: 2 605 584) using the ProteinLynx global server 2.0 (Micromass) and/or MASCOT server 2.1.0 (Matrix Science, London, UK) search engines. The research was carried out in all species. One missed cleavage *per* peptide was allowed, a mass tolerance of 100 ppm was used and some variable modifications were taken into account such as carbamidomethylation of cysteines and oxidation of methionines. Protein identification results were manually evaluated. The number of peptides analyzed and the percentage coverage of the total amino acid sequence were determined for each protein identified. The database was checked for redundancy and inspected for single proteins listed under multiple names. The molecular weight and p*I* of identified proteins were evaluated and verified relative to the electrophoretic mobility of the protein feature on the 2-D gel. Only identification results with coverage above 20% were confirmed as positive hits.

For ESI-MS and CID MS (MS/MS) analysis, peptides were extracted from gel pieces in 25 mM NH_3HCO_3, 50% v/v ACN, 5% v/v formic acid, and dried in a speed vacuum. After reconstitution in 5% v/v formic acid, samples were processed using ZipTip (OMIX, Variant) following manufacturer's instructions. ESI-MS and MS/MS were performed with a Q-TOF 2 mass spectrometer (Micromass) equipped with a Z-spray nanoflow electrospray ion (nanoESI) source and a high-pressure collision cell. For MS/MS studies the quadrupole was used to select the parent ions, which were subsequently fragmented in a hexapole collision cell using argon as the collision gas and an appropriate collision energy (typically 20–35 eV). Data acquisition was performed with MassLynx system 3.5 based on Windows NT. MS/MS data were processed with a maximum entropy data enhancement program, MaxEnt 3™ (Micromass). Amino acid sequences were manually deduced with the assistance of Micromass' peptide sequencing program PepSeq (mass tolerance: 0.3; fragment ion tolerance: 0.15; threshold: 0.3%; BioLynx, Micromass). Potential modifications considered included oxidation of methionines, cysteines modified with iodoacetamide.

Searches for protein identity from sequence data were performed using the BLASTP algorithm 2.0 using the Release 7.0 of the UniProt Knowledgebase (February 06) composed of the UniProtKB/Swiss-Prot Protein Knowledgebase release 49.0 and the UniProtKB/TrEMBL Protein Database release 32.0 containing 2 812 716 sequence entries (UniProtKB/Swiss-Prot section: 207 132; UniProtKB/TrEMBL section: 2 605 584). The database was checked for redundancy and inspected for single proteins listed under multiple names. The molecular weight and p*I* of identified proteins were evaluated and verified relative to the electrophoretic mobility of the protein feature on the 2-D gel.

Sequence comparisons were also carried out using TBLASTN 2.08 program of TIGR server (CMR database release 18.0, January 2006 and UFMG database, January 2006) with default search parameters. BLAST results with *E* values $>10^{-4}$ were not considered significant.

Identified proteins were further analyzed for the secretion using SignalP (SignalP 3.0), TatP (tatP 1.0), and SecretomeP (SecretomeP 2.0) programs.

2.4 Animal sera

Immunoproteomic analysis was performed with sera from two naturally infected cows and one 11-month-old calf infected intravenously with 10^8 CFU of MAP ATCC 19698, as described previously [22]. All three animals were confirmed infected at postmortem by bacterial culture and presented strong seroconversions in *M. phlei*-adsorbed LAM based [25], and commercial crude CE based, ELISAs (Pourquier, France). Sera used for Sp selection originated from three cattle infected intratracheally with 10^6 CFU of a low passage field strain of *M. bovis,* sampled and confirmed infected at week 13, postinfection by postmortem and bacterial culture, and presenting at that time strong serological responses in the LAM ELISA, as described previously [26].

Seventeen positive reference sera used in ELISA in addition to the two positive reference sera described above, equally originated from naturally infected cows shedding MAP at the time of sampling, as shown by fecal culture (Supporting Table 1). Among these, five tested negative in the MAP commercial kit of Pourquier. Control sera originated from 48 cattle randomly sampled in two *M. bovis*-

infected herds with no history of paratuberculosis. Four of these sera were positive in the MAP Pourquier test (Supporting Table 1).

2.5 Immunoproteomic analysis of MAP CF

MAP CF or CE (100 μg) was separated by 2-DE, as described above, and blotted onto NC membranes (Hybond ECL; AP Biotech), using a semidry blotting device (Trans-Blot SD-Dry Transfer Cell, BioRad) operated for 40 min at 500 mA and 25 V. The membranes were then washed three times for 5 min in 50 mM PBS, 0.5% v/v Tween-20 (immunoblot buffer, IB). IB containing 5% w/v BSA (blocking solution) was then added for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary bovine antiserum preadsorbed on MAP lipoarabinomannan and diluted 1000 × in IB containing 1% w/v BSA. Membranes were rinsed in IB (5 × 5 min) before incubation with the secondary antibody diluted in IB, 1% w/v BSA (1:2000; rabbit anti-bovine, HRP-conjugated; Pierce). Immunoreactive proteins were detected by a chemiluminescence detection kit (Lumi-light Western blotting substrate, Roche) following manufacturer's instructions, and identified by MS.

2.6 Antigen selection and cloning

Candidate antigens were selected based on two criteria: prospective Sp in BLAST searches, and antigenicity in immunoproteomic analysis. Specific proteins selected in the CF database were blasted against the complete *M. bovis* [27] and the unfinished *M. avium* subsp. *avium* genomes (TIGR server). Antigenic proteins were selected by immunoproteomic approach in MAP CF and CE. Only proteins recognized by at least two of the three MAP-positive sera and by none of the three sera of *M. bovis*-infected animals, were selected.

