Allegato 7

RELAZIONE DI PROGETTO
(A cura del coordinatore di progetto)

1. Tematica e Filiera	Activity line F-Production diseases-epidemiology, diagnostics and vaccination

2. Titolo	Development of Novel Diagnostic Strategies for the Ante-Mortem Immunodiagnosis of Bovine Tuberculosis and Johne's Disease

3. Acronimo	MYCOBACTDIAGNOSIS

4. Progetto	Bando	Affidamento diretto	Sportello
	[1] EMIDA-ERA-NET del 7 Marzo 2011	[2]	[3]

5. Durata (mesi)	36	Report[4] Intermedio† Finale X	Nota[5]

6. Dati finanziari	Finanziamento concesso totale (€)	Finanziamento ricevuto (€)	**Importo rendicontato (€)[6]**
	300.000,00	193.048,19	295.808,78

7. Coordinatore di progetto	Nome e COGNOME	Maria Pacciarini
	Qualifica	Dirigente Biologo
	Istituzione di appartenenza	Istituto Zooprofilattico Sperimentale della **Lombardia e dell'Emilia**-Romagna
	Indirizzo	via Bianchi 9, 25124 Brescia
	Tel/fax	0302290308/0302290369
	e-mail	maria.pacciarini@izsler.it

8.Ente coordinatore	Denominazione: IZSLER Indirizzo: Via Bianchi 9, 25124 Brescia Tel.:_03022901, Fax:_0302425251, e-mail: info@izsler.it Si confermano gli estremi bancari o di tesoreria già forniti per la concessione del contributo † si x † no, indicare IBAN ABI CAB BIC Responsabile amministrativo della rendicontazione finanziaria: Nome Giovanni Ziviani Tel/fax 030 2290293/030 2290547 Email contabgen@izsler.it

9. Sintesi del progetto (max 20 righe) *(può essere oggetto di pubblicazione)*	La tubercolosi bovina (bTB) e la malattia di Johne's (JD) sono causa di importanti perdite economiche nel settore agricolo in Europa. bTB e JD sono malattie croniche infiammatorie causate rispettivamente da *Mycobacterium bovis* (MB) e da *M. avium* subsp. *paratuberculosis* (MAP). Entrambe le malattie possono colpire numerose specie animali domestiche e selvatiche. Gli attuali test diagnostici sono basati sulla risposta immunitaria alla tubercolina aviare, bovina o Jonnina, costituite da estratti batterici grezzi che hanno grossi limiti di sensibilità e specificità. L'assenza di un adeguato metodo diagnostico per la diagnosi precoce di allevamenti infetti di JD interferisce pesantemente con il benessere degli animali. Inoltre le infezioni da JD possono interferire con la diagnosi di bTB nel caso di allevamenti con doppia infezione. Le basi comuni dell'immunobiologia di queste due infezioni consentono di proporre un approccio integrato per lo sviluppo di nuovi test diagnostici basati su piattaforme di saggi multipli. L'obiettivo finale del progetto è migliorare la diagnosi di entrambe le infezioni generando nuovi sistemi non compromessi in sensibilità e specificità nel caso di infezioni comuni di MB e MAP. Il raggiungimento degli obiettivi è previsto attraverso l'attuazione di quattro Work Packages: WP1: Validazione della sensibilità di antigeni (peptidi, proteine, lipidi di MB e MAP) con le piattaforme diagnostiche esistenti (test intradermico, test del γ-interferon (IFN-γ), test sierologici). WP2: Sviluppo di saggi diagnostici multipli per la diagnosi di bTB e JD. WP3: Identificazione e ottimizzazione di nuovi antigeni WP4: Studio dell'immunologia delle cellule T.

Parole chiave	

10. Relazione del progetto (*totale max. 10 pagine*)

10.1 Descrizione dei risultati in relazione agli obiettivi generali e specifici previsti nel periodo di riferimento (max. 2 pagine)

Nello specifico di questo progetto, il contributo del partner 7 (IZSLER) è previsto per WP1 e WP2, il contributo del partner 8 (IZS Venezie) è previsto per WP2. I dettagli sono riassunti nella tabella sottostante:

WP	Task	Attività/obiettivi specifici	Partner coinvolto
WP1: Validazione della sensibilità degli antigeni (peptidi, proteine, lipidi, di MB e MAP) con le piattaforme diagnostiche esistenti (test intradermico, test del γ-interferon, test sierologici)	Task 1.1: Validazione multinazionale degli antigeni in campioni di animali infetti con MB, MAP o con entrambe le infezioni.	1 Produzione di antigeni ricombinanti 2 Valutazione degli antigeni messi a disposizione dai partners nel test in-house Multiplex ELISA sviluppato presso l'IZSLER. 3 Raccolta di sieri di bovino e suino nero da aziende positive/negative per TB e/o JD.	Partner 7 (IZSLER)
WP2: Sviluppo di saggi diagnostici multipli per la diagnosi di bTB e JD.	Task 2.1: standardizzazione della metodica Luminex utilizzando antigeni di *Mycobacterium bovis* e *Mycobacterium avium* subsp. *paratuberculosis* resi disponibili dai partner consociati nel progetto.	1 Organizzazione di una banca dati dei sieri TB/JD. 2 Standardizzazione del test Luminex con gli antigeni TB.	Partner 7 (IZSLER)
WP2: Sviluppo di saggi diagnostici multipli per la diagnosi di bTB e JD.	Task 2.1: standardizzazione della metodica Luminex utilizzando antigeni di *Mycobacterium bovis* e *Mycobacterium avium* subsp. *paratuberculosis* resi disponibili dai partner consociati nel progetto.	Standardizzazione del test Luminex con gli antigeni MAP.	Partner 8 (IZS Venezie)

Riassunto risultati WP1 (Leader James McNair)

Lo scopo di questo WP è stato valutare gli antigeni MB e MAP messi a disposizione dai partners del progetto nei test in vivo e con le nuove tecnologie esistenti.

- Tutti i partners hanno partecipato fornendo antigeni, reagenti o effettuando i test previsti (Test intradermico, γ-interferon, sierologia).
- Alcuni antigeni di MB hanno confermato la loro elevata sensibilità e specificità nel test intradermico o nel test del γ-interferon. La sensibilità e la specificità dei reagenti MB non è stata compromessa nel corso delle infezioni sperimentali (doppia infezione con MB/MAP o infezione singola con MAP).
- Le performances (specificità e sensibilità) dei reagenti diagnostici di MB sono risultate superiori rispetto a quelle dei reagenti MAP con l'eccezione della sierologia con il composto L5P che ha mostrato una buona sensibilità e specificità nel rilevamento di animali infetti con MAP.

Risultati WP1 (Partner 7)

Come previsto nello schema del WP1, il partner 7 ha prodotto, purificato e distribuito gli antigeni ricombinanti ESAT 6, CFP10, MPB70 e MPB83 ai partners del progetto. Sono stati inoltre raccolti sieri e informazioni da bovini di aziende positive/negative per TB/JD sul territorio nazionale.

Per l'obiettivo specifico (Organizzazione di una banca dati dei sieri TB/JD) del WP2 sono state raccolte le informazioni maggiormente significative ed è stato organizzato un database contenente i sieri messi a

disposizione dai partners. I files con i dettagli dei sieri TB/JD sono stati pubblicati sul sito comune del progetto EMIDA (AHVLA-Sharepoint).

Riassunto risultati WP2 (Leader Maria Pacciarini)

Gli obiettivi di questo WP sono stati lo sviluppo di nuovi test per massimizzare il rilevamento di animali infetti

- Sfruttando la tecnologia della piattaforma Luminex sono stati sviluppati due test sierologici Multiplex per il bovino: uno per rilevare anticorpi anti-MB (TB Luminex) e uno per rilevare anticorpi anti-MAP (JD Luminex).
- I saggi TB Luminex applicati in campioni di riferimento positivi e negativi del bovino e del suino hanno mostrato una buona sensibilità e una elevata specificità. La valutazione di campioni positivi al MAP e negativi al MB non ha rilevato aumento del segnale aspecifico.
Il saggio JD Luminex ha mostrato una buona concordanza con i test ELISA convenzionali anche se non vi è stato un miglioramento significativo nella sensibilità.
- Nel corso del progetto è stato dimostrato che il rilevamento del γ-inteferon e della risposta sierologica può essere effettuato a partire da un'unica matrice (sovranatante campioni plasma), utilizzando la piattaforma Enferplex (Risultati Task 2.2 e Task 2.3 a carico del partner 1).

10.2 Attività svolte (max 7 pag)

Attività WP1 (Obiettivo specifico: produzione di antigeni ricombinanti)

Nel corso del kick-off meeting sono stati definiti gli antigeni messi a disposizione per il progetto EMIDA.
Nella tabella sono riassunti gli antigeni e il contributo dei singoli partners.

Task 1.1 Validare gli antigeni MB e MAP antigens negli animali infetti da MB e MAP

Task 1.1	Antigeni forniti	Partner ricevente
Partner 1	Peptidi derivati da ESAT-6, CFP10, Rv3615c, Rv3020c, Rv2346c	P1, P2, P5, P6, P7, P8, P9, P10, P11
Partner 2	Lipopentapeptide (L5P)	P1, P2, P5, P6, P7, P8, P9, P10, P11
Partner 3	PIM, glicolipidi	P1, P2, P5, P6, P7, P8, P9, P10, P11
Partner 7	MPB70, MPB83, CFP10, ESAT6	P1, P2, P5, P6, P8, P9, P10, P11
Partner 9	MAP3651C, MAP0268C, MAP1365	P1, P6

Nella 2° fase del progetto il partner 3 ha messo a disposizione i seguenti antigeni MAP: Ag1 (MAP0210c), Ag2 (MAP2942), Ag7 (MAP2609), MAP1272 e PPDjd non previsti nella tabella iniziale.

Come previsto il partner 7 ha prodotto e fornito i seguenti antigeni: ESAT6, CFP10, MPB70 and MPB83. Le proteine sono state espresse con una coda di 6 istidine (his-tag) nella porzione N terminale utilizzando il vettore di espressione pQE30 di *E. coli*. Tutte le proteine sono state purificate in condizioni denaturanti (Urea 8M). MPB70 e MPB83 sono state purificate utilizzando una colonna di affinità (Nikel affinity cromatography, Ni-NTA).
ESAT6 e CFP10 sono state purificate usando un protocollo di purificazione di tre step (colonna di affinità Ni-NTA, cromatografia a scambio ionico e gel filtrazione).
Nello specifico le proteine sono state richieste dai partners 1, 6, 8 e 11:
ESAT6, CFP10, MPB70 and MPB83 (0.5 mg/ogni proteina) sono state fornite al partner 1 (AHVLA) per la valutazione sulla piattaforma MSD.
ESAT6, CFP10, MPB70 e MPB83 (1 mg/ogni proteina) sono state fornite al partner 8 (IZSVE) per la piattaforma Luminex.
MPB70 e MPB83 (1 mg /ogni proteina) sono stati forniti al partner 11 (BRNO) per i test sierologici.
ESAT6, CFP10, MPB70 e MPB83 (1 mg/ogni proteina) sono state fornite al partner 6 (AFBI) da saggiare nel γ-interferon test e nel test IDT.

Attività WP1 (Obiettivo specifico: Raccolta di sieri di bovino e suino nero da aziende positive/negative per TB e/o JD).
Nel corso del progetto sono stati raccolti sieri bovini e suini da mettere a disposizione del consorzio EMIDA attraverso la banca dati sieri TB/JD pubblicata nel sito comune del progetto (Sharepoint, AHVLA):

[redacted]

e da 111 animali negativi per JD (raccolti da aziende con status negativo per JD da almeno 5 anni). Tutti i campioni sono stati prelevati in aziende negative localizzate in territorio ufficialmente indenne alla bTB (OTF).

- Sono stati prelevati 100 campioni di siero prelevati da bovino in 15 aziende del Sud Italia positive alla bTB con status JD ignoto.
- Sono stati prelevati 244 sieri suini: 59 prelevati da allevamenti con status positivo alla TB localizzati in Sicilia e positivi ad almeno un test in vivo o post mortem (IDT o γ-interferon o istologia/presenza VL o isolamento colturale di *M.bovis*), e 185 negativi alla TB (prelevati da allevamenti in territori OTF).

Attività WP1 (Obiettivo specifico: valutazione degli antigeni messi a disposizione dai partners nel test in-house Multiplex ELISA sviluppato presso **l'IZSLER**).
I partners hanno fornito i seguenti antigeni:
Partner 1 (AHVLA): antigeni ricombinanti Rv3615c, Rv3020c specifici per MB.
Partner 2 (INRA): L5P (composto idrosolubile 13 e analogo non-idrosolubile 4) specifico per MAP.
Gli antigeni ricombinanti di MB (Rv3615c, Rv3020c) sono stati valutati inizialmente con il test ELISA Multiplex su sieri di riferimento positivi e negativi con risultati incoraggianti. I risultati sono presentati in dettaglio nell'allegato tecnico (Final Report WP2).
L'antigene L5P è stato legato covalentemente a un set di biglie magnetiche utilizzando diversi protocolli di binding. La valutazione nel test Luminex ha mostrato problemi di background non facilmente risolvibile. I risultati sono presentati in dettaglio nell'allegato tecnico (Allegato tecnico report finale WP2).
Partner 3 (AFBI): Ag1 (MAP0210c), Ag2 (MAP2942), Ag7 (MAP2609), MAP1272 e PPDjd.

Attività WP2 (Task 2.1 Standardizzazione del test Luminex).
Introduzione
Lo scopo di questa task 2.1 è stata lo sviluppo di test sierologici con la piattaforma Luminex utilizzando gli antigeni prodotti e resi disponibili nel WP1.
Gli animali infetti da bTB sono principalmente rilevati da test basati sull'utilizzo della PPDb (test intradermico e test del γ-interferon). La sierologia, utilizzata in parallelo ai test basati sulla risposta cellulo-mediata, può potenzialmente massimizzare il rilevamento complessivo degli animali infetti.
Per quanto riguarda la TB, sono stati descritti un certo numero di antigeni, in grado di riconoscere anticorpi di animali infetti con MB che possono essere stimolati in diversi stadi dell'infezione (Bezos et al. 2014). Queste scoperte hanno portato allo sviluppo di test sierologici Multiplex che si basano sull'utilizzo combinato di più antigeni (Whelan et al. 2008; Bezos et al. 2014).
I programmi di controllo della JD sono basati sulla sierologia (test ELISA) che rilevano animali infetti con elevata sensibilità solo negli stadi avanzati dell'infezione (Verdugo et al. 2015). Nonostante ciò gli animali infetti possono eliminare MAP nel loro latte e feci e disseminare l'infezione prima che vi sia una risposta anticorpale rilevabile (Nielsen SS, 2008). Un approccio sierologico con più antigeni (Multiplex) potrebbe essere utile per migliorare il rilevamento di animali nelle prime fasi d'infezione di MAP.
Il sistema Luminex è una piattaforma ben nota (Elshal, McCoy, 2006) progettata per test multipli che può essere applicata per il rilevamento di anticorpi verso più target antigenici in un singolo saggio (Anderson et al. 2011). Lo scopo della Task 2.1 è stato esplorare l'utilizzo di questa tecnologia per lo sviluppo di un nuovo sistema diagnostico che combinasse la sierologia di MB e MAP. Un obiettivo associato a questa Task è stato lo sviluppo di un saggio Luminex per la sierologia di TB nel suino per una potenziale applicazione in territori (Sicilia) dove sono presenti allevamenti di suino nero allo stato brado o semi-brado (Di Marco et al. 2013; Barandiaran et al. 2015).

