Selection of a monoclonal antibody by ELISA, immunoblotting and Quartz Crystal Microbalance technology for immunohistochemical detection of Mycoplasma mycoides *subsp.* mycoides

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Summary
An immunohistochemical (IHC) technique was optimised using a monoclonal antibody (MAb) to detect *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*), the agent of Contagious Bovine Pleuropneumonia (CBPP), in sections of lung tissue. A panel of MAbs was produced and screened for *Mmm* specificity and for cross-reactivity against other mycoplasmas belonging and not belonging to the *Mycoplasma mycoides* cluster, using in parallel indirect ELISA (i-ELISA) and Immunoblotting (IB). Based on i-ELISA and IB characterization data, 1 MAb (clone 3G10E7) was selected and its highest affinity vs *Mmm* was confirmed by the Quartz Crystal Microbalance (QCM) technology. Afterwards, IHC analyses were conducted to compare MAb 3G10E7 vs rabbit *Mmm* specific hyperimmune serum using lung tissue sections of CBPP infected and CBPP negative animals. Results suggest that screening of MAbs using in parallel ELISA, IB, and QCM technology enables to select high affinity target specific MAbs. Immunohistochemical results demonstrated that MAb 3G10E7 improved IHC performances, showing reduced background staining and no cross-reactivity against *Mycoplasma bovis*, which is responsible of pneumonia in cattle.

Selezione mediante ELISA, immunoblotting e microbilancia a cristalli di quarzo di un anticorpo monoclonale e suo utilizzo in immunoistochimica per la ricerca di Mycoplasma mycoides *subsp.* mycoides

Parole chiave
Anticorpi monoclonali,
Biosensori,
Immunoistochimica,
Mycoplasma mycoides subsp. *mycoides*,
Microbilancia a cristalli di quarzo,
Pleuropolmonite contagiosa bovina.

Riassunto
In questo lavoro è stata ottimizzata una metodica immunoistochimica (IIC) per la ricerca, in sezioni di tessuto polmonare, di *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*), l'agente della pleuropolmonite contagiosa bovina (PPCB), utilizzando un anticorpo monoclonale (MAb). Un pannello di MAbs è stato prodotto e caratterizzato; la specificità dei MAbs verso *Mmm* e la cross-reattività verso altri micoplasmi appartenenti e non appartenenti al *Mycoplasma mycoides* cluster sono state testate utilizzando in parallelo l'ELISA indiretta (i-ELISA) e l'immunoblotting (IB). Dai risultati ottenuti in i-ELISA e IB, un MAb (clone 3G10E7) è stato selezionato e l'elevata affinità verso *Mmm* è stata confermata mediante la tecnica della microbilancia a cristalli di quarzo (MCQ). Successivamente, sono state effettuate analisi immunoistochimiche per comparare il MAb 3G10E7 e un siero iperimmune di coniglio specifico per *Mmm* utilizzando sezioni di tessuto polmonare di animali positivi per PPCB e di animali negativi. I risultati ottenuti suggeriscono che lo screening dei MAbs, effettuato utilizzando in parallelo l'ELISA, l'immunoblotting e la tecnologia MCQ, permette di selezionare un MAbs con alta affinità e specificità per l'antigene di interesse. I risultati dell'immunoistochimica hanno dimostrato che il MAb 3G10E7 migliora le prestazioni del metodo IIC, fornisce segnali di fondo più bassi e non presenta cross-reazioni verso *Mycoplasma bovis*, responsabile di polmonite nei bovini.

Introduction

Contagious Bovine Pleuropneumonia (CBPP) is a serious respiratory disease affecting ruminants of the *Bos* genus, mainly cattle and domestic buffaloes. Contagious Bovine Pleuropneumonia is caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) and is characterised by severe exudative inflammation involving lung and pleura. In acute cases, animals show respiratory distress with coughing and high fever; disease progression may result in animal death. A severe and usually monolateral fibrinous pleuropneumonia, with marbling and enlargement of interlobular septa, is observed at *post mortem* examination. In CBPP endemic areas, subacute or chronic forms and lung sequestra, mainly characterised by areas of necrotic parenchima surrounded by fibrotic tissue, are commonly detected (Provost *et al.* 1987, OIE 2014).