Genes encoding candidate antigen proteins were amplified by PCR from MAP ATCC 19698 genomic DNA [22] using primers derived from the sequences of MAP (Supporting Table 2) and were initially cloned in a V1J.ns-tPA vector (Merk Research Laboratories, PA, USA; Roupie V. *et al.*, manuscript in preparation). The integrity of cloned sequences was checked by sequence analysis.

Coding sequences were subsequently subcloned by PCR amplification (Expand High Fidelity PCR System, Roche), agarose gel purification (QIAkit PCR kit, Qiagen), and ligation into a pQE-80L (Qiagen) expression vector predigested with *BamH*I/*Hind*III. After ligation (T4 DNA ligase, Fermentas) and transformation into Top-10F′ chemically competent *E. coli* cells (Invitrogen) for expression, positive clones were screened on LB-ampicillin medium (100 μg/mL) and confirmed by the restriction enzyme digestion. The integrity of cloned sequences was checked by sequence analysis.

2.7 Expression and purification of candidate antigens

2.7.1 Expression of recombinant proteins

A 10 mL preculture of transformed *E. coli* containing the selected construct was grown to an OD of 0.6 at 600 nm, and used to inoculate a 100 mL volume of LB broth containing 50 μg/mL ampicillin. Cells were incubated at 37°C with shaking to reach the same OD. Recombinant protein expression was then induced by the adding 1 mM IPTG in overnight culture at 37°C, with shaking. Cells were harvested by centrifuging 15 min at 5000 × *g* at 4°C.

2.7.2 Extraction of recombinant proteins

Harvested cells were resuspended in 5 mL of lysis buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 8 M urea, 10 mM imidazole), and lysozyme (Novagen) was added to a final ratio of 50 kU/g of harvested cells. Samples were sonicated twice for 5 min on ice, and subsequently incubated for 15 min at room temperature under gentle agitation. Nucleic acids were digested by addition of 5 μL benzonase (25.0 U/μL, Novagen). Supernatants were finally clarified by ultracentrifugation at 110 000 × *g* at 4°C for 40 min.

2.7.3 Purification of recombinant proteins

A His-Select column (6.4 mL, Sigma) was used with a peristaltic pump set to a constant flow rate of 3 mL/min with a sample collector (FC250, Gilson) programmed to collect 3-mL fractions. The column was first washed with 45 mL of ultrapure water, followed by equilibration with 60 mL of lysis buffer. Samples were applied and fraction collection was started immediately. Nonbinding proteins were removed by washing with 45 mL of lysis buffer. A 10–300 mM linear gradient of imidazole in a total volume of 100 mL was used for protein elution. Contents of collected fractions were analyzed by SDS-PAGE followed by CBB staining. Fractions containing the recombinant protein were pooled and extensively dialyzed against 10 mM PBS, pH 7.2, containing 0.1 M urea. After concentration by ultrafiltration (Ultracel 5 kDa, Amicon, Millipore, USA) and protein quantification (BioRad protein Assay kit, BioRad), samples were stored at −80°C until use.

2.8 Antibody ELISA

Flat-bottom 96-well plates (Maxisorp, Nunc) were coated with 50 μL of each recombinant protein alone or in a pool diluted to 5 μg/mL in 37% formaldehyde, and left under a fume hood at room temperature to allow the solvent to evaporate. Unbound antigens were removed by washing each well five times with 100 μL of PBST (100 mM PBS, pH 7.2, 0.05% v/v Tween-20), and uncoated sites were blocked with 5% w/v casein hydrolysate in PBST for 1 h at 37°C. Fifty microliters of primary antibodies (bovine sera diluted 250 × in PBST, 1% w/v casein) were then added to the plate and incubated for

1 h at 37°C. The plate was washed five times with PBST and 50 μL of secondary antibody (HRP-conjugated goat anti-bovine immunoglobulin, Sigma, diluted 1/25.000 in PBST, 1% w/v casein), were added to each well for 1 h at 37°C. The plate was again washed five times with PBST and the peroxidase activity was detected by adding 75 μL of TMB (3,3′,5,5′-tetramethylbenzidine, liquid substrate system, Sigma). After 10 min incubation, the reaction was stopped by the addition of 35 μL of 1 N H_2SO_4, and ODs were read at 450 nm on a multiskan ascent microplate photometer (Thermo labsystem).

3 Results

3.1 MAP secretome analysis

SDS-PAGE and 2-DE of MAP CF were used to characterize the secretome of MAP. These analyses led to the construction of a database that could be exploited for antigen identification after immunoproteomic analysis, or for the prediction of protein antigenicity. The established database is accessible as Supporting Information (Supporting Table 3).

A total of 320 protein bands and spots stained by CBB in 1-D or 2-D gels were analyzed by MS (Fig. 1). MALDI-TOF and/or ESI MS/MS analysis allowed the definitive identification of 249 of them, corresponding to 125 different proteins. The remaining 71 spots did not yield interpretable data.

Out of the 125 proteins identified, only 35 presented a predicted signal sequence using the SignalP 3.0 software, and three additional proteins presented a tat secretion motif (CBS server; Fig. 2A). Using the SecretomeP software, a prediction approach based on different protein features rather than on sequential characteristic (CBS server), five more proteins were defined as putatively extracellular. Together, these three prediction tools revealed 43 putatively exported proteins among the 125 proteins identified, suggesting the apparent occurrence of contamination of the CF with intracellular proteins, possibly due to bacterial lysis during the 4-wk MAP culture. Nevertheless, it is now well established that mycobacteria dispose of Sec-independent protein secretion pathways [28, 29]. Additionally, chaperon proteins such as GroEL are not only functionally active in the cytoplasm [30] but can also be displayed at the bacterial cell surface [31–33].