Risultati (Premessa)
La piattaforma Luminex è stata utilizzata per sviluppare dei test sierologici per MB e MAP nel bovino e per il rilevamento dell'infezione di MB nel suino. I dettagli dello sviluppo e ottimizzazione dei test sono descritti nel nell'allegato tecnico (Final Report WP2) inviato al capofila del progetto. In questo riassunto descriviamo i principali risultati e conclusioni.

Risultati (TB Luminex nel bovino)

Dopo un rilevante lavoro di standardizzazione con tutti gli antigeni a disposizione comprendenti MPB70, MPB83, ESAT6, CFP10, Rv3020c, Rv3615c, PPDb, è stato possibile sviluppare un saggio Luminex Multiplex utilizzando MPB70, MPB83, ESAT6, e CFP10 per la sierologia della TB nel bovino, con una sensibilità e specificità

e sensibilità quando utilizza due antigeni per l'interpretazione positiva. In particolare il test non ha mostrato alcun aumento di segnale aspecifico analizzando 216 campioni negativi alla TB e positivi al MAP.

Per la ROC analisi e la definizione dei cut off sono stati utilizzati un totale di 1023 sieri classificati in 4 diversi gruppi, di cui 162 positivi alla TB e 861 negativi alla TB (Tabella 1).

Tabella 1 (Gruppi di campioni saggiati)

	JD	TB	
Gruppo 1	?	+	162
Gruppo 2	-	-	555
Gruppo 3	+	-	216
Gruppo 4	?	-	90
Totale			1023

In dettaglio:

- Gruppo 1: sieri da allevamenti positivi alla bTB /stato sconosciuto al JD. Tutti i sieri sono stati prelevati 15-20 giorni dopo il test intradermico singolo (IDT), da allevamenti positivi alla bTB localizzati nel Nord Italia (Trentino Alto Adige, Veneto, Lombardia) e Sud Italia (Calabria) da animali risultati positivi all'isolamento colturale di *M. bovis/M.caprae.*
- Gruppo 2: sieri da allevamenti negativi alla bTB/JD. Tutti i sieri sono stati prelevati da allevamenti "TB free" in territori ufficialmente indenni (OTF) (Germania e Nord Italia). Tutti gli allevamenti sono stati controllati per la presenza di JD per almeno 6 anni con sierologia ed isolamento colturale.
- Gruppo 3: sieri da allevamenti positivi al JD /negativi bTB. Tutti i campioni sono stati prelevati da allevamenti "TB free" in territori ufficialmente indenni (OTF) (Germania e Nord Italia) e confermati positivi al JD mediante isolamento colturale di MAP.
- Gruppo 4: sieri provenienti da allevamenti negativi alla bTB/ stato sconosciuto al JD prelevati da allevamenti "TB free" in Nord Italia.

L'analisi ROC (Receiver Operating Characteristic) è stata applicata (Zhou et al. 2009) per determinare i valori di cut off positivi/negativi. Fissando la specificità diagnostica (Sp) per ciascun antigene maggiore del 99%, è stata ottenuta, di conseguenza, una Sensibilità (Se) compresa dal 75,3% per MPB70 al 2,5% per Rv3020c. Le aree sotto la curva (AUC) sono state calcolate rispettivamente: 0,969, 0,932, 0,843 e 0, 668 per MPB70, MPB83, ESAT6 and CFP10. Gli antigeni Rv3615c e Rv3020c hanno mostrato una bassa Se e uno scarso valore di AUC, pertanto considerati poco affidabili per la discriminazione dei campioni positivi e negativi (Tabella 2) e sono stati esclusi dal test.

Tabella 2

	Cut-off	Sensibilità	CI (95%)	Specificità	CI (95%)	AUC
MPB70	30,5	0,753	0,679-0,817	0,999	0,994-1	0,969
MPB83	16,2	0,506	0,427-0,586	0,998	0,992.1	0,932
ESAT6	41,9	0,222	0,160.0,294	0,994	0,986-0,998	0,843
CFP10	35,5	0,111	0,067-0,169	1	0,993-1	0,668
RV3615c	38,1	0,086	0,048-0,141	0,991	0,982-0,996	0,594
RV3020c	0,774	0,025	0,006-0,062	0,991	0,981-0,995	0,653

La performance dell'intero test è stata calcolata con gli antigeni MPB70, MPB83, ESAT6 e CFP10 considerando uno o due antigeni positivi (Tabella 3). La Se del test Luminex TB Multiplex con un antigene è stata calcolata del 79% con una Sp del 99,1%. La combinazione di due antigeni positivi aumenta la Sp al 100% ma riduce la Se al 51,2% (Tabella 3). La performance del Luminex è comparabile al test ELISA "in-house" usato presso l'IZSLER

(Casto et al. 2010) e a quella di altri test sierologici che utilizzano uno o più antigeni ricombinanti (Souza et al. 2012; Whelan et al. 2008).

Tabella 3

1 antigene positivo	0,79	0,991
2 antigeni positivi	0,512	1

Il Test TB Luminex TB è stato in seguito applicato su 256 campioni di campo prelevati da allevamenti positivi alla TB localizzati in Sud Italia. I campioni possono essere suddivisi in 3 diversi gruppi riassunti in tabella 4: gruppo 1 positivo al test IDT e/o γ-interferon, gruppo 2 positivo alla PCR e/o presenza di lesioni (VL), e gruppo 3 (il più consistente) che comprende campioni negativi alla IDT e al γ-interferon senza informazioni sui test post-mortem (isolamento, PCR, presenza di VL).

Tabella 4

Gruppo	in-vivo test (IDT, IFNγ)	post-mortem test (VL, PCR)	N. campioni
1	+	+	19
2	+	?/-	51
3	-	?/-	186

In Tabella 5 sono presentati i risultati del test Luminex in parallelo con i test ELISA "in house" ottenuti considerando uno o due antigeni positivi.
Dei 186 sieri negativi al test in vivo, 46 sono risultati positivi al saggio TB-Luminex e 29 al test ELISA utilizzando l'interpretazione con un solo antigene positivo. La combinazione di 2 antigeni rileva rispettivamente 8 e 14 campioni positivi. La maggior parte della reattività è stata rilevata con MPB70 e MPB83. Non è stato possibile confermare la positività sierologica con esami post-mortem o ulteriori test in vivo.

Tabella 5

			Test LUMINEX				"In-house" test ELISA			
in-vivo test (IDT, IFNg)	post-mortem test (VL, PCR)	N. campioni	1 antigene positivo		2 antigeni positivi		1 antigene positivo		2 antigeni positivi	
			+	%	+	%	+	%	+	%
+	+	19	13	68,4	11	57,9	12	63,2	11	57,9
+	?/-	51	31	60,8	16	31,4	28	54,9	21	41,2
-	?/-	186	46	24,7	8	4,3	29	15,6	14	7,53

Conclusioni

E' stato standardizzato un saggio TB Luminex per la sierologia nel bovino con la possibilità di modulare sensibilità e specificità a seconda del numero di antigeni positivi utilizzati per l'interpretazione del test. Il test mostra una specificità molto alta specialmente con la combinazione di due antigeni per l'interpretazione positiva. In particolare il test non ha mostrato alcun aumento di aspecificità analizzando i 216 sieri negativi alla TB e positivi al MAP (Gruppo 3, Tabella 1).
Risultati preliminari su campioni da campo prelevati da allevamenti positivi alla TB (negativi ad altri test in vivo) mostrano un segnale positivo in particolare con gli antigeni MPB70 e MPB83. Malgrado l'elevata specificità del test non si può escludere una reattività aspecifica dovuta alla presenza di micobatteri ambientali visto le condizioni di allevamento degli animali dai quali i campioni sono stati prelevati (animali in stato di semi-libertà).

Il saggio Luminex è un sistema flessibile ora costituto da pochi antigeni di cui MPB70 e MPB83 sono i target diagnostici principali, che potrebbe essere potenzialmente implementato da altri antigeni eventualmente identificati per migliorare le performance del test.

Risultati (TB Luminex nel suino)

Il saggio TB-Luminex è stato stantardizzato utilizzando un totale di 244 sieri suini di cui 59 positivi alla TB prelevati da allevamenti con status positivo alla TB localizzati in Sicilia e positivi ad almeno un test in vivo o post mortem (IDT o γ-interferon o istologia/presenza VL o isolamento colturale di *M.bovis*), e 185 negativi alla TB (prelevati da allevamenti in territori OTF). Il prelievo di sieri positivi alla TB è stato fatto da un numero limitato di animali da allevamenti localizzati in Sicilia dove è stato possibile dimostrare la presenza di *M. bovis* (Di Marco et al. 2012).

La curva di analisi ROC è stata utilizzata per definire i valori cut off tra campioni positivi/negativi. Fissando la specificità diagnostica per ciascun antigene maggiore del 97,5% (Tabella 6), gli antigeni MPB83 e CFP10 mostrano una migliore Se, mentre MPB70 e CFP10 risultano meno immunogeni.

Table 6

	Cut-off	Sensibilità	CI (95%)	Specificità	CI (95%)	AUC
MPB70	99,9	0,661	0,526 - 0,779	0,978	0,946 - 0,994	0,928
MPB83	14,2	0,966	0,883 - 0,996	0,978	0,946 - 0,994	0,998
ESAT6	91,7	0,203	0,11 - 0,328	0,978	0,946 - 0,994	0,615
CFP10	12,9	0,864	0,75 - 0,94	0,984	0,953 - 0,997	0,927

Conclusioni.

La risposta umorale nel suino con il saggio Luminex si è mostrata diversa rispetto a quanto rilevato nel bovino. In particolare è stata osservata un'elevata immunogenicità degli antigeni MPB83 e CFP10 (rispettivamente 57 e 51 sieri positivi) e una minore reattività di MPB70 ed ESAT6 (rispettivamente 39 e 12 sieri positivi).

Questi dati possono essere spiegati da diverse ipotesi: la specie ospite può rispondere con modalità diverse ai diversi antigeni di *M. bovis* (Bezos et al. 2014); una diversa selezione della popolazione utilizzata per il prelievo dei campioni e per la definizione dei valori cut off.

Altre studi sono necessari per verificare i dati ottenuti, in particolare sarebbe necessario valutare l'immunogenicità degli antigeni anche con altri test sierologici.

Risultati (MAP Luminex nel bovino)

I dettagli dello sviluppo e ottimizzazione dei test sono descritti nell'allegato tecnico. In questo riassunto descriviamo i principali risultati e conclusioni.

Dopo un rilevante lavoro preliminare di valutazione degli antigeni MAP a disposizione comprendenti: L5P (composto 13), PPDjatcc, e le proteine ricombinanti Ag1 (MAP0210c), Ag2 (MAP2942), Ag7 (MAP2609), MAP1272 è stato possibile sviluppare un saggio Luminex Multiplex con gli antigeni Ag1 (MAP0210c), Ag2 (MAP2942) e Ag7 (MAP2609) per la sierologia di MAP nel bovino.

Per l'analisi ROC sono stati utilizzati un totale di 737 sieri classificati in 3 diversi gruppi: 227 positivi e 510 negativi al MAP (Tabella 7), tutti provenienti da allevamenti OTF:

Tabella 7

JD status allevamento	N° Totale campioni	Elisa IDEXX / IDVet	Isolamento colturale	N° campioni
positivo	227	-	+	165
		+	+	62
negativo	510	-	-	510

In dettaglio:

- Campioni positivi: sieri da animali positivi JD. Tutti i campioni sono stati prelevati da allevamenti "TB free" in territori OTF (Germania e Nord Italia) e confermati positivi JD mediante isolamento colturale di MAP.
- Campioni negativi: sieri prelevati da allevamenti TB free in territori OTF (Germania e Nord Italia). Tutti gli allevamenti sono stati controllati per la presenza di JD per più di 6 anni con sierologia e isolamento colturale con

risultati negativi.
I risultati sono stati utilizzati per l'analisi ROC, per il calcolo dei valori cut off e di Se e Sp per ciascun antigene.
La Se individuale varia dal 16.7% di Ag1 al 0.9.% di MAP1272 (Tabella 8). Poichè il MAP1272 ha mostrato di non avere alcuna capacità discriminatoria tra animali infetti e non infetti, si è deciso di escluderlo dal test Luminex.

Tabella 8

Antigene	Cut-off	Sensibilità	Specificità
Ag1	10132.3	0.167	0.994
Ag2	1357.3	0.106	1
Ag7	5894.8	0.097	1
MAP1272	25428.4	0.009	1

La performance dell'intero test è stata calcolata considerando uno o due antigeni positivi (Tabella 9). La positività ad un antigene mostra una Sp del 99.4% ed una Se del 23.8. Tale performance è comparabile all'ELISA commerciale che nella popolazione bovina ha una Se del 27.3%. La combinazione di 2 antigeni aumenta la Sp al 100% ma riduce la Se allo 8.4%. Si è deciso quindi di adottare esclusivamente il primo criterio di interpretazione.

Tabella 9

Criterio per la positività	Se	Sp
Almeno 1 antigene positivo	0.238	0.994
Almeno 2 antigeni positivi	0.084	1

Con questo protocollo si è visto che circa il 70% di tutti i campioni positivi è dovuto alla reattività con Ag1, indipendentemente dal fatto che lo stesso campione sia risultato positivo al test ELISA o positivo all'isolamento di MAP. Diversamente la reattività ad Ag2 e Ag7, si è mostrata correlata alla natura del campione poiché è stata osservata con frequenza maggiore nei campioni positivi al test ELISA.