Since rinderpest has been eradicated, CBPP represents the most important threat to cattle farming in sub-Saharian Africa. The economic impact is related to direct and indirect production losses and costs for the control of the disease (Tambi *et al.* 2006). Contagious Bovine Pleuropneumonia is included in the diseases notifiable to the World Organization of Animal Health (OIE); infected countries or zones are subjected to live animal export restrictions, as indicated in the OIE Terrestrial Animal Health Code (OIE 2014).

Between the 80's and early the 90's, CBPP outbreaks have occurred in Southern Europe, involving Portugal, Spain, France, and Italy. Disease was eradicated from Europe by 1999, through a policy of 'stamping out' of infected herds, animal movement restriction, and traceback. Surveillance was based on serological testing and *post mortem* inspection of slaughtered animals (Regalla *et al.* 1996, Giovannini *et al.* 2000).

Laboratory diagnosis of CBPP is based on indirect tests such as the complement fixation test (CFT), ELISA, and immunoblotting (IB), as well as direct methods such as culture and polymerase chain reaction (PCR) (OIE 2014). Even if not routinely used to diagnose CBPP, immunohistochemical (IHC) technique remained for long time among the diagnostic tests proposed to demonstrate the presence of *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) or its antigens (OIE 2008). In the 2001 Report of the EU Scientific Committee on Animal Health and Animal Welfare (European Commission 2001), PCR and IHC were considered tests of choice in presence of carcasses with suspect lesions, when serum was not available and mycoplasma culture from lung tissue was inconclusive because of the poor conditions of the carcass or for logistic difficulties.

Immunohistochemical technique has also been used to correlate histological lesions recorded in lung and kidney with the presence of *Mmm* (Ferronha *et al.* 1990, Rodriguez *et al.* 1996, Scanziani *et al.* 1997, Grieco *et al.* 2001). To date, molecular techniques (conventional PCR, PCR-REA and nested-PCR) are largely preferred to IHC for routine diagnosis. However, pathogenic investigations still rely on IHC, which enables to correlate the presence of the antigen with tissue pathological changes. One of the major limitations in development and optimization of IHC methods is the lack of highly antigen specific reagents (Rodriguez *et al.* 1996). In the past, IHC was largely based on the use of polyclonal antibodies, which pose major drawbacks as they are limited in availability. Animals are continuously required for antibody production, and there is a considerable batch to batch variability that affects test standardization. At the same time, in presence of lung tissue sections characterised by great quantity of fibrin, the use of rabbit hyperimmune polyclonal serum (RHS) still provides results of difficult interpretation because of high background staining due to unspecific binding to components of the fibrinous matrix. Non-specific staining has also been observed in lungs showing CBPP-like lesions (sequestra) and characterised by a subacute-chronic necrotizing fibrinous pneumonia, caused by *M. bovis* or by a combined infection of *M. bovis* and *Mannheimia haemolitica* (Radaelli *et al.* 2008).

Conversely, use of monoclonal antibodies (MAbs) guarantees large quantities of identical antibodies, ensuring reproducible results. Rabbit hyperimmune polyclonal serum was originally combined with the revealing system Peroxidase-Anti Peroxidase in order to detect *Mmm* by IHC (Bashiruddin *et al.* 1999). The technique has been further implemented using the Avidin-Biotin system (D'Angelo *et al.* 2010).

The MAb M92/20 produced by Ayling and colleagues, showing no background noise but some cross-reactivity with mycoplasmas belonging to the cluster, was used to test 11 CBPP affected lungs from Portuguese cattle. The immunohistochemical technique detected all, while the polymerase chain reaction and bacteriological culture detected 5 and 4 cases, respectively (Ayling *et al.* 1998). Though the sample size was small, it illustrated that IHC is a sensitive and robust test for CBPP diagnostic.

The aim of this study was the production of MAbs specific for *Mmm*, to optimize immunohistochemical detection of *Mmm* in lung tissue sections. Selection of MAbs was made by i-ELISA and IB combined with the innovative Quartz Crystal Microbalance (QCM) technology to assess MAb-antigen binding affinity. Quartz Crystal Microbalance is a sensitive balance capable of measuring changes in mass at a molecular level: molecules that bind to the quartz crystal increase the mass and cause a change in the vibration frequency, that is measured and used to characterise real-time molecular interactions, without labeling or chemical modifications (Marx 2003, Johansson 2010, Vashist and Vashist 2011).