Determination of the activity classes of the predicted nonsecreted proteins revealed that they belong predominantly to the energy metabolism class (Fig. 2B). This class of proteins is known to contain particularly stable proteins, thus explaining how light bacterial lysis might produce a significant contamination of CFs.

3.2 Identification of candidate antigens

Two different and complementary approaches were used for antigen identification, one based on sequence comparison with *M. bovis* and *M. avium* subsp. *avium* genome, and the other on an immunoproteomic approach, using sera from MAP-infected cattle.

Only 15 proteins out of the 125 CF proteins identified, were found to be absent from the *M. bovis* genome. These proteins could be particularly useful to discriminate infections by MAP and *M. bovis*, in areas where they overlap [9]. Further genomic comparison with public domain, complete or unfinished mycobacterial genomes, indicated that three proteins were prospectively fully specific of MAP (MAP2746, MAP2879c, and MAP3680c).

Alternatively, antigenic proteins were probed in MAP CE and CF by Western blot with sera from MAP-infected cattle (Fig. 3). Immunoreactive proteins were identified either by comparison with the master gel established previously for CF, or by the identification of the corresponding spot on a CBB-stained gel, by MS. As shown in Fig. 3, numerous proteins were detected using this approach. However, only 14 were simultaneously detected by at least two positive reference sera without being detected by any of the three sera from *M. bovis*-infected cattle.

Combination of both analyses resulted in the identification of 25 putative MAP-specific antigens (Table 1). A first pool of five of them, *i.e.* MAP1693c, MAP4308c and MAP0586c MAP3199, and MAP2677c selected for antigenicity and/or Sp was selected for cloning.

3.3 Purification and antigenicity of cloned candidate antigens

All five selected candidate antigens were successfully cloned into the pQE-80L expression vector (QIAGEN), and expressed in *E. coli* as histidine-tagged proteins. Except for MAP4308c, all were expressed as inclusion bodies, requiring 8 M urea for an efficient solubilization (Fig. 4). Nickel affinity chromatography using an imidazole gradient for protein elution, resulted in an efficient purification of the candidate antigens (Fig. 5). SDS-PAGE separation of eluted material followed by MS analysis revealed homogeneity of the purified antigens. No contaminating proteins of *E. coli* were identified in the purified samples by MS. The purification yield ranged from 0.7 to 2.5 mg of purified protein *per* gram of bacteria.

3.4 Antibody ELISA with purified candidate antigens

The conditions for an indirect antibody detection ELISA for the five candidate antigens were first optimized with four positive and three negative sera covering the whole range of responses (data not shown). Coating in formaldehyde, a 250-fold dilution of sera and casein as blocking agent were selected.

In a preliminary approach, the antibody responses against the five purified recombinant proteins were then evaluated in this ELISA using a representative panel of seven positive reference and three control sera (Fig. 6). A response