Conclusioni

In questo progetto è stato standardizzato un saggio JD Luminex per la sierologia nel bovino che utilizza tre antigeni ricombinanti specifici per MAP.
Analizzando un pannello di sieri di riferimento (positivi e negativi alla JD) Il test ha mostrato un'accuratezza simile a quella dei test ELISA commerciali.
Risultati preliminari ottenuti da campioni di campo prelevati da allevamenti positivi alla bTB (negativi ad altri test in vivo), infetti con MAP (negativi all'isolamento colturale), mostrano reattività principalmente con Ag1 (MAP0210c). La positività con Ag2 (MAP2942) e Ag7 (MAP2609) è stata osservata principalmente nei sieri positivi al test ELISA.
Si auspica che in futuro il test Luminex JD potrà essere implementato con altri antigeni specifici per MAP per migliorare le performance del test.

Referenze

Anderson, A., P. Wakeley, G. Wibberley, K. Webster, J. Sawyer. 2011. Development and evaluation of a Luminex multiplex serology assay to detect antibodies to bovine herpes virus 1, parainfluenza 3 virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods. J. Immunol. Methods. 366: 79-88.
Barandiaran, S., A. M. Perez, A. K. Gioffrè, V. M. Martinez, A. A. Cataldi, M. J. Zumarraga. 2014. Tuberculosis in swine co-infected with Mycobacterium avium subsp. hominissuis and Mycobacterium bovis in a cluster from Argentina. Epidemiol. Infect. 143: 966-974.
Bezos, J., C. Casal, B. Romero, B. Schroedere, R. Hardegger, A. J. Raeber, L. Lopez, P. Rueda. L. Dominguez. 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. Res. Vet. Science 97: 544-552.
Casto, B.,C. Donati, S. Zoppi, M. Pacciarini, M.B. Boniotti. 2010. A multi-antigen ELISA-test for serology detection of Mycobacterium bovis infection in cattle. European Society of Mycobacteriology, 31° Annual Congress, 4-7 July, Bled-Slovenia.
Di Marco, V., P. Mazzone, M. T. Capucchio, M. B. Boniotti, V. Aronica, M. Russo, M. Fiasconaro, N. Cifani, S. Corneli, E. Biasibetti, M. Biagetti, M. L. Pacciarini, M. Cagiola, P. Pasquali, C. Marianelli. 2012. Epidemiological

significance of the domestic black pig (Sus scrofa) in maintenance of bovine tuberculosis in Sicily. J. Clin. Microbiol. 50:1209-1218.
Elshal, M. F., J. P. McCoy. 2006. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. Methods 38: 317-323.
Lawson, S., J. Lunney, F. Zuckermann, F. Osorio, E. Nelson, C. Welbon, T. Clement, Y. Fang, S. Wong, K. [redacted]
development: assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. Vaccine 28: 5356-5364.
Nielsen SS. Transitions in diagnostic tests used for detection of Mycobacterium avium subsp. paratuberculosis infections in cattle. Vet Microbiol. 2008 Dec 10;132(3-4):274-82.
Souza, I. I., E. S. Melo, C. A. Ramos, T. A. Farias, A. L. Osorio, K. S. Jorge, C. E. Vidal, A. S. Silva, M. R. Silva, A. O. Pellegrin, A. O. Araujo. 2012. Screening of recombinant proteins as antigens in indirect ELISA for diagnosis of bovine tuberculosis. Springerplus 1: 77.
Verdugo C, Toft N, Nielsen SS. Within- and between-herd prevalence variation of Mycobacterium avium subsp. paratuberculosis infection among control programme herds in Denmark (2011-2013). Prev Vet Med. 2015 Oct 1;121(3-4):282-7
Whelan, C., E. Shuralev, G. O'Keeffe, P. Hyland, H. F. Kwok, P. Snoody, A. O'Brien, M. Connolly, P. Quinn, M. Groll, T. Warrerson, S. Call, K. Kenny, A. Duignan, M. J. Hamilton, B. M. Buddle, J. A. Johnston, W. C. Davies, S. A. Olwill, J. Clarke. 2008. Multiplex immunoassay for serological diagnosis of Mycobacterium bovis infection in cattle. Clinical and Vaccine Immunology 15: 1834-1838.
Zhou, Xiao-Hua, Donna K. McClish, and Nancy A. Obuchowski. Statistical methods in diagnostic medicine. Vol. 569. John Wiley & Sons, 2009.

10.3 Descrizione delle interazioni tra le UUOO partecipanti, eventuali collaborazioni esterne ed imprese (inserire diagramma) max 1 pag

La cooperazione all'interno del consorzio è stata essenziale per il raggiungimento degli obiettivi scientifici del progetto. Questo si rispecchia nell'interindipendenza dei WPs e nel coinvolgimento sinergico dei vari partners. L'ottenimento di quasi tutti i risultati è dipeso dagli input di tutti i partecipanti, in particolare per quanto riguarda la fornitura di antigeni, campioni e competenze. Il consorzio ha lavorato in un'atmosfera di attiva e integrata collaborazione.
Per quanto riguarda l'ottenimento degli obiettivi specifici del WP2, questo è stato possibile grazie all'attiva collaborazione con altri partner del consorzio che hanno fornito antigeni, campioni, sieri.
Nello specifico i partner 7 e 8 (IZSLER e IZS Venezie), hanno lavorato integrando le proprie attività e competenze per ottenere i risultati previsti nella task 2.1 del WP2. In particolare il partner 7 ha lavorato principalmente per lo sviluppo del test con piattaforma Luminex per la sierologia della TB in bovino e suino, il partner 8 per lo sviluppo del test con piattaforma Luminex per la sierologia del JD in bovino.
Il subcontratto con l'IZS della Sicilia ci ha permesso di avere sieri e informazioni da allevamenti di bovino e suino positivi alla Tubercolosi Bovina.

10.4 Ostacoli occorsi ed azioni correttive messe in atto (max 1 pag)

N° WP	Ostacolo occorso	Azione correttiva

1	Non è stato possibile utilizzare gli antigeni L5P e PPDj per lo sviluppo del test Luminex JD visto l'elevato segnale di background.	Per la standardizzazione del test Luminex JD sono stati utilizzati altri antigeni MAP forniti dal partner 3.

Timbro Istituzione	Il responsabile di gestione (o delegato)	Il coordinatore di progetto
	nome e cognome: Direttore Generale prof. Stefano Cinotti	*nome e cognome: Maria Pacciarini*
	firma	*firma*

ALLEGATO TECNICO MYCOBACTDIAGNOSIS-WP2 Final report

Partners involved in WP2:
P1 : Martin Vordermeier, Shelley Rhodes, Thomas Holder, AHVLA
P4 : John Clarke, Enfer
P7 : Maria Pacciarini, Beatrice Boniotti, Stefania Fontana, IZSLER
P8 : Elisabetta Stefani, Nicola Pozzato, Michele Gastaldelli, IZSVE

1. WP2 Tasks and partners involved

Task N°	Main Partner involved	Summary
2.1	7-8	To develop serology multiplex systems using the Luminex platform in conjunction with MB and MAP antigens tested in WP1. **Specific objective**: Standardization of Luminex test with the available antigens.
2.2	1	Development of a combined serology and cytokine multiplex based on IFNγ and other cytokines.
2.3	1	To transfer technology from Luminex and MSD research platforms to high throughput commercial Enferplex technology.

Task 2.1: Report (Partner 7-8)

1 Executive summary

- Exploiting the potentiality of multiplex platform technology, we have developed two Luminex test for serology in cattle with available MB and MAP antigens: one for TB and one for JD serology respectively.
- The TB Luminex Multiplex assay showed good sensitivity and a very high specificity especially when combination of two positive antigens is used for positive interpretation. No increase of unspecific signal was pointed out with MAP positive samples.
- The JD Luminex Multiplex showed good specificity but low sensitivity not better than ELISA conventional tests. Differently to the commercial ELISA test for JD, this test showed limited cross reactions in TB infected animals boosted with PPDb.
- In addition, we have standardized a Luminex assay for TB serology in swine for potential application in areas where domestic free or semi-free ranging pig has been described to be/as maintenance host of TB. Preliminary results showed a good Sensitivity and Specificity. Performance of the test and reactivity of antigens has to be verified with a larger number of samples.

Future work
- Combination of the existing TB and JD-Luminex test in a unique assay.
- Possible implementation of this flexible system with new TB and MAP antigens eventually identified to improve the performance of the test.

2 Background and objectives

Bovine tuberculosis (bTB) and Johne's disease (JD) are chronic inflammatory diseases caused by *Mycobacterium bovis* (MB) and *M. avium* subsp. *paratuberculosis*, respectively.
bTB infected animals are mainly detected by PPDb based tests such as Skin test or Gamma-interferon test (IFN-γ). Serological assays can integrate the performance of methods based on cellular response and help detection of the pathogen for control of TB in infected herds. Humoral immune response against *M. bovis* (MB) has a complex pattern of antibody production that could be elicited by several antigens at different stages of infection (Bezos et al. 2014). These findings have led to the development of multiplex antigen serology test (Whelan et al. 2008; Bezos et al.

2014).
JD control programmes are based on serology (ELISA tests) which detects infected animals with high sensitivity only at an advanced stage of infection (Verdugo et al, 2015). Nonetheless, infected animals can shed MAP in their milk and faeces and disseminate infection before there is a detectable antibody response (Nielsen SS, 2008). A multiplex antigen approach could be useful for improving detection of JD infected animals, at earlier stages of infection.
The Luminex is an established technology platform (Elshal, McCoy, 2006) designed for multiplex testing that can be applied for detection of antibody to multiple antigen targets in a single assay (Anderson et al. 2011). The aim of this task was to exploit this instrument for the development of a new diagnostic tool possibly combining the serology of MB and MAP. However, on the base of initial feasibility studies with MB and MAP available antigens it was possible to standardize two separate Luminex assays: one with MB and one with MAP recombinant antigens respectively.
In addition, a side objective of the main task 2.1 was the development of a Luminex assay for TB serology in swine for potential application in areas where domestic free or semi-free ranging pig has been described as maintenance host of TB (Di Marco et al. 2012; Barandiaran et al. 2015).

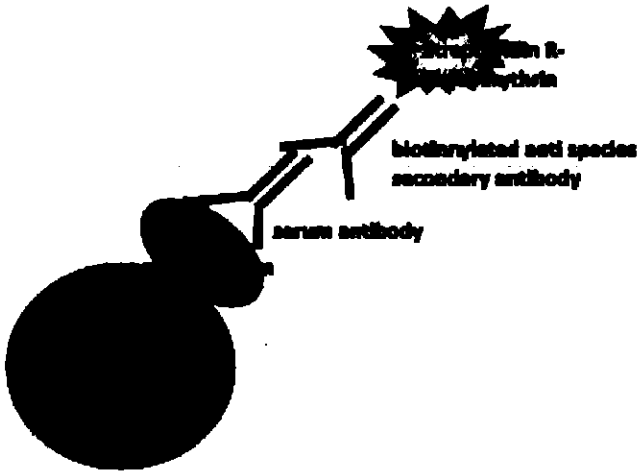
3 Development of Luminex immunoassays.

3.1 Introduction

Luminex [x Multi-Analyte Profiling (xMAP) technology] is a liquid suspension array instrument based on the use of polystyrene beads which can be covalently conjugated with protein antigens and used for direct or indirect detection of antibodies similarly to ELISA test.
Microspheres dyed with distinct proportions of red and near infrared fluorophores, define "spectral addresses" for each bead population. As a result, up to one hundred different detection reactions can be carried out simultaneously on the various bead populations in very small volumes. Interactions of sample analytes with each conjugated microsphere set are reported by Phycoerythrin, a common reporter fluorochrome, conjugated to a secondary detection reagent.
The dual-laser Luminex-100 instrument has a three colour fluorescence signal-detection system. Two colour are dedicated to microsphere classification, the third colour is used for measurement of the reporter fluorescence intensity.
The Luminex immunoassays developed for detection of MB and MAP antibodies were designed according to the scheme presented in Figure 1. Briefly:

1) Antigens are conjugated to different labelled fluorescence beads;
2) Antigen conjugated beads are incubated with serum samples;
3) Antibodies are detected by anti-species biotinylated secondary antibody;
4) The complex antigen-antibody-secondary antibody is detected by Streptavidin conjugated with Phycoerythrin and emission of fluorescence signal.

Figure 1



3.2 TB-Luminex test for serology in cattle (Partner 7).

MB antigens used in the test.

MB antigens evaluated in TB-Luminex test were:

MPB70, MPB83, CFP10, ESAT6 produced by partner 7, PPDb supplied by IZSUM (Perugia) and Rv3615 and RV3020c supplied by partner 1.

MPB70, MPB83, CFP10 and ESAT6 were His-tag proteins cloned in the expression vector pQE30. Protein purification was performed in denaturing condition (8M Urea) by using a Ni-NTA affinity column. ESAT6 and CFP10 were submitted to three purification steps:

- Ni-NTA affinity column;
- Anion exchange chromatography;
- Gel filtration.

Recombinant proteins were quantified by BCA Protein assay kit and Comassie PAGE.

PPDb was produced by Istituto Zooprofilattico of Umbria and Marche (IZSUM, Perugia) according to standard procedures (Annex B of EC 1226/2002).

Rv3615 and RV3020c were His-Tag proteins expressed and purified in *E. coli* supplied by Lionex Diagnostics and Therapeutics (Braunschweig, Germany).

Optimization of TB-Luminex test.

Antigens were covalently coupled to magnetic carboxyl-functionalized fluorescent polystyrene microspheres (Bio-Plex Pro Magnetic COOH beads, Bio-Rad) via a two-step carbodiimide reaction, according to the protocol provided by Luminex (http://www.luminexcorp.com/prod/groups/public/documents /lmnxcorp/ protein-coupling-protocol-magp.pdf).

At first microsphere conjugation was evaluated on different amount of antigens (1, 4, 8 and 12 µg). Best conditions were obtained by using 12 µg antigen /1,25X10^6 beads.

The efficiency of antigen coupling reaction was verified with Monoclonal antibody (Mab) anti-MPB70 (for MPB70 and MPB83 coupled beads), Mab anti-ESAT6 (for ESAT6 coupled beads) followed by a biotinylated secondary antibody (goat polyclonal anti-mouse IgG) and with a TB positive serum followed by a biotinylated secondary antibody (anti-bovine IGg1 Mab 1G10) (Brocchi et al. 2006) for CFP10, PPDb, Rv3615c and Rv3020c.

All Mabs used in this study were produced by standard procedures at IZSLER (unpublished results).