Materials and methods

Mycoplasma strains and antigens

Reference and field strains of the *Mycoplasma mycoides* cluster (*M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri* - *Mmc* -, *M. capricolum* subsp. *capripneumoniae* - *Mccp* -, *M. capricolum* subsp. *capricolum* - *Mcc* -, *M. leachii*) together with strains of *M. agalactiae* and *M. bovis* were grown from master seeds, stored at -20°C at the OIE CBPP Reference Laboratory of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' of Teramo (IZSAM) (Table I). After rapid thawing at 37°C, 1 ml of each master seed strain was inoculated into Pleuropneumonia-Like Organisms (PPLO) medium (Becton-Dickinson, Franklin Lakes, New Jersey, USA) and incubated from 2 to 4 days at 37°C. *Mycoplasma* cells were collected by centrifugation (9,000 × g for 40 minutes) and the pellets washed 3 times in 0.01 M phosphate buffered saline, pH 7.2 (PBS). After the last centrifugation, pellets were resuspended in PBS, heat-inactivated, and protein concentrations determined by the bicinchoninic acid method (BCA Protein Assay Kit, Thermo Scientific, Illinois, USA).

Immunization of mice

For the production of MAbs, BALB/c mice of 6/8 weeks of age were inoculated with *Mmm* strain 95 heat inactivated. Animal experimentation was carried out in compliance with Italian national law[1] implementing Directive 86/609/EEC of the Council of the European Communities on the protection of animals used for experimental and other scientific purposes[2]. The antigen, diluted to a protein concentration of 80 µg/ml, was emulsified with complete Freund adjuvant (CFA, Sigma, St. Louis, Missouri, USA) and administered intraperitoneally; 21 days later a second immunization was performed using the same concentration of antigen emulsified with incomplete Freund adjuvant (IFA, Sigma, St. Louis, Missouri, USA). Subsequently, on days 27, 30, and 54, inocula had a protein concentration of 80, 40, and 50 µg/ml in PBS, respectively. Finally, on day 67, 80 µg/ml of antigen in PBS was given. Three days later, the mice were sacrificed, the spleen collected and splenocytes subjected to cell fusion with murine myeloma cells Sp2/O-Ag-14 (ATCC CRL-1581™). The antibody-secreting hybridomas were screened by

[1] Decreto legislativo 27 gennaio 1992, n. 116. Attuazione della direttiva n. 86/609/CEE in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici. *Off J*, **40**, 18/02/1992.

[2] European Commission (EC) 1986. Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). *Off J*, **L 358**, 18/12/1986, 1-28.

Table I. Mycoplasma *strains used in the study.*

Mycoplasma strains		Country of origin	Year of isolation	Host	References
M. mycoides subsp. *mycoides* (*Mmm*)	95	Italy	1992	**Cattle**	Goncalves *et al.* 1998
	PG1	Africa	1931	Cattle	Cheng *et al.* 1995
	Afadé	Cameroon	1968	Cattle	Cheng *et al.* 1995
	B17	Chad	1967	Cattle	Cheng *et al.* 1995
	57/13	Italy	1992	Cattle	Goncalves *et al.* 1998
	T1/44	Tanzania	1952	Vaccine strain	Yaya *et al.* 2004
M. mycoides subsp. *capri* (*Mmc*)	NCTC10137	-	1950	Goat	-
	NCTC 11706	-	1955	Goat	-
	2269	Italy	2010	Goat	-
M. capricolum subsp. *capripneumoniae* (*Mccp*)	NCTC 10192 (F38)	-	1985	Goat	-
M. capricolum subsp. *capricolum* (*Mcc*)	NCTC 10154 (California kid)	-	1954	Goat	-
	W/17	Turkey	2006	Goat	-
M. leachii PG50	NCTC 10133	-	1970	Cattle	-
M. agalactiae	NCTC 10123	UK	1955	Goat	-
M. bovis	9573/08	Italy	2008	Cattle	-
	5810/07	Italy	2007	Cattle	-
	11003/08	Italy	2008	Cattle	-

i-ELISA vs *Mmm* strain 95 and the positive ones were cloned by the limiting dilution method (Luciani *et al.* 2006). Monoclonal antibodies were produced *in vitro* on a large scale by means of serial cultures of hybridomas and collection of the supernatants.

