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Identification of conserved *Mycoplasma agalactiae* surface antigens by immunoproteomics

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ABSTRACT

Contagious agalactia represents one of the most relevant infectious diseases of dairy sheep, with *Mycoplasma agalactiae* being the primary etiological agent. The early, sensitive, and specific identification of infected animals, as well as the development of efficient prophylactic tools, remain challenging. Here, we present a comprehensive characterization of *M. agalactiae* antigens focusing on those shared among different isolates. Leveraging on previous proteomic data obtained on individual strains, we adopted a strategy entailing sample pooling to optimize the identification of conserved proteins that induce an immune response. The liposoluble proteins from previously characterized field isolates and the type strain PG2^T were enriched by Triton X-114 fractionation, pooled, analysed by one-dimensional (1D) and two-dimensional (2D) electrophoresis, and subjected to western immunoblotting against sheep sera collected during natural infection with *M. agalactiae*. Immunodominant antigens were identified by Matrix-Assisted Laser Desorption-Time-Of-Flight-Mass Spectrometry (MALDI-TOF-MS). This combined immunoproteomic approach confirmed the role of several known immunogens, including P80, P48, and P40, and most variable surface proteins (Vpmas), and unveiled novel immunodominant, conserved antigens, including MAG_1000, MAG_2220, MAG_1980, phnD, MAG_4740, and MAG_2430. Genomic context, functional prediction, subcellular localization, and invariable expression of these proteins in all isolates suggest their possible involvement in bacterial pathogenicity and metabolism. Moreover, most of the identified antigens elicit a host humoral response since the early stages of infection, persisting for at least 270 days. The immunodominant, conserved antigen panel identified in this work supports the development of effective vaccines and diagnostic tools with higher sensitivity and specificity in all the natural infection stages.

1. Introduction

Contagious agalactia (CA) is one of the most severe infectious diseases of small ruminants, characterized by various clinical signs including mastitis and subsequent drop in milk production, arthritis, keratoconjunctivitis, and abortion (Contreras et al., 2007). In the acute phase of infection, animals display one or more of the characteristic clinical signs which usually spontaneously regress, leading to a chronic state (Pooladgar, 2014). CA is an OIE - listed disease with worldwide distribution and has a significant economic impact in the Mediterranean basin, where small ruminant farming is extensively practiced (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.07.03_CONT_AGALACT.pdf). The massive diffusion of CA is associated with many factors, such as poor husbandry practices, the inefficacy of antimicrobial therapies, and the absence of an efficient vaccine (Kumar et al., 2014).

Sensu stricto CA is caused by *Mycoplasma agalactiae* (*M. agalactiae*), a bacterial agent infecting primarily sheep and goats, but also some wild ruminant species (Contreras et al., 2007). CA remains endemic to several Countries of the World, and the development of effective prophylactic

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and diagnostic tools still represents a challenge. Both live attenuated and inactivated vaccines are available inducing humoral and cellular immune responses, but they have been shown to fail long-term protection (Buonavoglia et al., 2010, 2008, 1998; de la Fe et al., 2007; Foggie et al., 1971; Tola et al., 1999). Vaccination with live attenuated vaccine followed by a vaccine prepared by inactivating the mycoplasmas with saponin had some success, but its use is forbidden in the European Union (Agnone et al., 2013). A prototype DNA vaccine was developed based on *M. agalactiae* P48 and tested in mice where it induced both humoral and cellular immune responses. However, this vaccine has not yet been tested in the natural host (Chessa et al., 2009).

The detection of *M. agalactiae* with high-performance tests and affordable costs is also paramount for developing effective control strategies. Isolation of *M. agalactiae* on culture media from individual or bulk tank milk represents a relatively inexpensive and specific diagnostic method, but it is time-consuming and has several drawbacks, such as unsatisfactory sensitivity and applicability limited to lactation time. As an alternative, indirect Enzyme-Linked Immunosorbent Assay (ELISA) can be used both during lactation and in the dry period, allows the detection of latent infections, and is largely applicable even when mycoplasma is not shed in milk (Poumarat et al., 2012). Indirect ELISA tests for *M. agalactiae* have been developed using total bacterial extracts (Campos et al., 2009; Fusco et al., 2007; Kittelberger et al., 2006; Pépin et al., 2003; Rosati et al., 2000), with varying sensitivity and specificity (Poumarat et al., 2012). These tests suffer from the variability associated with intrinsic strain variation and with the quantitative and qualitative differences in antigenic protein expression during infection (Poumarat et al., 2012). Moreover, cross-reactivity with other *Mycoplasma* species may occur, creating challenges in result interpretation (Kumar et al., 2014). ELISA with recombinant proteins can reduce specificity issues, but to date only 2 recombinant assays have been described, based on P48 and P80/P55 (Fusco et al., 2007; Rosati et al., 2000).

Therefore, the identification of novel *M. agalactiae* antigens represents a necessary step for developing effective prophylactic and diagnostic tools. A limited number of *M. agalactiae* antigens has been reported, including the variable surface proteins (Vpma) (Razin et al., 1998), the *M. fermentans malp*-homologue P48 (Rosati et al., 2000, 1999), the surface-exposed nuclease MAG_5040 (Cacciotto et al., 2013), P80 (Tola et al., 1997), P40 (Fleury et al., 2002), P30 (Fleury et al., 2001), and AvgC (Santona et al., 2002). In the past, characterization of the *M. agalactiae* antigenic repertoire has been approached using one-dimensional electrophoresis, but a systematic study is still missing (Tola et al., 1997).