A feasibility study of TB-Luminex multiplex test was carried out with a panel of 15 TB positive and 15 TB negative sera evaluating, possible signal interference between conjugated beads in individual and multiple reactions. PPDb showed competition with the positivity level of the other beads. Therefore PPDb was removed from the beadset. The remaining antigens did not show interference between each other in the multiplex reaction. Therefore we decided to include in the TB beadset: MPB70, MPB83, CFP10, ESAT6, RV3615, RV3020.

Optimization of assay condition was defined after testing different parameters of Luminex test, specifically:

- Different assay buffer composition: 1) Phosphate buffered saline (PBS) 1X pH 7.4 only; 2) PBS 1X + 0,05% Tween-20 + 1% yeast extract; 3) PBS-TBN (PBS 1X + 0,1% BSA + 0,02% Tween-20 + 0,05% Sodium Azide) in combination with streptavidin R-Phycoerythrin dilution buffer PBS 1X or PBS 1X + 0,05% Tween-20.
- Different incubation time: 1 hour vs 30 minutes of incubation time for sera, for biotinylated secondary antibody, for streptavidin R-Phycoerythrin;
- Different dilutions of sera (1/10, 1/50, 1/100);
- Different dilutions of biotinylated secondary antibody (1/500, 1/1000, 1/2500, 1/5000, 1/10000);

Following test optimization, TB-Luminex assay was carried out according the protocol provided by Luminex ("Luminex-xMAP_Cookbook_2nd_edition", 07.2014, Luminex Corporation, Austin TX USA) with the followings specification: assay buffer: PBS 1X + 0,05% Tween-20 + 1% yeast extract; wash buffer: PBS 1X + Tween 0,05%; dilution 1/1000 of biotinylated secondary antibody (anti-bovine Mab 1G10); streptavidin R-Phycoerythrin (2 µg/ml in 100 µl/well PBS 1X).

The dilution of serum samples, determined after optimization, was 1/100.

In all the experiments we used PBS 1X and fetal calf serum as negative controls and two known TB positive sera as positive controls.
All samples were analyzed in duplicate and average readings calculated. The Mean Fluorescence [redacted] from the sample signals.
The variation of fluorescence among different experiments, was normalized by using a relative ratio calculation of (MFI) values respect to the value of the positive control used in each run (sample/positive control X 100).

Performance of the test.
A total of 1023 sera classified in 4 different groups, 162 TB positive and 861 TB negative (Table 1) were analyzed by TB-Luminex assay:

Table 1

	JD	TB	
Group 1	?	+	162
Group 2	-	-	555
Group 3	+	-	216
Group 4	?	-	90
Total			1023

In details:
- Group 1: sera from TB positive herds/unknown JD status. All sera were collected 15-20 days post single intradermal test (IDT), from TB positive herds localized in North (Trentino Alto Adige, Veneto, Lombardia) and South Italy (Calabria) from *M. bovis/M.caprae* culture positive animals.
- Group 2: sera from TB/JD negative herds. All the sera were collected from TB free herds in Officially TB free territories (OTF) (Germany and North Italy). All herds had been controlled for JD for more than six years with serology and culture isolation test.
- Group 3: sera from JD positive/TB negative herds. All the samples were collected in TB free herds in OTF areas (Germany and North Italy) and confirmed positive to JD by culture isolation of MAP.
- Group 4: sera from TB negative/JD unknown status collected from TB free herds in North Italy.

Receiver Operating Characteristic (ROC) curve analysis (Zhou et al. 2009) was used to determine the positive/negative cut off values. Fixing the diagnostic Specificity (Sp) for each antigen higher than 99% consequently, a Sensitivity (Se) ranging from 75,3% for MPB70 to 2,5% for Rv3020c was obtained. The areas under the curve (AUC) were 0,969, 0,932, 0,843 and 0, 668 for MPB70, MPB83, ESAT6 and CFP10 respectively. Rv3615c and Rv3020c showed a low Se and a poor AUC value and were not considered reliable for discrimination of positive and negative samples.

Table 2

	Cut-off	Sensitivity	CI (95%)	Specificity	CI (95%)	AUC
MPB70	30,5	0,753	0,679-0,817	0,999	0,994-1	0,969
MPB83	16,2	0,506	0,427-0,586	0,998	0,992.1	0,932
ESAT6	41,9	0,222	0,160.0,294	0,994	0,986-0,998	0,843
CFP10	35,5	0,111	0,067-0,169	1	0,993-1	0,668
RV3615c	38,1	0,086	0,048-0,141	0,991	0,982-0,996	0,594
RV3020c	0,774	0,025	0,006-0,062	0,991	0,981-0,995	0,653

In Figure 2 A-F are shown the boxplots with the distribution of TB negative and TB positive sera obtained for each antigen.

Figure 2 A

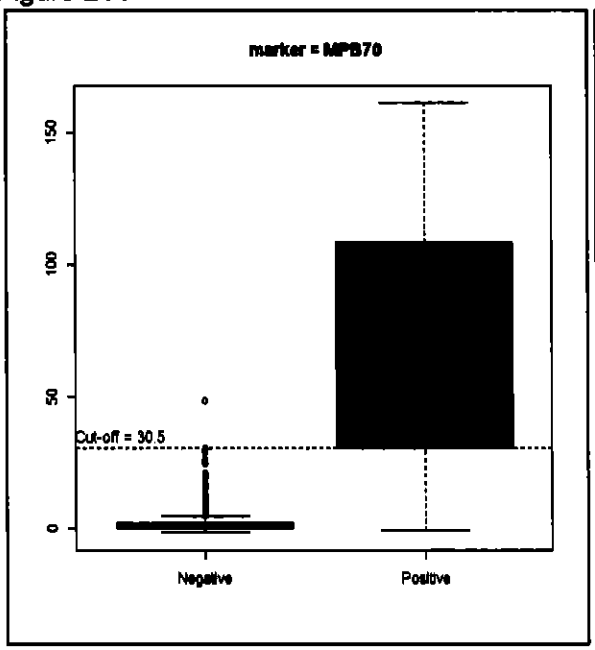


Figure 2 B

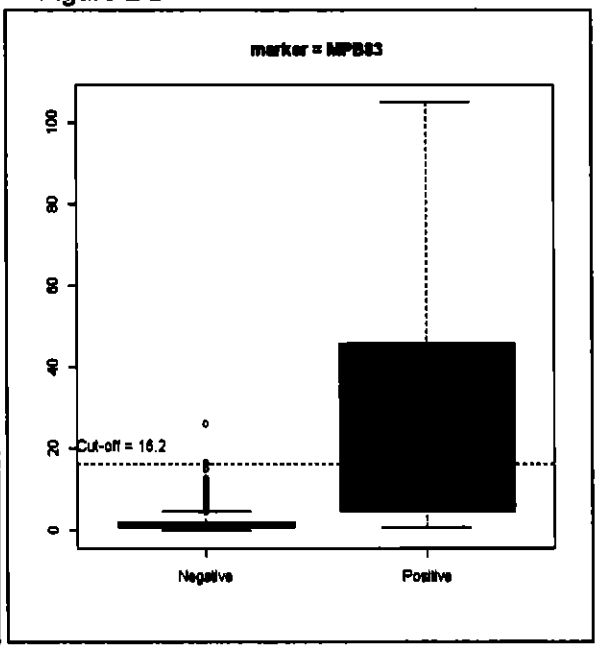


Figure 2 C

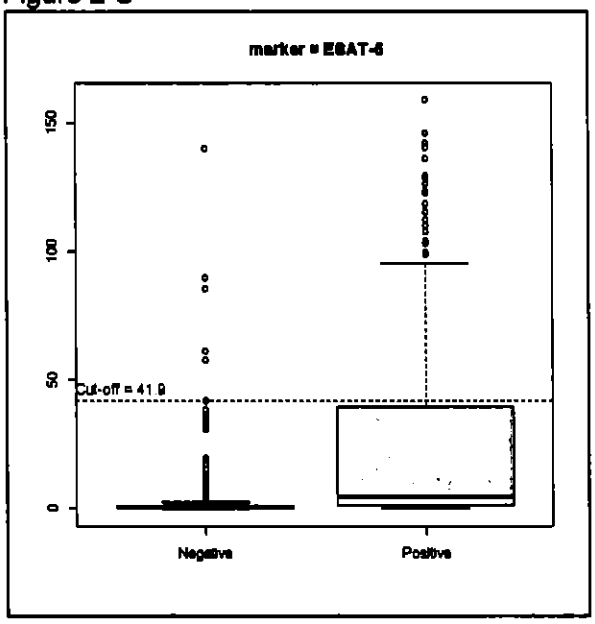


Figure 2 D

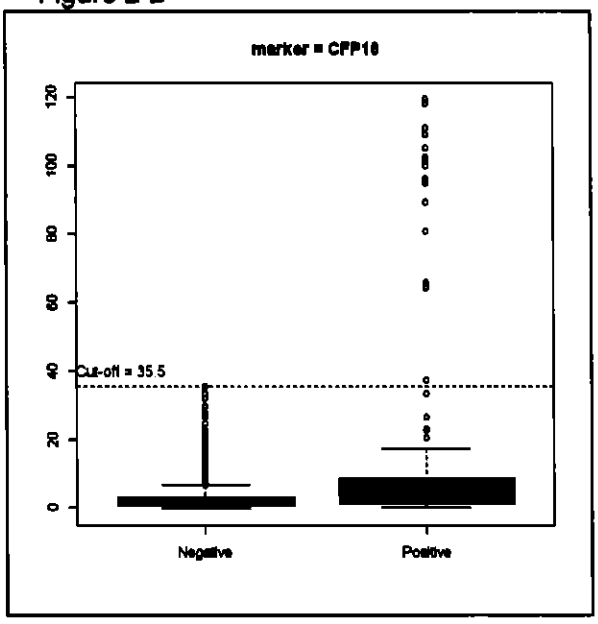


Figure 2 E

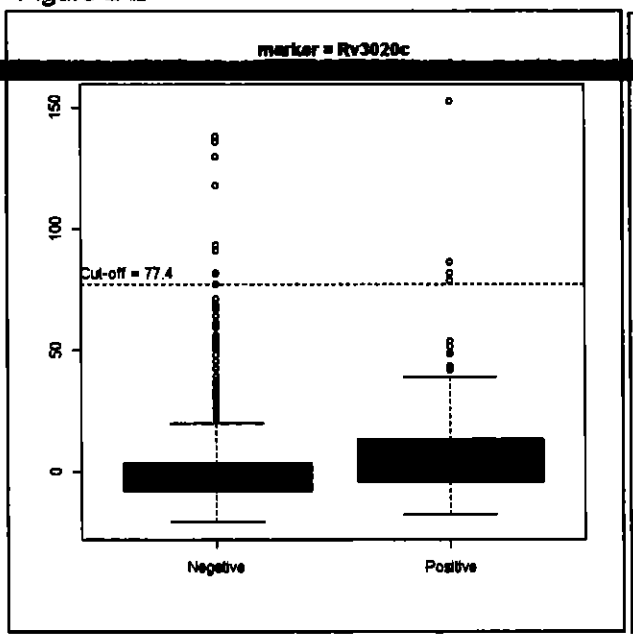
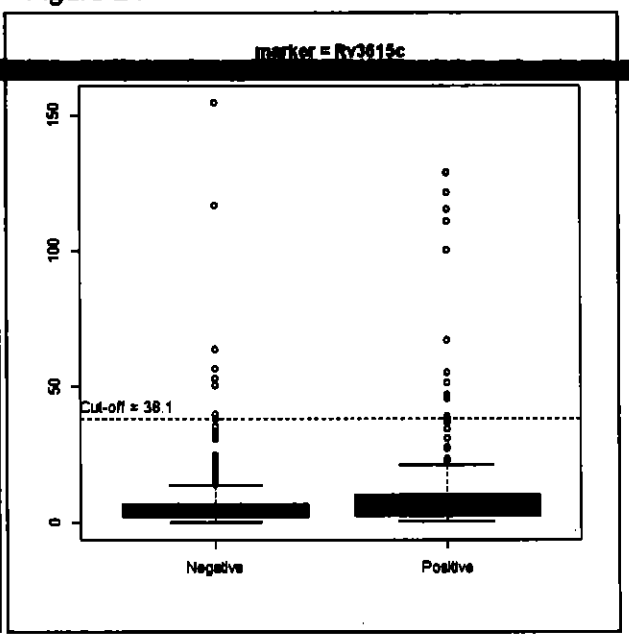


Figure 2 F



The performance of the whole multi-antigen TB-Luminex test was calculated with MPB70, MPB83, ESAT6 and CFP10 considering either one or two positive antigens (Table 3). The Se of Multiplex TB-Luminex assay with one antigen was calculated 79% with Sp of 99,1%. The combination of two positive antigens increases the Sp to 100% but reduces the Se to 51,2% (Table 3). The performance is comparable to the "in-house" ELISA test used at IZSLER presented in Table 4 (Casto et al. 2010) and to other serological assays using two or more recombinant antigens (Souza et al. 2012; Whelan et al. 2008).

Table 3

LUMINEX multiple test	**Sensitivity**	**Specificity**
1 positive antigen	0,79	0,991
2 positive antigens	0,512	1

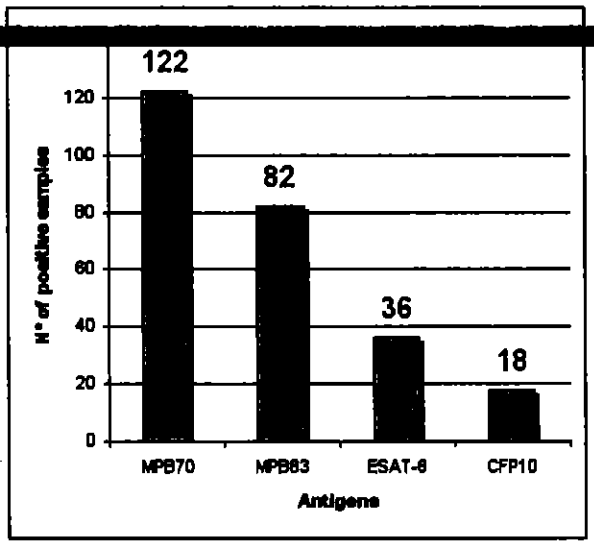
Table 4

"In-house" IZSLER ELISA multiple test	**Sensitivity**	**Specificity**
1 positive antigen	0,742	0,949
2 positive antigens	0,613	0,998

Reactivity to the antigens and comparison with in house ELISA test.