Characterization of MAbs vs *Mmm*

Monoclonal antibodies were isotyped using the Mouse-Typer Isotyping Panel (Bio-Rad, Hercules, California, USA). Cross-reaction of selected MAbs against a panel of mycoplasmas was assessed by i-ELISA. Briefly, 96-well microplates (PolySorp, NUNC, Roskilde, DK) were coated with 10 µg/ml of the different mycoplasma antigens in order to test hybridoma supernatants. As secondary antibody, ECL anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare, Uppsala, Sweden) was used; the 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma, St. Louis, Missouri, USA) was adopted as chromogenic substrate. Reading was performed with a biophotometer (Bio-Rad, Hercules, California, USA) at a wavelength of 450 nm. Based on the values of optical density (OD_{450nm}), the following levels of positivity were assigned: + + + strong ($OD_{450nm} \geq 2.5$), + + moderate ($2.5 \geq OD_{450nm} \geq 1.5$), + weak ($1.5 \geq OD_{450nm} \geq 0.3$), - absent ($OD_{450nm} \leq 0.3$). Monoclonal antibodies that were most reactive in i-ELISA against the strains of the *mycoides* cluster and not reactive vs strains not belonging to the cluster, were characterised by IB test. For IB analysis, MAbs with IgM isotype were purified on affinity chromatography using a HiTrap IgM Purification HP column (GE Healthcare, Uppsala, Sweden). Only 1 of the MAbs, with isotype IgG2a, was purified by a column HiTrap rProtein A FF (GE Healthcare, Uppsala, Sweden) according to the manufacturer instructions.

The different mycoplasma antigens (2.5 µg/well) were subjected to electrophoretic separation in SDS-PAGE with NuPAGE 10% Bis-Tris Gels Mini (Life Technologies, Carlsbad, California, USA) and transferred onto nitrocellulose membrane with iBlot Dry Blotting System (Life Technologies, Carlsbad, California, USA). After blocking with 5% skimmed milk in 0.01 M phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST), membranes were incubated with the purified MAbs. The detection of immune complexes was performed using the ECL anti-mouse IgG conjugated with horseradish peroxidase (GE Healthcare, Uppsala, SW) and a chemiluminescent substrate (ECL Select Western Blotting Detection Reagent, GE Healthcare, Uppsala, SW). The analysis of the results was performed using the Quantity One Quantitation Software version 4.3 (Bio-Rad, Hercules, California, USA). Monoclonal antibodies showing 1 or more immunoreactive bands for each mycoplasma strain were considered positive for that particular antigen. The results of the i-ELISA and IB were analysed by performing the 2 tests in parallel. The MAbs anti-*Mmm* were selected according to the following criteria: positivity to both i-ELISA and IB for all the analysed *Mmm* strains, maximum intensity of the ELISA reaction to *Mmm*, absence of cross-reaction, in i-ELISA and IB, against all the other mycoplasmas object of the study and different from *Mmm* and *Mmc*.

Measurement of MAb/antigen binding affinity by QCM

Based on MAb characterization analyses, MAb 3G10E7 was selected for preliminary studies of antigenic affinity conducted with the Attana Cell 200 Biosensor (Attana, Stockholm, Sweden), able to measure the molecular interactions in real time using QCM technology. The MAb 3G10E7 was immobilised on Attana LNB carboxyl chip by the Amine Coupling Kit (Attana, Stockholm, Sweden): after activation of the carboxyl groups, the MAb 3G10E7 (diluted to a concentration of 25 µg/ml in 10 mM HEPES Buffer Saline containing 0.05% Tween 20, running buffer) was injected, at a flow rate of 10 µl/min, in the Channel A of the instrument. Then, the remaining active groups were deactivated. The surface of the Channel B was activated and deactivated in the absence of MAb 3G10E7, in order to highlight the presence of any non-specific binding with a negative reference chip; *Mmm* strain 95 (positive control), *Mcc* California kid, *Mccp* F38 and *M. bovis* 9573/08 (negative controls) were diluted in the running buffer at a concentration of 50 µg/ml and injected in channel A (activated chip) and in channel B (reference negative chip) at a flow rate of 5 µl/ml. The experiment was performed in triplicate and the affinity values were quantified in terms of frequency variation (Hz). After each antigen injection, chips surface was regenerated using 50 mM NaOH for 10 seconds. The data generated for each experiment were collected by Attester 3.1 software and analyzed with Clamp XP software.

Immunohistochemistry

The IHC was performed on histological sections of 37 paraffin-embedded lung tissue blocks obtained from 20 animals experimentally infected with *Mmm*, showing typical CBPP pathological lesions confirmed by the isolation of *Mmm* (Scacchia *et al.* 2007).