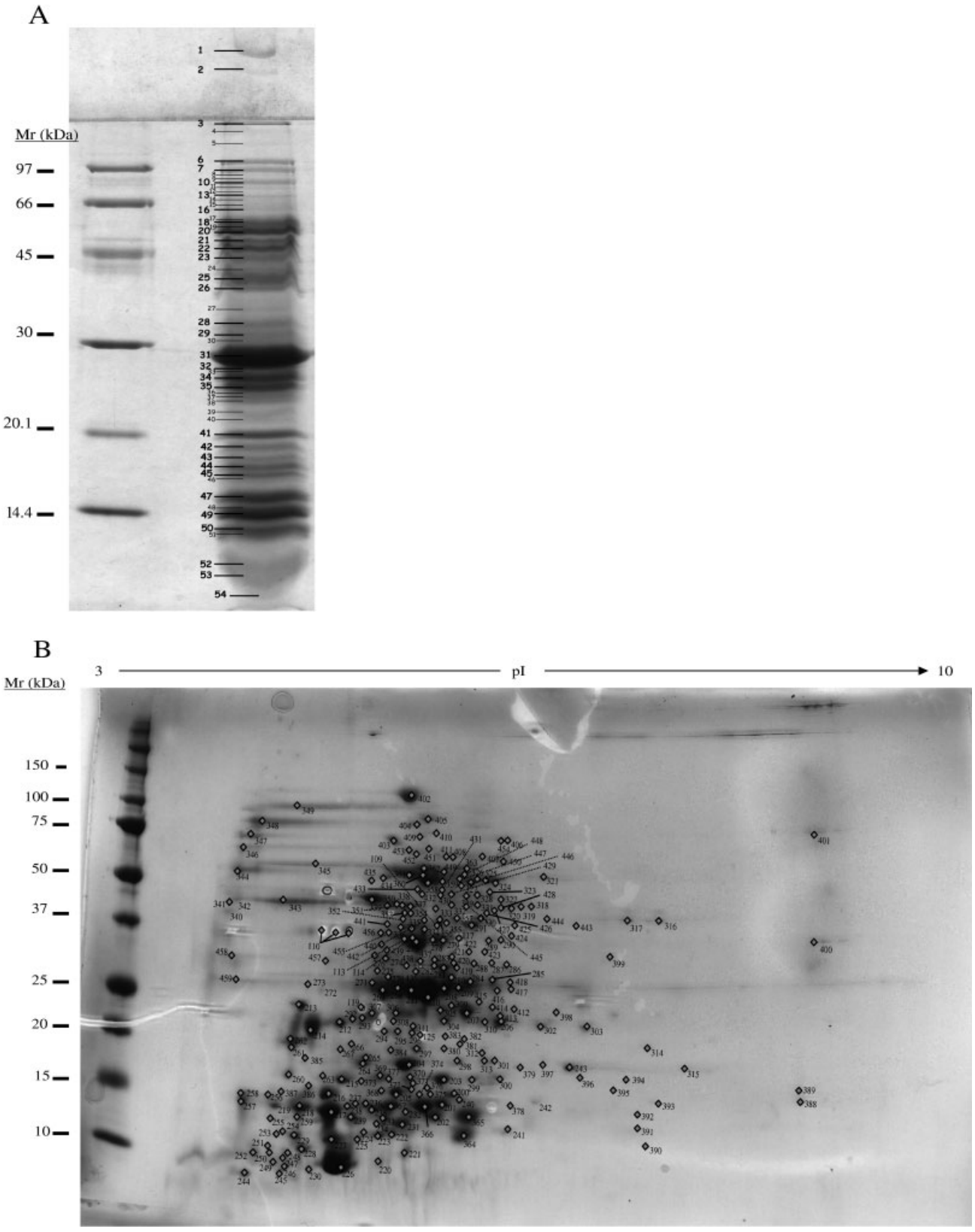

Figure 1. Master gels of MAP CF. Proteins of MAP CF were separated by SDS-PAGE (A) or 2-DE (B) and stained by CBB. Systematic proteic band/spots excision and analysis led to the construction of the first MAP CF database presented as Supporting Information.

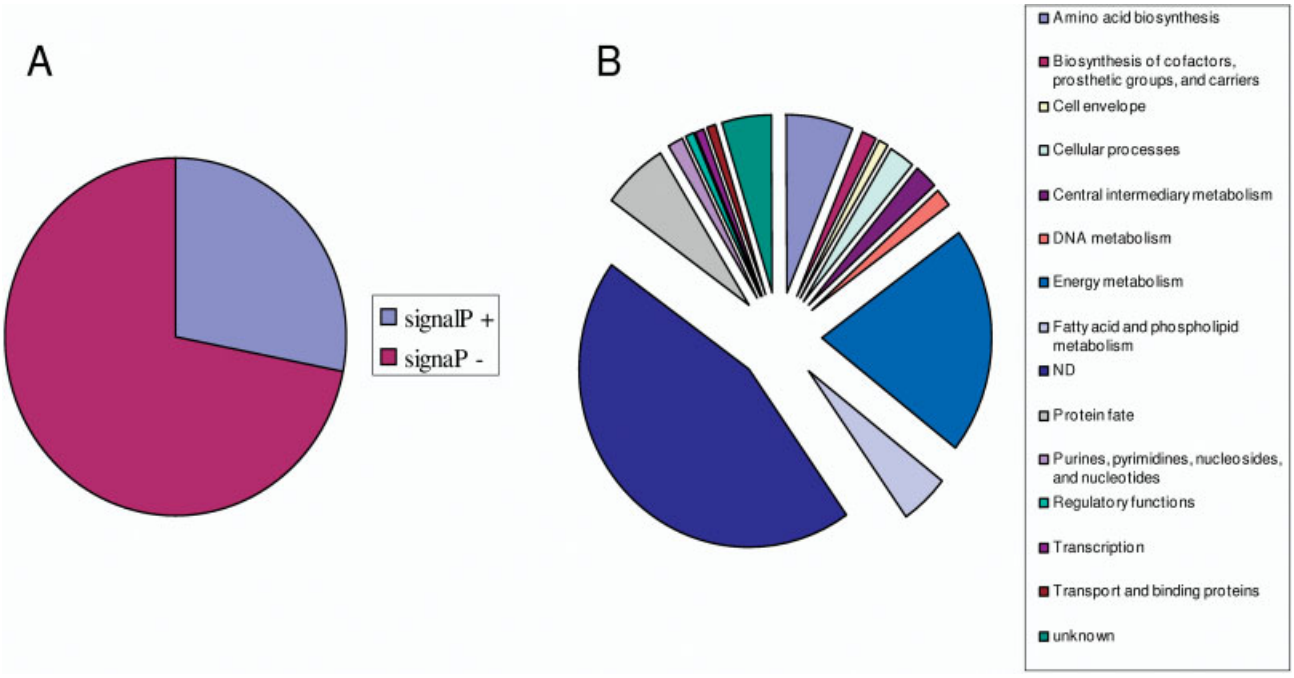

Figure 2. MAP CF database analysis. (A) SignalP prediction software (CBS server) was used to predict secretion signal sequences for each protein. Only 35 out of 125 identified proteins presented such a sequence. Use of supplemental prediction tools (SecretomeP and tatP, CBS server) increased this value to 43 potentially secreted proteins.

was considered positive if above the maximum value obtained with the control sera. MAP0586c and MAP3199 remained undetected by the panel of sera used. Reversely, MAP1693c was detected by three sera and MAP4308c and MAP2677c by five sera each. Moreover, when the latter three antigens were tested as a single pool, all seven reference sera tested positive (Supporting Fig. 1).