The reactivity of sera in TB-Luminex test was different for each antigen. Of the 162 sera confirmed by *M. bovis* culture isolation and in vivo test, 128 had a positive reaction in TB-Luminex test with one or more proteins. In particular, 122 reacted with MBP70, 82 with MPB83 while ESAT6 and CFP10 showed a lower immunogenicity (positive to 36 and 18 sera respectively) (Figure 3). Interestingly MPB70 showed a better sensitivity respect to MPB83 which is usually reported to be the main potential diagnostic target (Bezos et al. 2014).

Figure 3



Of the 128 Luminex positive sera, most of them (83) were positive to 2 or more antigens, the remaining 45 were positive only to one antigen: 40 with MPB70, 2 with MPB83 and 3 with ESAT6. The reactivity of MPB70, MPB83, ESAT6 and CFP10 in TB-Luminex assay was compared with the "in house" ELISA test with the 162 TB positive sera. The results showed a good level of agreement for MPB70, MPB83 and CFP10 (Table 5) while ESAT6 showed a lower sensitivity in TB-Luminex test (36 sample) respect to ELISA test (61).

Table 5

	Reactivity to Antigens	
ANTIGEN	**Luminex Test**	**Elisa Test**
MPB70	122	127
MPB83	82	75
ESAT6	36	61
CFP10	18	23

Within and between assay variation.
To demonstrate within and between assay variations, 4 known positive control sera were diluted (1/100, 1/400, 1/1600, 1/6400). They were tested in 3 separate independent multiplex assays with three replicates per assay, making a total of 9 replicates for each sample and dilution (Lawson et al. 2010).
The within variation coefficient values for each bead-set ranging from a minimum of 1,20% to a maximum of 10,81% are in agreement with the precision values indicated by Luminex manual (Luminex Corporation, Austin TX USA).
As expected the inter-assay has higher coefficient values ranging from 4,84% to 27,17% that are still included in the parameters of acceptability.

Application of TB-Luminex test in TB positive herds.
Luminex test was applied to additional 256 serum samples collected from TB positive herds located in South of Italy with no data available on culture isolation test. Samples were divided into 3 different categories that are summarized in Table 6: one group positive to IFN-γ and/or IDT, one

group positive to PCR and/or TB-like lesions (VL) and one group (the largest one) including samples negative to IDT and γ-interferon test with unknown data on post-mortem test (isolation, PCR, presence of lesions).

Table 6

	In-vivo test (IDT, IFNg)	**post-mortem** test (VL, PCR)	N. samples
Group 1	+	+	19
Group 2	+	?/-	51
Group 3	-	?/-	186

In Table 7 are presented the results of Luminex test in parallel with the "in house" ELISA test obtained considering one or two positive antigens.
Of the 186 sera negative to in vivo test, 46 were found positive to TB-Luminex assay and 29 to IZSLER ELISA test by using one positive antigen interpretation. Combination of two antigens revealed 8 and 14 positive samples respectively. Most of the reactivity was found with MPB70 and MPB83 antigens. The two serological tests showed a concordance in 20 out of the 46 positive samples detected with one antigen and in 6 of the 14 samples detected with two antigens. The serological positivity could not be confirmed by post-mortem investigation or by additional in vivo test.

Table 7

			LUMINEX multiple test				**IZS"In-house" IZSLER ELISA multiple test**			
In-vivo test (IDT, IFNg)	**post-mortem** test (VL, PCR)	N. samples	1 positive antigen		2 positive antigens		1 positive antigen		2 positive antigens	
			+	%	+	%	+	%	+	%
+	+	19	13	68,4	11	57,9	12	63,2	11	57,9
+	?/-	51	31	60,8	16	31,4	28	54,9	21	41,2
-	?/-	186	46	24,7	8	4,3	29	15,6	14	7,53

Conclusions
We have standardized a TB-Luminex Multiplex assay for serology in cattle with the possibility to modulate sensitivity and specificity depending of the number of positive antigens used for interpretation of the test. The test showed a very high specificity especially when two positive antigens are used for positive interpretation. In particular the test did not show any increase of unspecific signal by analyzing the 216 sera negative to TB and positive to MAP (group 3, Table 1).
Preliminary results on field samples collected from TB positive herds (negative to other in vivo test) show reactivity mainly with MPB70 and MPB83 antigens. Despite the high Sp of the test, we cannot exclude an unspecific reactivity due to the presence of environmental mycobacteria considering the husbandry condition of animals from which samples were taken (Calabria region, semi-free ranging animals).
Luminex assay is a flexible system which now include MPB70 and MPB83 as main potential diagnostic targets, but it could be potentially implemented by additional antigens eventually identified to improve the performance of the test.

3.3 TB-Luminex test for serology in swine (Partner 7).

TB antigens and Optimization of the test.

At the beginning we include in the TB bead set: MPB70, MPB83, CFP10, ESAT6, Rv3615c and RV3020c. However the high reactivity of RV3615c and Rv3020c antigens with all negative swine sera and the difficulties to find serum negative controls lead us to exclude them from the TB bead panel used in the consecutive experiments.

Optimization of assay condition was defined after testing different parameters of TB-Luminex test, specifically:

- Different assay buffer composition: 1) PBS 1X + 0,05% Tween-20 + 1% Yeast Extract; 2) PBS 1X + 0,05% Tween + 1% Casein + 0,05% Proclyn; 3) PBS 1X + 0,05% Tween-20 +1% Casein + 0,05% Proclyn 1%+ mouse serum; 4) PBS 1X + 0,05% Tween-20 +1% Casein + 0,05% Proclyn 1%+ 1% *E. coli* lysate in combination with wash buffer PBS 1X + 0,05% Tween-20.
- Different dilutions of sera (1/50, 1/100, 1/200);
- Different dilutions of biotinylated secondary antibody (1/5000, 1/7500, 1/8000, 1/10000, 1/12.000);

Following test optimization, Luminex assay was carried out according the protocol provided by Luminex ("Luminex-xMAP_Cookbook_2nd_edition", 07.2014, Luminex Corporation, (Austin TX USA) with the followings specification: assay buffer: PBS 1X + 0,05% Tween-20 +1% Casein, 1% Mouse Serum, 0,05% Proclyn; wash buffer: PBS 1X + 0,05% Tween-20; dilution 1/5000 of biotinylated secondary antibody (goat anti-swine polyclonal serum); streptavidin R-Phycoerythrin (2 µg/ml in 100 µl/well PBS 1X).

The dilution of serum samples, determined after optimization, was 1/50.

In all the experiments we used PBS 1X + 0,05% Tween-20 +1% Casein + 0,05% Proclyn 1%+ mouse serum and a known negative serum as negative controls, and a pool of 6 known TB positive sera as positive controls.

All samples were analyzed in duplicate. The readings and value normalization were performed as described for TB-Luminex test applied for cattle serology.

Performance of the test.

A total of 244 swine sera of which, 59 TB positive collected from TB positive herd status located in Sicily and positive to at least one in vivo or post mortem test (IDT or γ-interferon or histology/macroscopic lesions or *M.bovis* culture isolation), and 185 TB negative (collected from North Italy herds in OTF territories), were analysed by TB-Luminex assay. Collection of TB positive swine sera was possible on a limited number of animals from herds located in Sicily where it was demonstrated the presence of *M. bovis* (Di Marco et al. 2012).

Receiver Operating Characteristic (ROC) curve analysis (Zhou et al. 2009) was used to determine the positive/negative cut off values. Fixing the diagnostic Specificity (Sp) for each antigen higher than 97,5% (Table 8), the best Se with MPB83 and CFP10 and a lower immunogenicity for MPB70 and CFP10 were obtained.

Table 8

	Cut-off	Sensitivity	CI (95%)	Specificity	CI (95%)	AUC
MPB70	99,9	0,661	0,526 - 0,779	0,978	0,946 - 0,994	0,928
MPB83	14,2	0,966	0,883 - 0,996	0,978	0,946 - 0,994	0,998
ESAT6	91,7	0,203	0,11 - 0,328	0,978	0,946 - 0,994	0,615
CFP10	12,9	0,864	0,75 - 0,94	0,984	0,953 - 0,997	0,927

In Figure 4 A-D are shown the boxplots with the distribution of TB negative and TB positive sera obtained for each antigen.

Figure 4 A

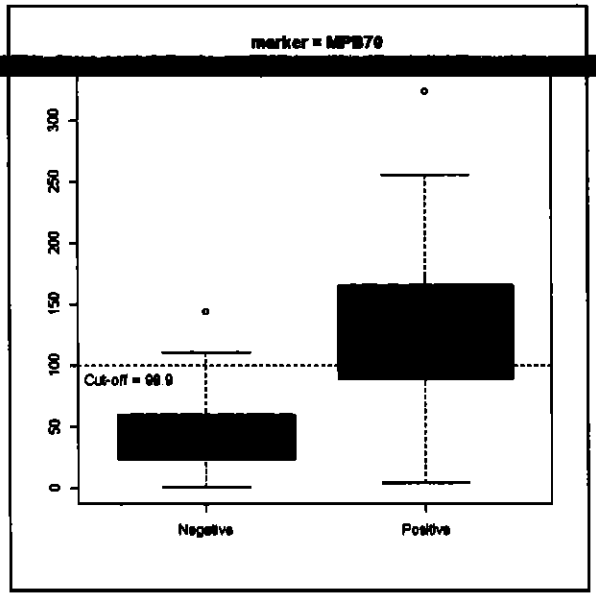


Figure 4 B

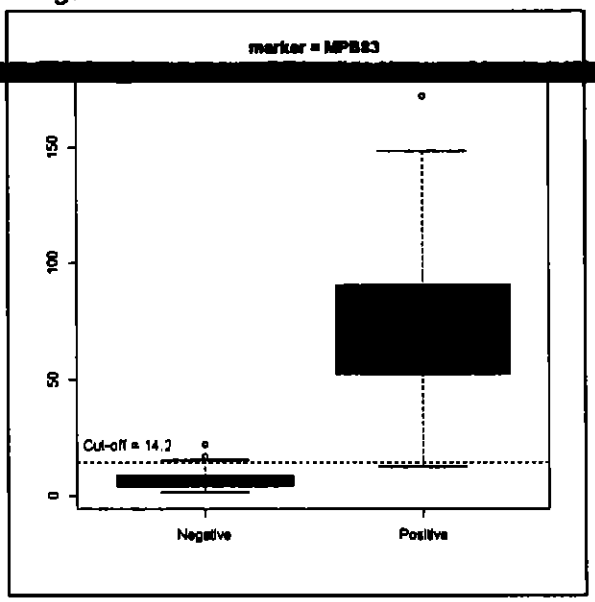


Figure 4 C

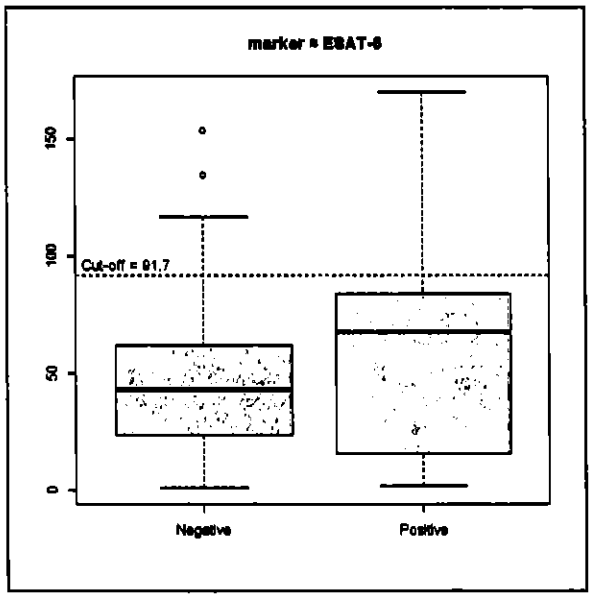
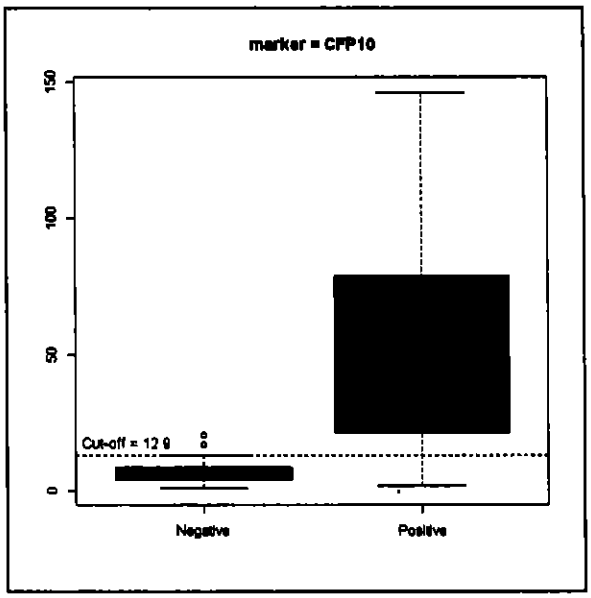


Figure 4 D



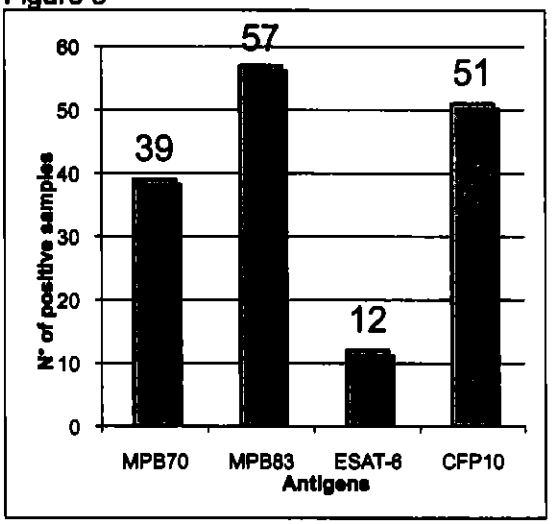
The performance of the whole multi-antigen TB-Luminex test were calculated: 100% for Se and 96,2% for Sp considering one positive antigen and 86,4% (Se) and 97,8% (Sp) considering two positive antigens (Table 9).

Table 9

LUMINEX multiple test	**Sensitivity**	**Specificity**
1 positive antigen	1	0,962
2 positive antigens	0,864	0,978

Reactivity to the antigens and comparison with in house ELISA test.
The reactivity of sera in TB-Luminex test was different for each antigen. All the 59 sera positive at least to one in vivo or post mortem test had a positive reaction in TB-Luminex test with one or more proteins. In particular 57 reacted with MBP83, 51 with CFP10, 39 with MPB83 while ESAT-6 showed a lower immunogenicity (positive to 12 sera) (Figure 5). The performance was comparable to the "in-house" ELISA test used at IZSLER for MPB70, MPB83 and ESAT-6. Interestingly CFP10 showed a higher reactivity in TB-Luminex respect to ELISA test.