Considering these issues, in a previous study we characterized the differential surface proteome of *M. agalactiae* strains by combining two-dimensional polyacrylamide gel-electrophoresis followed by mass spectrometry (2D-PAGE/MS) and one-dimensional gel electrophoresis followed by tandem mass spectrometry (GeLC/MS-MS) (Cacciotto et al., 2010). Here, we leveraged also the individual strain information generated in that study to identify which conserved proteins induce an immune response. Using an antigen pooling strategy, we subjected a *M. agalactiae* membrane protein preparation including the strains analysed in Cacciotto et al., 2010, to one-dimensional and two-dimensional western immunoblotting with sera collected longitudinally along 270 days from naturally infected sheep. The identity of the resulting immunodominant antigens was then confirmed by Matrix-Assisted Laser Desorption Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS).

2. Materials and methods

2.1. Bacterial culture

Four *M. agalactiae* strains were used in this study: the PG2^{T} reference strain and 3 field isolates (F1, F2, and F3) recovered from outbreaks that occurred independently in 3 locations of Sardinia, Italy (Benetutti: 40°27′23.52″N /9°10′05.82″E, in 2012/2019; Bortigali: 40°16′53.36″N /8°50′16.75″E; and Nurri: 39°42′40.61″N/9°13′49.45″E, in 2009). Two of the three field samples had been subjected to an extensive comparative proteomics study, namely F2 (Bortigali) and F3 (Nurri). Detailed information is available in Cacciotto et al., 2010. Mycoplasmas were cultured either on blood agar plates or in PPLO liquid medium, both supplemented with 20 % heat-inactivated horse serum and 500 μg/mL ampicillin, as previously described (Cacciotto et al., 2010). After reaching the logarithmic phase, cultures were centrifuged at 10.000 x g at 4 °C; pellets were washed 3 times with PBS and stored at −80 °C until use.

2.2. Mycoplasma species identification

Mycoplasma field isolates were identified by specific PCR. Briefly, DNA was extracted from mycoplasmas with the DNeasy Blood & Tissue Kit (Qiagen), quantified, and analysed by PCR with FS1-FS2 primers (Tola et al., 1996). PCR products were resolved in agarose gel electrophoresis and images were acquired with a Gel Doc™ EZ Gel Documentation System (Bio Rad).

2.3. Natural infection of sheep and collection of sera

Sheep sera used in this work were obtained in the course of natural infection in a previous work (Cacciotto et al., 2013). Briefly, 10 sheep from a CA-free flock in which the absence of *M. agalactiae* was checked by bacterial isolation, rP48 ELISA, and western blotting, were moved by the shepherd into a CA-affected flock in close contact with symptomatic positive animals. Sheep were monitored for 270 days, and sera were collected at T0 (before the introduction) and every 15 days from all animals. Five sheep were used as negative controls and sampled at the same timeframe in the original flock.

2.4. Protein fractionation and quantification

Mycoplasma liposoluble proteins were enriched as previously described (Cacciotto et al., 2010). Briefly, *M. agalactiae* pellets derived from 40 mL of liquid culture were resuspended with PBS and subject to Triton X-114 phase partitioning. After phase separation, liposoluble proteins were quantified with EZQ™ Protein Quantitation Kit (Invitrogen).

2.5. SDS-PAGE

M. agalactiae proteins were diluted in Laemmli buffer, and SDS-PAGE was performed on 8% or 10 % polyacrylamide gels on a Protean Tetra Cell (Bio-Rad) following manufacturer instructions. After the run, gels were stained with SimplyBlue™ Protein SafeStain (Invitrogen). Replicate gels were subjected to western immunoblotting.

2.6. 2-D PAGE

2-D PAGE was conducted as previously described (Cacciotto et al., 2010). Briefly, Triton X-114 fractions were precipitated with methanol-chloroform and resuspended in a 2-D PAGE suitable lysis buffer. Then, 18 cm IPG strips (GE Healthcare, pH 3–10 NL) were passively rehydrated overnight with 150 μg protein samples and then focused on an IPGphor (GE Healthcare) for a total of 60,000 Vh. After focusing, strips were equilibrated, reduced, and alkylated. The SDS-PAGE was carried out on polyacrylamide gradient gels (10–18%) in an EttanDALT six electrophoresis system (GE Healthcare). Subsequently, gels were silver stained with a mass-compatible method (Chevallet et al., 2006), and images were acquired with an Image Scanner (GE Healthcare). Replicate gels were blotted into nitrocellulose for 2D immunodetection.

2.7. Western immunoblotting

Enriched liposoluble proteins from PG2^T and from the 3 field isolates were pooled to identify conserved proteins that induce an immune response. Pools were resolved both by SDS-PAGE and 2D PAGE. Subsequently, proteins were transferred onto nitrocellulose membranes with a Mini-Trans-Blot Cell (Bio-Rad) at 250 mA for 1 h (1D-WB), or with a Trans-Blot® Plus Cell (Bio-Rad) at 0.15 A O/N at 4 °C (2D-WB). After blotting, membranes were blocked with PBS-T containing 5% skim milk and incubated for 1 h with individual sera collected from naturally infected sheep, or pooled sera, in both cases diluted 1:1000 in PBS-T containing 2% skim milk. After 3 washes with PBS-T, membranes were incubated with anti-sheep IgG secondary HRP-conjugated antibodies (Southern Biotech) diluted 1:50.000 in PBS-T containing 2% skim milk. After 5 washing steps, membranes were developed with Chemiluminescent Peroxidase Substrate (Sigma-Aldrich) and images were acquired with a VersaDoc MP 4000 Imaging System (Bio Rad).