Tissue sections of paraffin-embedded lungs collected from 6 healthy animals were used as negative controls; presence of *Mmm* was excluded by bacteriological culture and PCR analyses (Bashiruddin *et al.* 1999). Histological sections of 5 paraffin-embedded lung tissue blocks, collected from an animal infected with *M. bovis*, were also included in the present study. Pneumonia caused by *M. bovis* and *Mannheimia haemolytica* had been confirmed by culture and PCR (Bashiruddin *et al.* 2005). Either RHS anti *Mmm* strain B17, produced at IZSAM (Ferronha *et al.* 1988, Santini *et al.* 1992), or the MAb 3G10E7 supernatant were used as primary antibodies in the IHC method, after antigen retrieval by enzymatic digestion with trypsin 0.01% in 0.15 M Tris buffered solution, pH 7.8 at 37°C. The RHS and the MAb were diluted 1:2,560 and 1:10 in 0.15 M Tris buffered solution, pH 7.6, containing 1% bovine serum albumin (BSA, Sigma, St. Louis, Missouri, USA), respectively. A rabbit serum negative for *Mmm* and Dulbecco Modified Eagle Medium (DMEM, Sigma, St. Louis, Missouri, USA) were used as negative controls for RHS and MAb 3G10E7, respectively. Streptavidin Biotin Peroxidase Complex (StreptABC-Perox, DAKO, Glostrup, Denmark) was used as detection system (D'Angelo *et al.* 2010).

Results

Characterization of MAbs vs *Mmm*

Forty three clones secreting MAbs vs *Mmm* strain 95 were obtained, among them, 7 MAbs showing in i-ELISA the highest values of optical density were selected. Six MAbs were IgM and 1 (2C6E6) was IgG2a. Results of cross-reactions vs the panel of mycoplasma antigens, checked by i-ELISA, indicated that MAbs 3G10D6 and 3G10E7 were the most immunoreactive against *Mmm*, but no cross-reactions were observed against the other analysed mycoplasmas. On the contrary, RHS reacted with all the *Mmm* strains, with the 3 strains of *Mmc*, with *Mccp*, and with the 2 strains of *Mcc* under test (Table II).

Immunoblotting results showed different patterns of reactivity of MAbs 3G10D6 and 3G10E7 against the strains of the *mycoides* cluster and strains not belonging to the cluster. Both MAbs recognized all *Mmm* strains. At the same time they also showed a weak reaction against *Mmc* strain NCTC 11706. Interestingly, no reactivity was observed vs *Mmc* strain NCTC 10137 and the field strain 2269. However, conversely to the MAb 3G10D6, the MAb 3G10E7 did not cross-react with *M. leachii* PG50 (Table III).

Measurement of MAb/antigen binding affinity by QCM

Analysis conducted on Attana Cell 200 Biosensor confirmed a strong binding affinity between MAb 3G10E7 and *Mmm* strain 95 (positive control), highlighted by a change in frequency of 90 Hz in all the three experiments done; no binding was detected between MAb 3G10E7 and the three negative controls (*Mcc* California kid, *Mccp* F38

Table II. *Reactivity of MAbs and rabbit hyperimmune polyclonal serum (RHS) vs different* Mycoplasma *strains.* The intensity of the reaction is expressed in i-ELISA based on the values of optical density (OD_{450nm}).

Strains	MAbs							RHS
	3B2E4	3B2B6	5D3D5	2B5C4	3G10D6	3G10E7	2C6E6	
Mmm 95	+	++	++	++	+++	+++	+	+++
Mmm PG1	+	+	++	+	+++	+++	++	++
Mmm Afadè	+	++	++	++	+++	+++	+	++
Mmm B17	+	+	++	+	+++	+++	+	++
Mmm 57/13	+	+	++	++	+++	+++	+	+++
Mmm T1/44	+	+	++	++	++	++	+	+
Mmc (NCTC 10137)	++	-	-	-	-	-	-	++
Mmc (NCTC 11706)	++	-	-	-	-	-	-	++
***Mmc* 2269**	**+**	**+**	**+**	**-**	**-**	**-**	**-**	**++**
Mccp F38 (NCTC 10192)	+	++	++	-	-	-	-	+
Mcc California kid (NCTC 10154)	++	++	++	-	-	-	-	+
***Mcc* W/17**	**+**	**++**	**++**	**-**	**-**	**-**	**-**	**+**
M. leachii PG50 (NCTC 10133)	+	+	+	-	-	-	-	-
M. agalactiae (NCTC 10123)	+	++	++	-	-	-	-	-
M. bovis 9573/08	+	++	++	-	-	-	-	-
***M. bovis* 5810/07**	**+**	**+**	**++**	**-**	**-**	**-**	**-**	**-**
***M. bovis* 11003/08**	**+**	**+**	**++**	**-**	**-**	**-**	**-**	**-**