Figure 5



Conclusions.
The detection of TB humoral response in swine in TB-Luminex assay was different compared to cattle serology. In particular we obtained a high immunogenicity with MPB83 and CFP10 (57 and 51 sera were positive respectively) and lower reactivity with MBP70 and ESAT6 (39 and 12 respectively). These data can be explained by different hypothesis: host species can respond in different way to the different antigens of *M. bovis* (Bezos et al. 2014), different choice of TB positive serum population (for cattle confirmed by *M. bovis* culture isolation, for swine considering at least one in vivo or post mortem positive test). In fact *M. bovis* isolation confirmed sera could have been mainly collected from animals in advanced stages of TB infection while sera positive only to in vivo test (IDT or γ-interferon) could have been collected at different stages of pathogen infection including the earliest, influencing differently the reactivity response to the various antigens.
Further analysis would be necessary to assess these data including evaluation of antigen immunogenicity with other serology test.

3.4 MAP-Luminex test for serology in cattle (Partner 8).

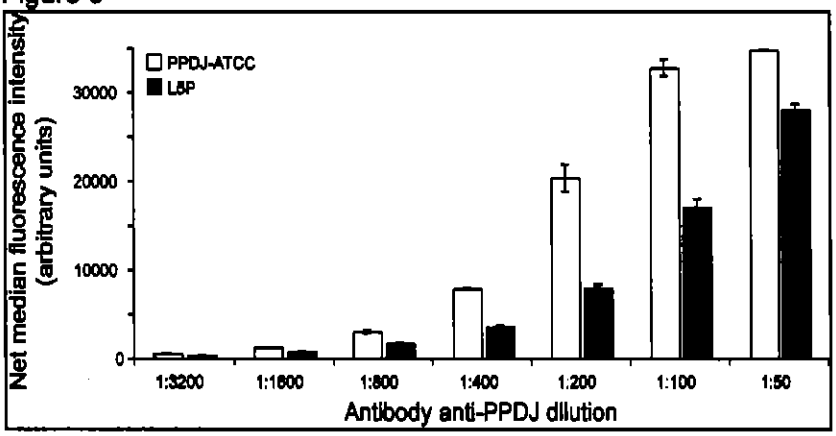
MAP antigens used in the test

MAP antigens evaluated in JD-Luminex test were:
L5P (compound 13) supplied by Partner 2;
PPDjatcc and PPDjb produced by IZSUM (Perugia, Italy) and supplied by Partner 7;
Ag1 (MAP0210c), Ag2 (MAP2942), Ag7 (MAP2609), MAP1272 recombinant proteins and PPDjd supplied by Partner 3 in Dec 2014 (not included in the initial WP1 table of MAP available antigens).

Evaluation of L5P and PPDjs

We prepared stocks of beads coupled to L5P (hydrosoluble compound 13) and to the 3 different PPDj available. Coupling was performed by means of the standard protocol described above for TB antigens or using the chemical spacer adipic acid dihydrazide (ADH), which allows proteins to be anchored through their COOH groups. Coupling reactions were validated with the Luminex platform using serial dilutions of polyclonal rabbit anti-PPDJ antibody. In these conditions we did not observe any background noise-related problems (Figure 6).

Figure 6



Evaluation of bead-coupled antigens with field serum samples revealed background problems with both standard and ADH-coupled beads, which showed unspecific signal also in the blank reaction (beads+anti-bovine biotinylated antibody). In order to verify if these problems could be due to direct/indirect unspecific binding of the biotinylated antibody to the surface of the beads, we decided to test different coupling procedures and assay protocols. We proved that high background did not depend on the amount of antigen utilized for coupling as lowering it down to 0,1 µg did not significantly affect unspecific noise of the assay. We compared 3 different anti-bovine biotinylated antibodies: 1G10 mouse monoclonal antibody from Partner 7 (IZSLER, Italy), a goat polyclonal antibody from ThermoScientific® and a sheep polyclonal antibody from AbD Serotec®. These antibodies were tested on L5P- and PPDj-coupled beads with MAP-positive and negative field samples. All of the antibodies utilized showed high background noise that did not allow discriminating positive from negative samples. In order to improve blocking of the beads, we tested different assay buffers prepared by substituting the yeast extract present in the standard buffer with BSA (1%) or skim milk (3%) and, in parallel, we increased to 0,5 M the concentration of NaCl present in PBS (PBS-HS) to increase stringency of the binding reaction. Although the background noise was lowered by the use BSA and skim milk, both in PBS and in PBS-HS, the fluorescence of positive samples was strongly dimmed too so that the assay performance worsened respect to the standard assay conditions. In addition, we tested an assay buffer from Prime Diagnostics (Wageningen, NL) but we did not observe any substantial improvements. Blocking was also carried out by pre-incubation of the beads in assay buffer containing 3% skim milk or 5% MAP-negative ovine serum, however this step did not help too. All of these results seem to indicate that the observed background depends on the nature of the antigen, as no

background problems were revealed using beads conjugated to TB and MAP-specific recombinant antigens or analysing serum from immunized rabbits.

Optimization of Luminex test with MAP recombinant antigens for serology in cattle.
On December 2014, we received 4 MAP recombinant proteins from Partner 3:
- Ag1, MAP0210c (1.8 mg/ml);
- Ag2, MAP2942 (3.56 mg/ml);
- Ag7, MAP2609 (1.43 mg/ml);
- MAP1272 (0.8 mg/ml).

Each one was coupled to a specific bead set by the standard coupling protocol and the binding efficiency was assessed as explained above. A group of positive and negative samples was then tested with Luminex in simplex and multiplex assays to reveal potential signal interference among the different bead sets. We determined that the co-presence of the 4 recombinant antigens did not hamper the assay performance.
For the analysis of the samples we adopted the same protocol explained in paragraph 3.1, introducing some minor changes:

1. in order to reduce potential unspecific signals deriving from antibodies targeting different mycobacteria other than MAP, the serum samples were pre-treated with *Mycobacterium phlei* by 30-45 min incubation in Buffer 6 from ID SCREEN® PARATUBERCULOSIS INDIRECT ELISA kit (IDVet, France) (5 µl serum in 45 µl Buffer 6);
2. plate reading was carried out in "high" acquisition mode (photomultiplier set at ~ 730 Volt);
3. the results were expressed as net median fluorescence intensity.

Therefore the final JD bead panel included: Ag1, Ag2, Ag7 and MAP1272.

ROC analysis and performance of the test.
We proceeded to evaluate the JD bead panel with additional serum samples and perform the ROC analysis with new data.
We analyzed a total of 737 sera classified in 3 different groups, 227 MAP positive and 510 JD negative (Table 10), all belonging to TB officially free herds:

Table 10

JD herd status	Total samples	Elisa IDEXX / IDVet	Isolation by culture	No. samples
positive	227	-	+	165
		+	+	62
negative	510	-	-	510

In details:
- Positive: sera from JD animals from JD infected herds. All the samples were collected in TB free herds in OTF areas (Germany and North Italy) and confirmed positive to JD by culture isolation of MAP
- Negative: sera were collected from TB free herds in Officially TB free territories (OTF) (Germany and North Italy). All the herds have been controlled for paratuberculosis with negative results for more than six years by serology and culture isolation

The results were used to perform ROC (Receiver Operating Characteristic) analysis and calculate the cut off value, sensitivity (Se) and specificity (Sp) for each antigen (Figure 7-10: A). The histograms show the distribution of the quantitative results obtained with each antigen analysing sera from JD negative and positive animals (Figure 7-10: B).

Figure 7. Results for Ag1 (MAP0210c)

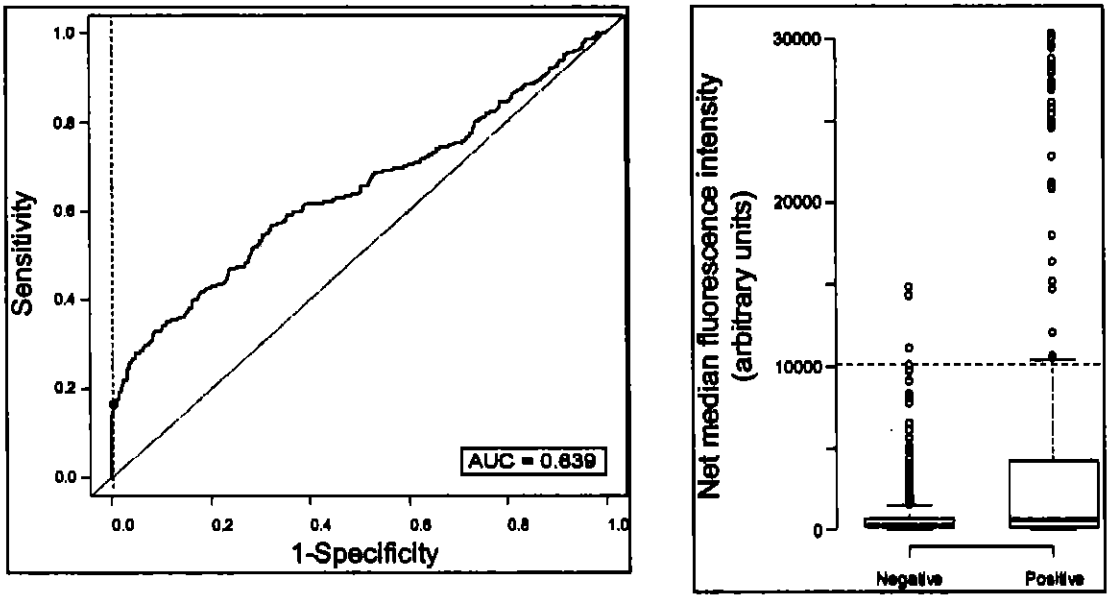


Figure 8. Results for Ag2 (MAP2942)

A

B

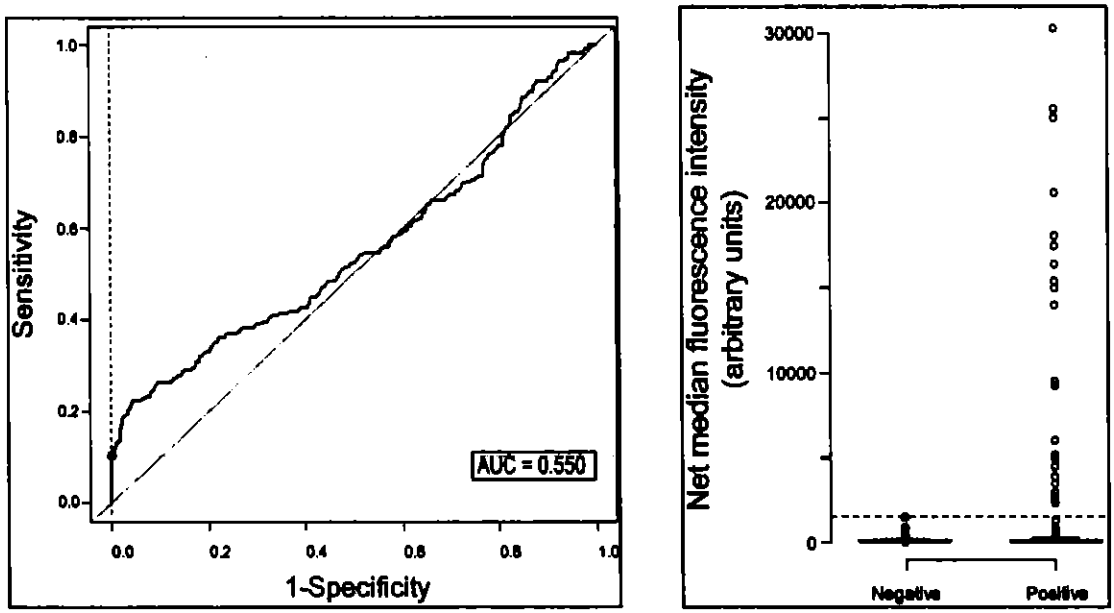


Figure 9. Results for Ag7 (MAP2609)

A B

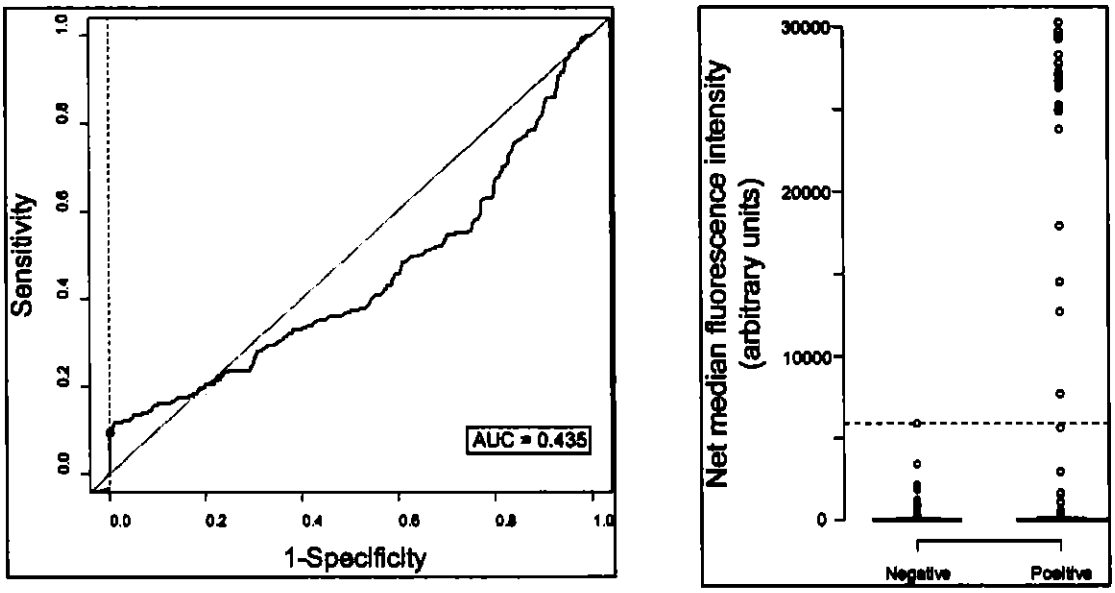
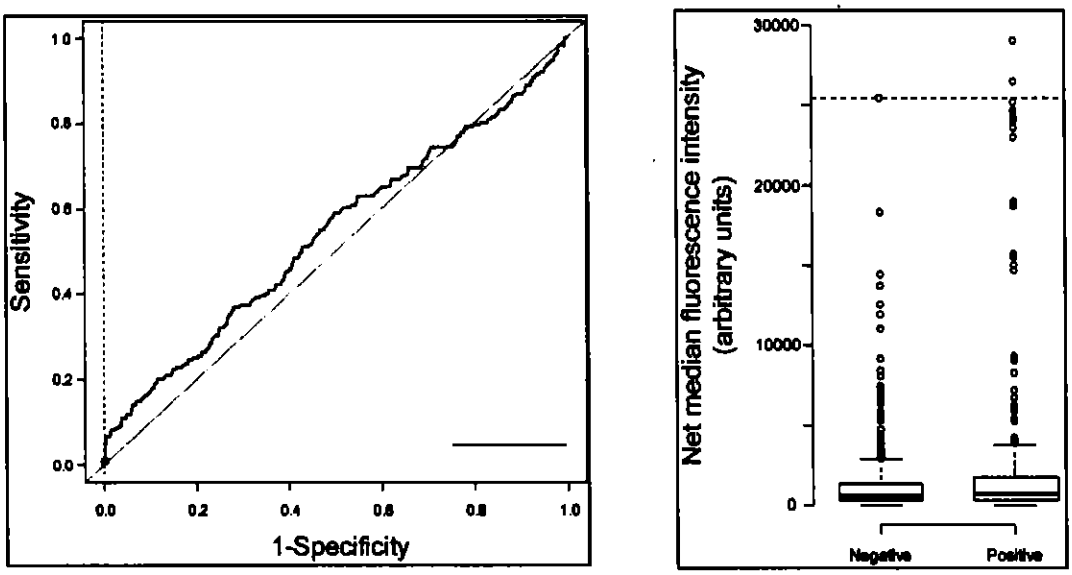


Figure 10 Results for MAP1272

A B



We determined the optimal cut-off values fixing a minimum Sp of 99,4% for each antigen and considering the importance of detecting the majority of infected animals with the maximum likelihood of having no false positive results.