2.8. Mass spectrometry identification of proteins

Immunoreactive protein spots were identified in 2-D PAGE gels by taking reference points of the blotted membrane after temporary staining with Ponceau S solution, scanning the images, and then using ImageMaster 2D Platinum v7.0 software (GE Healthcare) for spot matching. The spots of interest were manually excised from gels and subjected to destaining and tryptic digestion as described previously (Cacciotto et al., 2010). Peptide mixtures were dried in a Concentrator Plus apparatus (Eppendorf), resuspended in 0.2 % formic acid and then mixed with an equal volume of a-cyano-4-hydroxycynnamic acid as matrix (10 mg/mL in acetonitrile/0.2 % TFA) (70:30, v/v), applied to the metallic sample plate, and air dried. Mass spectra were recorded on a MALDI micro MX (Waters, Manchester, UK) equipped with a reflectron analyser and used in delayed extraction mode; raw data, reported as monoisotopic masses, were then introduced into in-house MASCOT peptide mass fingerprinting search program (Version 2.2, Matrix Science, Boston, MA) and used for protein identification as described previously (Cacciotto et al., 2010). Search parameters were as follows: peptide tolerance 50 ppm, enzyme trypsin.

2.9. Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3. Results

3.1. One dimensional analysis of bacterial liposoluble proteins profiles and host antibody dynamics

The liposoluble protein profiles of isolates F1, F2, and F3 and of the type strain PG2^T are summarized in Fig. 1. Qualitative and quantitative differences were observed. Specifically, the field isolate F2 displayed a profile similar to the PG2^T type strain, consistently with what was previously observed by 2D-DIGE (Cacciotto et al., 2010). The field isolate F3 was considerably different from the type strain and from F2, while it was more similar to F1, even if several differences in protein profiles were observed between the two isolates.

Fig. 2 reports the reactivity of pooled liposoluble protein preparations obtained from the 3 *M. agalactiae* isolates and the PG2^T strain against pooled sheep sera collected at selected infection times. Pooled sera recognized the same bands at the selected time-points, with few variations during infection. The pre-infection pooled sera 0dpi (days post infection) did not show any reactivity by 1D-WB, as expected. Overall, the reactivity of *M. agalactiae* liposoluble protein pools with pooled sera varied over time. Fifteen days after the sheep were moved into the infected flock, only a few faint bands (at about 50 kDa and 15 kDa) could be appreciated by 1D-WB. As the infections progressed,

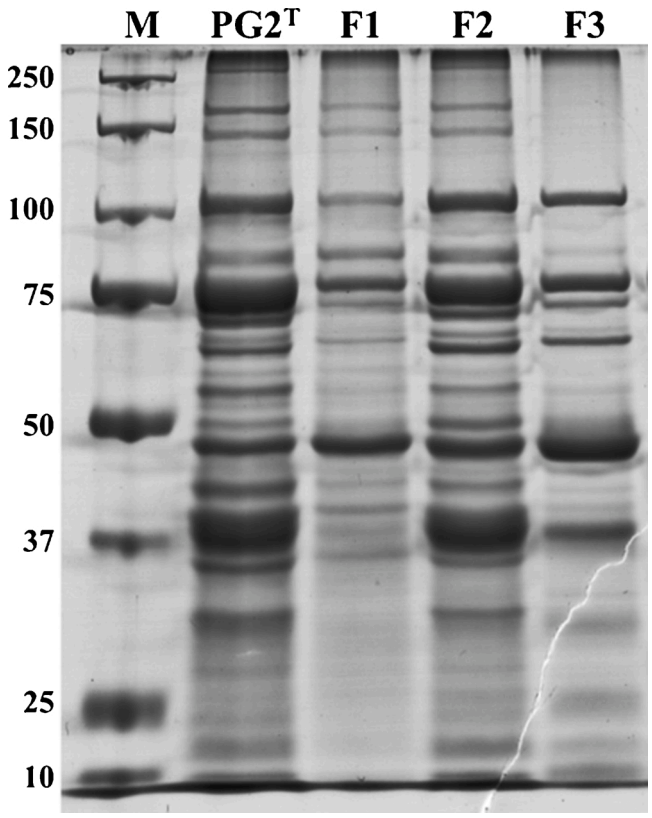

Fig. 1. SDS-PAGE of *M. agalactiae* PG2^T, F1, F2, and F3 liposoluble protein fractions. M: Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad). Molecular weight marker sizes are indicated on the left (kDa).