The combination of MAP1693c, MAP4308c, and MAP2677c was therefore selected for performance testing on the entire panel of sera, *i.e.* 19 sera from MAP fecal culture positive animals, and 48 control sera from animals of *M. bovis*-infected herds. In receiver operating characteristic analysis (Fig. 7), the area under the curve and its standard error were found equal to 0.993 (95% confidence interval, 0.981–1.006) and 0.007, respectively. The optimal cut-off value of 0.704 was determined as being associated with the maximal likelihood ratio of 45.47, resulting in a relative test Se of 94.74% and Sp of 97.92% calculated from the limited samples analyzed. At this cut-off value, three positive reference sera tested negative (Table 2). Among these false negative sera, one (4415) was also negative in Pourquier test. The four potentially *M. bovis*-infected animals that tested positive in Pourquier test, remained negative in our test. Finally, our test permitted to detect four MAP-infected animals that consistently scored as negative in the commercial Pourquier test (Table 2). The likelihood ratio for the Pourquier test was equal to 8.8 on this serum panel.

4 Discussion

Bovine paratuberculosis is a fatal chronic disease responsible for important economic losses in the dairy and beef cattle industries [2]. In the United States, its prevalence is estimated to exceed 20% [34]. In Belgium, most recent data from the paratuberculosis monitoring program indicate a herd prevalence rate of 27% (23.9–30.2%, CI 95%) for the 2004–2005 period using a commercial antibody detection ELISA (Luc De Meulemeester, personal communication). Moreover, the possible involvement of MAP in Crohn's disease is still a matter of debate [35–41]. Better control programs are clearly needed to restrict this insidious infection. Unfortunately, the currently available diagnostic tools are inadequate and limit the feasibility of any large-scale control programs [1]. Indeed, fecal culture, the current gold standard test, requires as long as 15 wk to complete while existing serological tests commercially available have variable sensitivities ranging from 10 to 80%, depending on the product and especially the infection stage [5, 7]. Taken together, this has led to increased efforts by several research groups to develop more efficient paratuberculosis diagnostic assays [4, 9–14, 21, 42, 43]. In this study, we report on results obtained through a postgenomic approach to identify new antigens that could potentially be used for the serological diagnosis of Johne's disease.

In a first approach, we established a database of proteins found in CF from MAP grown as a surface pellicle on synthetic Sauton medium. This particular subproteome is

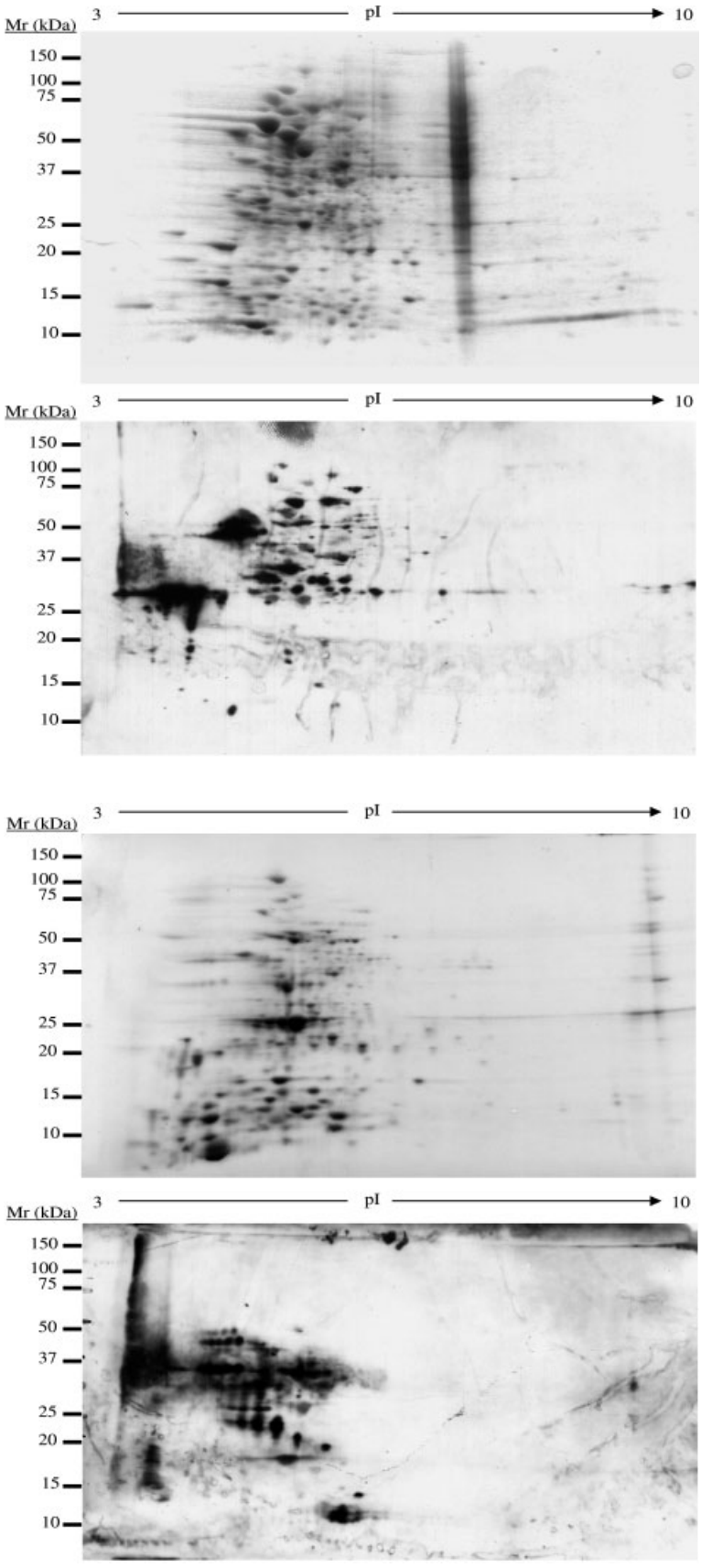

Figure 3. Immunoproteomic analysis of MAP CE and CF. MAP CE (A) and CF (B) were separated by 2-DE (upper panel) and submitted to Western blot using sera of MAP-infected cattle (lower panel). Sp of antigenic proteins was determined using sera from three *M. bovis*-infected cattle.