The individual Se ranged from 16.7% of Ag1 to 0.9% for MAP1272 (Table 11). Since MAP1272 clearly showed no discriminatory power between infected and non-infected animals, we decided to exclude it from the rest of the study.

Table 11

Antigen	Cut-off	Sensitivity	Specificity
Ag1	10132.3	0.167	0.994
Ag2	1357.3	0.106	1
Ag7	5894.8	0.097	1
MAP1272	25428.4	0.009	1

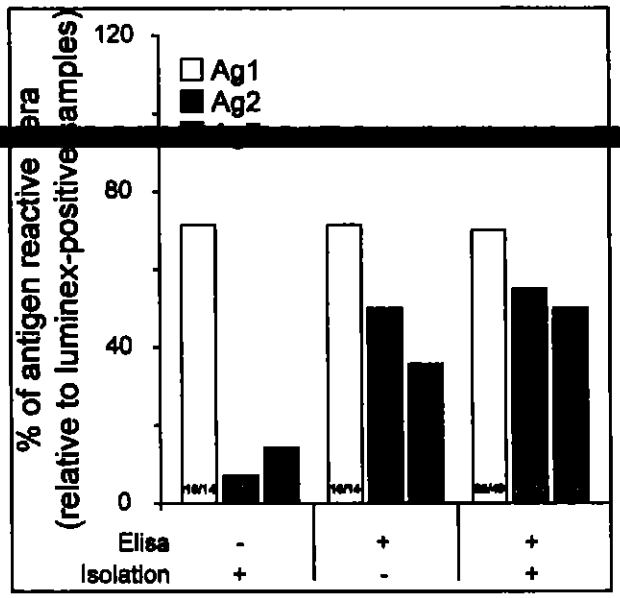
Se and Sp of the whole multi-antigen JD-Luminex test were also determined considering one or two positive antigens among Ag1, Ag2 and Ag7 (Table 12). The positivity to one antigen showed a Sp of 99.4% and a Se of 23.8%. Such performance is comparable to commercial ELISA that in this population showed a Se of 27.3%.The combination of two positive antigens increased the Sp to 100% but reduced the Se to the 8.4%. The first criterion for positivity calling was adopted in the analysis of the samples tested in the rest of this study.

Table 12

Criterion for positivity calling	Sensitivity	Specificity
At least 1 positive antigen	0.238	0.994
At least 2 positive antigens	0.084	1

According to this protocol, about 70% of all positive callings were due to reactivity of Ag1-coupled beads, independently of whether the same samples were tested positive by ELISA or were from animals tested MAP positive via cultural isolation. Differently, reactivity of Ag2- and Ag7- coupled beads was shown to depend on the nature of the samples since it was observed with higher frequency (about 50%) in sera tested positive by ELISA.

Figure 11. Differential reactivity of sera to Luminex- JD antigen panel


% of antigen reactive sera
(relative to luminex-positive samples)
120
80
40
0
Ag1
Ag2
16/14
16/14
Elisa - + +
Isolation + - +

Field evaluation of the JD-Luminex

In order to deepen the evaluation of the JD-Luminex assay, we tested a panel of 189 sera **from** different populations (Table 13).

Table 13

Individual testings		Herd status		
***M. bovis* culture isolation**	**MAP culture isolation**	**TB**	**JD**	**No. animals**
+	?	+	?	72
?	?	+	?	44
-	-	-	+	43
-	?	-	+ (JD-vaccinated)	20
				Total: 189

A subset of samples from TB positive animals of unknown paratuberculosis status, employed for Luminex-TB performance evaluation, was tested with the JD-Luminex test in parallel with a commercial ELISA for paratuberculosis (IDEXX Paratuberculosis Screening Ab). The results are shown in Table 14 and demonstrate a low correlation between the 2 tests ($\kappa = 0.023$).

Table 14: TB confirmed cattle from herds TB infected/JD unknown status

	ELISA positive	**ELISA negative**	**Total**
Luminex positive	3	1	4
Luminex negative	42	26	68
Total	45	27	72

Similar results were obtained testing animals of TB unknown status belonging to TB infected herds (Table 15). These results markedly differ from those obtained with samples from officially free TB herds, employed for ROC analysis (Table 10), in which ELISA and the JD-Luminex test showed similar sensitivity. These differences could be attributed to IGRA boosting carried out in cattle from TB infected herds 15 days prior to blood sampling that might cause cross reactions with the whole MAP cell coated plates of commercial paratuberculosis ELISA. On the other hand TB positive sera showed no reaction with specific MAP antigens of the JD-Luminex assay.

Table 15: TB unknown cattle from herds TB infected/JD unknown status

	ELISA positive	**ELISA negative**	**Total**
Luminex positive	1	2	3
Luminex negative	11	30	41
Total	12	32	44

Table 16 represents the results of the assay conducted on sera from JD-seropositive/faecal culture negative animals from JD-infected herds. Among these samples, roughly a third tested positive with the JD-Luminex assay. Despite the small number of specimens analysed, we can speculate that many ELISA positive animals reacts to MAP antigens other than the ones present in the JD-Luminex assay.

Table 16: JD seropositive/culture negative cattle from JD infected/OTF herds

	ELISA positive	**ELISA negative**	**Total**
Luminex positive	14	0	14
Luminex negative	29	0	29
Total	43	0	43

Similar results were observed when we analyzed sera from animals vaccinated with an inactivated strain of Map. In a herd of 20 animals, only 6 out 10 Elisa-positive samples tested positive with the JD-Luminex assay. The observed difference in performance between the 2 serology tests might be attributed, not only to a reduced sensitivity of the JD-Luminex assay, but also to the production by vaccinated animals of a vaccine-specific repertoire of antibodies, different respect to the one present in naturally infected animals and more reactive towards the whole Map extract absorbed on ELISA plates.

Table 17: JD-vaccinated cattle from JD infected/OTF herds

	ELISA positive	**ELISA negative**	**Total**
Luminex positive	6	0	6
Luminex negative	4	10	14
Total	10	10	20

Conclusions

We have standardized a JD-Luminex Multiplex assay for serology in cattle with three recombinant specific MAP antigens. The test showed accuracy similar to commercial ELISA test in a panel of truly positive and negative sera. However, in sera from PPDb boosted animals of unknown JD status, it demonstrated a significantly lower sensitivity respect to ELISA.

Preliminary results on field samples collected from TB positive herds (negative to other in vivo test), JD infected herds (negative to faecal culture) show reactivity mainly with Ag1 (MAP0210c) antigen, while reactivity to Ag2 (MAP2942) and Ag7 (MAP2609) was observed mainly in ELISA positive sera.

As resulted in this project, JD-Luminex assay needs to be further implemented with additional antigens in order to improve the performance of the test.

3.5. References

Anderson, A., R. Wakeley, G. Wibberley, K. Webster, J. Sawyer. 2011. Development and evaluation of a Luminex multiplex serology assay to detect antibodies to bovine herpes virus 1, parainfluenza 3 virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods. J. Immunol. Methods. 366: 79-88.

Barandiaran, S., A. M. Perez, A. K. Gioffrè, V. M. Martinez, A. A. Cataldi, M. J. Zumarraga. 2014. Tuberculosis in swine co-infected with *Mycobacterium avium* subsp. *hominissuis* and *Mycobacterium bovis* in a cluster from Argentina. Epidemiol. Infect. 143: 966-974.

Bezos, J., C. Casal, B. Romero, B. Schroedere, R. Hardegger, A. J. Raeber, L. Lopez, P. Rueda. L. Dominguez. 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. Res. Vet. Science 97: 544-552.

Brocchi E., I. E. Bergmann, A. Dekker, D. J. Paton, D. J. Sammin, M. Greiner, S. Grazioli, F. De Simone, H. Yadin, B. Haas, N. Bulut, V. Malirat, E. Neitzert, N. Goris, S. Parida, K. Sorensen, K. De Clercq. 2006. Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. Vaccine. 24(47-48):6966-79

Casto, B.,C. Donati, S. Zoppi, M. Pacciarini, M.B. Boniotti. 2010. A multi-antigen ELISA-test for serology detection of Mycobacterium bovis infection in cattle. European Society of Mycobacteriology, 31° Annual Congress, 4-7 July, Bled-Slovenia.

Di Marco, V., P. Mazzone, M. T. Capucchio, M. B. Boniotti, V. Aronica, M. Russo, M. Fiasconaro, N. Cifani, S. Corneli, E. Biasibetti, M. Biagetti, M. L. Pacciarini, M. Cagiola, P. Pasquali, C. Marianelli. 2012. Epidemiological significance of the domestic black pig (*Sus scrofa*) in maintenance of bovine tuberculosis in Sicily. J. Clin. Microbiol. 50:1209-1218.

Lawson, S., J. Lunney, F. Zuckermann, F. Osorio, E. Nelson, C. Welbon, T. Clement, Y. Fang, S. Wong, K. Kulas, J. C. Hennings. 2010. Development of an 8-plex Luminex assay to detect swine cytokines for vaccine development: assessment of immunity after porcine reproductive and respiratory syndrome virus (PRRSV) vaccination. Vaccine 28: 5356-5364.

Nielsen SS. Transitions in diagnostic tests used for detection of Mycobacterium avium subsp. paratuberculosis infections in cattle. Vet Microbiol. 2008 Dec 10;132(3-4):274-82.

Souza, I. I., E. S. Melo, C. A. Ramos, T. A. Farias, A. L. Osorio, K. S. Jorge, C. E. Vidal, A. S. Silva, M. R. Silva, A. O. Pellegrin, A. O. Araujo. 2012. Screening of recombinant proteins as antigens in indirect ELISA for diagnosis of bovine tuberculosis. Springerplus 1: 77.

Verdugo C, Toft N, Nielsen SS. Within- and between-herd prevalence variation of *Mycobacterium avium* subsp. *paratuberculosis* infection among control programme herds in Denmark (2011-2013). Prev Vet Med. 2015 Oct 1;121(3-4):282-7

Whelan, C., E. Shuralev, G. O'Keeffe, P. Hyland, H. F. Kwok, P. Snoody, A. O'Brien, M. Connolly, P. Quinn, M. Groll, T. Warrerson, S. Call, K. Kenny, A. Dulgnan, M. J. Hamilton, B. M. Buddle, J. A. Johnston, W. C. Davies, S. A. Olwill, J. Clarke. 2008. Multiplex immunoassay for serological diagnosis of *Mycobacterium bovis* infection in cattle. Clinical and Vaccine Immunology 15: 1834-1838.

Zhou, Xiao-Hua, Donna K. McClish, and Nancy A. Obuchowski. *Statistical methods in diagnostic medicine*. Vol. 569. John Wiley & Sons, 2009.

Task 2.2 and 2.3: Report (Partner 1)

- We have provided evidence that the detection of IFNy and serological responses can be undertaken using the same sample matrix is feasible (IGRA plasma supernatants).
- We have optimised conditions for the MSD platform in preparation for future multiplex application using this system.
- We have provided proof of concept that a multiplexed IFNg/antibody response assay can be developed using the Enferplex platform.
- However, due to plasma dilution effects on IFNg detection and heterophilicity in detecting antibody responses, we observed a loss of sensitivity compared to standard Bovigam IGRA ELISA.
- Further development resulted in a test using the Enferplex system that detects on one test plate, IFNg as effectively as the Bovigam ELISA as well as antibody responses from the same sample (plasma supernatants). Whilst not a true IGRA/serology multiplex system, this test will still provide significant cost savings by reducing staff time and the need for collecting different samples for serology and IGRA.

Future work:

- Addition of reagents detecting other diagnostically important cytokines or chemokines.
- Test validation on a larger number of samples.

2 Background and objectives:

Bovine tuberculosis is a slow-progressing disease in most animals with a potential latency phase which will increase the possibility of persistent cases in infected herds if the detection of infected animals is not maximized by the application of ante-mortem diagnosis. It is known that that parallel application of interferon-gamma release assays (IGRA) and intradermal tuberculin test will increase the overall sensitivity of detecting infected cattle by up to 20 %. Therefore the phase 'two tests are better than one' has been coined. Further, studies have also shown that an additional proportion of animals that escape detection by both tuberculin skin test and IGRA can be detected by serology. Thus, 'three tests can be better than two'. However, resource limitations will most likely not allow the use of three technically independent tests. Thus, our work package proposed to overcome such limitations by developing a combined IGRA-serology platform that detects both read-out parameters using the same sample matrix (plasma) and assay platform. Thus, in this work package we first optimized the use of plasma supernatants from IGRA whole blood cultures for the detection of mycobacterial antibodies. This work was done using the versatile MSD test platform. Once these conditions had been optimized, the combined serum antibody-IGRA test system was transferred to the Enferplex test system [1] that is already in use and being marketed as a serology assay for bovine TB (Enferplex TB).