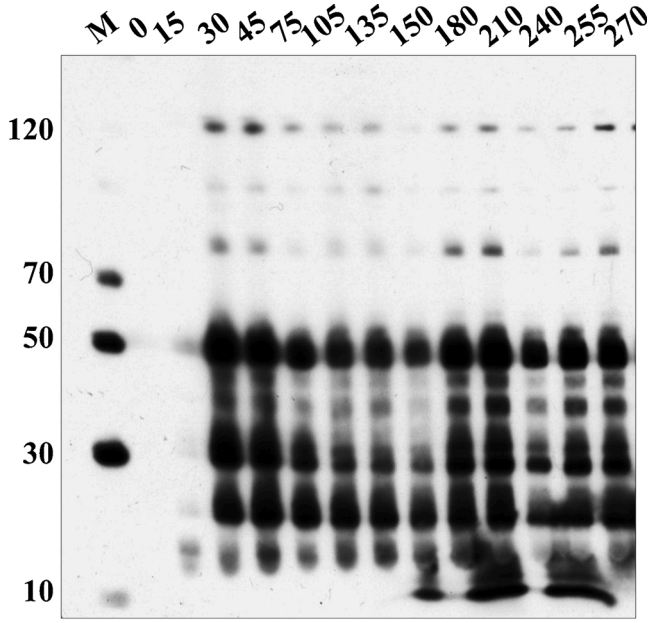

Fig. 2. 1D-WB showing the dynamics of host humoral response during *M. agalactiae* natural infection. A pool of sheep sera collected at representative time points were tested against pooled liposoluble proteins obtained from the 3 *M. agalactiae* isolates analysed and the PG2^T strain. Numbers above lanes indicate the time-points expressed as days post infection (dpi). M: MagicMark™ XP Western Protein Standard (Invitrogen). Molecular weight marker sizes are indicated on the left (kDa).

antibodies against other mycoplasma antigens were appreciable. In detail, after 30 days, a complex pattern was detected. Eight immunoreactive bands ranging from 120 kDa to 15 kDa could be observed, with 3 immunodominant bands approximately weighing 50 kDa, 30 kDa, and 25 kDa (Fig. 2). After 30dpi the immunoreactive band profile did not show significant variations, and it was so up to 270dpi. However, between 75dpi and 150dpi the antibody titre decreased, while a transient increase in signal intensity could be appreciated at 180dpi and 210dpi.

To tentatively identify the antigens, 1D-WB profiles were compared to the SDS-PAGE profile (Fig. 3) previously characterized by GeLC-MS/MS (Cacciotto et al., 2010). However, we failed to match immunoreactive bands with the proteins previously identified with this approach. Indeed, the SDS-PAGE profile comprised 34 main bands, and most contained multiple proteins (Cacciotto et al., 2010), making it impossible to discriminate the contribution of the single proteins within a specific band. Also, immunodominant antigens masked the signals produced by weaker immunogens with similar MW.

3.2. D-PAGE and 2D-western immunoblotting analysis of M. agalactiae liposoluble proteins

To overcome the failures related to the 1D-approach, two-dimensional western immunoblotting (2D-WB) immunoproteomics experiments were performed. Fig. 4 illustrates the 2D-WB pattern obtained when testing *M. agalactiae* liposoluble pooled proteins against pooled sheep sera collected at 30, 150, 210, and 270dpi.

2D-WB produced highly reproducible profiles, efficiently comparable to the 2D-PAGE maps, enabling spot excision and identification by MALDI-TOF-MS. This led to the identification of 15 immunogenic proteins, all represented in trains of spots with the same MW but different pI, probably indicating post-translational modifications (Fig. 5). Protein IDs, pI, MW, identification scores, and predicted subcellular localization are listed in Table 1. Among the 15 identified antigens, 9 corresponded to known *M. agalactiae* immunogens (P80, P48, P40, and all the Vpmas) already identified in previous works (Chopra-Dewasthaly et al., 2008; Fleury et al., 2002; Rosati et al., 1999; Tola et al., 2001). Interestingly, we identified 6 *M. agalactiae* proteins (MAG_1000, MAG_2220, MAG_1980, phnD, MAG_4740, and MAG_2430) as antigens for the first time. All the identified immunogenic proteins were classified as "lipoproteins" based on predicted signal-peptidase cleavage sites.

The comparison of 1D-WBs and 2D-WBs confirmed the presence of multiple proteins with similar MW with a potential contribution to a 1D signal (Fig. 6). Indeed, the 2D resolution of proteins uncovered the contribution of different proteins to the positive signal located in the same 1D band. On the one hand, the immunoreactive bands at about 110 and 80 kDa seen in 1D-WB produced single trains of spots when 2D methods were applied, corresponding to MAG_1000 and P80, respectively. On the other hand, immunoreactive bands comprised between 50 and 20 kDa, when resolved in 2D-WB revealed the presence of different proteins contributing to the 1D-WB signal. More specifically, 3 different proteins were identified as antigens at about 50 kDa (phnD, P48, and MAG_1980) and 40 kDa (P40, VpmaY, and VpmaW), while the band at about 30 kDa contained at least 2 immunoreactive proteins (VpmaD and MAG_2430).

4. Discussion

The success of measures and strategies to control infectious diseases strictly depends on establishing reliable high-throughput diagnostic and prophylactic tools. Despite the identification of a limited number of antigens and their use in developing recombinant diagnostic indirect tests, the control of contagious agalactia remains a challenge in endemic regions around the world. One of the main issues in developing high-performance diagnostic and prophylactic tools for CA is identifying an adequate set of antigenic proteins, conserved among different field strains and constantly expressed during infection.