+++ = strong ($OD_{450nm} \geq 2.5$); ++ = moderate ($2.5 \geq OD_{450nm} \geq 1.5$); + = weak ($1.5 \geq OD_{450nm} \geq 0.3$); - = absent ($OD_{450nm} \leq 0.3$).

and *M. bovis* 9573/08), for which the change in frequency was 5 Hz. Moreover, no binding of *Mmm* strain 95 and the three negative controls with the reference negative chip was recorded.

Table III. *Cross-reaction of MAbs 3G10D6 and 3G10E7 assessed by immunoblotting vs different* Mycoplasma *antigens.*

Strains	MAbs	
	3G10D6	3G10E7
Mmm 95	pos	pos
Mmm PG1	pos	pos
Mmm Afadè	pos	pos
Mmm B17	pos	pos
Mmm 57/13	pos	pos
Mmm T1/44	pos	pos
Mmc (NCTC 10137)	neg	neg
Mmc (NCTC 11706)	pos	pos
Mccp F38 (NCTC 10192)	neg	neg
Mcc California kid (NCTC 10154)	neg	neg
M. leachii PG50 (NCTC 10133)	pos	neg
M. agalactiae (NCTC 10123)	neg	neg
M. bovis (9573/08)	neg	neg

pos = positive; neg = negative.

Immunohistochemistry

The results of IHC tests are shown in Table IV. Specific *Mmm*-immune reactivity (IR) was detected using either RHS or MAb 3G10E7. However, the tests carried out with the RHS showed a homogeneous, non-specific and diffuse IR on fibrin (Figure 1) and onto the fibers of the connective tissue septa. In a lung section, such deposition led to an inconclusive result. Conversely, using the MAb 3G10E7 the presence of *Mmm* was highlighted by an intense and granular staining limited to the cytoplasm of alveolar macrophages (Figure 2), in the debris of necrotic areas, in the inflammatory cells of fibrotic septa and at perivasal level (Figure 3). Furthermore, while using MAb 3G10E7 no cross-IR was observed on sections of lung infected by *M. bovis* and *Mannheimia haemolytica* (Figure 4A), RHS gave a distinctly non-specific staining (Figure 4B). With regard to the sections of the lungs of healthy animals, there was no evidence of IR with both MAb 3G10E7 and RHS.

Discussion

The aim of this study was the production of a panel of MAbs to improve the IHC method for detection of *Mmm* in tissue samples when CBPP is suspected.

Table IV. *Bovine lung tissue sections tested by immunohistochemistry using rabbit* Mmm *hyperimmune serum or MAb 3G10E7.*

	N° of animals	N° of sections	Rabbit *Mmm* hyperimmune serum			MAb 3G10E7		
			Positive	Negative	Inconclusive	Positive	Negative	Inconclusive
Mmm positive	20	37	36	0	1	37	0	0
M. bovis positive	1	5	0	0	5	0	5	0
Mmm negative	6	6	0	6	0	0	6	0

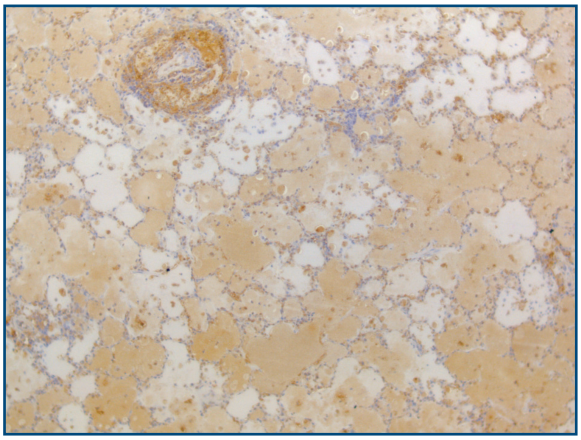

Figure 1. *Contagious Bovine Pleuropneumonia infected lung: immunohistochemical technique staining for* Mycoplasma mycoides *subsp.* mycoides *using rabbit hyperimmune serum.* Immuno-reactivity in the cytoplasm of alveolar macrophages and background staining of the intra-alveolar fibrin (200 ×magnification).