3 Results:

3.1 Task 2.2. Optimization of assay conditions using the Meso Scale Discovery (MSD) platform

The MSD platform (Meso Scale Discovery, Gaithersburg, MD) has been optimized for the serological detection of antibodies against MPB70 and MPB83, within WP1 of this project. In this work package we aimed to define the conditions in which both serological and IGRA responses could be detected simultaneously using IGRA plasma supernatants. Therefore during the initial optimization work we used stimulated cell supernatant samples from both MB infected and non-infected herds. The infected animals used were naturally infected, skin test positive animals currently housed at AHVLA Weybridge.

Initial optimisation:
MPB70 and MPB83 are major serological targets following infection of cattle with *Mycobacterium bovis* (MB)[2]. Consequently, full length recombinant MBP70 and MPB83 proteins (Llonex, Braunschweig, Germany) and IFNy detection antibody (Mabtech ELISPOT, MABTECH, Stockholm, Sweden) were coated overnight in PBS onto MSD detection plates. It became clear that a dilution factor would be required to detect antibody responses in plasma supernatants as a "blocking effect" was seen when serum samples were tested neat.

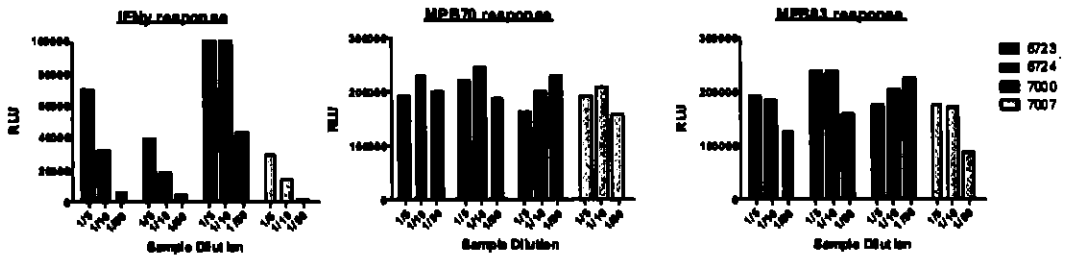


Figure 1. Optimization of plasma supernatant dilution for the detection of INFg and antibody responses.

The results in figure 1 show the responses from 24hr stimulated whole blood from four TB reactor cattle. IFNy responses are derived after 24 hour stimulation with bovine tuberculin (PPDB). Serology responses were determined using the non-stimulated medium control plasma supernatants from these cultures as we were confronted, when we measured serology response using PPDB stimulated supernatants, by a large reduction in responses to both MPB70 and MPB83 most likely due to antibody absorption to these antigens contained in PPDB3 (data not shown). IGRA responses at the same dilutions were measured in PPD stimulated plasma to determine whether higher plasma dilutions would impact the sensitivity of detecting IFNg. Plasma were tested at 1/5, 1/10 and 1/50 dilutions. Significant reductions in the IFNy signals were observed the more we diluted the plasma supernatants. In contrast, the effects on detecting serum responses were less pronounced (figure 1). We found a dilution of 1/10 to be optimal to measure serological responses as both at 1/5 and 1/50 dilutions we observed a reduction in signal strength. Thus, we provisionally decided to apply this plasma dilution in the assays to detect INFg and antibody responses simultaneously.

As we also observed a reduction in signal of the IGRA response when we diluted the plasma supernatants 1/5 and above compared to the responses observed with ½ diluted plasma supernatants normally used for Bovigam IGRA, we investigated whether longer cultures beyond the standard 24 h or pre-diluted whole blood would restore the signal strength without affecting the signal strength of the antibody responses. This could indeed be achieved by extending the culture

of both 1/5 and 1/10 diluted whole blood beyond 4 days, with MBP 70 or MPB83-specific antibodies still detectable at undiminished signal strength (data not shown). However, it was deemed impractical for routine use to extend the culture period for such a significant time. Subsequently, the further assay development, described in the following paragraph, was undertaken with plasma diluted 1/10.

Evaluation of assay using the MSD platform:
Having defined the conditions that can be applied to measure IGRA and humoral responses in the same samples, we applied this protocol to a larger number of animals to provide further data of proofing the concept that this approach could be developed into a practical test. This was carried out using the MSD system before we transferred the method to the Enferplex platform. These data would also allow multiplexing of the test in the MSD platform at a later stage (this was beyond the scope of this work package). Please note that this test was not multiplexed but performed in individual wells of the MSD plate

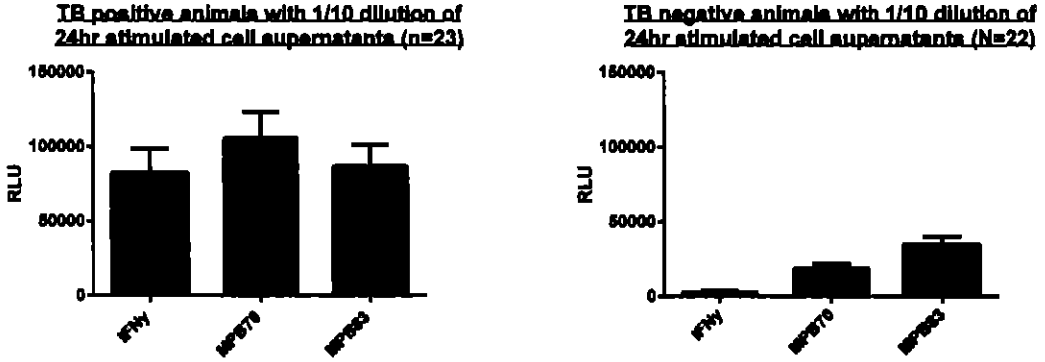


Figure 2. Results obtained using samples from naturally *M. bovis* infected skin test reactor animals (n=23) and non-infected control animals (n=22). Results are expressed as mean relative light units (RLU) ± SD. The plasma supernatants used for IFNg determination derived from whole blood cultures stimulated with PPD-B, antibody responses were determined using plasma from medium control cultures.

Figure 2 summarises the results obtained after determining IGRA and MB specific serum responses to MPB70 and MBP83 in samples collected from naturally MB infected cattle as well as uninfected control cattle.

Table 1. Summary of ROC analysis for the multiplex system

	Cut-off value (RLU)	Sensitivity	95% CI range	Specificity	95% CI range
IFNγ PPD-B	17143	91.3	72.0 to 98.9%	100%	84.6 to 100%
MPB70	51925	56.5	34.5 to 76.8%	100%	84.6 to 100%
MPB83	97187	30.4	13.2 to 52.9%	100%	84.6 to 100%

To define cut-offs for positivity and to determine the sensitivity of the serum and IGRA tests performed on the MSD platform, ROC analyses were performed. Table 1 summarises the outcomes of these analyses. As the objective of this approach was to establish tests that display comparable specificity to the standard SICCT skin test, the specificity was set at 100%. With this specificity setting, we observed good sensitivity (91.3 %) for IGRA and 56.6 and 30.4 % when measuring antibody responses to MPB70 and MPB83, respectively. These values are comparable to those seen in either Bovigam IGRA or conventional serum tests using MPB70 (data not shown). The sensitivity of MPB83 is lower but this was expected as a number of our negative sample set showed cross- reactivity to MPB83, probably due to concurrent infection with environmental mycobacteria or *M. a. paratuberculosis* (data not shown). We also included the antigens Rv3020c and Rv3651c the serological test. However, responses to these antigens were low and of no

practical value (data not shown. NB. These two antigens were previously defined as potent stimulators of IFNg responses[4]).
Thus, we could demonstrate with these data that both humoral and cell-mediated responses could be determined from plasma supernatants generated from whole blood IGRA cultures without apparent loss of sensitivity or specificity despite using higher sample dilutions for measuring IFNg responses than applied in the gold standard Bovigam IGRA. This is most likely due to the more sensitive read-out system of the MSD platform compared to conventional ELISA readers. The next task (2.3) was to transfer this test to the Enferplex platform and to develop a multiplex assay based on this system.

3.2 Task 2.3. Transfer of test system to Enferplex platform

The Enferplex technology utilises a BioDot plate printer to 'print' antigens to wells of 96-well transparent ELISA plates and a camera based detection device produced by Quansys to measure chemiluminescence [1]. This platform allows up to 9 antigens to be printed onto individual wells.

The initial experiments were performed detecting IFNy and antibody responses in separate individual wells. The results of these preliminary results were encouraging as we were able to transfer the method from the MSD platform to the Enferplex system applying the same plasma dilutions (1/10) as optimised on the MSD platform (data not shown). These preliminary results also allowed the calculation of cut-offs for positivity based on the mean RLU of medium control wells plus 2 times SD. We therefore proceeded to multiplex this assay by printing MPB70, MPB83 and the coating anti-bovine IFNg monoclonal antibody onto the same wells of transparent ELISA plates. However when the detection antigens/antibodies were combined we experienced a major technical problem with non-specific reactions occurred in all of the IFNy spots regardless whether plasma supernatants were applied or not. We hypothesised that these reactions were caused by so-called heterophilic antibodies in the polyclonal anti-bovine IgG sera used to detect serological responses. Such antibodies react with immunoglobulin of different species and thus would react with the coating anti-anti-bovine IFNg specific mouse monoclonal antibody. In order to overcome this we modified the system by replacing the polyclonal anti-bovine IgG antibody with a mouse monoclonal anti-bovine IgG and also explored the use of the Bovigam sample diluent to replace the antibody diluent as this reagent is designed to block such heterophilic responses. These measures successfully reduced the noise in our system, although it did not fully remove the background reactions caused by heterophilic antibody recognition (data not shown). However, the noise reduction was substantial enough so that we were able to continue development of this multiplex test.

Thus, this multiplex test was applied to 48 plasma samples which were acquired from the GB Bovigam TB surveillance laboratory. These samples originated from skin-test negative but Bovigam IFNy positive animals with a range of Bovigam OD values representing weak to very strong responding animals. The results showed a reduction in sensitivity when compared to the Bovigam test, with only 45% (22/48) of the samples which gave positive Bovigam results being positive on the multiplex system. This could be explained by higher plasma dilution factor which we had to use compared to Bovigam (1/2) to optimise the serological responses. After discussion with scientists at Enfer we concluded that multiplexing IFNg testing and serology on their system and with these conditions would be challenging, and suffer from a significant loss of overall sensitivity. Thus, we decided to maintain separate wells for measuring cytokine responses (in this case IFNg, at ½ plasma dilutions), and measuring antibody responses (as multiplex system) in another well using plasma dilutions of 1/50. This approach also removed the issue of heterophilicity.

Forty-four samples were obtained from skin test negative cattle that underwent routine Bovigam surveillance testing. Table 2 summarised the comparison of the two test format. 57 % (25/44) of these samples tested positive in the Bovgiam test, 52 % (23/44) were positive when the test was

performed using the Enferplex platform, with 91 % (40/44) of samples giving identical results in both test formats. 2 samples were Bovigam only positive, 1 was enferplex only positive.

Table 2. Comparison of results of IFNg determination using Bovigam and Enferplex platforms.

	Enferplex format		
Bovigam	Positive	Negative	
Positive	22	3	25
Negative	1	18	19
	23	21	44

Next we compared quantitatively the signal strengths obtained with the two test platforms. The results are shown in figure 3 demonstrate a high degree of statistically significant correlation between the signals generated in the two test platforms (Spearman r = 0.9301, P < 0.0001).

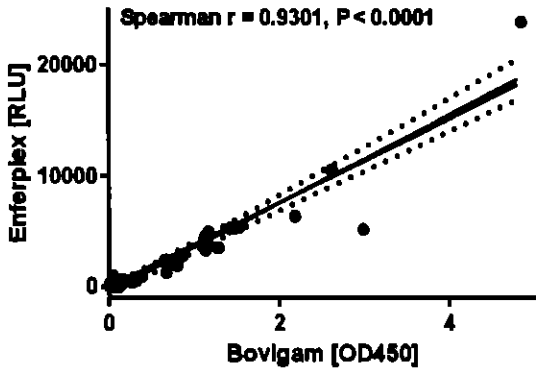


Figure 3. Comparison of IGRA signal strengths when determined on Bovigam or Enferplex platforms. Statistical analysis: correlation using non-parametric Spearman correlation and linear regression analysis (GraphPad, San Diego, CA). Solid line: Best fit line of linear regression analysis; dotted lines represent 95 % CI band of best fit line.

Taken together, these results demonstrated that the Enferplex system can be modified to measure IFNg in plasma supernatants from whole blood cultures as well as with the Bovigam ELISA. This system could be extended to measure other cytokines or chemokines of diagnostic interested (for example IP-10 or IL-2) in a multiplex combination.

These samples were also examined using the Enferplex system to detect humoral responses MPB70 and MPB83. For this analysis, 1/50 dilutions of the plasma supernatants from the medium control whole blood culture wells were used. The results indicated that 11 % (5/44), 9 % (4/44) and 16 % (7/44) of these samples responded to MBP70, MBP83 alone, or in parallel, respectively. These responder frequencies are low. However, this herd had all skin test reactors already removed and thus it is likely that only animals at early stages of infection remained, and such animals are considered to present with low antibody responses to MB antigens. Importantly, one of the serology-positive animals was IGRA negative, suggesting that the application of skin test, IGRA and serology in parallel could identify additional animals compared to application of IGRA and tuberculin skin test alone. However, future larger studies need to be undertaken to demonstrate the veracity of this observation statistically.

3.3 References:

1 **Whelan, C.** *et al.* Multiplex immunoassay for serological diagnosis of bovine infection in cattle. *Clinical and vaccine immunology : CVI* **15**, 1834-1838, doi:10.1128/CVI.00238-08 (2008).

2 **Marassi**, C. D. *et al.* The use of MPB70 and MPB83 to distinguish between bovine tuberculosis and paratuberculosis. *Comparative immunology, microbiology and infectious diseases* **33**, 485-489, doi:10.1016/j.cimid.2009.08.003 (2010).

3 **Borsuk**, S., Newcombe, J., Mendum, T. A., Dellagostin, O. A. & McFadden, J. Identification of proteins from tuberculin purified protein derivative (PPD) by LC-MS/MS. *Tuberculosis* **89**, 423-430, doi:10.1016/j.tube.2009.07.003 (2009).

4 **Sidders**, B. *et al.* Screening of highly expressed mycobacterial genes identifies Rv3615c as a useful differential diagnostic antigen for the Mycobacterium tuberculosis complex. *Infection and immunity* **76**, 3932-3939, doi:10.1128/IAI.00150-08 (2008).