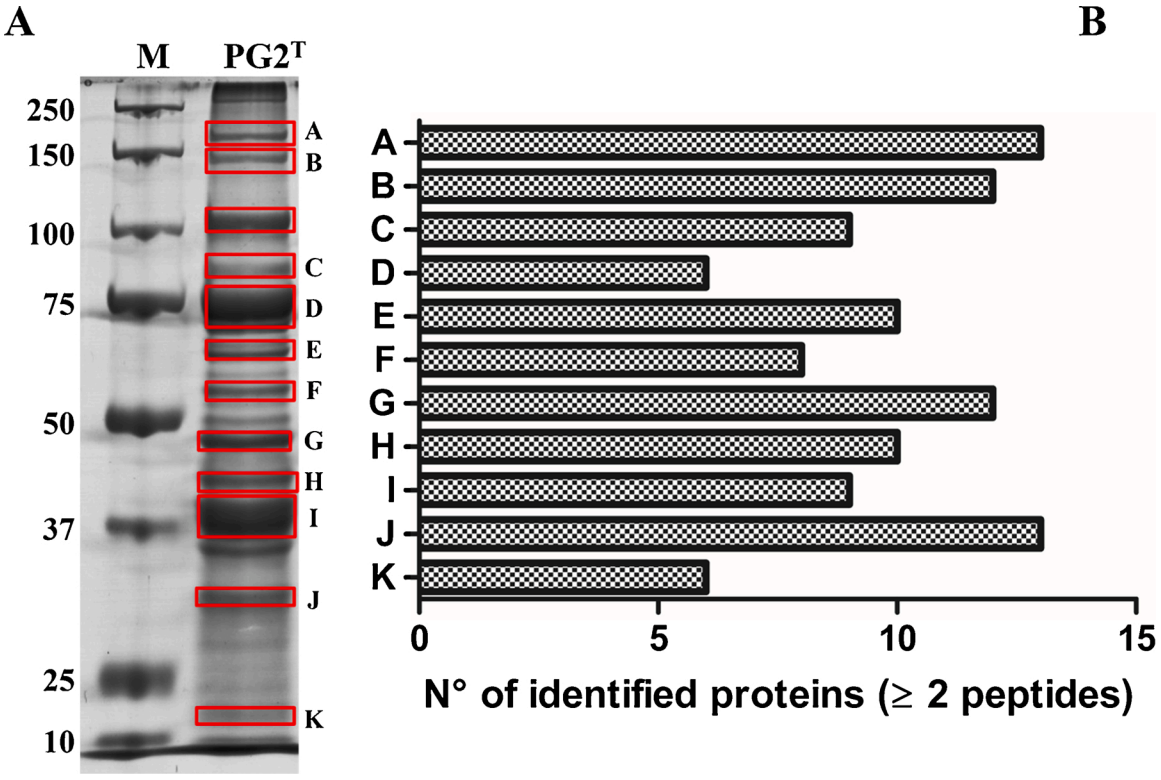

Fig. 3. Number of proteins identified in representative bands of the *M. agalactiae* PG2^T liposoluble proteins profile. A: SDS-PAGE of the *M. agalactiae* type strain liposoluble proteins (PG2^T); Molecular weight marker sizes are indicated on the left (kDa). B: number of proteins identified in selected bands marked with red rectangle in A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

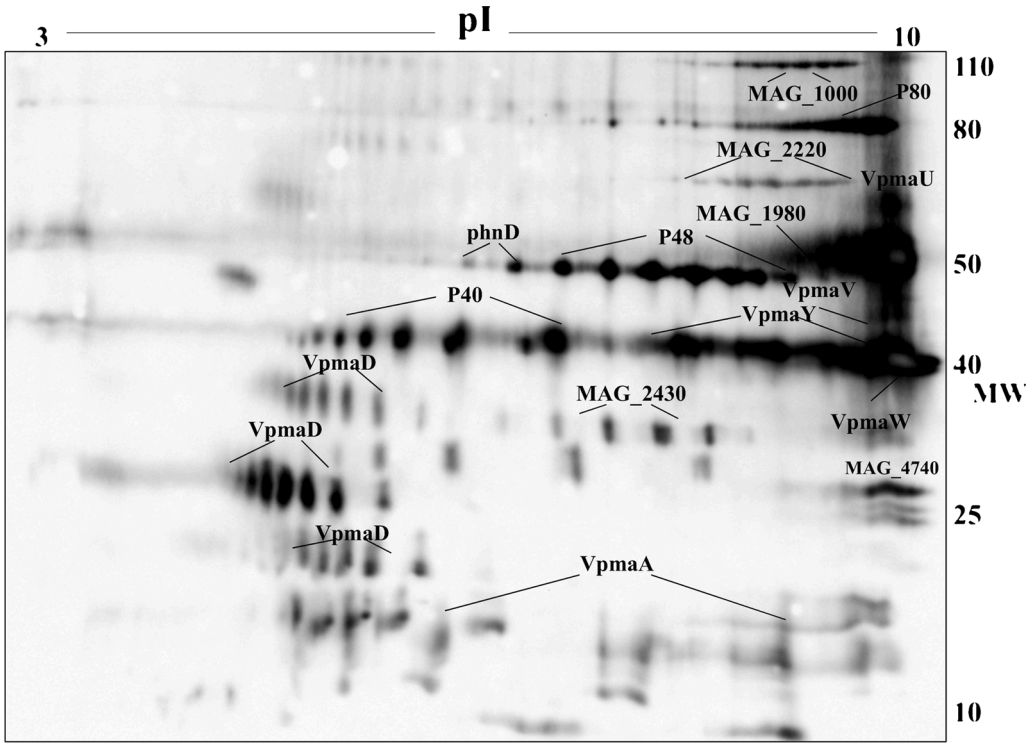

Fig. 4. 2D-WB. Reactivity of pooled sera collected from naturally infected sheep against pooled liposoluble *M. agalactiae* proteins belonging to the 4 different strains. pI: isoelectric point; MW: molecular weight (kDa).