Table 1. Database of 25 potential candidate antigens, Sp; as determined by proteomic analysis of MAP CF, antigenicity was determined by immunoproteomic analysis of MAP CF and CE

Name	Reference	Similarity/putative function	Specificity	Present in CF	Antigenicity
MAP0139c	CF206	Putative PadR-like family transcriptional regulator	X	X	
MAP0494	Mptb0142; Mptb0156	Putative oxidoreductase, *Streptomyces coelicolor*	X		X
MAP0586c	CF027; CF274	Possible transglycosylase SLT domain, *Prochlorococcus marinus* strain MIT 9313	X	X	X
MAP0740c	CF458	None	X	X	
MAP0796c	CF222	None	X	X	
MAP0907	CF136; Mptb0099; Mptb0143; CF280	Probable oxidoreductase, *Bordetella pertussis*		X	X
MAP1168c	Mptb0121	Putative oxidoreductase SAV780, *Streptomyces avermitilis*	X		X
MAP1438c	Mptb0048	BII4284 protein, *Bradyrhizobium japonicum*	X		X
MAP1562c	CF201	None	X	X	
MAP1693c	CF041; CF264; CF106, CF126, Mptb0136	Peptidyl-prolyl *cis–trans* isomerase, *S. coelicolor*	X	X	X
MAP2411	CF205	Pyridoxamine 5′-phosphate oxidase	X	X	
MAP2677c	CF040; CF233	Hypothetical protein SCO4486, *S. coelicolor*	X	X	
MAP2746	CF215	None	X	X	
MAP2770	CF107	27 kDa lipoprotein antigen precursor, *Mycobacterium intracellulare*		X	X
MAP2963c	Mptb0076	Hypothetical protein, *S. avermitilis*	X		X
MAP3199	CF036	Alternative splicing variant of microtubule-associated protein tau, *Bos taurus*	X	X	
MAP3385	Mptb0094	Conserved hypothetical protein, *Mycobacterium bovis*			X
MAP3486	CF352	Possible L-lactate 2-monooxygenase?	X	X	
MAP3547c	Mptb0136; CF341	Hypothetical protein, uncultured bacterium 581	X	X	X
MAP3680c	CF319	Formate dehydrogenase	X	X	
MAP3731c	Mptb0012	ATP binding protein of ABC transporter, *Bifidobacterium longum*	X		X
MAP3804	CF118; Mptb0035; CF032	Possible beta-1,3-glucanase, *M. bovis*		X	X
MAP4056c	CF128	Possible conserved secreted protein, *M. bovis*		X	X
MAP4096	CF038	Putative NADH dehydrogenase/NAD(P)H nitroreductase AF1167, *Archaeoglubus fulgidus*	X	X	
MAP4308c	CF281; CF282; CF283; CF028; Mptb0074; Mptb0097; Mptb0137; Mptb0141; Mptb0151	Fructose-bisphosphate aldolase class I, *Synechocystis* sp. strain PCC 6803	X	X	X

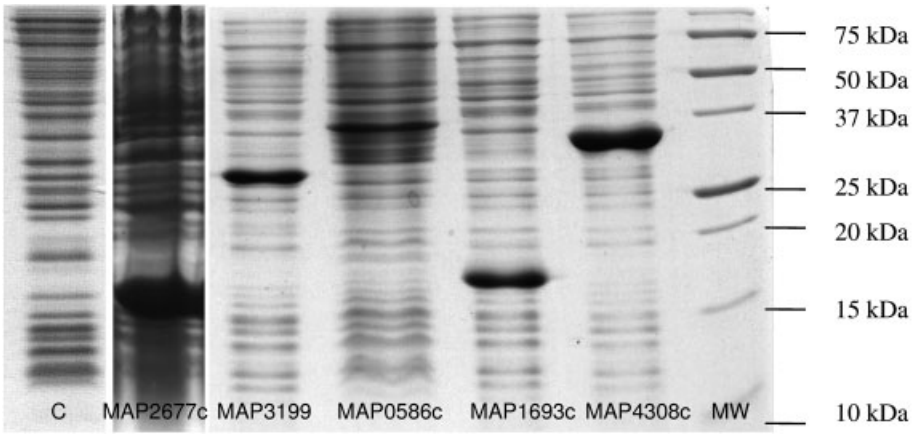

Figure 4. Expression of recombinant proteins. Five candidate antigens were cloned into the *E. coli* pQE-80L expression vector and expressed upon IPTG induction. Except for MAP4308c, all recombinant proteins were found in inclusion bodies. C, noninduced control; MW, molecular weight marker.