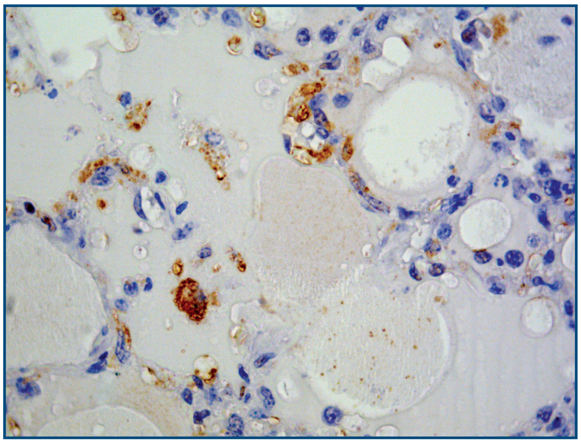

Figure 2. *Contagious Bovine Pleuropneumonia infected lung: immunohistochemical technique staining for* Mycoplasma mycoides *subsp.* mycoides *using monoclonal antibody 3G10E7.* Immuno-reactivity in the cytoplasm of alveolar macrophages (600 × magnification).

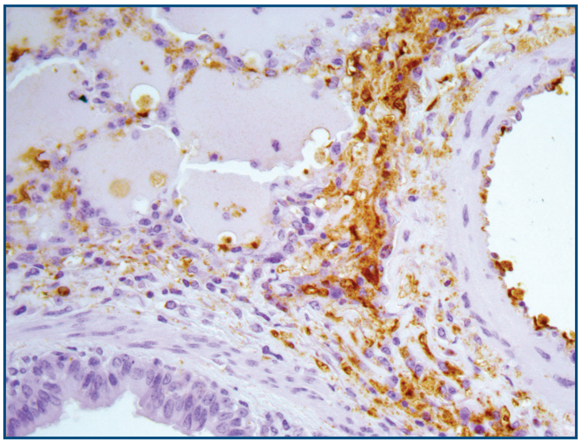

Figure 3. *Contagious Bovine Pleuropneumonia infected lung: immunohistochemical technique staining for* Mycoplasma mycoides *subsp.* mycoides *using monoclonal antibody 3G10E7.* Immuno-reactivity around vessel (400 × magnification).

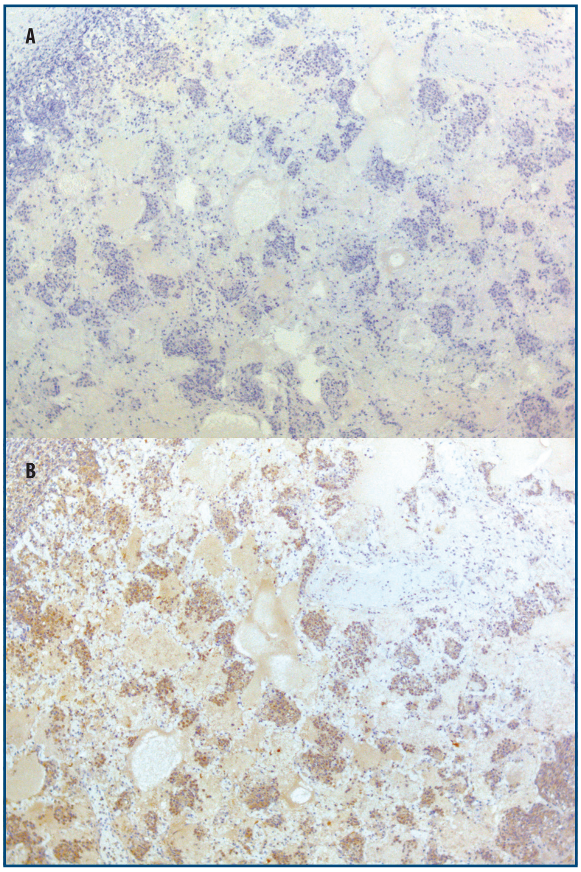

Figure 4. M. bovis *and* Mannheimia haemolytica *infected lung: immunohistochemical technique staining for* Mycoplasma mycoides *subsp.* mycoides. **A.** No immune-reactivity using monoclonal antibody 3G10E7 (200 × magnification). **B.** Non-specific staining using the rabbit hyperimmune serum (200 × magnification).