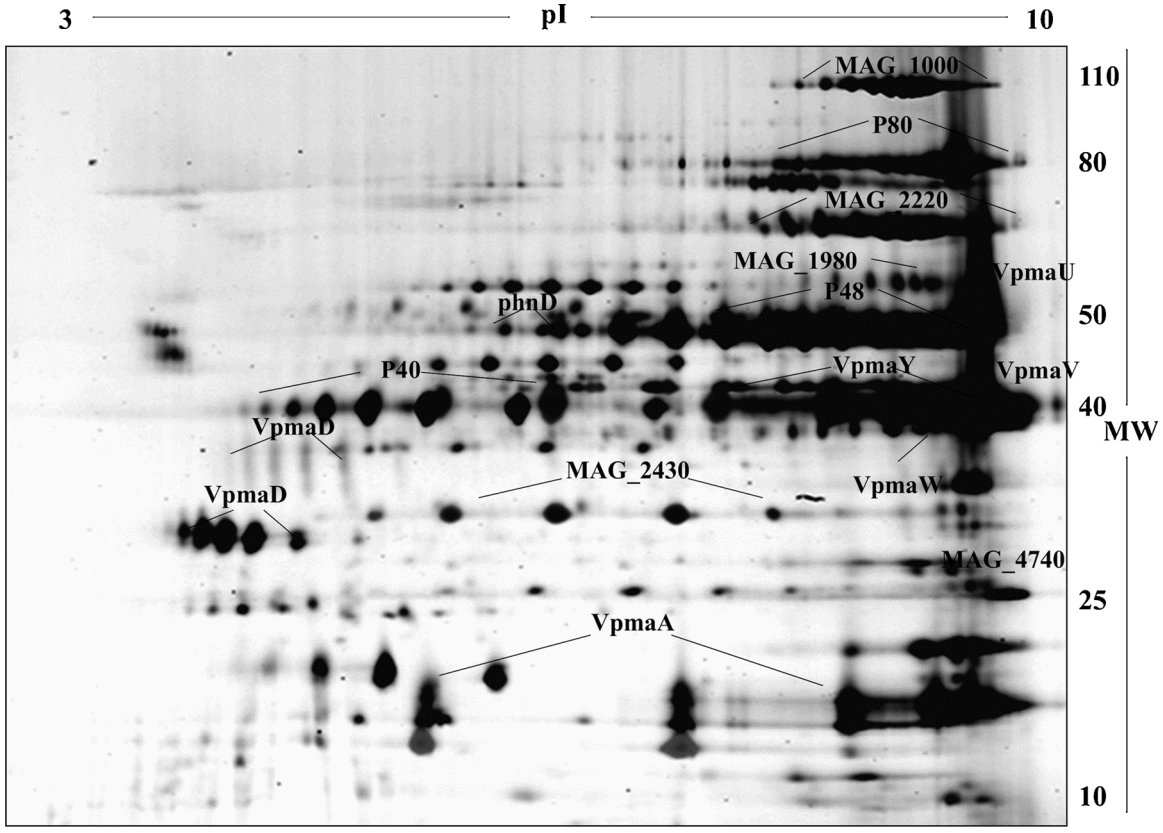

Fig. 5. 2D-PAGE of pooled *M. agalactiae* lipoproteins belonging to 4 different strains. pI: isoelectric point; MW: molecular weight (kDa).

Table 1
Protein IDs, locus, MS scores, function, and predicted cellular location of *M. agalactiae* antigens identified by 2-D PAGE/MS.

Protein name	Old locus tag	New locus tag	Ncbi protein id	MW (kDa)	pI	Mascot score	Queries	Peptide match (sequence coverage, %)	Function	Subcellular localization
Hypotetical protein MAG_1000	MAG_1000	MAG_RS00535	WP_011949281.1	109.73	8.66	155	21	25%	ABC transporter	Lipoprotein
P80	MAG_5030	MAG_RS02550	WP_011949669.1	81.02	9.12	219	23	35%	ABC transporter	Lipoprotein
Hypothetical lipoprotein MAG_2220	MAG_2220	MAG_RS01155	WP_011949401.1	69.57	8.78	149	13	33%		Lipoprotein
Variable surface lipoprotein U	MAG_7090	MAG_RS03610	WP_011949862.1	46.99	9.26	56	2	4%		Lipoprotein
Lipoprotein, MAG_1980	MAG_1980	MAG_RS00995	WP_011949377.1	53.79	8.98	73	9	23%		Lipoprotein
P48	MAG_0120	MAG_RS00075	WP_011949195.1	51.23	7.19	137	11	33%	ABC transporter	Lipoprotein
Alkylphosphonate ABC transporter substrate-binding protein	MAG_2690	MAG_RS01410	WP_011949445.1	49.74	6.99	95	9	22%	ABC transporter	Lipoprotein
P40	MAG_2410	MAG_RS01280	WP_011949418.1	37.10	8.20	141	11	37%		Lipoprotein
Variable surface lipoprotein Y	MAG_7080			37.53	8.81	142	13	38%		Lipoprotein
Variable surface lipoprotein V	MAG_7050			37.36	9.25	108	7	14%		Lipoprotein
Variable surface lipoprotein W	MAG_7060			35.47	9.54	127	10	30%		Lipoprotein
Lipoprotein MAG_4740	MAG_4740	MAG_RS02415	WP_011949643.1	25.45	9.47	71	8	37%		Lipoprotein
Variable surface lipoprotein D	MAG_7100	MAG_RS03615	WP_011949863.1	36.61	9.53	77	8	21%		Lipoprotein
Variable surface lipoprotein A	MAG_7070	MAG_RS03595	WP_011949860.1	24.77	8.33	85	5			Lipoprotein
Lipoprotein, MAG_2430	MAG_2430	MAG_RS01295	WP_011949420.1	33.86	8.29	120	10	36%		Lipoprotein