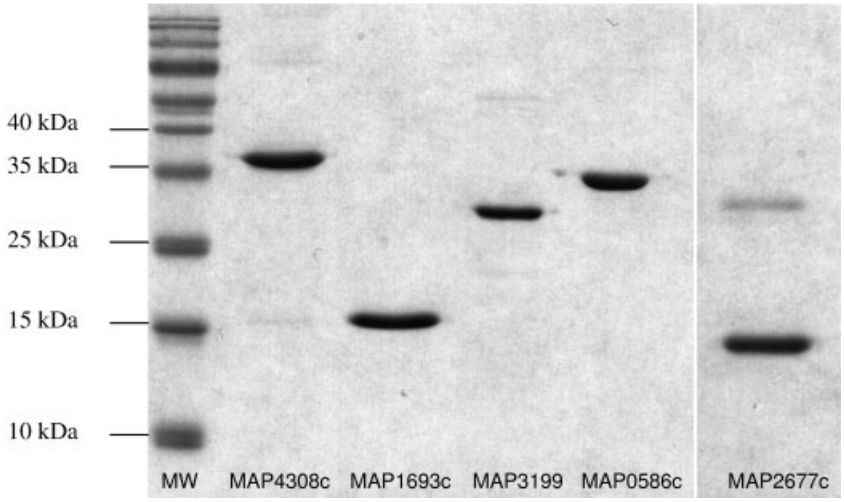

Figure 5. Recombinant protein purification. The five candidate antigens were purified by IMAC using a linear imidazole gradient for protein elution. This procedure yielded highly efficient purification as revealed by systematic MS analysis.

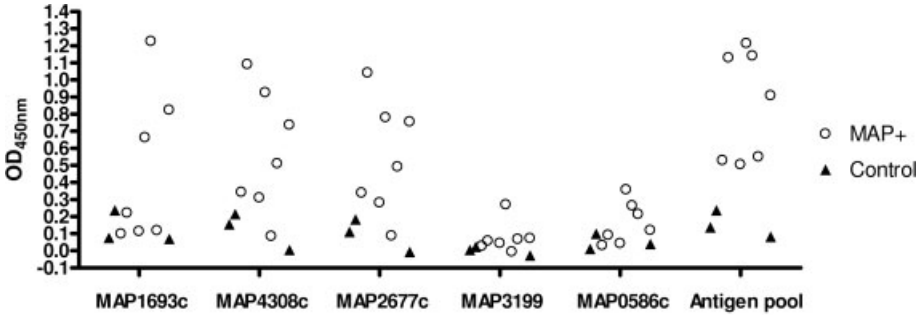

Figure 6. Antigenicity of selected antigens. Antigenicity of the five purified candidate antigens was analyzed by ELISA using sera from seven fecal culture-positive MAP animals and three cattle experimentally infected by *M. bovis*. Presented ODs result from subtraction, for each serum, of signal obtained with antigens omission.

indeed recognized as an abundant source of immunoactive proteins [22, 44, 45]. Proteins were identified using uni- and bidimensional SDS-PAGE. The use of both types of electrophoresis was motivated by the eventual loss of information in 2-DE associated with solubility problems or the p*I* range. In our study six proteins, MAP3199, MAP1588c, MAP1643, MAP1661c MAP3570c, and MAP4096, were only identified by 1-DE, clearly showing the added value of this kind of analysis.

The sequences of the proteins identified in the MAP CFs were analyzed using three prediction algorithms for secretion signals. The results of this analysis suggest contamination of the CF with nonsecreted proteins partially due to bacterial lysis. This finding must however be put into perspective on four grounds. First, the percentage of secreted proteins predicted in MAP CF (34.4%) remains significantly higher than the 1.32% of secreted proteins predicted from the whole *Mycobacterium tuberculosis* genome [46]. Second, accuracy of bioinformatic predictions of subcellular localization of prokaryotic proteins always remains below 95% leaving 5% of incorrect prediction [47]. Third, the majority of predicted nonsecreted proteins in our sample belonged to the activity class of energy metabolism, known to contain particularly stable proteins. This could explain how moderate cell lysis could lead to significant contaminations of our CF with cell components. Fourth, mycobacteria are known to use alternative Sec-independent proteins secretion pathways such as the Snm and SecA, involved in the secretion of ESAT6/CFP10 complexes [29] and of SodA [48], respectively. Furthermore, chaperone proteins from the GroEL family are predicted to be located in the cytoplasm [30], based on the fact that no member of this family possesses any secretion signal sequence or other known motif that would suggest its export. Yet, these chaperonins are reported to be additionally located extracytoplasmically in pathogenic bacteria [31–33].

Proteins exported by Sec-independent pathways could not be identified by the prediction softwares used in this study, possibly leading to an overestimation of the so-called nonsecreted cell lysis contaminating proteins in our CFs. In conclusion, we believe that the MAP CF studied here remains relatively representative of the MAP secretome.

An important characteristic of the diagnostic proteins we have identified is their Sp. Indeed, all proteins of the database were compared to the complete *M. bovis* and the unfinished *M. avium* subsp. *avium* genome sequences, using BLAST. Only 15 CF proteins were absent from *M. bovis* genome and three proteins (MAP2746, MAP2879c, and MAP3680c) out of 125 identified in MAP CF seemed to be fully specific for MAP. These proteins were selected with the possible value for differential paratuberculosis diagnosis.

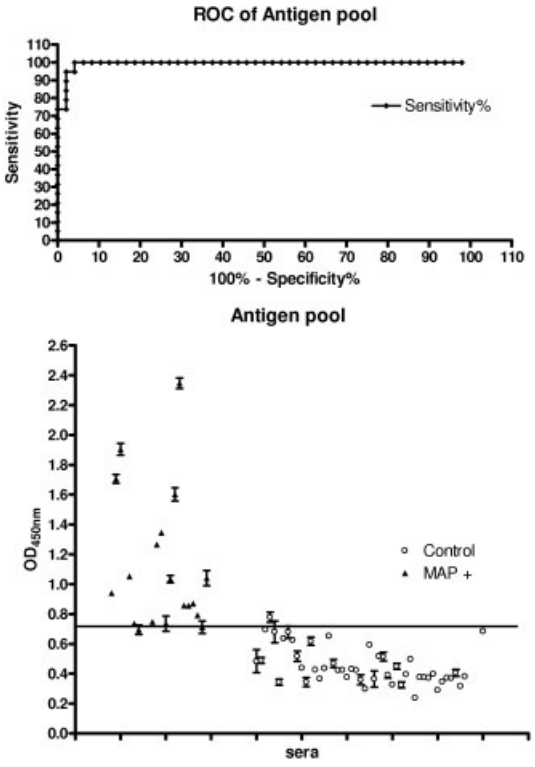

Figure 7. ROC analysis of the antigen's diagnostic performance in ELISA. ROC analysis performed on a panel of sera from 19 MAP positive and 48 control animals, using a combination of MAP1693c, MAP4308c, and MAP2677c, allowed calculation of Se and Sp values at cut-off 0.704 as 94.74 and 97.92%, respectively.