Our approach for MAbs characterisation was based on a combination of i-ELISA and IB methods against homologous *Mmm* strains and potentially cross-reactive heterologous mycoplasma antigens (*Mmc*, *Mccp* F38, *Mcc*, *M. leachii* PG50, *M. agalactiae* and *M. bovis*) so to select a panel of MAbs showing the best diagnostic performances. It is known that in some cases i-ELISA and IB give conflicting results and this is due to differences in antigen preparation. In this study, a heat-denatured antigen was used for i-ELISA, while antigens denatured by heat and chemical substances, as reducing agents (β-mercaptoethanol or dithiothreitol) and surfactants (SDS), were used in SDS-PAGE and IB. In particular, reducing agents break protein disulfide bonds and surfactants cause protein unfolding and subsequent loss of tertiary and quaternary structures. This results in the disappearance of antigen conformational epitopes and in the unmasking of linear epitopes recognized by antibodies. However, in our work, this approach proved to be efficacious to identify 1 MAb (clone 3G10E7), which reacted exclusively to *Mmm* strains both in i-ELISA and IB. The reactivity of MAb 3G10E7 vs *Mmc* NCTC 11706, observed with IB, was expected because the *Mmm* and *Mmc* share genomic and antigenic features (Thiaucourt *et al.* 2011). Previous studies demonstrated that bovine infection with *Mmc* in natural condition is very improbable, although the endotracheal intubation of *Mmc* in *Trypanosoma congolense* immunodepressed animals may determine fibrinous pleuropneumonia (Rosendal 1981, Rosendal 1983, Ajuwape *et al.* 2006).

The Attana biosensor, used in parallel with i-ELISA and IB, allowed us to evaluate the binding affinity of the selected MAb vs its antigen (*Mmm* strain 95), confirming the high specificity for *Mmm* and the lack of affinity for *Mccp* and *Mcc*, belonging to the *Mycoplasma mycoides* cluster, and for *M. bovis*, thus further confirming the results obtained by i-ELISA and IB. The ability to have information on the mechanisms of interaction between the molecules, in particular between antigens and antibodies, as well as the ability to measure the degree of affinity of these interactions could be very useful in the screening of MAbs and increases the possibility to select antibodies with the best properties for the use in diagnostic tests, such as the highest affinity vs target antigens, the slowest off-rate and the shortest incubation times (Johansson 2010).

Similarly to what has been previously reported by Rodriguez and colleagues (Rodriguez *et al.* 1996) and Ayling and colleagues (Ayling *et al.* 1998), we also observed a clear improvement of the IHC method with a reduced background staining using our MAb 3G10E7. In addition, we also demonstrated that cross-reactivity of MAb 3G10E7 is exclusively directed against some strains of *Mmc*, but not against other mycoplasmas not belonging to the *mycoides* cluster. Furthermore, specificity of MAb 3G10E7 for *Mmm* observed by i-ELISA, IB, and QCM was also confirmed in sections of lung infected by *M. bovis* and *Mannheimia haemolytica*, also responsible of pneumonia in cattle.

The IHC is able to locate both the whole pathogen and mycoplasma fragments that retain the antigenic power. Hence, IHC may still represent, from a diagnostic point of view, an important albeit not innovative tool in presence of infected animals treated with antibiotics. In Africa, the use of antibiotics is increasingly frequent in order to reduce clinical symptomatology and losses due to the disease. It has been shown that in some cases antibiotic treatment may resolve infection and even lead to recovery (Yaya *et al.* 2004, Huebschle *et al.* 2006) even if incorrect application may lead to antibiotic resistance and development of clinically healthy and serologically negative carriers. Antibiotic treatment may mask pathological changes and may make difficult microbiological isolation of the mycoplasmas. Molecular techniques remain the preferred method to confirm the diagnosis of the disease when the organism is isolated from clinical samples. However, such tecniques could also give inconclusive results when applied directly to tissue samples, because of the presence of PCR inhibitors. Thus, IHC may become the elective choice to assess the presence of mycoplasmas or their fragments in tissue samples.

The identification of a MAb anti-*Mmm* more specific than rabbit polyclonal antibodies, offers new perspective in studying disease pathogenic mechanism and host immune response, allowing to better correlate the presence of the pathogen within the tissue lesions and cellular and humoral components of the hosts immune response involved in the pathological process.

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