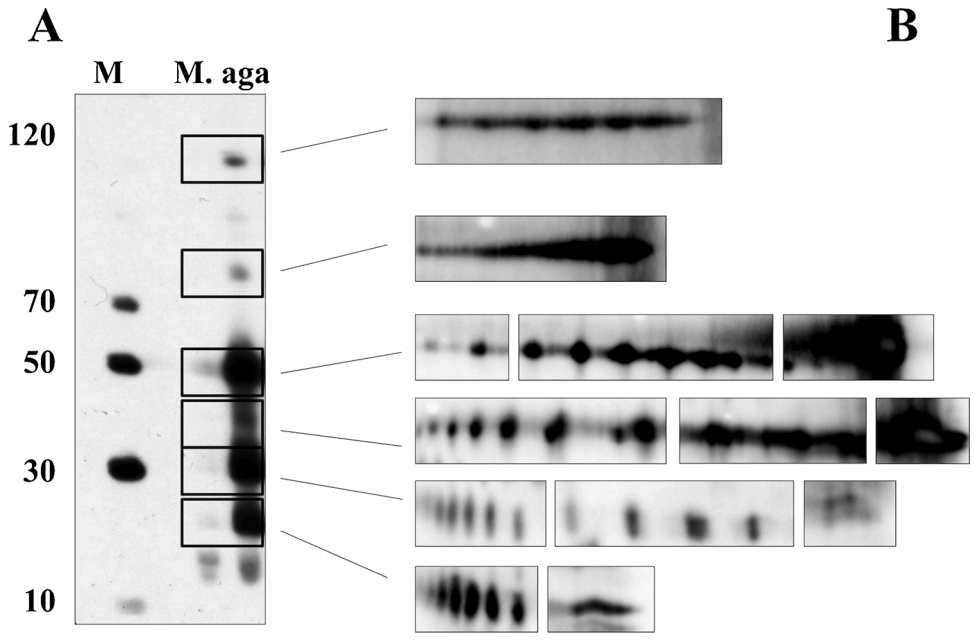

Fig. 6. 1D-WB (A) and 2D-WB (B) of pooled *M. agalactiae* lipoproteins belonging to 4 different strains tested with a pool of sera collected from naturally infected sheep (M. aga). M: Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad). Molecular weight marker sizes are indicated on the left (kDa).

Since mycoplasmas do not have a cell wall, their membrane proteins play a crucial role in pathogenesis by mediating interaction with the host cells and are often conserved and constantly expressed among different isolates (Alberti et al., 2008, 2006; Cacciotto et al., 2010). Lipoproteins represent a large proportion of the mycoplasma surface proteome and are involved in a number of functions, such as adhesions to host cells, nutrients uptake, immunomodulation, and induction and digestion of NETs (Browning et al., 2011; Cacciotto et al., 2013; Sharma et al., 2015; Cacciotto et al., 2016, 2019).

In a previous study, our group characterized the *M. agalactiae* membrane proteome by 2-D PAGE/MS and GeLC/MS—MS and systematically identified proteins contained in the liposoluble fraction (Cacciotto et al., 2010). Moreover, in the same paper, we compared the F2 and F3 field isolates (Bortigali and Nurri, respectively) employing 2D-DIGE, identifying conserved and differentially expressed proteins among these isolates and the type strain. Here, we applied a 2-D gel-based immunoproteomic approach to shed light on their immunogenic properties, and by comparison with the previous study, we identified antigens conserved among strains. Compared to more innovative methods, such as shotgun proteomics, 2-D gel-based methods have the

advantage of providing additional information for the characterization of the host immune response by allowing separation and visualization of proteoforms with different sizes and charges (Dwivedi et al., 2016).

Therefore, this approach allowed us to establish the contribution to the *M. agalactiae* immunoproteome of selected isoforms sharing similar MW but having different isoelectric points. Among the identified proteins, several already described antigens, such as P80, P48, P40, and the Vpmas, were confirmed to play a role in the specific host immune response.

P80 is a lipoprotein of about 81 kDa identified by Tola and collaborators and used to develop a diagnostic ELISA (Fusco et al., 2007; Tola et al., 2001); moreover, it was detected by 2-PAGE and GeLC-MS/MS analysis in different *M. agalactiae* field strains (Cacciotto et al., 2010). Here, the reactivity of P80 with sera collected from naturally infected sheep along the course of infection is reported for the first time. Interestingly, while P80 produced a signal weaker than expected by 1D-WB, it appeared immunodominant in 2D-WB. This difference may be due to band intensity flattening upon CCD camera optimization in 1D-WB.

P48 is an already established immunodominant antigen, and a P48-based ELISA has been developed and commercialized (Rosati et al., 2000, 1999). Also, the *p48* gene was used to develop a prototype DNA vaccine for CA (Chessa et al., 2009). Similar to P80, P48 was identified in the surface proteome of *M. agalactiae* by 2-D PAGE and GeLC-MS/MS (Cacciotto et al., 2010). Here, we highlight that P48 is recognized very early upon infection, as demonstrated by the appearance of a highly immunoreactive band in 1D-WB and spots in 2D-WB, at about 50 kDa. However, it should be noticed that P48 is not the only protein present at 48 kDa (at least 9 different proteins are present according to MS), and that 2D-WB allowed to establish its contribution to immunogenicity, which could not be extrapolated by 1D-WB only. Therefore, we recommend the use of 2D-WB to clearly identify the contribution of single proteins to immunogenicity, which could be misinterpreted by 1D studies. The same applies to other SDS-PAGE bands, where multiple proteins with similar MW co-migrate and can be identified by MS.