Table 2. Comparison of results obtained from our ELISA and the commercial Pourquier test with the 19 positive reference and 48 control sera

			Pourquier test	
			Positive	Negative
Pooled antigen ELISA	MAP + sera	Positive	12	4
		Negative	2	1
	Control sera	Positive	0	2
		Negative	4	42

In a complementary approach, we used immunoproteomic tools to resolve by 2-DE proteins from CE and CF and analyzed them for antigenicity by Western blot using sera from MAP- infected cattle. Sera from three Johne's disease confirmed cases were used and allowed identification of more than 40 antigenic proteins. Only proteins reacting with at least two out of the three positive sera were selected for further study. Additionally, antigenic proteins crossreacting with sera from *M. bovis*-infected cattle were discarded. As a result, 14 antigenic proteins were selected using immunoproteomic analysis.

Combining the data obtained in these two complementary approaches, we established a database of 25 candidate antigens based on their Sp and/or antigenicity. Three of them, MAP1693c, MAP4308c, and MAP0586c, were selected because they were both specific and antigenic. Two others proteins, MAP3199 and MAP2677c, were chosen because of their Sp.

It is interesting to note that, using a similar approach, Cho *et al.* [11] also selected MAP1693c and MAP4308c as potential antigens. The independent finding, by two groups, of the same immunodominant targets in MAP CF is particularly encouraging. In contrast, other proteins described as antigenic in our study were not detected by these authors, reinforcing the idea that one universal antigen probably does not exist and that an efficient diagnostic test of bovine paratuberculosis will require a combination of antigens.

The final purpose of our study was to characterize antigens able to discriminate sera from MAP-infected and uninfected cattle in a serological assay. We chose the ELISA format rather than immunodiffusion or latex beads agglutination tests because of easier handling, potential for robotization, automation of output data analysis and result interpretation. Speer *et al.* [42] have shown that the treatment of antigens with 37% formaldehyde improves the S/N significantly. We also observed a slight increase in this ratio when antigens were coated in the presence of 37% formaldehyde rather than sodium carbonate buffer (data not shown) probably due to better antigen fixation and epitope protection by formaldehyde [49].

Three out of our five candidate antigens appear to be antigenic and complementary in our ELISA assay. Indeed, while two did not yield any signal with our serum panel, none of the others three was capable yet to detect all culture-confirmed MAP-infected animals on its own. These three antigens were therefore tested in combination. Receiver operating characteristic analysis of results obtained with the pooled MAP1693c, MAP4308c, and MAP2677c permitted us to determine a cut-off value corresponding to a relative Se of 94.74% and an Sp of 97.92% for that test on the panel of sera tested. These results were clearly better than those obtained with the commercial Pourquier test on the same serum panel, with an Se of 73.68% and an Sp of 91.67% respectively. Indeed, only three animals were incorrectly classified as negative. Among these, one was also negative in the Pourquier test. In contrast, four sera from culture-positive animals that scored negative in the Pourquier test were positive with our test. Moreover, four sera of the control group scored falsely positive in the Pourquier test as a result of cross-reactivity with *M. bovis* antigens. The likelihood ratio decreased from 45.47 with our test to 8.8 with the Pourquier test. Finally, the use of selected antigens, produced in *E. coli*, rather than of crude protein extracts or culture-derived antigens has the advantage of avoiding the culture of slow growing pathogenic mycobacteria. Moreover, the kind of standardized antigenic preparation and test described here can be easily and cost effectively scaled up for industrial purpose.

We are now entering a validation phase of a diagnostic kit using a combination of MAP1693c, MAP4308c, and MAP2677c with a large panel of sera from MAP-infected and healthy animals. Their potential as immune targets for use in IFN-γ-based diagnosis and vaccination is further investigated. Analysis of vaccine and diagnostic potential of the remaining candidate antigens of our database is also in progress.

Finally, it could be of interest to complete the database of potential candidate antigens described here by the use of a nondenaturating and complementary procedure. Indeed, conditions of the immunoproteomic approach (sample denaturation, loss of a few proteins in 2-DE conditions) may impair the identification of particular antigens. An immunocapture procedure in which antigens could be isolated from a CE or a CF based on their reactivity with an immobilized serum of MAP-infected cattle could lead to identification of novel candidate antigens. This kind of approach has already been successfully applied for other diseases [50].

In conclusion, using a proteomic approach, we have established a useful database of potential antigens for the diagnosis of bovine paratuberculosis. Using this database, three protein antigens have been identified that, when used in combination, resulted in an ELISA test that is equally sensitive and more specific than the existing commercial test.

This work was supported by Wallon Government (Belgium; Recherche Initiative5). R. Wattiez is a Research Associate of the National Funds for Scientific Research. V. Roupie holds an FRIA Scholarship. The authors thank Catherine S'Heeren for her technical assistance.

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