P40 is a protein already described in the literature as an antigenic lipoprotein involved in *M. agalactiae* adhesion to the host cells (Fleury et al., 2001). P40 has a MW of about 37 kDa and was detected in different bands by GeLC-MS/MS (Cacciotto et al., 2010). We confirm the antigenic nature of P40 by 2D-WB, as P40 was efficiently recognized by pooled sera collected from infected sheep.

All the 5 variable membrane lipoproteins (Vpma) were confirmed as immunodominant antigens. VpmaU, VpmaY, VpmaV, and VpmaW are present in spot trains with the same MW and different pI, while VpmaD and VpmaA also exhibit size variations. Notably, although the lower MW proteoforms of VpmaA and VpmaD show an appreciable 2D-WB signal, the corresponding 2-D PAGE spots are faint, suggesting a low expression level. As already hypothesized, we observe quantitative differences in the expression of the different isoforms of each Vpma in the different field isolates and in the reference strain under laboratory conditions. Notably, all the isoforms are recognized by naturally infected sheep sera, indicating the presence of conserved domains. Vpmas are known to play a crucial role in *M. agalactiae* infection, facilitating evasion from host immune response by creating a highly variable antigenic shield. Furthermore, Vpmas are involved in cell adhesion and invasion (Chopra-Dewasthaly et al., 2008; Hegde et al., 2018). However, due to their extremely high variability, Vpmas are not good candidates for diagnostic tool development.

Notably, we identified here 6 novel *M. agalactiae* antigens (MAG_1000, MAG_2220, MAG_1980, phnD, MAG_4740, and MAG_2430), and we demonstrated their *in vivo* expression at different stages of natural infection. All these newly identified antigens were previously annotated as “hypothetical proteins”, and their *in vitro* expression was already demonstrated (Cacciotto et al., 2010). MAG_1000 showed the highest MW and was easily identified by 1D-WB and SDS-PAGE as a well-defined band at 110 kDa. Since no other proteins could be appreciated by 2-D PAGE at the same MW, and considering that MS analysis demonstrated that MAG_1000 is the most abundant protein at 110 kDa (Supplementary table), we speculate that the 1D-WB signal is fully due to this protein. Considering the persistence of antibodies reacting against MAG_1000 up to 270dpi, MAG_1000 represents an ideal candidate for developing dedicated diagnostic and prophylactic tools.

PhnD is the substrate-binding protein of an alkyl phosphonate ABC transporter (Sirand-Pugnet et al., 2007) identified by GeLC-MS/MS as a protein weighing about 50 kDa (Supplementary table). 1D-WB failed to identify phnD as an antigen due to the presence of several proteins with the same MW, such as P48. However, the antigenicity of phnD was revealed by 2D-WB, by resolving P48 and phnD into distinct immunoreactive spots.

Among the identified antigens, at least 4 out of 15 proteins (P80, P48, MAG_1000, and phnD) are putative substrate-binding proteins, according to protein homology and to their location upstream of ABC transporters (Cacciotto et al., 2010; Rosati et al., 1999; Sirand-Pugnet et al., 2007). A possible role as components of ABC transporter has been suggested for P80 and P48 (Fusco et al., 2007; Rosati et al., 2000, 1999).

We failed to identify by 2D-WB MAG_5040, a *M. agalactiae* antigen previously described in the literature (Cacciotto et al., 2013). However, MAG_5040 is expressed at very low levels *in vitro*, and its 2D spot may have been hidden by the signal produced by VpmaY, an abundant protein with a very high signal in 2D-WB.

Despite its limitations, the usefulness of 2D- based methods in identifying antigenic proteins is not questioned, and systematic 2D-based studies should be integrated with a top-down approach to identify single antigens expressed at low levels. This latter approach, which carries several drawbacks due to the different codon-utilization of mycoplasmas hampering protein expression, is advantageous for characterizing single antigens but is not recommended for systematic studies.

Based on data previously obtained by 2D-DIGE (Cacciotto et al., 2010) P48, P80, P40, MAG_1000, phnD, MAG_2220, MAG_1980, and MAG_4740 are expressed in all studied isolates and are therefore putative conserved antigens and recommended candidates for the development of dedicated diagnostic and prophylactic tools. In turn, Vmpas are the most variable antigens, with variations in size and phase and therefore not constantly expressed in all isolates. The same applies to MAG_2430 lipoprotein, which expression is absent in the F3 strain.

Concluding, this work represents the first attempt towards a systematic description of the complex antigenic mosaic of *M. agalactiae* by using a 2D-immunoproteomic approach, overcoming the previous 1D-based attempts (Fusco et al., 2007). Future studies based on more sensitive comparative proteomic approaches and including a higher number of strains are recommended to strengthen our findings.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. This study was approved by the Ethics Committee of the University of Sassari. Blood samples were collected by a veterinary practitioner authorized by the National Health System, after obtaining permission from the sheep owner. Animals where moved and transported by the shepherd during routine management of the flock in accordance with D.P.R. 8 Febbraio 1954, n. 320.

Declaration of Competing Interest

The authors have no conflicts of interest that are directly relevant to the content of this article.

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Appendix A. Supplementary data

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